Translational Medicine Research
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Translational Research in Stroke

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Translational Medicine Research

Aims and Scope

In collaboration with National Infrastructures for Translational Medicine (Shanghai), the largest translational medicine research center in China, the book series "Translational Medicine Research" offers a state-of-the-art resource for physicians and researchers alike who are interested in the rapidly evolving field of translational medicine. It features original and observational investigations in the broad fields of laboratory, clinical and public health research, providing practical and up-to-date information on significant research from all subspecialties of medicine and broadening readers' horizons, from bench to bed and bed to bench.

With a focus on global interdisciplinary academic collaboration, the series aims to expedite the translation of scientific discovery into new or improved standards of management and health outcomes practice.

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Produced in close collaboration with National Infrastructures for Translational Medicine (Shanghai), the largest translational medicine research center in China, the book series offers a state-of-the-art resource for physicians and researchers alike who are interested in the rapidly evolving field of translational medicine. Prof. Zhu Chen, the Editor-in-Chief of the series, is a hematologist at Shanghai Jiao Tong University, China's former Minister of Health, and chairman of the center's scientific advisory board.

More information about this series at http://www.springer.com/series/13024

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Contents

Part I
Stroke Epidemiology-Diagnosis

Chapter 1
Stroke Therapy Development Successes: Research Guidelines and Embolic Stroke Models for Monotherapy and Adjuvant Therapy Development

Paul A. Lapchak

Abstract Acute ischemic stroke patients now have the benefit of receiving either thrombolytic therapy or endovascular procedure therapy, alone or with an approved thrombolytic to significantly improve clinical outcome measured using the modified Rankin scale. Most recent data indicate that large vessel occlusion stroke victims can achieve a modified Rankin scale score of 0–2, clinically normal, 3–12 months after treatment. Like tissue plasminogen activator (rt-PA), endovascular procedures will be of benefit to a small well-defined patient population, because it appears that a small ischemic core and large penumbral substrate are a prerequisite for neuroprotection and functional recovery. Endovascular procedures and thrombolysis both effectively target reperfusion of the penumbra to promote cell survival, but neither therapy actually targets a specific mechanism directly involved in the ischemic cascade, a complex series of temporally regulated events that result in differential death of cellular populations in the brain. With our constantly evolving understanding of cell death mechanisms, we now have the opportunity to develop neuroprotective or cytoprotective strategies to use as adjuvant therapy with endovascular procedures and rt-PA to further promote function and potentially repair ischemia-damaged cellular pathways. The benefit of testing novel neuroprotective and cytoprotective therapies as an adjuvant in the patient population defined above will take advantage of both large penumbra and reperfusion of the penumbra. Progress to develop new therapies will be inextricably linked to standardization of translational research practices, complete transparency of data, and adherence to STAIR, RIGOR, ARRIVE, and VOW guidelines. Moreover, because the target population will be undergoing reperfusion therapies, the recommendation is for translational research to be conducted in standardized and validated embolic stroke models.

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Abbreviations

ARRIVE	Animals in Research: Reporting In Vivo Experiments
ASPECTS	Alberta Stroke Program Early CT Score
BBB	Blood-brain barrier
CT	Computed tomography
ECASS	European Cooperative Acute Stroke Study
EPITHET	Echoplanar Imaging Thrombolysis Evaluation Trial
ESCAPE	Endovascular Treatment for Small Core and Proximal Occlusion Ischemic Stroke
EXTEND-IA	Extending the Time for Thrombolysis in Emergency Neurological Deficits-Intra-Arterial
GBD	Global Burden of Disease
GLP	Good laboratory practice
HERMES	Highly Effective Reperfusion evaluated in Multiple Endovascular Stroke Trials
ICER	Incremental cost-effectiveness ratio
IST-3	Third International Stroke Trial
MERCI	Mechanical Embolus Removal in Cerebral Ischemia
MR CLEAN	Multicenter Randomized Clinical Trial of Endovascular Treatment for Acute Ischemic Stroke in the Netherlands
mRS	Modified Rankin scale
NIHSS	National Institutes of Health Stroke Scale
NINDS	National Institute of Neurological Disorders and Stroke
OHS	Oxford Handicap scores
PWI/DWI	Perfusion-weighted image/diffusion-weighted image
QALYs	Quality-adjusted life-years
REVASCAT	Endovascular Revascularization With Solitaire Device Versus Best Medical Therapy in Anterior Circulation Stroke Within 8 Hours
rt-PA	Tissue plasminogen activator
STAIR	Stroke therapy academic industry roundtable
SWIFT PRIME	Solitaire With the Intention For Thrombectomy as PRIMary Endovascular Treatment
THRACE	THRombectomie des Arteres CErebrales
TIMS-China	Thrombolysis Implementation and Monitor of acute ischemic Stroke in China
VOW	Visions and opportunities workshop

1.1 Introduction

Stroke, acute ischemic stroke, and hemorrhagic stroke are still a critical problem worldwide. In the United States, per recent statistics, ischemic stroke incidence has been maintained at approximately 795,000 victims between 2009 and 2016) [1–4]. Stroke is the fifth leading cause of mortality and leading cause of adult morbidity in the United States [4]. However in China, stroke is more prevalent than in the United States with an incidence rate of 301–517 per 100,000 [5]. The Global Burden of Disease (GBD Stroke) atlas and Demographic and Epidemiologic Drivers paper [6] document that worldwide stroke burden continues to increase, and the increase is negatively threatening and affecting sustainability. The GBD estimated that there were 25.7 million stroke survivors in 2013 [7]; a 2;1 ratio of ischemic/hemorrhagic stroke and that there were estimated 10.3 million first strokes worldwide. Interestingly, there was a significant 50.2% increase in ischemic stroke-related deaths measured between 1990 and 2013 [7]. In recent global stroke incidence statistics, there was an incidence of 10.3–16.9 million strokes annually, with at least 5.9 million stroke-related deaths, and 25–33 million stroke survivors needing a neuroprotective or neuroregenerative therapy.

1.2 Chronological Successes

Even though government sources, private agencies, and industry have committed billions of stroke research dollars [8] over the last 50 years, there have only been two major successes in the treatment of acute ischemic stroke, thrombolysis and endovascular procedures. Since these treatments now form a solid basis for treating stroke patients, the primary clinical data will be reviewed in detail in this chapter.

1.2.1 Thrombolysis

Tissue plasminogen activator (rt-PA; Activase) gained Food and Drug Administration approval in 1996 and is now widely accepted as a pharmacological standard-of-care therapy for ischemic stroke. Activase is effective up to 4.5 h after a stroke [9, 10], but efficacy is somewhat dependent upon the comorbidities of individual stroke patients [11, 12]. rt-PA may be beneficial in up to 50% of patients provided that the drug is the first-line treatment option [12] with or without endovascular procedures. Despite being recognized as a standard-of-care therapy, statistics have suggested that less than 10% of stroke patients are being treated with rt-PA in the United States [13–17].

Table 1.1 Thrombolysis: efficacy analysis

		mRS 90-day outcome						
		No symptoms ----------► death						
Clinical trial	Treatment group	0	1	2	3	4	5	6
NINDS rt-PA (1995) Ref. [12]	Control (312)	26		25		27		21
	Intervention (312)	39		21		23		17
ECASS (2008) Refs [9, 18, 19]	Control (403)	21.8	23.3	16.4	11.4	13.7	5.2	8.2
	Intervention (418)	27.5	24.9	14.1	9.3	9.3	8.1	6.7
EPITHET (2008) Ref [20]	Control [49]	6	18	26	14	24	6	14
	Intervention [51]	12	24	8	12	10	10	25
IST-3 (2012)[a] Ref [21]	Control (1520)	8	13	14	13	9	17	27
	Intervention (1515)	9	15	13	16	8	13	27

mRS modified Rankin scale (%)

[a]*OHS* Oxford Handicap scores at 6 months, mRS/OHS score of 0–2 indicates functional independence

1.2.2 Select rt-PA Clinical Trials

Table 1.1 presents cumulative efficacy data from the original National Institute of Neurological Disorders and Stroke (NINDS) rt-PA clinical trial [12], the European Cooperative Acute Stroke Study (ECASS) trial (i.e., ECASS III) [9, 18, 19], the Echoplanar Imaging Thrombolysis Evaluation Trial (EPITHET) trial [20], and the Third International Stroke Trial (IST-3) [21]. The data directly compares standardized 90-day outcome on either the modified Rankin scale (mRS) or Oxford Handicap scores (OHS).

In the NINDS trial, it was established that intravenous rt-PA significantly improved neurological outcome in approximately 160 patients out of 1,000 treated and that the effect was prominent when given within 3 h of stroke onset. However, after a 6-h delay, rt-PA was without significant efficacy, and neurological outcome was not different from that in the placebo group. There were a few important findings in the NINDS trials; one finding indicated that rt-PA was effective not only for cardioembolic stroke but also potentially useful for athero-embolic and small vessel lacunar stroke. Statistically with respect to efficacy in different stroke populations, in lacunar stroke patients, 62% of patients improved with rt-PA compared to 41.5% with placebo, and 38% of cardioembolic stroke patients improved with rt-PA compared to 29% with placebo. The efficacy of rt-PA in lacunar stroke may have accounted for the fact that infarct volume was not predictive of outcome in the NINDS rt-PA stroke trial. Zunker et al. [22] have now confirmed that there is demonstrable and similar efficacy of rt-PA in cardioembolic, thromboembolic large vessel, and in lacunar stroke it was comparable [22]. In the NINDS trial, the absolute change (increase) varied dependent upon the type of stroke patient treated with thrombolysis.

In the ECASS III trial [9, 18, 19], the safety and efficacy of rt-PA were studied in patients when rt-PA was administered 3–4.5 h following an ischemic stroke. The trial enrolled 418 patients in the rt-PA group and 403 to placebo. rt-PA was effective in various subgroups, including older patients, and the effectiveness was independent of the severity of stroke at baseline. The trial showed that there was a significant shift in the mRS score 0–2 in the rt-PA-treated group (66.5% of patients) compared to 61.5% in the control group, representing an absolute change of 5% (see Table 1.1).

In the EPITHET trial [20], 51 rt-PA-treated patients and 49 placebo-treated patients were included in 90-day mRS endpoint analysis. Patients were enrolled and randomized within 3–6 h of stroke onset. The trial concluded that rt-PA increased reperfusion in patients who had PWI/DWI mismatch, and there was a notable improved clinical outcome (see Table 1.1).

In the IST-3 trial, another extended treatment window trial was mounted and 3035 patients were enrolled within 6 h of a stroke [21]. rt-PA within 6 h did not significantly alter mortality rate within the first 6 months, but rt-PA did increase death within the first week. There was a significant shift in ordinal OHS scores comparing placebo to rt-PA.

As recently described by Wardlaw and colleagues [23], administration of a thrombolytic is most efficacious when provided within 3 h of a stroke (significance level, $p < 0.0001$; six trials, $n = 1{,}779$). Additional eight intravenous rt-PA clinical trials enrolling 6,729 patients also provided data that thrombolysis can promote significant ($p = 0.0006$) improvement in patients when administered up to 6 h following a stroke. More recently, Lees et al., as part of the Stroke Thrombolysis Trialists' Collaborators Group, used a database of 6,756 patients characterized by age 71 ± 13 years, National Institutes of Health Stroke Scale (NIHSS) baseline score of 12 ± 7, and treatment delay 4.0 ± 1.2 h. Odds ratio for improvement when comparing mRS indicated a falloff for efficacy when 0–4 vs 4–6 and 0–5 vs 6 was compared. The report emphasized that there is significant efficacy of rt-PA when used up to 4.5 h after a stroke, but early use is best for greatest improvement.

1.2.3 Thrombolysis Cost-Effectiveness (USA Compared to China)

Between 1990 and 2010, the financial burden of stroke in the United States has escalated from $29 billion to 74 billion [24–26]. Cost analysis from the ECASS III rt-PA trial demonstrated an incremental cost benefit of $6255 per quality-adjusted life-years (QALYs) for victims less than 65 years old and $35,813 per QALY for victims above 65 years old, a benefit that was dependent upon NIHSS admission scores: NIHSS 0–9, the benefit was $16,322 per QALY; NIHSS 10–19 increased to $37,462 per QALY, and in patients with high NIHSS scores ≥20, there was low cost benefit ($2432 per QALY). Cost analysis of rt-PA use within 3–4.5 h after stroke

onset shows incremental benefit in patients with NIHSS scores of 0–19, compared to no treatment [27].

The cost-effectiveness of thrombolysis in China has recently been calculated or estimated by Pan et al. [28], as part of the Thrombolysis Implementation and Monitor of acute ischemic Stroke in China (TIMS-China) study. This study had one severe limitation; the efficacy of rt-PA treatment was based on the pooled analyses from the studies in developed countries (ECASS, ATLANTIS, NINDS, and EPITHET). Not surprisingly, when directly comparing rt-PA treatment in patients to no treatment within 4.5 h, there was a short-term gain of 0.101 QALYs at a cost of CNY 9520 (US$1460), yielding an incremental cost-effectiveness ratio (ICER) of CNY 94,300 (US$14,500) per QALY gained within 2 years. The calculation model also indicated a long-term gain of 0.422 QALYs at a cost of CNY 6530 (US$1000). The ICER was then CNY 15,500 (US$2,380) per QALY gained within 30 years. The study showed that rt-PA treatment is cost-effective if the initial threshold payment of CNY 105,000 (US$16,200) per QALY could be achieved.

Taken together, cost-effectiveness analysis of rt-PA utilization within 3–4.5 h of stroke onset clearly shows incremental benefit in patients with NIHSS scores of 0–19, compared to no treatment [27]. Moreover, this translates into significant QALY benefit for the stroke victim [27].

1.2.4 Endovascular Procedures

The introduction and effective use of new devices from a variety of manufacturers have provided stroke patients with large vessel occlusions a new opportunity to dramatically improve after a stroke [29–34]. The Mechanical Embolus Removal in Cerebral Ischemia (MERCI retriever) is from Concentric Medical Inc., Mountain View, CA, United States, and the Solitaire FR Revascularization Device is from Ev3/Covidien, Paris, France, a retrievable stent.

Herein we review the results from six primary endovascular procedure trials that were conducted worldwide with previously unrealized success for stroke victims:

1. Multicenter Randomized Clinical Trial of Endovascular Treatment for Acute Ischemic Stroke in the Netherlands (MR. CLEAN) [35]
2. Endovascular Treatment for Small Core and Proximal Occlusion Ischemic Stroke (ESCAPE) [30]
3. Endovascular Revascularization With Solitaire Device Versus Best Medical Therapy in Anterior Circulation Stroke Within 8 Hours (REVASCAT) [32]
4. Solitaire With the Intention For Thrombectomy as PRIMary Endovascular Treatment (SWIFT PRIME) [33]
5. Trial and *Extending* the Time for Thrombolysis in Emergency Neurological Deficits-Intra-Arterial (EXTEND-IA) [36].
6. THRombectomie des Arteres CErebrales (THRACE) [37]

Table 1.2 Endovascular procedures – cumulative results

Clinical trial designation		Modified Rankin scale (% per tier) 90-day outcome No symptoms ----------▶ death						
Study	Treatment (# of patients)	0	1	2	3	4	5	6
MR CLEAN [35]	Control (267)	0	6	13	16	30	12	22
	Intervention (233)	3	9	21	18	22	6	21
ESCAPE [30]	Control (150)	7	10	12	15	24	12	19
	Intervention (165)	15	21	18	16	13	7	10
REVASCAT [32]	Control [102]	5.8	6.8	15.5	19.4	16.5	20.4	15.5
	Intervention [102]	6.8	17.5	19.4	18.4	7.8	11.7	18.4
SWIFT PRIME [33]	Control [97]	9	11	16	17	22	26	
	Intervention [97]	17	26	17	12	15	12	
EXTEND-IA [36]	Control [35]	17	11	11	11	17	11	20
	Intervention [35]	26	26	20	17	3	0	9
THRACE [37]	Control (202)	11.9	16.3	13.9	12.4	27.7	4.5	13.4
	Intervention (200)	15.5	19.5	18	12.5	17	5.5	12

Table 1.3 Embolectomy in rt-PA-ineligible patients

Clinical trial designation		Modified Rankin scale (% per tier) 90-day outcome No symptoms ----------▶ death						
Study	Treatment	0	1	2	3	4	5	6
rt-PA eligible	Control (565)	5.1	8.1	13.8	17.5	23.7	13.3	18.4
	Intervention (525)	9.9	17.1	19.4	16.6	17.3	5.9	13.7
rt-PA ineligible	Control [80]	3.6	6.2	12.5	8.7	31.2	15.0	22.5
	Intervention [107]	10.2	15.7	17.6	18.5	7.4	7.4	23.1

In the endovascular procedure and clinical trials, in some percentage of patients, thrombolysis with either rt-PA or urokinase was administered 2 h before the thrombectomy procedure. In the same trials, some percentage of the patients were rt-PA ineligible (see Tables 1.2 and 1.3). The majority of the clinical trials were designed to directly compare rt-PA with rt-PA and endovascular procedure. However, some additional data was gleaned for embolectomy alone in patients ineligible for rt-PA.

In all trials, including the most recently published THRACE trial article, the thrombolytic was administered 85–180 min after enrollment, and endovascular procedures were conducted in the embolectomy arm within 87–345 min. In all trials, when thrombolysis was used, the thrombolytic was administered well within current FDA-approved guidelines. Moreover, in the embolectomy arm, the initiation of "thrombolysis" occurred well before the procedure.

1.2.4.1 Summary of MR CLEAN

The MR CLEAN clinical trial [35] enrolled 500 patients (233 mechanical thrombectomy and thrombolytic/267 either rt-PA maximum dose of 90 mg or 1.2 million IU of urokinase) with a proximal artery occlusion in the anterior cerebral circulation within 6 h after symptom onset. As defined by the enrollment criteria, as a measure of a small ischemic core and large penumbra, median Alberta Stroke Program Early CT Score (ASPECTS) on CT was 9, with an interquartile range of 7–10 in the embolectomy arm and 8–10 in the thrombolytic arm. The patient population was quite diverse with inclusion of standard comorbidities: diabetes mellitus (thrombolytic 12.7%; thrombectomy 14.6%), hypertension (42.1–48.3%), and atrial fibrillation (thrombolytic 28.3%; thrombectomy 25.8%). This comparison study used mechanical treatment in 83.7% of patients: retrievable stents were used in 81.5% of patients, and other devices were used in 2.1% patients assigned to the intra-arterial thrombolytic treatment.

A direct comparison of the two treatment groups indicated that there was no difference in reperfusion percentage between the two groups: 58.7% (TICI score of 2b or 3) vs. 57.5% (mAOL score of 2 or 3), but there was additional benefit and a statistically significant difference in the rate of functional independence (mRS 0–2) in the combined intervention (32.6% vs. 19.1%) group, representing an absolute difference of 13.5%.

1.2.4.2 Summary of ESCAPE

The ESCAPE clinical trial [30] enrolled a total of 316 patients (238 patients received thrombolysis, 120 in the embolectomy [retrievable stents or balloon catheters for suction clot removal] plus IV rt-PA arm, and 118 in the rt-PA control group). Patients were enrolled with a proximal intracranial occlusion in the anterior circulation (ICA and M1 middle cerebral artery (MCA), or M1 or M2 MCA segments) and moderate-to-good collateral circulation. The median ASPECTS on CT was 9 for both groups. Patients were enrolled up to 12 h after symptom onset within a median time of 110 min for the embolectomy arm and 125 min for the thrombolytic arm. The median time from CT head to the first noted reperfusion was 84 min for embolectomy, defined as the first visualization of reflow in the MCA, which in most patients was coincident with the deployment of a retrievable stent. For embolectomy, the median time from symptom onset to groin puncture was 51 min in the intervention group, and rt-PA was initiated within 110 min. In the control group, IV rt-PA was administered within 125 min from stroke onset. The median time of stroke onset to the first reperfusion was 241 min in the embolectomy arm. Both groups included 52.1–52.7% of females, and the majority of enrolled patients had hypertension (63.6–72%) or were diabetic (20–26%).

In the embolectomy/thrombolytic group, 53% of patients achieved mRS of 0–2 (90 days) compared to only 29.3% in the rt-PA group demonstrating additional benefit of combination therapy. This benefit was associated with more patients

achieving reperfusion in the embolectomy/rt-PA group 72.4% compared to 31.2% in the rt-PA group. There was an equal response of males and females in this study [common odds ratio (95% Cl) of 2.5 (1.4–4.5 male) to 2.6 (1.5–4.4 female) favoring embolectomy/rt-PA].

1.2.4.3 Summary of REVASCAT

The REVASCAT trial [32] enrolled 206 patients (103 embolectomy/rt-PA and 103 rt-PA) with a proximal anterior circulation occlusion within 8 h of stroke symptom onset. The median time for groin puncture was 269 min for the embolectomy arm (Solitaire stent retriever) plus 117.5 min for rt-PA compared to 105 min for the rt-PA arm. The trial was designed to include patients with a small ischemic core and large penumbra [median ASPECTS on CT was 7 in the embolectomy arm; 8 in the rt-PA arm]. The study enrolled in the thrombectomy/rt-PA and rt-PA groups, respectively, 53.4/52.4% male participant, including 21.4/18.4% diabetic patients, 60.2/69.9% with hypertension, and 34.0/35.9% with atrial fibrillation.

The rates of revascularization in the embolectomy/rt-PA group were reported to be 66; the reperfusion rate was lower when compared to other recent embolectomy trials. Nevertheless, as a measure of functional independence (mRS 0–2) at 90 days, 43.7% of patients in the thrombectomy/rt-PA arm and 28.1% in the rt-PA were clinically normal. Adjusted odds ratio for one-point improvement of 1.7 (1.05–2.8) was in favor of embolectomy/rt-PA.

Recently, Davalos et al. [38] reported on 1-year clinical follow-up of patients originally enrolled in the REVASCAT trial. Let's recall that at 3 months, 43.7% of patients were normal. The result at 12 months was the same at 3 months: 44% had improved functional independence, and quality of life was superior in the thrombectomy group.

1.2.4.4 Summary of SWIFT PRIME

The SWIFT PRIME trial [33] enrolled 196 patients (98 patients in the embolectomy [Solitaire revascularization device] plus IV rt-PA arm/98 patients in the IV rt-PA arm). Patients were enrolled with a proximal anterior intracranial circulation occlusion in the absence of large ischemic core lesions and were randomized within 6 h after stroke symptom onset. The median ASPECTS on CT was 9, with interquartile range of 7–10 in the embolectomy/rt-PA arm and 8–10 in the rt-PA arm. In the embolectomy group, the median time from stroke onset to groin puncture was 224 min. In SWIFT PRIME, the majority of patients had one or more comorbidities: hypertension (embolectomy/rt-PA 67%/ rt-PA 58%), diabetes (embolectomy/rt-PA 12%/ rt-PA 15%), and atrial fibrillation (embolectomy/rt-PA 36%/ rt-PA 39%). Using mRS as a measure, the study demonstrated a significant improvement in functional independence (range 0–2) at 90 days and greatly enhanced reperfusion in patients in the embolectomy/rt-PA treatment group (83% at 27 h) and 40% (at 27 h)

in the rt-PA group. Sixty percent of patients treated with embolectomy/rt-PA had a mRS score of 0–2 compared with standard rt-PA therapy (35%).

1.2.4.5 Summary of EXTEND-IA

The EXTEND-IA clinical trial [36] enrolled 70 patients (35 embolectomy[Solitaire FR (flow restoration) stent retriever]/rt-PA/35 rt-PA), with patients receiving rt-PA within 4.5 h of stroke symptom onset. Embolectomy was initiated within a median time of 248 min from stroke onset to modified TICI2b or 3 or completion of the procedure (interquartile range 204–277) for endovascular therapy in combination with rt-PA (within 127 min median) compared to a median time of 145 min for rt-PA. The median time from stroke to groin puncture was 210 min (166–251 min) and 74 min from initiation of rt-PA to groin puncture.

Enrollment criteria included an occlusion of the internal carotid or MCA and evidence of salvageable brain tissue and ischemic core of less than 70 ml on CT perfusion imaging. Embolectomy was initiated within 6 h of stroke onset and completed within 8 h of onset. Significant clinical efficacy was documented after enrolling 35 patients in each of the two treatment groups. There were equal numbers of male and female patients enrolled in the trial (49% male/51% female in both groups), and the stroke population had a history of comorbidities: hypertension (thrombectomy/rt-PA 60%; rt-PA 66%), diabetes (thrombectomy/rt-PA 6%; rt-PA 23%), and atrial fibrillation (thrombectomy/rt-PA 34%; rt-PA 31%).

The EXTEND-IA trial was stopped at interim analysis because of a significant benefit in the endovascular/rt-PA therapy arm and release of the positive MR CLEAN trial. With embolectomy, 94% of patients achieved recanalization at 24 h, 89% had >90% reperfusion at 24 h, and 71% of patients achieved mRS of 0–2 at 90 days compared to 40% in rt-PA arm. In rt-PA patients, 34% of patients had extensive reperfusion at 24 h and a lower recanalization rate at the same time point (43% vs. 94% in the endovascular group).

1.2.4.6 Summary of THRACE

The THRombectomie des Arteres CErebrales (THRACE) clinical trial [37] enrolled 204 patients to the embolectomy/rt-PA group and 208 patients to the rt-PA group. For enrollment in THRACE, patients with proximal artery occlusions (internal carotid artery, M1 MCA, or superior third of the basilar artery) were randomized within 4 h of stroke for rt-PA and 5 h for thrombectomy/rt-PA and had initial NIHSS scores of 10–25. In this trial, the rt-PA group had 50% male/50% female while the rt-PA/embolectomy group had 57% male/43% female. The population has common comorbidities: hypertension (47% thrombectomy/rt-PA/57% rt-PA), type 2 diabetes (8% thrombectomy/rt-PA/17% rt-PA), and hypercholesterolemia (45% thrombectomy/rt-PA/58% rt-PA).

Median time for IV rt-PA was 150 min in the combination group and 153 min with rt-PA, and embolectomy was initiated within 250 min. In the thrombectomy/rt-PA group, at 3 months, 53% of patients had mRS 0–2 compared to 42% in the rt-PA group, and in a subgroup of thrombectomy/rt-PA patients, 69% had good reperfusion (mTICI grade 2b or 3).

1.2.4.7 Cumulative Summary

Key criteria have been identified for embolectomy or thrombectomy to be a method to achieve significant clinical improvement in thrombolysed patients. First, patients with large vessel occlusions must have sufficient salvageable brain tissue (i.e., large penumbra) with small infarct areas and have ASPECTS score between 7 and 10. However, meta-analysis published by the Highly Effective Reperfusion evaluated in Multiple Endovascular Stroke Trials (HERMES) collaboration [39] suggested that optimal reperfusion outcome is achieved when ASPECTS was 6–8 or 9–10 indicating a significant amount of penumbra present. The best outcomes were also when the embolus was located in either the internal carotid artery (ICA) or M1 MCA. The timing of embolectomy is not that critical as long as intervention was initiated ≤5 h according to recent studies. Interestingly, there were no significant gender differences, both males and females responded to the treatment. However, there may be age-dependent improvement that needs further clarification. Certain studies demonstrated benefit in patients 50–80 years of age, but less benefit between 18–49 years of age.

Retrospective analysis of the embolectomy trial database indicates that embolectomy without adjuvant thrombolysis in patients ineligible for rt-PA is beneficial [34] based upon mRS scores at 90 days (Table 1.3). Significant clinical benefit was observed in patients with ASPECTS scores of 8–9 [34] indicative of large penumbral areas as a physical "substrate" for therapy. In rt-PA-ineligible patients, 43.5% of patients were mRS 0–2 in the embolectomy group compared to 22.3% in the control arm.

1.2.4.8 Patient Selection and Treatment

For all trials, patients were included in the embolectomy trial if the infarct core was small and if there was brain tissue to salvage. The six trials cited above lacked standardized measurement or visualization of stroke regions and "penumbra." In the REVASCAT trial, computed tomography (CT) was utilized with ASPECTS, and patients with a score <7 were excluded. In the EXTEND trial, ASPECTS was also used and patients with a score of 6–10 were included. In the EXTEND-IA trial, CT angiography was used, and automated (RAPID) CT perfusion imaging was used to identify salvageable brain tissue. The MR CLEAN trial used CT, angiography (CTA), MRI, or digital subtraction angiography (DSA) for patient enrollment. In the stroke patient population in the United States, it is estimated that 30–35% of

patients have large vessel occlusions [12, 40, 41], but the average ASPECTS score for the patient population is not known. This is a potential strict limitation of the use of thrombolysis/embolectomy.

1.2.5 Cost-Effectiveness

Powers et al. [42], in the recent AHA/ASA guidelines, state that patients eligible for IV rt-PA should receive the thrombolytic whether or not endovascular procedures can be performed [42]. As reviewed in Sect. 2.3, thrombolysis is cost-effective for the long-term health of the patient.

The cost-effectiveness of endovascular procedures, most in combination with thrombolysis, has been reviewed in a series of recent articles [43–45]. The calculated incremental cost-effectiveness ratio (ICER) is $11,990 per QALYs for thrombectomy plus IV thrombolytic [43]. Using a Markov model, Aronsson and colleagues [44] also indicated that thrombectomy with thrombolysis increased QALY by 0.99 years with a cost saving of $221 per patient. The third cost-analysis study found that endovascular procedures over IV rt-PA alone were more than $163,000 amounting to more than $8 billion for every 50,000 patients treated [45]. Lobotesis et al. [46] suggested that after the initial commitment, the high cost of endovascular procedures with thrombolysis can somewhat be offset by enhanced quality of life and health status, and this could represent $103,530 USD benefit per patient. Another article, using the Markov model and directly comparing the cost-effectiveness of thrombectomy to IV thrombolysis, showed that thrombectomy was much more expensive than thrombolysis ($2,520 increase), but thrombectomy was associated with a cost-effective ratio of $11,990 per QALY gained by the patient since it significantly improved independence [47, 48].

1.3 Future Stroke Therapy Development

Six positive embolectomy trials have now produced reproducible clinical efficacy results. As intimated previously in the chapter, one existing problem with the endovascular/thrombolytic approach is that a small percentage of the stroke population will be eligible for the therapy, because of the current LVO and ASPECTS requirements, and then only an overall smaller percentage will be clinically normal at outcome. As discussed by numerous investigators in the literature over the last 2 years, the future of stroke treatment will utilize a multidisciplinary approach to provide the best care to patients [49–53]. Because the ischemic cascade is complex [8, 54–57], and there is differential death of cellular populations in the brain that is temporally regulated, there is ample opportunity to develop neuroprotective or cytoprotective compounds to use as adjuvant therapy with endovascular procedures and rt-PA. The

standardization of research and research practices to accomplish this goal is briefly discussed in the following section.

1.3.1 Translational Research to Complement Existing Clinical Practice

Due to severe funding limitations for extensive exploratory translational research, scientists now have the conundrum of how to best develop a new therapeutic strategy. Strategic choices must be made regarding the most useful potential targets within the ischemic cascade, and the neurovascular unit, the best animal model to parallel embolic stroke in patients, and the methodology used to assess and advance a new therapy. While it is becoming clear that high-quality translational studies must incorporate current standard-of-practice guidelines, the guidelines need to be well defined and accessible to the stroke research community. We have recently started documenting guidelines in a series of articles in *Translational Stroke Research*, the preeminent journal for the advancement of stroke therapies, models, and ideas [8, 58–61]. The most recent article, in the form of a letter, attempted to comprehensively review the most important stroke research guidelines. For the benefit of readers of this translational research volume, and since the focus is research, the research guidelines will be briefly reviewed herein: they include STAIR [62], RIGOR [63–65], ARRIVE [66–73], and VOW [74, 75] guidelines (see Table 1.4). Readers are encouraged to review the primary original publications for all guidelines [62–75] and a summary by Marbacher with some useful website links [76].

As shown in Table 1.4, multiple aspects of therapy development are emphasized, and they have been tiered into three groups, with some important features being duplicated in two tiers:

1. *Basic science principles*: The first tier of guidelines relates to basic science practices that should be applied to all scientific research conduct independent of the disease target population. These are heavily based upon the RIGOR guidelines of 2012, which called for complete transparency of research practices [61, 63, 65]. Crucial to the idea of transparency are inclusion of conflict of interest statements and identification of funding source (s), if any, since these often lead to investigator bias. Additionally, RIGOR has now been identified by a series of requirements including (1) power analysis for all major endpoints, (2) extensive blinding of studies, (3) concealed randomization of all treatment groups to be tested prior to animal enrollment in the study, and (4) application of appropriate statistical analysis for the endpoints being measured. Investigators should apply good laboratory practice principles to all studies and archive data in a form for scientific audit [58, 59, 78].
2. *Data management*: Data management has been discussed in detail by Lapchak and Zhang [59]. As shown in the table, data management includes archiving of all data components, and the main purpose is to have the data available for

Table 1.4 Guideline summary (Modified from Lapchak and Zhang [58])

	Guideline	STAIR [62, 77]	ARRIVE [66–73]	RIGOR [61, 63, 65]	VOW [74, 75]
Tier 1: Basic science principles					
1	Allocation concealment	X		X	
2	Blinding (all aspects of study)	X		X	
3	Conflict of interest statement	X		X	
4	Data audits				X
5	Data publication (−ve/+ve/neutral)			X	X
6	Funding source (conflict)			X	
7	Good laboratory practice			X	X
8	Inclusion/exclusion criteria	X		X	
9	Power analysis (sample size)	X		X	X
10	Randomization	X		X	X
11	Statistical analysis method			X	X
Tier 2: Data management					
1	Archived data				X[1]
2	Data audits				X
3	Data publication (−ve/+ve/neutral)			X	X
Tier 3: Stroke drug development priorities					
1	Acute outcome	X			
2	Animal (strain and source)		X		
3	Assay/model rationale/validation			X	
4	Combination studies (i.e., rt-PA/ urokinase/streptokinase)	X[2]	X[2]	X[2]	X[2]
5	Comorbidity: aging	X			X
6	Comorbidity: diabetes	X			X
7	Comorbidity: hypertension	X			X
8	Dose-response curve	X	X		
9	Ethical/humane		X		
10	Gender analysis	X			X
11	Good laboratory practice			X	X
12	Long-term outcome	X			X
13	Multiple laboratories	X			X
14	Multiple species	X	X		
15	Physiological monitoring	X	X		
16	Reproducibility			X	X
17	Route of exposure (oral, sc, iv)	X			
18	Optimal species	X[2]	X[2]	X[2]	X[2]
19	Therapeutic window	X			X
20	Toxicity	X			

X[1]: Since data audits were introduced, data must be archived
X[2]: See Sect. 4 for a discussion of embolic stroke models and species

Table 1.5 Comparison of embolic stroke models for drug development

Measure	Rabbit embolic stroke	Rat embolic stroke
Analgesic interference	No	Yes
Anesthesia interference	No	Yes
Cognitive measure	No	Yes
Combination therapy analysis(rt-PA + add-on)	Yes	Yes
Heterogeneous population	Yes	No
Long-term behavior endpoint	Yes (21 days)	Yes (months)
Motor function measure	Yes	Yes
Positive control (rt-PA)	Yes	Yes
Short-term behavior endpoint	Yes	Yes

scientific audit and importantly publication. While most journals do not currently require raw data, or a great deal of information regarding the circumstances under which data was accumulated, in the very near future, there will be an emphasis on adherence to GLP and complete transparency so that worldwide research is reproducible.

3. *Stroke drug development priorities*: Extensive discussion of this topic is well beyond this chapter and will be covered in some detail by other invited chapter for this translational research volume. However, a few comments are required with respect to the direction of stroke research in this new era. With the emergence of endovascular procedures [53, 79] as a clinically efficacious and viable therapy for stroke patients, translational stroke research should be focused on drug and therapy development in embolic stroke models. This idea is not new and has been proposed in the literature previously [80–84].

Embolism by a blood clot initiates a sequence of ischemic events that cannot be fully recapitulated by any other methods of ischemia induction [80, 85]. Independent of the type or location of stroke, the "clot" is responsible for the interruption of cerebral blood flow, thereby severely altering tissue metabolism, in part by depleting cellular energy stores in the immediate core [86–89]. Reduced blood flow and severe oxygen deficiency lead to an ischemic brain area comprising a central core of severely ischemic tissue that is irreversible, surrounded by a tissue zone consisting of moderately ischemic tissue with preserved cellular metabolism and viability. The insult also triggers a cascade of excitotoxicity, free radical formation, blood-brain barrier injury, and extended inflammatory processes. In acute ischemic stroke, there is a region of salvageable tissue commonly referred to as the "penumbra" [90, 91]. Interestingly, both treatments currently used in stroke patients enhance reperfusion and may be most effective when there is a large penumbral area, an apparent substrate for an effect. Thus, perhaps it is time to recognize that stroke research commonly done in common animal models primarily using intraluminal suture occlusion may not represent the best model or approach to treat a human disease process initiated by a blood clot. There are two validated embolic alternatives to consider for stroke drug therapy, and these should be used in parallel for drug efficacy screening (see Table 1.5).

1.3.2 Option 1: Rabbit Embolic Stroke Model [81, 82, 92–95]

This model has the benefit that it was the model used for validation of efficacy of rt-PA that is now FDA approved. The model builds upon a statistical analysis technique to directly assess drug efficacy compared to a vehicle control, with a positive control group, rt-PA, included in studies. Briefly, the benefits of therapy testing in the RSCEM bioassay are outlined in Sects. 3.2.1, 3.2.2, 3.2.3, 3.2.4, 3.2.5, and 3.2.6.

1.3.2.1 No Anesthetic or Analgesic Interference [81]

Rabbits are allowed to recover from the effects of inhaled anesthesia (i.e., either halothane or isoflurane) before embolization. Thus, the absence of anesthesia during the embolization procedure avoids potential anesthetic interference with the ischemic cascade [96–104], interactions that can confound data and drug efficacy interpretation. Analgesics are also not administered because of the potential to interfere with the ischemic stroke cascade [97].

1.3.2.2 Use of a Heterogeneous Population Endpoint

Clinical trial data derived from randomized trials such as the original NINDS rt-PA trial [12] included patients with NIHSS scores of 1–32 and 1–37 [12] in the placebo and active drug groups, respectively. This clinical trial patient population encompassed a widespread heterogeneity within each experimental study group. The RSCEM model is based upon embolization with a suspension of non-autologous blood clots [97, 105, 106]. Because the immediate behavioral response shows heterogeneity, the stroke population is similar to that enrolled in clinical trials.

1.3.2.3 Clinically Relevant Endpoint

The use of clinical rating scores or a clinically relevant endpoint is an advantageous primary endpoint to use when developing a novel therapeutic [81, 97]]. For RSCEM analysis, a statistical method was adopted [107–109] to take advantage of the heterogeneous population being studied. While secondary endpoint is utilized, the primary endpoint used when assessing treatment efficacy in the RSCEM is behavioral functional, which is based upon motor function components of the NIHSS [110].

1.3.2.4 Positive Controlled Studies

For drug efficacy testing, it is essential to utilize an embolic stroke model where a current FDA-approved therapy rt-PA can be used as a positive control to ensure the validity of model and data [12]. Translational research studies done without the use of a positive control have limited drug development value.

1.3.2.5 Combination Drug Testing for Efficacy and Safety

The RSCEM has the benefit of being used to study new therapies in combination with rt-PA either coincident with rt-PA or following rt-PA administration to simulate current clinical trial design. The combination studies can be used to determine if there are interactions with rt-PA to either inhibit or enhance rt-PA efficacy and whether the interactions are safe [111–119] with respect to hemorrhage incidence, morbidity, and mortality [120, 121].

1.3.2.6 Cost-Effective Analysis

Because clinical trial development of a new stroke therapy is associated with costs in the hundreds of million dollars and significant ethical concerns that patients will not be negatively impacted or harmed by therapy administration [8], it is important that extensive preclinical efficacy and safety analysis be conducted. The RSCEM model has been established as the model for advancing drugs to a clinical trial [81, 97].

1.3.3 Option 2: Rodent Embolic Stroke Model [122, 123]

The rodent embolic stroke model that is not commonly or extensively used for stroke research does has the benefit of both short- and long-term behavioral analysis using a variety of tasks such as the cylinder test, Barnes maze, Morris water maze, and rotameter [124–127]. The model can contribute significant data for testing of new therapeutic strategies to attenuate cognitive deficits following ischemia. Moreover, because of the small size of the brain, infarct analysis using TTC for early analysis or H&E for delayed analysis is easily accomplished [128, 129]. Unfortunately, the concerns of extensive spontaneous recovery in the rat model, the need to exclude up to 50% of the population for technical difficulties, absence of stroke response, high response variability, and poor reproducibility between strains can complicate drug testing and analysis (see [130–147]). Nevertheless, an established and validated embolic model in rats should be used as an initial screen for drug development.

1.4 Conclusions

Reperfusion therapies now effectively treat clinical deficits in a small percentage of acute ischemic stroke patients. Rational drug discovery efforts should now quickly move forward and build upon this exciting progress in the stroke field and use established and validated rabbit and rodent embolic stroke models to develop adjuvant therapies to be used in combination with reperfusion therapies. All preclinical and translational stroke studies should incorporate the STAIR, RIGOR, ARRIVE, and VOW guidelines, especially those related to GLP, blinding, and randomization.

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Chapter 2
Recent Success with Endovascular Stroke Therapy

Konark Malhotra and David S. Liebeskind

Abstract Stroke is the fifth leading cause of disability and mortality, with ischemic stroke as the major contributor of the total number of cases. The clinical outcome of ischemic stroke primarily depends on timely reperfusion of the ischemic territory. Intravenous thrombolysis has been the mainstay medical therapy for decades, though it may have limited efficacy in patients with more extensive clot burden in proximal arterial occlusion. Various endovascular trials in recent past failed to demonstrate the clinical benefit over medical therapy alone. Five randomized controlled trials published in 2015 demonstrated superior efficacy and improved clinical outcomes of endovascular reperfusion over medical therapy alone. Recent success of endovascular therapy has established a new standard of care for select ischemic stroke patients.

Keywords Stroke • Endovascular • Mechanical thrombectomy • Neuroimaging • Vessel occlusion • Thrombolysis

Abbreviations

ASPECTS	Alberta stroke program early computed tomography score
EMS	Emergency management of stroke
FR	Flow restoration
IA	Intra-arterial
IV	Intravenous
LVO	Large-vessel occlusion

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MELT	Middle cerebral artery embolism local fibrinolytic intervention trial
MERCI	Mechanical embolus removal in cerebral ischemia
MRI	Magnetic resonance imaging
MT	Mechanical thrombectomy
NCCT	Non-contrast computed tomography
PROACT	Prolyse in Acute Cerebral Thromboembolism
sICH	Symptomatic intracranial hemorrhage
TICI	Thrombolysis in cerebral infarction
TIMI	Thrombolysis in myocardial infarction

2.1 Introduction

Acute ischemic stroke (AIS) has high recurrence rates and may result in significant morbidity and mortality [1, 2]. It involves disruption of blood flow from arterial occlusion and accounts for 85–87% of all stroke cases [3]. Large-vessel occlusion (LVO) accounts for 40–45% of AIS cases and is associated with poor clinical outcomes [4]. The number of LVO cases seems to be increasing and may continue to increase in the future due to the increased prevalence of atrial fibrillation and cervical carotid artery disease in the older population.

Until recently, intravenous (IV) thrombolysis was the preferred medical therapy for AIS patients. Poor revascularization and reperfusion rates, however, were noted in proximal LVO cases and resulted in worse clinical outcomes. Endovascular procedures in this selective cohort demonstrated better clinical outcomes yet failed to demonstrate its superiority to medical therapy in earlier clinical trials [5–7]. After a streak of multiple neutral trials performed until 2013, five randomized controlled trials were conducted that involved meticulous selection of patients, advanced neuroimaging, and endovascular procedural techniques, and demonstrated the superiority of endovascular procedures over medical therapy alone. In this chapter, we summarize the evolution of endovascular trials, critical aspects that culminated in the success of recent mechanical thrombectomy (MT) trials, and its implication for future stroke therapy.

2.2 Evolution of Endovascular Techniques

The evolution of endovascular techniques in cerebrovascular disease began in the early 1980s. Intra-arterial (IA) thrombolytic drugs were introduced to assess and reproduce similar benefits as were observed with cardiovascular interventions [8, 9]. Investigators also compared IA thrombolytics with IV administration of antiplatelet and anticoagulant drugs, and demonstrated improved survival rates with the former techniques [10]. This further led to the conduct of larger randomized controlled trials to investigate the efficacy of IA endovascular techniques.

Various IA thrombolytic agents were investigated in the initial endovascular trials. Patients were randomized to receive IA thrombolytics including recombinant prourokinase (r-proUK) in Prolyse in Acute Cerebral Thromboembolism (PROACT) trial, r-proUK + heparin vs. heparin in PROACT-II trial, and urokinase in Middle Cerebral Artery Embolism Local Fibrinolytic Intervention Trial (MELT). Emergency management of stroke (EMS) bridging trial was a small pilot trial that compared IV + IA alteplase with placebo + IA cohorts. The trial showed better recanalization rates with combined IV+IA group; however, no difference was observed in the rates of clinical outcome among the cohorts. EMS bridging trial sparked the conductance of interventional management of stroke (IMS) I trial, a major trial designed to assess the futility of endovascular procedures, which compared the efficacy of IV+IA therapy and the control group from National Institute of Neurological Disorders and Stroke (NINDS) study. The results showed a significant difference in clinical outcomes at 3 months in favor of combined IV+IA therapy. These trials established the platform to orchestrate large randomized controlled trials comparing endovascular therapy with IV alteplase alone.

2.3 Endovascular Trials: Path to Success

2.3.1 First-Generation Neutral Trials

Three major trials were published in 2013 that compared the efficacy of endovascular procedures with standard medical therapy [5–7]. These trials assessed the clinical outcome of AIS patients using modified Rankin scale (mRS), with mRS 0–2 correlating to functional independence at 3 months. All the trials concluded with a lack of significant difference for endovascular therapy on comparison to standard treatment (including IV tPA). However, after major scrutiny, several faults in the methodology and conductance were observed with these trials. Few major factors that were involved in negative results with these trials were poor patient selection, lack of LVO confirmation using vessel imaging, enrollment in trials over many years, and predominant utilization of first-generation MT devices.

Although these clinical trials faced heavy criticism related to such methodology, pivotal conclusions were drawn in post hoc analyses. The efficacy of IV tPA was directly related to the thrombus burden and the site of vessel occlusion, and reduced recanalization rates were observed with proximal large-vessel occlusions [11, 12]. There was no increased risk of hemorrhagic conversion observed with concurrent treatment of IV tPA followed by endovascular therapy. Time to revascularization was determined to be a critical factor for improved clinical outcomes at 3 months [13]. Lastly, on comparison to first-generation MT devices, Trevo and Solitaire flow restoration (FR) devices demonstrated superiority with the ease of procedural techniques and rates of clinical outcomes [14, 15].

2.3.2 Second-Generation Landmark Trials

Five randomized controlled trials were conducted in AIS patients with proximal LVO in anterior circulation and compared the efficacy of endovascular procedures with medical therapy alone [16–20]. All the trials aimed to overcome the limitations of their predecessor endovascular trials to evaluate for a superior therapeutic option in AIS patients. These trials incorporated multimodal imaging to assess infarct core and vessel imaging for LVO, utilized second-generation stent-retriever devices, and introduced parallel workforce model for minimal time to reperfusion. The positive results of these landmark trials resulted in early termination of few other trials after their successful completion of interim analysis [21, 22].

Various meta-analyses for endovascular trials, including three predecessor neutral trials and five recent positive trials, have been performed [23]. The authors demonstrated improved clinical outcomes with endovascular therapy for patients with angiographic LVO confirmation and the use of state-of-the-art stent retrieval devices. Although no significant difference was observed for symptomatic intracranial hemorrhage (sICH) and mortality at 3 months, a significant improvement was noted in functional independence (90-day mRS of 0–2). Successive completion and rapid publication of second-generation endovascular trials have established the efficacy of MT as compared to medical therapy alone. These randomized controlled trials have revolutionized the current stroke management, with MT now being considered as a standard of care for all eligible AIS patients presenting with severe deficits.

2.4 Factors Involved in Success of Endovascular Trials

Prior to the commencement of second-generation endovascular trials, the design of first-generation trials was scrutinized to assess the pitfalls in their methodology. Apart from a multitude of other factors, the major factors that resulted in the success of recent endovascular trials are discussed in this section.

2.4.1 Patient Selection Based on Neuroimaging

Initial neuroimaging performed in the management of AIS patients has progressively evolved over the last few decades. The first-generation endovascular trials used non-contrast computed tomography (NCCT) or magnetic resonance imaging (MRI) to assess ischemic or hemorrhagic changes. However, recent trials incorporated multimodal imaging that includes vascular imaging for vessel patency and perfusion studies providing a snapshot of blood flow abnormalities in AIS patients. Few trials have also utilized innovative imaging techniques such as multiphasic CT angiography (CTA) to definitively assess collateral status.

Imaging modalities have been pivotal in the selection of stroke patients for recent endovascular trials [24]. Baseline neuroimaging involving NCCT or MRI has been replaced by more comprehensive and multimodal imaging techniques. Recent endovascular trials utilized the Alberta stroke program early computed tomography score (ASPECTS) to assess early ischemic changes on baseline NCCT. Various studies have demonstrated ASPECTS ≥7 associated with better clinical outcomes as compared to lower scores [25]. To include patients based on early ischemic injury, ESCAPE [16] and SWIFTPRIME [20] trialists used ASPECTS ≥6, while REVASCAT [19] investigators preferred ASPECTS ≥7 on NCCT and ASPECTS ≥6 on DWI-MRI sequence.

The assessment of vessel patency remains the most crucial data prior to the consideration of MT procedure. However, majority of first-generation endovascular trials did not perform vessel imaging to confirm the presence of a proximal LVO in their patients. There could very likely be absence of a proximal LVO in patients who got randomized into endovascular group of first-generation trials. Similarly, many patients with undetected proximal LVO could have been randomized into medical therapy group and demonstrated poor functional independence rates. Various trials have investigated the response of IV tPA for proximal LVO cases and demonstrated poor response rates [26]. As expected, better recanalization rate has been shown to occur with MT approach for proximal LVO cases [27, 28]. This major limitation was overcome in all the recent endovascular trials with an inclusion criterion of vascular imaging involving either CTA or MR angiography.

The recent studies also utilized advanced multimodal imaging to assess the perfusion and collateral status. Collaterals play a crucial role in procedural and clinical outcome of AIS patients [29]. Good baseline collateral supply to the ischemic territory increases reperfusion, reduces infarct core, and improves clinical outcome [30]. To assess the collateral status, ESCAPE trialists utilized multiphase CTA to capture snapshot images of vascular supply during arterial, venous, and delayed venous phases [16]. However, the assessment of perfusion status, which differentiates salvageable tissue from infarct core, remains paramount. Few studies have used "target mismatch profile" and demonstrated improved clinical outcomes in AIS patients selected beyond 3 h for endovascular techniques [31–33]. EXTEND-IA and SWIFTPRIME investigators used RAPID software [34] to assess the size of ischemic territory and excluded patients with large infarct core size. The second-generation trials concluded that patients with poor collateral status and large infarct core have poor outcome, irrespective of complete revascularization.

2.4.2 State-of-the-Art Mechanical Thrombectomy Devices

Various options for endovascular therapy have emerged over the last decade including intra-arterial fibrinolysis, mechanical aspiration, mechanical clot retrieval, and angioplasty. Mechanical clot removal devices have shown promise in the past and were cleared by FDA for recanalization in AIS patients. These devices include

Mechanical Embolus Removal in Cerebral Ischemia (MERCI) clot retrieval system, mechanical clot aspiration with the Penumbra System, and stent retrieval FR devices including Trevo and Solitaire. Due to the technical flaws with old mechanical clot removal devices, stent retrievers were introduced and revolutionized the modern era of MT in AIS patients, mostly recently culminating with two stent retrieval devices approved by the FDA to improve the clinical outcome of patients with AIS.

Stent retrievers aka stentrievers are the state-of-the-art mechanical clot removal devices with an excellent safety profile. The technical advantages with stentrievers include (a) multiple-point engagement of thrombus with the stent rendering mechanical stability and (b) concurrent entrapment of thrombus within the struts during the expansion of stent allowing instant restoration of blood flow [14, 15, 35]. These advantages were assessed and compared with previous MERCI retriever device in two randomized controlled trials, Solitaire With Intention For Thrombectomy (SWIFT) and Trevo versus MERCI retrievers for thrombectomy revascularization of large-vessel occlusions in acute ischemic stroke (TREVO-2) [14, 15]. Patients enrolled in the stentriever group demonstrated >80% recanalization rates and better functional independence at 3 months. Additionally, MERCI devices were associated with higher complication rates including vessel perforation and intracerebral hemorrhage as compared to stentrievers.

2.4.3 Quality Metrics for Revascularization

Various scoring systems have emerged for the assessment of revascularization and reperfusion in endovascular procedures. Thrombolysis in cerebral infarction (TICI) was initially introduced as a modification to its cardiac counterpart of thrombolysis in myocardial infarction (TIMI), to assess intracranial angiographic scores for revascularization [36]. TICI grading system, ranging from 0 (no reperfusion) to 3 (full reperfusion), has been validated to assess the revascularization status during endovascular procedures, with a score of TICI ≥2b or 3 associated with good reperfusion [37]. The majority of the prior endovascular trials used TIMI grading system, except TREVO-EU [38], TREVO 2 [15], IMS III [5], and MR RESCUE [6] that utilized TICI scoring system to assess the revascularization status. However, all the second-generation landmark trials incorporated TICI or modified TICI (mTICI) grading system. The second-generation endovascular trials utilized TICI ≥2b or 3 as a desired goal and observed a mean rate of 69% in endovascular as compared to 34% in the control groups. This concluded that mechanical clot retrieval performed with stentrievers results in successful reperfusion in twice the number of patients as compared to medical therapy alone.

2.4.4 Speed of Revascularization

The concept of "time is brain" is well versed in the stroke community. However, recent modification of the age-old concept with the "tissue is brain" has bolstered the success of second-generation endovascular trials. Revascularization and reperfusion are independent factors for functional clinical outcomes in AIS patient [39], with early and faster reperfusion further associated with lower disability rates. Rapid evaluation of AIS patients, focus to procure selective neuroimaging sequences, and speedy administration of IV tPA while the patient is in a scanner suite were few of the critical factors that expedited the reperfusion process in second-generation endovascular trials. Detection of critical stenosis or LVO on vessel imaging further leads to the activation of neurointerventional team for revascularization procedures.

Time to reperfusion remains a critical factor during endovascular procedures as it is directly correlated to the rates of clinical outcome. Authors of the IMS III trial studied this major time metric and observed their mean time to reperfusion as 325 min [5]. This correlated to 41.1% good outcome for their respective cohort treated within 300 min, while only 26.5% had good outcome when the duration of endovascular procedure exceeded 360 min [13]. Significant workflow delays involved with the first-generation endovascular trials likely cumulated to the poor clinical outcomes. However, critical observation and meticulous correction of these workflow delays bolstered the parallel workforce model in recent endovascular trials.

The second-generation endovascular trials emphasized on speedy evaluation for rapid reperfusion, both in the emergency department and the neurointerventional suite. ESCAPE [16] and SWIFTPRIME [20] trials established a goal for imaging to groin puncture time of 60 min and 70 min, respectively, while EXTEND-IA [18] proposed groin puncture to occur within 6 h of symptom onset. Trials that emphasized on rapid parallel workflow model [16, 18, 20] demonstrated highest rates of reperfusion, as compared to trials that overlooked this precarious aspect [17, 19]. The trialists also emphasized over the total procedural time and strived to reduce the total time from groin puncture to the clot removal or final pass with the FR devices. Parallel and target-oriented workforce with rapid revascularization remains critical toward the success of these landmark trials.

2.5 Future Trends

Recent success of second-generation trials has established endovascular procedures as the standard of care for proximal LVO in anterior circulation ischemic stroke. Significant improvement in clinical outcomes led to the emergence of endovascular therapy as the superior option for eligible patient population. However, implementation of MT as standard of care in routine clinic practice would require multidisciplinary effort and a parallel workflow model.

Health-care system in the future would need further optimization to deliver rapid endovascular therapy for AIS patients. Paramedical staff and primary stroke centers stand at the forefront to redirect the eligible AIS patients for endovascular therapy toward comprehensive stroke centers that are well equipped with endovascular facilities. Utilization of screening scales to identify LVO cases would need to be validated across pre-hospital levels and especially among the paramedics, as they are the initial responders [40–43]. However, with the recent emergence of mobile stroke units, even physicians would be at the forefront in the chain of acute stroke management.

Generalizability of endovascular procedures to distal vascular occlusions in anterior circulation, basilar artery occlusion in posterior circulation, and in delayed presentation of patients remains to be determined. Various endovascular trials are currently under investigation to address these important issues. The BASICS [44] trial is currently investigating the efficacy of endovascular and IV thrombolytic therapies in patients with basilar artery occlusion. Around one-quarter of AIS patients present late from their symptom onset or wake up with symptoms and are considered ineligible for either IV or to the most part of IA therapies. Utilization of advanced neuroimaging to identify eligible patient profiles likely to benefit with endovascular procedures remains paramount [45]. Various clinical trials including DAWN [46], POSITIVE [47], RESTORE [48], and DEFUSE-3 [49] are investigating these concepts with the use of novel imaging and procedural techniques in this patient cohort, to maximize the clinical benefit with endovascular procedures.

2.6 Conclusion

Recent success of endovascular trials has demonstrated the superiority of mechanical thrombectomy and established a standard of care for anterior circulation ischemic stroke patients. Rapid evaluation, utilization of multimodal imaging to select eligible patients, use of stentrievers, and minimization of the duration of endovascular procedures have been the key factors for the success of second-generation endovascular trials. Future trials are expected to expand the current knowledge of endovascular techniques for patients with distal or posterior vessel occlusions and who wake up with stroke-like symptoms.

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Chapter 3
Stroke Epidemiology

Chad M. Miller

Abstract Stroke is a leading cause of mortality and disability worldwide. Stroke may be broken into ischemic and hemorrhagic subtypes, with the former occurring at a rate four to six times that of hemorrhagic strokes. Intracerebral hemorrhage includes both non-traumatic intraparenchymal hemorrhage and subarachnoid hemorrhage resulting from cerebral aneurysms and other vascular disorders. Stroke types share many common causes, the majority of which are modifiable and form the rationale for primary and secondary stroke prevention. Hypertension, diabetes mellitus, hypercholesterolemia, obesity, smoking, and physical inactivity pose the greatest risks for stroke. Among non-modifiable risk, race, age, gender, and genetic disorders frequently impact the rate of stroke. Public awareness of signs, symptoms, and causes of stroke is improving but still remains as substantial barriers to improved population health.

Keywords Stroke • Epidemiology • Ischemic stroke • Hemorrhagic stroke • Subarachnoid hemorrhage • Intraparenchymal hemorrhage

Abbreviations

aSAH	Aneurysmal subarachnoid hemorrhage
CAA	Cerebral amyloid angiopathy
DM	Diabetes mellitus
ICH	Intracerebral hemorrhage
IS	Ischemic stroke
LDL	Low-density lipoprotein
PROGRESS	Perindopril Protection Against Recurrent Stroke Study
TIA	Transient ischemic attack
TOAST	Trial of ORG 10172 in Acute Stroke Treatment
US	United States

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3.1 Introduction

Stroke is a term used to refer to an acute injury to the brain from a vascular cause that results in permanent neuronal injury and functional disability. Strokes are commonly divided into ischemic (IS) and hemorrhagic subtypes. IS arise from occlusion or flow-limiting stenosis of cerebral vessels that result in inadequate blood flow to sustain neuronal viability. Hemorrhagic strokes may result from rupture of an aneurysm (aSAH) or other non-traumatic causes including chronic hypertension (spontaneous), arteriovenous malformations (AVM), cerebral amyloid angiopathy (CAA), coagulopathy induced, and those resulted from illicit drug abuse. Non-aneurysmal hemorrhagic strokes are often collectively classified as intracerebral hemorrhages (ICH). Stroke most frequently affects individuals age 55–75 years [1]. The mean age of IS victims is older than that of aSAH and ICH patients.

3.1.1 Ischemic Stroke

IS accounts for roughly 85% of all strokes and affects nearly 800,000 people annually in the United States (US) [2]. Seventy-seven percent of these patients are first-time victims, and over six million Americans are living with disability resulting from IS [2]. Ischemic symptoms that resolve prior to resulting in radiologic injury or permanent impairment are termed transient ischemic attacks (TIAs). The true incidence of TIAs is difficult to determine but is thought to be substantially more common than IS [3]. Patients suffering TIAs have a 90-day risk of stroke of 10.5% [4, 5]. Therefore, TIA occurrence is regarded as a harbinger of IS and is managed aggressively in attempts to minimize subsequent cerebral infarction. IS tend to affect men more commonly than women [1]. Mean age of stroke in a large Dutch IS cohort was 65.6 years, and 56% of the victims were male [1]. IS in patients under the age of 55 is more likely to affect nonwhite males. IS subtypes are commonly classified according to the Trial of ORG 10172 in Acute Stoke Treatment (TOAST) criteria [6]. TOAST subtypes include large artery atherosclerosis, small-vessel disease, cardioembolic, other determined cause, and undetermined cause. Other determined causes of stroke include arterial dissection, coagulopathy, and sickle cell disease [7]. Typical distribution of TOAST subtypes may be found in Table 3.1.

3.1.2 Hemorrhagic Stroke

ICH and aSAH account for 55–60,000 strokes in the United States each year [8]. While this number comprises 8–15% of all strokes in western countries, eastern countries, such as Japan and Korea, experience a notably higher portion of ICH

Table 3.1 Relative distribution of ischemic stroke subtypes utilizing the TOAST criteria

	Number of ischemic strokes (%)	Median age at stroke onset
Large-vessel atherosclerosis	815 (25)	68 years
Small-vessel disease	632 (19)	66 years
Cardioembolic stroke	467 (14)	72 years
Other determined causes	214 (7)	51 years
Undetermined cause	1130 (34)	66 years

Mean age at time of stroke event is also provided (D)

(18–24%) [9, 10]. The rate of ICH in low and middle socioeconomic countries is twice as much as seen in developed countries (22 vs. 10/100,000) [11]. Spontaneous ICH is the most common cause of ICH and results from lipohyalinosis of small cerebral vessels due to chronic hypertension. Spontaneous ICH occurs in characteristic areas of the brain (pons, cerebellum, basal ganglia, thalamus, and occasional white matter) where delicate perforating vessels emanate directly from larger capacitance cerebral arteries. When ICH occurs in patients who are younger, female, non-smokers, with the absence of coagulopathy or hypertension, non-spontaneous etiologies of ICH should be suspected. Moyamoya, AVMs, tumors, and cerebral vein thrombosis are other causes of ICH. aSAH is less common than ICH, and its incidence varies considerably throughout the world and among populations within the same country. In general, aSAH is more prevalent in lower socioeconomic countries [12]. In the United States, aSAH risk ranges from 2 to 16 per 100,000 persons [12]. It is estimated that 1 in 20–30 adults harbors a cerebral aneurysm, a quarter of whom will experience aSAH throughout their lifetime [13].

3.2 Stroke Morbidity and Mortality

IS is the fourth leading cause of death in the United States and the second most common cause of death worldwide [14, 15]. Survivors of stroke are commonly left with substantial disability, resulting in cost exceeding $73 billion annually in the United States alone [7]. This correlates to a mean lifetime patient cost of $140,048 [7]. According to the Framingham Heart Study, 15–30% of patients are permanently disabled after IS. One fifth of all patients continue to require institutional care 3 months after stroke occurrence. About half of all men and women suffering IS prior to age 65 will die within 8 years following their event [16]. Despite the consequence of IS, there is a lack of public awareness regarding appropriate response to stroke symptoms. Fewer than one half of all emergency medicine service calls for stroke are made within the first hour after symptom onset [17]. Furthermore, only 53% of stroke patients arrive to medical care via ambulance [18].

Outcome for ICH is even more concerning. Case fatality rates at 1 month approach 40% and increase to over 50% within the first year after hemorrhage [11].

Table 3.2 Hunt and Hess grade and correlating mortality as a result of aneurysmal subarachnoid hemorrhage from a study conducted at a large single high-volume health system (T)

Hunt and Hess grade	Hunt and Hess description	In-hospital mortality
1 and 2	Severe HA, nuchal rigidity, cranial nerve palsy or better	3%
3	Drowsy, confused, mild focal deficit	9%
4	Stuporous, moderate to severe weakness	24%
5	Deep coma, decerebrate posturing,	71%

Twenty percent of ICH patients deteriorate en route to medical care, and another 15–23% deteriorate within the first hours of hospitalization [19]. Much of this deterioration is related to expansion of the hemorrhage volume. Hematoma enlargement of at least one third of initial volume is seen in 28–38% of ICH within the first 3 h [20, 21]. While case fatality rates may be improving with better initial care, withdrawal of care remains the leading cause of early death [19]. ICH outcome is adversely driven by increased hemorrhage volume, greater patient age, intraventricular hemorrhagic extension, hemorrhage location, contrast extravasation, and initial examination as assessed by Glasgow Coma Scale or National Institute of Health Stroke Scale scores [11]. Furthermore, anticoagulant-associated hemorrhage is associated with greater and longer risk of volume expansion, and 76% of patients on anticoagulants die or become functionally dependent [22].

aSAH victims are also at great risk for poor recovery. One quarter of all aSAH patients die acutely, and half of the survivors are left with substantial disability [12]. For those living until hospital admission, one third will die, and one sixth will recover with severe disability [23]. In the United States, 8–20% of survivors are left with persistent dependence [12]. Intellectual impairment following aSAH is likely underestimated given the insensitivity of commonly utilized clinical scales for cognitive dysfunction. Mortality appears to be greater in women and in African-Americans, Native Americans, and Asian/Pacific Islanders [12]. Likelihood of recovery after aSAH is influenced by rehemorrhage, age, coexisting illness, cerebral edema, intraventricular hemorrhage, fever, infection, and disseminated intravascular coagulation [12]. Despite the overall grim prognosis following aSAH, mortality is quite variable depending upon presenting acuity. Table 3.2 correlates in-hospital mortality rates for a cohort of patients treated at Columbia Presbyterian Hospital [24]. Case fatality rates for aSAH have improved over the past decades. Treatment at a medical center managing high aSAH volumes is associated with improved clinical outcomes [25]. Aneurysm recurrence and rehemorrhage are factors for long-term prognosis. Completely obliterated aneurysms have a small short-term (5 year) risk of rerupture [12]. The risk for partially or incompletely treated aneurysms is still being defined but is considered to be greater.

3.3 Risk Factors

The various types of stroke share common risk factors. It is estimated that 80–90% of risk for IS and ICH can be traced to a common set of modifiable conditions including hypertension, tobacco use, obesity, physical inactivity, diabetes mellitus (DM), alcohol intake, and hyperlipidemia [26]. Chief among vascular risks is hypertension, which affects two thirds of Americans over the age of 65 [7]. Increased severity of elevated blood pressure correlates with greater risk, though causality has been established even for blood pressures in the prehypertensive range [7]. Use of antihypertensive medications is efficacious and has been shown to reduce stroke by up to 32% [27, 28]. Over 10% of Americans suffer from DM, which increased risk of stroke by two to six times [7]. On average, two of three diabetics die as a result of stroke or coronary artery disease [7]. Tight glycemic control will lower stroke and cardiovascular disease rates by 60% [7]. Elevated low-density lipoprotein (LDL >130 mg/dl) is an independent risk for ischemic stroke. This risk is particularly impacted by the use of cholesterol-lowering statin drugs, which lower LDL by 30–50% and stroke rates by 21% [7]. Dietary management has tremendous effect on stroke risk. Excess weight and high alcohol consumption are contributors to stroke. African-Americans, in particular, appear to disproportionately suffer from the cerebrovascular consequences of elevated salt intake [7]. Conversely, risk of stroke is reduced by 6% with each daily serving of fruits and vegetables. Physical inactivity is a major modifiable cause of stroke. Moderate-intensity exercise for at least 150 min each week is recommended to improve these risks.

Substantial research has explored the risk related to IS. Risk for IS doubles for every decade beyond the age of 55 years. Hypertension is seen in over half of all IS patients regardless of age but also is more prevalent with advanced age [1]. The same is true of hyperlipidemia and DM, though risk of stroke appears to peak at age 75 for these conditions. The Perindopril Protection Against Recurrent Stroke Study (PROGRESS) showed that a mean reduction in blood pressure of 12/5 mmHg resulted in a 43% lowering of IS risk [29]. Similarly, diabetics benefit from improved glycemic control with a goal HgA1C of <7% [7]. The 8% of the population with DM are recommended to adhere to tighter blood pressure (<130/80 mmHg) and lipid control compared to those at risk for stroke without diabetes [7]. Tobacco use is a more prevalent risk among younger individuals suffering IS and appears to double the risk of stroke [1]. Comparable risk is seen in those exposed to second-hand smoke. Risks for IS are reduced by one half by 1 year following smoking interruption and return to near non-smoking levels after 5 years of cessation [1]. Modifiable risks for stroke are more common in large- and small-vessel etiologies compared to cardioembolic sources [1]. Atrial fibrillation is the most common risk for cardioembolic stroke, resulting in 75,000 strokes annually in the United States [1]. The risk of stroke with non-valvular atrial fibrillation is increased four- to five-fold [30]. Patients with dilated cardiomyopathy and mural thrombus are also at elevated risk. Non-modifiable factors also play a role in stroke risk. A family history of stroke can increase prevalence by 30% [7]. Men have higher rates of IS than

Table 3.3 Adjusted risk of ICH for common causes of spontaneous intracerebral hemorrhage (L)

Risk factor	Adjusted OR	95% CI
Hypertension	5.71	(3.61–9.05)
Diabetes	2.40	(1.15–5.01)
Postmenopausal	2.50	(1.06–5.88)
Current cigarette smoking	1.58	(1.02–2.44)
Alcoholic drinks >2 per day	2.23	(1.16–4.32)
Caffeinated drinks >5 per day	1.73	(1.08–2.79)
Caffeine (in drugs)	3.55	(1.24–10.20)

OR odds ratio, *CI* confidence interval

women, and each has increased propensity for stroke after age 55. Hyperhomocysteinemia, antiphospholipid antibodies, sickle cell disease, and other coagulopathies represent additional unmodifiable risk.

As with IS, men suffer from ICH at greater rates than women. Hypertension is the leading risk for ICH and appears to correlate more closely with risk of deep hemorrhages [11]. Only 50–60% of patients with cortical hemorrhages have a history of hypertension [8]. Hypertension, advanced age, and deep hematoma location are risk factors for recurrent ICH [19]. Cerebral microhemorrhages on magnetic resonance gradient echo imaging may be a risk for ICH and are commonly associated with CAA [11]. CAA is tied to recurrence of cortical ICH [19]. Dual antiplatelet therapy carries an increased probability of ICH, and benefits of utility must be weighed against these dangers. Type of ICH may be impacted by race. Most initial and recurrent ICH in whites are lobar, whereas deep hemorrhage is more common in Asians [31]. Younger patients with ICH are less commonly white [1]. ICH in endogenous Africans tends to occur at an earlier age and with worse outcome compared to African-Americans and European Americans [32]. Therefore, in addition to race, environmental components appear to drive stroke risk. Relative risks for ICH are compared in Table 3.3.

Contrary to IS and ICH, women are at greater risk for aSAH (1.24 × rate) and are more likely to have multiple aneurysms than male counterparts [33]. In one Dutch study, nearly 74% of the cohort were women [1]. Hypertension, tobacco use, history of prior aSAH, and use of sympathomimetic drugs are additional contributors to aneurysm rupture [7]. aSAH also is unique in that onset of stroke occurs at a younger age compared to other stroke types. Over 80% of aneurysm ruptures occur in patients age 55 years and younger [15]. Correlations have been drawn between aneurysm size and location and rupture rate. Larger aneurysms and those located in the vertebrobasilar system have a greater tendency to cause hemorrhage [12]. For anterior circulation aneurysms, a size of 7 mm has been identified as a threshold for increased rupture risk [34]. Despite this correlation, the vast majority of aneurysms (70%) that rupture are <7 mm in diameter. While aneurysm size does predict likelihood of rupture, female sex and a history of smoking appear to be more predictive indicators of risk [13]. Aneurysm shape is likely an additional factor impacting rupture rate [12, 35]. Tobacco use triples the risk of aneurysm rupture. After cessation, prior smokers still appear to have an increased risk for aSAH [36]. Familial

aneurysms are considered when a patient has two or more first-degree relatives with a history of cerebral aneurysms. While a predisposition to aneurysm formation has been seen within families, the implication of this observation for monitoring and treatment is less clear. In a Finnish trial of 91 families with 2 or more aneurysms, 8.7% of individuals were found to have an aneurysm in families without clear heritable conditions [23]. A similar portion (9.1%) were found to have autosomal dominant polycystic kidney disease. A history of fibromuscular dysplasia confers similar risk for aneurysm formation (7%) [2].

3.4 Conclusion

IS, ICH, and aSAH are a diverse group of disorders that share common causes. Most of the risks for these conditions are modifiable, and concerted efforts at risk modification are appropriate considering their prevalence and the substantial disability resulting from their occurrence. Worldwide, significant disparities exist regarding the prevalence of stroke and the outcomes of those who suffer from stroke. Significant gaps exist regarding patient awareness of stroke signs, symptoms, and related health conditions.

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Chapter 4
Pathophysiology of Ischemic Stroke

Yongfang Li and Guo-Yuan Yang

Abstract In this chapter, we summarize the characteristics of pathophysiological changes after ischemic stroke. Ischemic stroke is a rapid occurring and developing disease, which is caused by one or several cerebral artery occlusions. The occlusion could be due to the thrombus or thrombosis. The immediate change after ischemic stroke includes ion balance destroyed such as Na^+–K^+ pump dysfunction, calcium overload, acid-sensing ion channel opening, and peri-infarct depolarization. The following change is metabolism failure such as decrease of ATP and pH levels, increase of excitotoxicity response, reduced protein synthesis, and disturbed phosphatase activity. After then, free radical release, inflammatory response, apoptosis, necrosis, autophagy, and blood-brain barrier disruption could occur based on the duration and severity of ischemia. During this process, the body defense system could be activated, and angiogenesis, neurogenesis, and oligodendrogenesis could simultaneously be induced. Finally, we also discuss briefly how to manage ischemic brain injury based on the pathophysiological changes.

Keywords Acid-sensing ion channels • Apoptosis • Autophagy • Blood-brain barrier • Excitotoxicity • Energy metabolism • Ionic homeostasis • Ischemic stroke • Necrosis • Pathophysiology • Peri-infarct depolarization

Abbreviations

3-MA	3-Methyladenine
AIF	Apoptosis-inducing factor
AMPARs	Amino-3-hydroxy-5-methyl-4-isoxanole propionate receptors

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ASICs	Acid-sensing ion channels
Bax	Bcl-2-associated X protein
BBB	Blood-brain barrier
Bcl-2	B-cell lymphoma 2
Bid	BH3 Interacting-domain death agonist
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
DAPK1	Death-associated protein kinase 1
DCE-MRI	Dynamic contrast-enhanced magnetic resonance imaging
Drp1	Dynamin-related protein 1
EndoG	Endonuclease G
LC3-II	Microtubule-associated light chain 3 II protein
LMP	Lysosomal membrane permeabilization
MMPs	Matrix metalloproteinases
mPTP	Mitochondrial permeability transition pore
NCXs	Na^{+}-Ca^{2+} exchangers
NMDARs	N-Methyl-D-aspartate receptors
nNOS	Neuronal nitric oxide synthase
PARP-1	Poly(ADP-ribose) polymerases-1
PSD-95	Postsynaptic density-95 protein
PUMA	p53-upregulated modulator of apoptosis
ROS	Reactive oxygen species
Smac	Second mitochondria-derived activator of caspase
SUMOylation	Small ubiquitin-like modifier conjugation
tMCAO	Transient middle cerebral artery occlusion
TNF-α	Tumor necrosis factor-α

4.1 Introduction

Stroke is a serious worldwide disease characterized with high morbidity, mortality, and healthcare costs and brings a heavy burden to society especially in low- to middle-income countries [1]. In the United States, the direct and indirect cost of stroke reached $33.0 billion in 2011–2012 [2]. Ischemic stroke consists of approximately 80% in stroke with bulk of disability-adjusted life years lost [1, 3]. Consequently, this justifies the need for in-depth pathophysiological mechanism explorations and investigations of ischemic stroke to search for better and optimal preventive management and therapeutic treatment. Indeed, scientists have advanced the understanding of basic pathophysiology of ischemic stroke including the initiations and components of ischemic cascade and the development of irreversible neuronal damage in the past decades. However, the pathophysiological studies mainly come from experimental and animal researches, which may differ from human results and limit their clinical applications. Tremendous prior clinical trials attempting to put a wide range of purported acute ischemic therapies into clinical settings

faced challenges and bleak outcomes, which only was proved to be efficient in animal models [4].

In this chapter, we try to elucidate the pathophysiological changes and mechanisms of individual component of ischemic stroke insult as detailed as possible. Although the human body itself could autonomously activate endogenous protective procedures involving angiogenesis, neurogenesis, oligodendrogenesis, remodeling of neurovascular unit, and neuronal network formation, these processes are generally subtle and far from enough to support the repair and recovery of ischemic stroke.

4.2 Altered Ion Balance

4.2.1 Ionic Changes Following Ischemic Stroke over Time

Ionic homeostasis plays a critical role in normal neuronal functions including maintaining resting membrane potential; generating action potential, synaptic transmission, neurotransmitter transporting; and providing intracellular ionic microenvironment for macromolecular normal functioning. Under ischemic conditions, such steady ionic homeostasis could not be sustained with excessive Na^+, Ca^{2+}, Cl^- influx, and intracellular K^+ efflux.

4.2.1.1 Na^+-K^+ Pump Dysfunction and Increased Intracellular Na^+

Na^+-K^+ pump (also named Na^+-K^+ ATPase) is an active transporter pumping three Na^+ out and two K^+ into the cell with ATP hydrolysis in each cycle, which largely contributes to the Na^+ and K^+ electrochemical gradient across the cell membrane [5]. Na^+-K^+ pump was generally inhibited due to the greatly decreased ATP level during ischemia and the free radical generation in reperfusion period [6–9]. In a rat transient middle cerebral artery occlusion (tMCAO) model, the Na^+–K^+ ATPase activity was reduced to its minimum at 48 h and upregulated at the third day and seventh day in the penumbra after tMCAO [7]. Interventions aiming to reduce the inhibition of Na^+-K^+ ATPase showed a neuroprotective potential [6, 10]. Na^+-K^+ pump dysfunction caused excessive extracellular sodium ions entering into the cell driven by the powerful electrochemical gradients and consequently increased intracellular Na^+ concentration [11, 12], which may just partially account for increased cellular Na^+ [13]. Additionally, the voltage-gated sodium channels were activated by hypoxia-induced depolarization and nonselective cation channels; Na^+/H^+ exchangers were also involved in the pathways in ischemia to trigger intracellular Na^+ elevation [14]. With the increase of intracellular sodium ions, the water concomitantly entered the cell resulting in cellular swelling and cell injury.

4.2.1.2 Calcium Overload

Studies showed that intracellular calcium concentration was markedly elevated in ischemia including cytosolic and mitochondrial calcium [12], which may be referred as "calcium overload." In the early phase of ischemia, with the rapidly declined ATP level and increased intracellular Na^+, the cell membrane Na^+–Ca^{2+} exchangers (NCXs) were reversely activated [15, 16] and followed by cell membrane depolarization, and the voltage-gated calcium channels opened [14], together causing the intracellular calcium to rise. Meanwhile, glutamate accumulated in the extracellular space and activated the inotropic glutamate receptors permeable to calcium, which was mainly via N-methyl-D-aspartate receptors (NMDARs) to mediate the calcium influx [17, 18]. The calcium released from the endoplasmic reticulum and mitochondria could also partially contribute to the increase of intracellular Ca^{2+} [14]. Noteworthy, the mitochondrial matrix calcium started to accumulate when cytosolic Ca^{2+} raised up to a certain point [19]. In the late phase of ischemia and reperfusion period, the secondary intracellular calcium elevation might be due to the release from the endoplasmic reticulum and the opening of reactive oxygen species (ROS)-sensitive nonselective cation channels [14]. However, detailed mechanisms behind this phenomenon are really complicated and need further investigations. For example, the role of NCXs seems to be a double-edged sword: by extruding Ca^{2+} in ischemia, they reduced intracellular calcium level and protected neurons from death [20, 21], whereas inhibition of NCX1 attenuated brain damage after 90-min tMCAO [22].

4.2.1.3 Acid-Sensing Ion Channels

Acid-sensing ion channels (ASICs) are proton-gated cation channels belonging to the degenerin/epithelial sodium channel superfamily and widely expressed on neurons of peripheral and central nervous systems, and ASICs could be activated by ischemic-induced acidosis [23, 24]. Up to date, four genes (ACCN1-ACCN4) have been identified to encode six different ASIC subunits (ASIC1a, 1b, 2a, 2b, 3, and 4) and three subunits consisting of homomeric and/or heteromeric functional ASICs. Currently, the investigations of ASICs in ischemic mainly focus on ASIC1a due to its predominant expression in the brain, its high sensitivity to proton, and its permeability to both sodium and calcium. Emerging evidences showed that ASIC1a was responsible for acidosis-mediated neuronal injury in a glutamate-independent manner in global and focal ischemia [25–29] and might couple the acidotoxicity and excitotoxicity together [30–32]. Selectively pharmacological blockade with psalmotoxin 1 or gene knockout of ASIC1a attenuated acidosis-induced neuronal injury and brain infarct volume via reducing intracellular calcium overload and exhibited more potent neuroprotective role compared with NMDAR block in ischemic stroke [25, 27, 28]. Provocative evidences indicated that under hypoxia/ischemic conditions, acidotoxicity and excitotoxicity might not be mutually exclusive entities. Actually, these two pathophysiological processes had a really complicated

interaction and might promote each other resulting in even severe cerebral injury. For instance, ASIC1a activation might be associated with protein kinase A-dependent phosphorylation of NMDA receptor subunit NR1 [28] and promoted NMDAR function aggravating neuronal damage [31]. Moreover, the combination of ASIC1a and NMDAR inhibition showed a synergistic neuroprotective effect [27, 28]. In turn, the activation of NMDARs containing NR2B could enhance the ASIC1a current through the phosphorylation of increased Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activity [30]. Correspondingly, the activation of ASIC1a propagated the calcium influx by modifying calcium-permeable amino-3-hydroxy-5-methyl-4-isoxanole propionate receptors (AMPARs) into harmful plasticity [32]. However, studies on ASIC2a demonstrated a potentially beneficial role in the ischemic environment [33, 34].

4.2.2 Peri-infarct Depolarization

Peri-infarct depolarization was first observed in ischemic penumbral area exhibiting spontaneously spreading depolarization of neurons and astrocytes [35], and later it was identified in patients with ischemic stroke and subarachnoid hemorrhage [36, 37]. Recently, increasing attentions with respect to the correlation between peri-infarct depolarization with the infarct volume progression and neuronal injury have been received. This peri-infarct depolarization could propagate widely at a speed of several millimeters per minute during acute ischemic phase and lead to a declined electrocorticographic activity [35, 38, 39]. Essentially, peri-infarct depolarization was generally considered to be triggered by ischemia-induced ion redistribution especially the potassium and excitatory amino acid release [35]. Investigations in experimental ischemic models indicated that the increased number and prolonged duration of peri-infarct depolarization were associated with infarct volume expansion and delayed neuronal deterioration in ischemic penumbra [40–42]. The accumulation of this depolarization indicated a consistent pathophysiological process and predicted focal pathology development. These may be partially explained by that peri-infarct depolarization exerted an extra strain on metabolically compromised cell, where the cell lifted its metabolic workload and shifted into anaerobic glycolysis to reverse this depolarization status. On the other hand, it could aggravate tissue hypoxia and reduce tissue reperfusion within the hypo-perfusion regions [35, 37, 42, 43]. Interventions to limit peri-infarct depolarization showed a beneficial role to increased cerebral blood flow [44–46]. Interestingly, reducing its occurrence via prolonged anesthesia couldn't provide protective effect in the absence of restoring reperfusion or enough collateral circulation in a rat focal ischemia model [39]. Collectively, these results suggested that modulating peri-infarct depolarization in ischemic penumbral with enough blood supply may hopefully promote ischemia recovery.

4.3 Altered Cell Energy and Metabolism Activity

4.3.1 Decreased ATP and pH Levels

Cerebral tissues are in high demand of oxygen and energy consumption to keep the brain in good work; they are almost entirely depending on sufficient blood flow due to the poor capacity of glucose/glycogen and energy reserve. Once the supply of brain blood flow is interrupted and not restored promptly, ischemic injury occurs. Oxygen and glucose deprivation during ischemia primarily inhibits the mitochondria functions including the electron transport chain and oxidative phosphorylation and then followed by rapid decrease of high-energy phosphates and a major consequence of the fall in ATP within 2–5 min [47–49]. The level of ATP maintained in the early first 2 min under ischemic conditions was largely associated with the intracellular storage of phosphocreatine [49]. Under ischemic conditions, the cell energy metabolism shifts into glycolytic pathway and the by-products lactate and protons from ATP hydrolysis began to accumulate, consequently contributing to a general pH decrease in brain tissue. Extracellular pH could drop to 6.5–6.0 or below 6.0 in severe ischemia within minutes following occlusion [50, 51]. A high-temporal-resolution simultaneous ^{1}H-^{31}P magnetic resonance spectroscopy was used to dynamically examine the cerebral lactate, ATP, phosphocreatine, pH, and neuronal field potential activity in a 12-min four-blood-vessel occlusion of whole-brain ischemia rat model. The results showed that lactate rapidly decreased with the dramatically reduced blood oxygenation level, and then the cellular ATP started to decreased at 2.2 min after occlusion; ATP recovered relatively slow to 80% baseline level within 10 min and neuronal field potential activity recovered to 70% of baseline, while the pH and lactate began to recover within 2 min during reperfusion [49].

4.3.2 Excitotoxicity Response to Ischemic Stroke

Excitotoxicity is the process of neuronal damage and death; it is generally induced by excessive extracellular accumulation of excitatory amino acids especially the neurotransmitter glutamate, which has been found to participate in the pathogenesis of many diseases including stroke [52, 53]. Excitotoxicity is the primary mechanism of ischemic insult during acute ischemic phase, and glutamate-induced excitotoxicity largely mediated by ionotropic NMDARs is the main source of neuronal excitotoxicity [52]. In the context of cerebral ischemia, energy depletion and membrane depolarization result in massive excitatory neurons releasing glutamate, concomitantly with the compromised uptake of glutamate from neurons and glial cells; these together contribute to extensive accumulation of extracellular glutamate. Additionally, the increased extracellular glutamate activates adjacent neurons via NMADRs leading to calcium influx and subsequent synaptic glutamate release; thus, neuronal excitotoxicity further spread to the surrounding neurons. Numerous

evidence has demonstrated that NMDAR activation in ischemia was inversely associated with infarct size and could induce delayed neuronal death that occurred hours or days in penumbra. However, reducing glutamate release or inhibiting NMDAR activation showed neuroprotective effects [52, 54, 55]. Glutamate-induced excitotoxicity is largely mediated by excessively elevated intracellular calcium through NMDARs. Emerging evidence has suggested that the cytoplasmic C-terminal domains of NMDARs could directly or indirectly bind to downstream death-signaling proteins [52, 53, 56, 57]. The neurotoxicity of elevated intracellular calcium includes (1) induction of mitochondrial depolarization partially via calcium uptake through mitochondrial calcium uniporter and subsequent neuronal death [58, 59]; (2) activation of associated DNA enzymes, proteases, and phospholipases causing DNA, protein, and phospholipid degradation [60–62]; and (3) activation of downstream lethal responses such as oxygen free radical production, nitrosative stress induction, protein S-nitrosylation, and calpain-mediated proteolysis [52]. For example, the death-associated protein kinase 1 (DAPK1) was dephosphorylated and activated by NMDAR-mediated activation of calcineurin consequently initiating neuronal apoptosis [63]. Furthermore, activated DAPK1 could augment intracellular calcium influx and excitotoxicity via enhancing NMDAR function by phosphorylating and recruiting GluN2B subunit-containing receptors [57]. Interrupting this interaction of GluN2B-DAPK1 with an interference peptide Tat-NR2B-CT containing the GluN2B C-terminal phosphorylation site could effectively mitigate DAPK1-induced phosphorylation of GluN2B-containing receptors and block calcium influx, which provided neuroprotection against excitotoxicity both in vitro and in vivo ischemic models [57]. Moreover, excessive calcium influx could activate neuronal nitric oxide synthase (nNOS), and activated GluN2B-containing receptors could promote GluN2B-conjugated nNOS translocation from the cytoplasm to membrane producing large amounts of neurotoxic nitric oxide [64, 65]. Later studies found that the postsynaptic density-95 protein (PSD-95), a scaffolding protein located in the postsynaptic membrane, could specifically bind nNOS to the C-terminal of GluN2B and bring nNOS physically proximal to the NMDAR channel pore, which allowed enough calcium to efficiently activate nNOS [66, 67]. Disrupting NMDAR/PSD-95/nNOS interaction with carefully designed drugs such as Tat-NR2B9c, IC87201, and ZL006 prevented nNOS activation by GluN2B-containing receptors, which attenuated neuronal damage in vitro and reduced infarct volume and neurological deficits in vivo [65, 68–70]. Excitingly, Tat-NR2B9c peptide has been shown to be effective in reducing iatrogenic stroke infarct in a second phase of the intracranial aneurism clinical trial (ClinicalTrial.gov NCT00728182) [71]. The evidence suggested that targeting NMDARs downstream, death-associated signaling events is of great therapeutic potential and applications in ischemic stroke. In addition to NMDAR-induced excitotoxicity, the calcium-permeable AMPK receptor has also been implicated in contributing to ischemic injury via promoting calcium influx and zinc release from presynaptic vesicles [72]. Blocking the calcium-permeable AMPK receptors with a selective antagonist, 1-naphthyl acetyl spermine, partially attenuated intracellular free zinc elevation and rescued hippocampal CA1 neurons from ischemia-induced cell death in rats subjected to four-vessel occlusion [73]. Blocking free zinc with

zinc ion chelator reduced infarct volume and neuron apoptosis in rat focal ischemia model. Oxygen-glucose deprivation showed that free zinc could induce excitotoxicity, oxidative stress, and inflammation which was associated with neuron death [74].

4.3.3 Global Inhibition of Protein Synthesis and Changes in Protein Kinase and Phosphatases

Protein synthesis is a complicated cellular process requiring energy supply, relatively stable intracellular hemostasis, and integral associated protein and RNA systems. Under ischemia, the protein synthesis was globally inhibited, while some certain deleterious proteins such as inducible NOS and cyclooxygenase-2 were reversely upregulated. The inhibition of protein synthesis was detected at 1 h after occlusion and persisted to 12 h after tMCAO in rats [75]. Another study showed that the level of protein synthesis already began to decrease when blood flow declined to 80% of baseline [76]. After perfusion in global ischemia, protein synthesis recovered completely or near completely in the cell surviving regions over 12–48 h, whereas the cell destined to die regions couldn't recover to normal synthesis levels [77, 78]. Using a novel wide-field synchrotron radiation Fourier transform infrared imaging in combination with traditional immunocytochemistry detecting brain tissue slices from a two-vessel occlusion rats, a decreased total protein level along with increased protein aggregation and oxidative stress was observed in CA1 pyramidal neurons at 1 day after global ischemia, and the neurons progressed to lose cellular integrity the next day [79]. These evidence suggested that an irreversible inhibition of protein synthesis was highly associated with ischemia-induced neuronal death. Even though the protein synthesis was sensitive to energy depletion and intracellular ion changes, synthesis depression still sustained after reperfusion especially in penumbra and selectively vulnerable regions [78, 80]. Translation inhibition [81, 82] and post-translation processing dysfunction [83] might explain for this phenomenon. For example, the phosphorylation of eukaryotic initiation factor2 alpha could initiate translation arrest in reperfused neurons at an early phase of reperfusion [84]. The abnormal cytoplasm aggregations of protein were examined using electron microscopy in penumbral neurons after 4-h reperfusion following 2-h focal ischemia. The results showed that the protein aggregations consisted of ribosomal proteins, eukaryotic initiation factors, and co-translational chaperones, and exhibited high levels of ubiquitination, which possibly resulted in neuronal death [85]. Proteomics analysis of cytoplasmic Triton X-100-insoluble aggregates revealed that these ubiquitinated proteins were composed of three initiation factors, two elongation factors, and five chaperons in global ischemic mice model [83]. Furthermore, these aggregations of ubiquitinated protein were triggered mostly following reperfusion rather than during ischemia phase; it started to accumulate at 5 min and peaked at 15 min after reperfusion [86]. Interestingly, only minor increase of protein aggregates was observed in the infarct core of striatum after 24-h

reperfusion [86]. These findings were in concomitant with the observations that the cytoplasmic ribonucleoprotein aggregates accumulated in integrated neurons but not in apoptotic cells in the hippocampal CA1 region at 48-h reperfusion following ischemia [82].

Importantly, recent studies on protein small ubiquitin-like modifier (SUMO) conjugation named SUMOylation, a process of protein post-translation modification in regulating proteins' subcellular localization, stability, and function, showed that the global SUMOylation level especially SUMO2/SUMO3 conjugation was remarkably increased both in vitro [87] and in vivo [88–90] ischemic insult. It is considered an endogenous protective response against hypoxia/ischemic stress. Overexpression of SUMO-conjugating enzyme Ubc9 increased tissue SUMOylation which was reversely correlated with the infarction size in a permanent MCAO transgenic mouse model [91]. In a 10-min forebrain ischemia of SUMO1–SUMO3 knockdown (SUMO-KD) mice specifically silencing SUMO expression in neurons, the transgenic mice exhibited more profound neurological function deficits compared to wild-type groups. SUMO1–SUMO3 knockdown also led to distinct post-ischemic gene expression profiles. Extracellular matrix receptor interaction, endocytosis, and focal adhesion pathways only showed up in SUMO-KD mice, and the TGF-beta, Toll-like receptor, and Wnt signaling were only identified in wild-type mice after ischemia [92].

The past two decades have enormous investigations on the roles of protein kinase or phosphatase inactivation or activation playing in signal transduction, protein alterations and synthesis, gene expression, and cellular death in ischemia insults [93–98]. These protein kinases and phosphatases include protein kinase c (PKC), CaMKII, protein kinase a, mitogen-activated protein kinases, extracellular signal-regulated kinase, AMP-activated protein kinase, and calmodulin-dependent phosphatase [93–98]. For instance, the expression and activity of protein kinase c mainly decreased after ischemia, but sometimes increased partly due to the differences in animal models, severity, and duration of ischemia-reperfusion [93]. Accumulating evidences showed that PKC was involved in glutamate-induced excitotoxic cell damage during ischemia and delayed neuronal apoptosis after reperfusion and inflammation [93, 97].

4.4 Increased Free Radical Generation During and After Ischemia

The formation of free radicals including nitric oxide, reactive oxygen species, and peroxynitrite has been shown to increase following ischemia and even to a great extent after reperfusion. It seems that multiple and sophisticated mechanisms including dysfunction of mitochondria; relatively increased activity of prooxidant enzymes like xanthine oxidase and nicotinamide adenine dinucleotide phosphate oxidase against antioxidant enzymes like superoxide dismutase, glutathione

peroxidase, and catalase; activation of nitric oxide synthase; metabolism of accumulated arachidonic acid via cyclooxygenase-2; and cascade of inflammation were involved [61, 99]. Increased free radicals dramatically damaged cellular lipid, protein, and DNA at a molecule level and further impacted mitochondrial function and membrane structure leading to cell damage and death, which was also defined as oxidative stress [100]. For more detailed elucidation of free radicals in ischemia, readers can refer to Chap. 13 in this book.

4.5 Cell Changes and Death During and After Ischemic Stroke

Following ischemia onset, the core or center of the brain ischemic territory tissue also called ischemic core experienced the most dramatic blood flow depletion. As a result, energy metabolism dysfunction, ionic disruption, excitotoxicity, and protein synthesis inhibition occurred. The cells mainly undergo necrotic cell death characterized by organelle dilation, cytoplasm swelling and vacuolization, and membrane dissolution with inflammation response [101]. Surrounding the ischemic core is a border zone of less severely affected tissue named ischemic penumbra with collateral blood supply which remains metabolically viable; cells mainly undergo apoptotic cell death characterized by cytoplasm shrinkage, chromatin compaction,nuclear condensation, pyknosis, and apoptotic body formation in this area [101, 102]. Compared with the necrotic cell death within minutes after ischemia, the apoptotic cell death is delayed by several hours or even days which is also called delayed neuronal cell death, which is the potential therapeutic target for neuroprotection. Additionally, another form of cell death observed in ischemia, autophagy-related cell death, receives increasing attentions and investigations with regard to its therapeutic potentials. Autophagy-related cell death is characterized by excessive cytoplasmic vacuolization and a series of autophagosomes and autolysosomes with cytoplasmic component engulfment [103, 104]. Studies showed that both apoptosis and autophagy were activated in ischemic penumbra; they could both present in the same cell and are tightly associated and interacted with each other [105].

4.5.1 Apoptotic Cell Death

Apoptosis refers to cell morphological changes following programmed cell death. Apoptosis is an active cell death process requiring energy and expression of new proteins, which occurs predominantly in ischemic penumbra where collateral blood and energy supply is sufficient to support apoptosis [102]. There are commonly two apoptotic pathways including the intrinsic pathway and the extrinsic pathway. The former is triggered by intracellular proapoptotic molecules, and the latter is

activated by extracellular signal molecules like Fas and tumor necrosis factor-α (TNF-α). The B-cell lymphoma 2 (Bcl-2) family proteins interact with intrinsic apoptotic pathway containing proapoptotic members like Bcl-2-associated X protein (Bax), BH3 interacting-domain death agonist (Bid), and p53-upregulated modulator of apoptosis (PUMA) and antiapoptotic members like Bcl-2 by sequestering proapoptotic members [104].

In brain ischemia, intracellular calcium elevated dramatically as discussed before and could subsequently initiate intrinsic apoptotic pathway via activating calpains and promoting irreversible opening of mitochondrial permeability transition pore (mPTP) [102]. Activated calpains cleaved Bid resulting in its truncated active form (tBid), which could also be activated by caspase-8. Then the tBid translocated to mitochondria and interacted with proapoptotic proteins subsequently antagonizing the neutralization role of antiapoptotic proteins like Bcl-2, which led to the opening of mPTP [102] and the release of cytoplasm c, second mitochondria-derived activator of caspase (Smac), apoptosis-inducing factor (AIF), and endonuclease G (EndoG) from mitochondria to cytoplasm [106]. Depletion of Bid gene showed reduced neuronal injury in vitro [107] and attenuated the infarction in vivo [108]. In addition, reactive oxygen species produced in mitochondria following ischemia and reperfusion could also induce these apoptotic proteins releasing [102]. Released cytoplasm c could interact with adapter protein apoptotic protease-activating factor-1 (Apaf-1) and recruit procaspase-9 further forming an active apoptosome with dATP exchanging, consequently activating caspase-9 and subsequent activation of caspase-3 [104]. Smac suppressed the inhibition of IAP (inhibitor of apoptosis protein) family members on caspase-3, caspase-7, and caspase-9, hence initiating caspase-dependent apoptotic pathway. AIF is normally located in mitochondria inner membrane, but AIF was released from mitochondria, translocated into nucleus, and triggered the activation of poly(ADP-ribose) polymerase-1 (PARP-1, a DNA-repairing nuclear enzyme) after ischemia, which initiated apoptosis in a caspase-independent pathway and resulted in DNA fragmentation [109]. Recent study found that macrophage migration inhibitory factor (MIF) had a nuclease activity and acted as a PARP1-dependent AIF-associated nuclease to induce DNA degradation and further stimulate PARP1 activation, which was recruited by AIF and mediated cell death induced by PARP1 [110]. Similar to AIF, EndoG was translocated from mitochondria into nucleus rapidly after MCAO [111, 112] and mediated the cleavage of DNA in a caspase-dependent pathway [113]. Furthermore, large-scale DNA fragment and oxidative stress activated p53, which was markedly upregulated in ischemia [102]. Then p53 initiated apoptosis in two different approaches: (1) promoting proapoptotic molecules expression such as PUMA and Fas receptor and inhibiting antiapoptotic proteins expression at a transcriptional level and (2) inducing mitochondrial dysfunction, which preceded the transcriptional pathway [104]. Apart from intrinsic apoptosis pathway, the extrinsic proapoptotic molecules like Fas and TNF-α were also implicated in ischemic cell death, which could initiate caspase-8-caspase-3 cascade pathway via binding to Fas receptor or tumor necrosis factor receptor 1, respectively [102].

4.5.2 Necrotic Cell Death

Necrosis is an irreversible and detrimental process of cell death primarily due to energy depletion and metabolism failure; it is excessively engaged in ischemia and reperfusion injuries. In stroke, cytosolic calcium overload, ionic imbalances, excitotoxicity, mitochondria dysfunction, and oxidative stress interacted with each other and together contributed to cell necrosis [114, 115]. For instance, extensive highly reactive oxygen species and reactive nitrogen species formation especially in reperfusion period and glutamate-mediated excitotoxicity could induce extensive PARP-1 activation, which further consumed and depleted its substrate nicotinamide adenine dinucleotide and possible ATP to an extreme extent thus contributing to necrosis [116]. Pharmacological inhibition or gene depletion of PARP1 in focal ischemia model reduced ischemic cell death and showed a neuroprotective effect [117, 118]. Excessive intracellular calcium could activate calpain, which then promoted lysosomal membrane permeabilization (LMP) and the leakage of lysosomal hydrolases into cytosol causing hippocampal neuronal necrosis in a nonhuman primate global ischemia model [119]. This calpain-mediated LMP may possibly be due to the cleavage of its substrates lysosomal-associated membrane protein 2 and 70-kDa heat shock protein, which stabilized the lysosomal membrane [120, 121]. Additionally, activated calpain could degrade neuronal cytoskeleton proteins such as α-fodrin and induce cell death.

Recently, the conventional concept that necrosis is purely a passive and uncontrolled process has been challenged by emerging evidence suggesting that specific signaling pathways were indeed participated in necrosis. Modulating these pathways provided protective effect against necrotic cell death [114, 122]. Consequently, this form of necrosis involving certain signaling pathways was referred to as "programmed necrosis" or necroptosis, which was triggered by death receptor signaling when apoptosis was compromised or downregulated. A growing evidence showed that suppressing this programmed necrosis with necrostatin-1 conferred a neuroprotective effect in delayed ischemia injury in vivo [123–125]. In brain ischemia-reperfusion injury, a novel mitochondrial p53-CypD (mPTP regulator cyclophilin D) axis contributing to oxidative stress-induced necrosis was identified, and inhibition of this complex formation reduced the infraction in ischemic mouse model [122]. Another study showed that in the context of oxidative stress, the p53-mediated mitochondrial necrosis pathway required the assistant of dynamin-related protein 1 (Drp1), which stabilized p53 and promoted p53 translocation to mitochondria via binding to p53 [126]. Pharmacologically inhibiting Drp1 translocation into mitochondria with P110 increased ATP level by over 50%, decreased the infarction volume, and enhanced neurologic function compared to the control in ischemic rat model [126].

4.5.3 Autophagy in Cell Death

Autophagy is a lysosome-dependent pathway that degrades cytoplasm long-lived proteins and organelles such as mitochondria, endoplasmic reticulum components, and peroxisomes to maintain metabolic stability and cell viability [104]. Autophagy consists of three different forms and mechanisms including microautophagy, chaperone-mediated autophagy, and macroautophagy; the latter is the most investigated and thus discussed here. When the cell is exposed to nutrition starvation and other physiological conditions, the process is moderate and protective in a self-eating manner. However, under pathological situations like ischemia-hypoxia, autophagy could be excessively and persistently activated, thus inversely promoting cell death either via apoptosis-dependent pathway or apoptosis-independent pathway [104]. In cerebral ischemia, the existence of autophagic vacuoles was first observed using an electron microscopy in hippocampal CA1 neurons at day 3 following ischemia [127]. Later on, accumulating evidence demonstrated that an increased formation of autophagosomes and autolysosomes and activation of autophagy were involved in ischemic insult. The role of autophagy in cell death might largely depend on the cellular contexts, intensity, and duration of autophagy induction [103, 128]. In a permanent MCAO rat model, the expression of LC3-II (microtubule-associated light chain 3 II protein, a protein essential for autophagosome formation and frequently used marker for autophagy) and cathepsin B (a predominant lysosomal enzyme) was upregulated in ischemia tissue. The results suggested an activated autophagy. Intraventricular delivery of 3-methyladenine (3-MA, an autophagy inhibitor) immediately after ischemia significantly reduced infarct volume, brain edema, and neurological deficits [129]. An in vivo imaging of autophagy in a 60-min tMCAO GFP-LC3 transgene mouse model observed that the fluorescent signal peaked at day 1 after tMCAO, and neurons displayed more activation of autophagy compared with astrocytes, which predominantly presented in penumbra [130]. In a rat neonatal cerebral ischemia model, intraventricular administration of 3-MA significantly attenuated the infarct volume even when delivered 4 h after the onset of ischemia and showed a potent neuroprotection compared with pharmacological caspase inhibition of apoptosis [131]. However, inhibition of autophagy via intraventricular delivering 3-MA or wortmannin 20 min before hypoxia-ischemia aggravated neuronal death and brain injury while activating autophagy by rapamycin showed a neuroprotective role [132]. Similar results were also observed in other literatures demonstrating that the protection role of ischemic postconditioning was associated with inhibition of autophagy, while ischemic preconditioning was associated with activation of autophagy [133, 134]. Additionally, autophagy was particularly implicated in neurotoxicity induced by NMDA, and inhibition of autophagy showed a protective role [135–137]. Primary cultured hippocampal neurons challenged with NMDA showed an increased autophagy activation and cell death numbers in a concentration-dependent manner. Increased autophagy triggered apoptosis when NMDA reached up to a certain level; this might be mediated by the activation of NR2B subunit and resultant inhibition of

PI3K-AKT-mTOR pathway (a pathway involving in inhibition of initiation of autophagy) which could be suppressed by insulin-like growth factor-1 [135]. Another excitotoxicity model using kainate as the glutamate receptor activator in rats led to cortical neuron death largely in an apoptosis-independent pathway, whereas this form of cell death was reduced via inhibition of autophagosome formation with 3-MA or genetic knockdown of beclin-1 and Atg7 [138].

4.6 Blood-Brain Barrier Pathophysiology Following Ischemic Stroke

The blood-brain barrier (BBB) is a highly selective and dynamic semipermeable membrane barrier structure. Brain microvascular endothelial cells are connected together by tight junctions, pericytes, basement membrane, and astrocyte end-feet, which together with neurons and microglia form a well-organized neurovascular unit [139]. The structural and functional integrity of BBB is critical to the maintenance of brain homeostasis. Disruption of BBB has been shown to actively participate in the pathophysiology of ischemic stroke involving cerebral vasogenic edema, immune cell infiltration, leakage of toxic substances into the brain, and hemorrhagic transformation, which worsen ischemic tissue injury. Studies in animal models of stroke suggested that the disruption of BBB showed a biphasic course where the early phase occurred within hours after ischemia, followed by partial closing and then the second delayed phased between 24 and 72 h following ischemia-reperfusion [140–142]. However, recent investigations with tighter experiment design and more advanced dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) casted a controversy on this traditional interpretation demonstrating a continuous disruption of BBB lasting for days to weeks without any closure [143, 144]. These discrepancies may be due to the differences in detecting methods, stroke models, or experimental designs. Interestingly, a clinical study in human acute ischemic stroke using DCE-MRI showed the continually elevated permeability of BBB with the greatest leakage at 6–48 h after ischemic onset [145]. Further studies are still warranted to explore the exact temporal file of BBB disruption in ischemia for better understanding and searching for potential therapeutic time points and targets.

The specialized brain endothelial cells and paracellular junctions including tight junctions and adherens junctions are the foundation and key constituents maintaining the high resistance and impermeability of BBB [139]. In the early phase of ischemia, dysfunction and structural alterations of brain endothelial cells accompanied with redistribution and loosening of paracellular junctions were the early events in BBB leakage [146, 147]. Moreover, ischemia caused the downregulation of tight junction proteins such as occludin and zonula occludens-1 [148], which might be partially mediated by matrix metalloproteinases (MMPs) [147]. Notably, recent studies suggested that the increased transcytosis in endothelial cells occurred before paracellular barrier disruption, which contributed to early compromised

permeability of BBB [146, 149]. Astrocyte end-feet was also found to swell early after ischemia [150]. Pericytes were shown to regulate BBB by modulating endothelial gene expression and astrocyte end-feet polarization [151] and protect the integrity of BBB by stabilizing the tight junction protein and reducing endothelial cell apoptosis in hypoxic insult [152]. Similarly, pericytes were observed to detach from the basement membrane and migrate from microvascular wall early in ischemia, which could augment the leakage of BBB [153]. Importantly, the degradation of basement membrane components by proteases including MMPs released from endothelial cells, astrocytes, pericytes, microglia, and recruited leukocytes played a critical role in BBB disruption; MMP-9 and MMP-2 were the primary contributors in ischemia [154]. Pharmacological inhibition or genetic depletion of MMP-9 exerted a protective role against ischemic injury by reducing BBB damage and brain edema [155, 156]. Vascular endothelial growth factor (VEGF) was also involved in aggravating BBB disruption via modulating MMP-9 activity in early ischemic phase; inhibition of endogenous VEGF in this acute phase reduced BBB permeability [157, 158]. In reperfusion period and late stage of ischemia, ischemia-reperfusion injury and inflammatory response were the key contributors to BBB disruption [159, 160].

4.7 Inflammation in Ischemic Stroke

Numerous studies have demonstrated that inflammatory response was an important component of pathophysiology during ischemic brain injury [160–162]. The investigation on inflammatory response in ischemia is of great value for the development of anti-inflammatory therapy [163, 164]. The most related molecules involved in inflammatory response include cytokines, chemokines, and adhesion molecules. Cytokines are important inflammatory factors in cell signaling in the brain, which include IL-1, IL-2, IL-6, IL-8, IL-10, IL-17, IL-18, TNF-α, and interferon-γ [162, 165]. Chemokines are a family of small cytokines and signaling proteins secreted by cells. Studies showed that CXC, CC subfamilies, and MMPs were involved in ischemic brain injury [166]. Cell adhesion molecules (CAMs) are proteins located on the cell surface mediating cell-to-cell adhesion or cell binding with extracellular matrix, which includes integrins, immunoglobulin superfamily CAMs, cadherins, and selectins [167]. Similarly, it is noted that many brain cells are involved in inflammatory response such as microglia/microphages, astrocytes, endothelial cells, and neurons. Microglia is the most interested cell type due to the different function of microglia subtype [168]. For detailed information on this topic, you can go to Chap. 10 in this book.

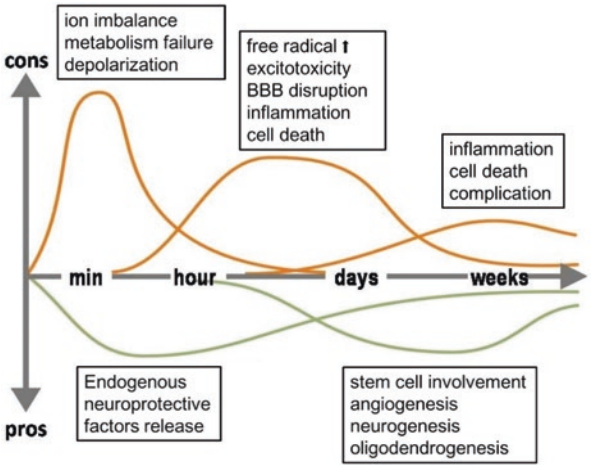

Fig. 4.1 The schematic chart illustrates brief pathophysiological changes after cerebral ischemia

4.8 Summary

The pathophysiological changes after cerebral ischemia are dynamic based on the duration and the severity of ischemic brain injury. On one hand, within minutes to hours, ischemia caused Na+-K+ pump dysfunction, calcium overload, acid-sensing ion channel opening, peri-infarct depolarization, and metabolism failure. From hours to days, free radical and excessive inflammatory factors are released. These harmful substances induced blood-brain barrier disruption and brain edema formation. Consequently, a large number of neuronal cells as well as brain glial cells died. On the other hand, ischemic injury induced endogenous defense system to activate and nerve growth factors to be upregulated. These helpful substances exerted neuroprotection function. In addition, neural stem cells from SVZ and bone marrow-derived mesenchymal stem cells from circulating blood proliferated and migrated toward the ischemic regions, which could then promote angiogenesis, neurogenesis, and oligodendrogenesis to help recovery (see Fig. 4.1).

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Chapter 5
Pathophysiology of Hemorrhagic Stroke

Zhongsong Shi

Abstract Intracerebral hemorrhage (ICH) is a devastating form of cerebrovascular disorder with a high mortality and morbidity. Clinical studies have recently risen to improve outcome for patients with intracerebral hemorrhage. More recent data indicate the mechanisms of brain injury after intracerebral hemorrhage. In this chapter, we focus on the new evidence of inflammation, blood–brain barrier (BBB) dysfunction, and oxidative stress mechanisms for intracerebral hemorrhage-induced brain injury. Several therapeutic targets for intracerebral hemorrhage are now being pursued and will be identified to translate into clinical trials. Thus, understanding the mechanisms underlying intracerebral hemorrhage and identification of novel therapeutic targets play an important role in translational research work in the development of new therapies for intracerebral hemorrhage.

Keywords Blood–brain barrier • Inflammation • Intracerebral hemorrhage • Oxidative stress • Secondary brain injury • Translational research

Abbreviations

AQP4	Aquaporin-4
BBB	Blood–brain barrier
HMGB1	High-mobility group box 1
ICH	Intracerebral hemorrhage
IL	Interleukin
MMP	Matrix metalloproteinase
NOX	NADPH oxidase
ROS	Reactive oxygen species

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TLR	Toll-like receptor
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
ZO-1	Zonula occludens-1

5.1 Introduction

Intracerebral hemorrhage (ICH) is a devastating form of cerebrovascular disorder with a high mortality and morbidity and accounts for 10–15% of all strokes [1–3]. The incidence of ICH is expected to increase due to an increasing elderly population [4]. Clinical studies have recently risen to improve outcome for patients with ICH [5–7]. Several therapeutic targets for ICH are now being pursued and will be identified to translate into clinical trials. Thus, understanding the mechanisms underlying ICH and identification of novel therapeutic targets play an important role in translational research work in the development of new therapies for ICH.

5.2 Brain Injury After ICH

5.2.1 Primary Brain Injury After ICH

An ICH produces physical disruption to the brain architecture within the first few hours after the onset of bleeding. During this initial damage, little can be done to ameliorate the primary brain injury [1]. Nearly one third ICH patients experience hematoma expansion during the first day [8–10]. Hypertension may influence hemorrhage growth and contribute to brain injury [1, 11, 12]. Hemorrhagic volume and hematoma expansion are the key factors of ICH outcome.

5.2.2 Secondary Brain Injury After ICH

Secondary brain injury caused by a cascade of events contributes to neurological deterioration in patients with ICH [13–15]. Blood components, including red blood cells, coagulation factors, complement components, and immunoglobulins, activate cytotoxic, excitotoxic, oxidative, and inflammatory pathways [16, 17]. Thrombin is produced in the brain immediately after ICH as an essential component of the hemostatic cascade. Thrombin influences endothelial cells, astrocytes, neurons, and microglia. Thrombin, hemoglobin, and iron from the hematoma are considered as major contributors to second brain injury following ICH [1, 18–21]. Increasing evidence has shown that many mechanisms are involved in the secondary brain

damage after ICH, such as the development of edema, free radicals, inflammation, and direct cellular toxicity of the degradation by-products of the hematoma. Recently, more attempts to identify novel therapeutic targets have focused on the mechanisms responsible for secondary brain injury following ICH [14, 15, 22, 23].

The mechanisms and preclinical studies of brain injury after ICH have been extensively reviewed [22–28]. In this chapter, we focus on the new evidence of inflammation, blood–brain barrier (BBB) dysfunction, and oxidative stress mechanisms in ICH-induced brain injury.

5.3 Inflammation in ICH

Inflammation plays a critical role in the brain injury following ICH. At the early stage of ICH, inflammatory signaling triggered by hematoma components causes inflammatory cell infiltration, microglial activation, and release of pro-inflammatory mediators, eventually resulting in cell death and brain injury. In the acute phase, inhibition of the inflammatory response after ICH may reduce the brain injury [22, 25, 26].

5.3.1 NLRP3 Inflammasome

The inflammasome plays an important role in the regulation of inflammation as an intracellular multiple protein complex. NLR family, pyrin domain containing three (NLRP3) inflammasomes, a key component of the innate immune system, can sense multiple stimulus, such as microbial invasion and tissue injury. The NLRP3 inflammasome facilitates caspase-1 and interleukin (IL)-1β processing, which amplifies the inflammatory response [29, 30].

After ICH, microglial activation and leukocyte infiltration enhance the production of pro-inflammatory cytokines. IL-1β is considered as a pivotal therapeutic target for ICH. Inhibition of caspase-1, the converting enzyme of active IL-1β, reduced brain injury in the animal model of ICH [25, 26]. NLRP3 inflammasome was activated as early as 3 h following ICH in a mouse model. NLRP3 knockdown reduced brain edema, IL-1b levels, and neutrophil infiltration in the peri-hematoma which improved neurological functions following ICH. Mitochondrial reactive oxygen species (ROS) is a major trigger of NLRP3 inflammasome activation. NLRP3 inflammasome contributes to inflammatory activation after ICH, which increases caspase-1 and IL-1b, and promotes neutrophil infiltration [31]. Cordycepin, a nucleoside derivative extracted from *Cordyceps militaris*, has been shown to possess anti-inflammatory effect. In mice ICH model, cordycepin reduced brain edema and peri-hematoma tissue injury. Besides, it also ameliorated neurological deficits, which is accompanied by the downregulation of NLRP3 inflammasome and a reduction of IL-1β. Cordycepin may confer neuroprotective effect through the suppression of NLRP3 inflammasome activation [32].

To explore the specific microRNA which could regulate the NLRP3 inflammasome after ICH, Yang and colleagues identified microRNA-223 downregulated NLRP3 to inhibit inflammation through caspase-1 and IL-1β, reduce brain edema, and improve neurological functions after ICH. MicroRNA-223 is an important regulator of microglial activation and inflammation after ICH by direct targeting NLRP3 [33]. In addition, neuroinflammation contributes to the pathogenesis of early brain injury after subarachnoid hemorrhage. In a rat model of subarachnoid hemorrhage, inhibition of the P2X7R/NLRP3 inflammasome axis reduced brain edema and improved neurological function by inhibiting caspase-1 activation and subsequent mature IL-1β/IL-18 production. NLRP3 inflammasome also contributes to neuroinflammation and brain injury following subarachnoid hemorrhage [34].

In summary, inhibition of the NLRP3 inflammasome reduced the inflammatory response following ICH, which may represent a novel therapeutic intervention for ICH patients. The roles and mechanisms of NLRP3 inflammasome in ICH will need further study.

5.3.2 Toll-Like Receptors

Toll-like receptors (TLRs), a group of class I transmembrane proteins, recognize distinct pathogen-associated and damage-associated molecular patterns. TLRs play a critical role in innate immunity and inflammatory responses [35]. TLR4 is expressed in neurons, astrocytes, and microglia. TLR4 is activated by many endogenous proteins that act as danger signals in the setting of injury. Many of these endogenous TLR4 ligands are present in the central nervous system after ICH.

TLR4 expression was significantly up-regulated within several hours following ICH in a rat model [36]. TLR4-deficient mice markedly decreased peri-hematoma inflammation, reduced recruitment of neutrophils and monocytes, and improved functional outcome after ICH. In a mouse model of ICH, TLR4 antagonist reduced inflammatory injury and neurological deficits through decreased expression of signaling molecules downstream of TLR4. TLR4 signaling plays a critical role in peri-hematoma inflammation and secondary injury following ICH in the animal model [37, 38]. In patients with ICH, the expression of TLR4 and TLR2 was increased in peripheral monocytes which was associated with a poor clinical outcome. In consistent with animal studies, both TLR4 and TLR2 may contribute to ICH-induced brain injury [39].

In both in vivo and in vitro models of ICH, Wang and colleagues identified that TLR2 formed a heterodimer with TLR4 and mediated ICH-induced inflammatory injury. TLR2/TLR4 heterodimer, induced by hemoglobin, initiates inflammatory injury in ICH [40]. Hemoglobin from the hematoma after ICH induced the formation of TLR2/TLR4 heterodimer and subsequently activated IL-23 secretion by infiltrating macrophages. This macrophage-derived IL-23 increased brain edema and exacerbated the secondary brain injury in a mice ICH model. Sparstolonin B, a

monomer component of Chinese traditional herbs, disrupted the formation of TLR2/TLR4 heterodimer and alleviated secondary brain injury following ICH [15].

TLR4 signaling pathway increased both astrocyte-derived and serum hepcidin, resulting in the inhibition of brain iron efflux into circulation after ICH. Increased hepcidin reduced iron efflux channels via binding to the brain ferroportin of microvascular endothelial cells [41].

Hemorrhagic transformation is the main hemorrhagic complication of revascularization therapies with both intravenous thrombolysis and endovascular thrombectomy for patients with acute ischemic stroke [42, 43]. In mice model, the presence of TLR4 increased hemorrhage severity and bleeding area and exacerbated BBB damage after delayed tPA administration, by increasing matrix metalloproteinase (MMP)-9 expression. TLR4 absence decreased hemorrhagic transformation after delayed tPA administration [44].

In summary, therapeutic inhibition of TLR4 signaling in the acute phase after ICH improves outcome in preclinical studies and may be a potential therapeutic target for patients with ICH. Interfering with the assembly of the TLR2/TLR4 heterodimer may be a novel target for developing effective treatment of ICH. In addition, TLR4 inhibition may be a promising therapeutic target to prevent tPA-induced hemorrhagic transformation and to increase the number of acute ischemic stroke patients that benefit from revascularization therapy.

5.3.3 High-Mobility Group Box 1

High-mobility group box 1 (HMGB1), a typical of the damage-associated molecular pattern family, plays an important pro-inflammatory action once released into the extracellular space from cellular nuclei. The secreted HMGB1 stimulates the receptor for advanced glycation end products and TLR-2 and TLR-4 to trigger the inflammation, which are expressed in peripheral macrophages and microglia as well as neurons in the brain [45, 46].

HMGB1 levels increased in peri-hematoma regions in subacute phase after ICH in animal models [47–49]. The serum levels of HMGB1 dramatically increased in patients with ICH and associated with stroke severity [50]. HMGB1 acts as an early pro-inflammatory cytokine to mediate ICH-induced inflammation injury.

The HMGB1 inhibitors, i.e., ethyl pyruvate and glycyrrhizin, exert their anti-HMGB1 activity and contribute to lessen the ICH-induced inflammatory damage in animal models. Ethyl pyruvate reduces brain edema and secondary injury and improves the functional outcome in the rat ICH model. Ethyl pyruvate exerts anti-inflammatory and anti-apoptotic effects via inhibiting the HMGB1/TLR4/NF-kB pathway and decreasing the release of HMGB1 [51]. Glycyrrhizin reduces brain edema, inhibits neuronal death, and improves neurological functions after ICH in rats by inhibiting the interaction between HMGB1 and its receptor [46].

In rat ICH model, anti-HMGB1 mAb inhibits the release of HMGB1 into the peri-hematoma region, reduces serum HMGB1 levels, decreases brain edema, and ameliorates ICH-induced secondary injury. Anti-HMGB1 mAb decreases the microglia activation and the expression of inflammation-related factors after ICH and protects BBB integrity [52, 53].

In summary, HMGB1 play a critical role in the inflammatory responses following ICH, which subsequently produce the microglial activation, cytokine expression, and BBB disruption. Therapeutic inhibition of HMGB1 by HMGB1 inhibitors or anti-HMGB1 mAb may be a novel therapeutic strategy to reduce secondary brain injury after ICH.

5.4 Blood-Brain Barrier in ICH

The blood–brain barrier (BBB) or neurovascular unit is formed by vascular endothelial cells, basement membrane, pericytes, extracellular matrix, astrocytes, and neurons [54, 55]. The BBB plays an important role in maintaining the microenvironment of the central nervous system [56, 57]. Loss of BBB integrity is a hallmark of ICH-induced brain injury, which results in vasogenic brain edema, the influx of leukocytes and potentially neuroactive compounds into the peri-hematoma brain [58–60]. The extent of BBB breakdown has been directly correlated with prognosis after ICH [61].

5.4.1 BBB Disruption Following ICH

BBB dysfunction following ICH has been investigated both in clinical studies and in animal models. ICH animal models were established by intracerebral injection of blood or collagenase [19]. BBB disruption after ICH can be assessed with multimodal MRI. In collagenase-induced ICH mouse model, vascular permeability measured by Evans blue extravasation on days 1, 3, and 7 correlated with the T1-gadolinium results, both of which peaked on day 3. Peri-hematoma vasogenic edema may be attributable to microglial activation, iron deposition, and BBB breakdown [62]. Acute and delayed vascular rupture occurs in patients with ICH. Contrast agent penetrates the brain in the hyperacute phase after ICH, which is an indicator of BBB damage [63].

5.4.2 Tight Junction Proteins in ICH

Many factors have been implicated in BBB disruption. Tight junction proteins mediate the gate functions of the BBB [64]. Claudin-5, zonula occludens-1 (ZO-1), and occludin are the main constituents of tight junction proteins that maintain BBB

integrity in brain endothelial cells. BBB permeability was severely affected in the claudin-5 knockout mice in MRI analyses [65]. Conversely, up-regulated expression of claudin-5 prevented the development of hemorrhagic transformation and reduced brain edema [66]. Insulin rapidly increased tight junction integrity via the PI-3 K/AKT/GSK-3β signaling in an in vitro model of human BBB [67]. Blocking GSK3β-mediated signaling significantly increased the expression of ZO-1 and occludin, maintaining the integrity of the BBB [68].

Blood occludin level reflects as a potential biomarker for early BBB damage [69]. In rat ICH model, the bone marrow mesenchymal stromal cell improved neurological recovery and lowered brain water content. Marrow mesenchymal stromal cell increased ZO-1 expression and protected BBB by reducing levels of pro-inflammatory factors and inhibiting inducible nitric oxide synthase expression and peroxynitrite formation in peri-hematoma regions [70].

The level of Dickkopf-1 in brain tissue significantly increased after ICH. Small interfering RNA targeting Dickkopf-1 treatment significantly reduced BBB disruption and brain edema, as well as neurological deficits by upregulating the expression of ZO-1 and increasing the transcription of Wnt-1 mRNA [71]. Sphingosine-1-phosphate receptor significantly reduced Evans blue extravasation after ICH and increased expression of occludin and ZO-1 suggesting BBB integrity protection [72]. Inhibition of P2X7 receptor alleviated brain water content and prevented ICH-induced BBB disruption via inhibiting the expressions of occluding and ZO-1 as well as RhoA activity [73]. In addition, nano-curcumi reduced BBB disruption by increasing the expression of ZO-1, occluding, and claudin-5 [74]. So, there is much evidence indicating a deficiency of tight junction proteins results in BBB disruption in ICH.

5.4.3 BBB Disruption and Inflammation Following ICH

Inflammation contributes to the destruction of BBB integrity with activation of microglia and induction of inflammatory cytokines after ICH [26]. TLR4 significantly increased the expression of MMP-9 and involved in hemorrhagic transformation [44]. In TLR2 knockout mice model, the decreased BBB disruption reduced neutrophil infiltration and MMP-9 expression after ICH [75]. Administered intravenously with mesenchymal stem cells reduced the degree of BBB leakage and decreased the levels of IL-1β, IL-6, and tumor necrosis factor-inducible protein (TNF-α) following ICH. The protective effect of mesenchymal stem cells on the BBB integrity in ICH rat model was also associated with the increased expression of tumor necrosis stimulated gene 6 [76].

Advanced glycation end product signaling plays an important role in the integrity of BBB. Advanced glycation end product antagonists reduced BBB disruption and the levels of IL-1β, IL-6, and MMP-9 [77]. Translocator protein 18 kDa ligand improved BBB integrity and diminished cell death after ICH by reducing leukocyte infiltration into the brain and production of IL-6 and TNF-α in microglia [78].

Intravenous injection of anti-HMGB1 mAb resulted in maintaining BBB integrity and reducing brain edema. The neuroprotective effects of anti-HMGB1 mAb were associated with decreased oxidative stress and the expression of IL-1β, IL-6, and TNF-α [52]. CD163-deficient mice have less BBB dysfunction, hematoma volume, and tissue injury in the early phase of ICH [79].

BBB dis-integrity and the extent of brain edema were attenuated in ICH mice with lentivirus encoding microRNA-132 injection. The expression levels of the cytokines IL-6, IL-1β, and TNF-α in the ipsilateral hemisphere were significantly reduced due to the overexpression of microRNA-132. MicroRNA-132 plays an important role in protecting the BBB integrity by alleviating inflammatory reactions after ICH [80].

Apolipoprotein E-mimetic peptide treatment significantly decreased the degradation of tight junction proteins and endothelial cell apoptosis. Apolipoprotein E-mimetic peptide inhibited the pro-inflammatory activators of MMP-9, such as IL-1β, IL-6, and TNF-α, thereby ameliorating BBB disruption [81]. In mice ICH model, the expression of von Willebrand factor (vWF) increased in the plasma and peri-hematoma regions. vWF treatment increased the expression of pro-inflammatory mediators and activation of MMP-9 and intercellular adhesion molecule 1 and decreased pericyte coverage. vWF treatment caused the elevated myeloperoxidase and recruitment of neutrophils and microglia. Inhibiting vWF reduced BBB damage and edema formation and improved neurological function [82].

5.4.4 Endothelial Cells Following ICH

The contraction of stress fibers in the endothelium, one of the components of BBB, leads to the formation of intercellular gaps between endothelial cells and increases the permeability of BBB. ICH induces formation of stress fibers. Inhibition of stress fiber formation preserves BBB, ameliorates brain edema, and improves neurological functions by inhibiting platelet-derived growth factor receptor-β and its downstream in mice after ICH [83].

The up-regulated expression of annexin A1 in brain microvascular endothelial cells prevented BBB disruption following ICH in threonine 24 and serine 27 phosphorylation-dependent manners [84]. In the collagenase-induced ICH model, cilostazol attenuated BBB disruption and inhibited endothelial cell death and the expression of collagen type 4, laminin, and vascular endothelial and N-cadherins [85]. The levels of nuclear β-catenin and claudin-1 expression in brain vascular endothelial cells of ICH patients significantly decreased. Endothelial cell-specific inactivation of β-catenin resulted in BBB breakdown and inhibited the expression of tight junction proteins claudin-1 and claudin-3 in adult brain endothelial cells [86].

5.4.5 *Aquaporin-4 Following ICH*

Aquaporin-4 (AQP4) is predominantly expressed in the astrocyte foot processes surrounding capillaries as one of the main ingredients of BBB [87, 88]. AQP4 plays an important role in maintaining BBB integrity [89, 90]. The expression of AQP4 significantly increases from 3 h after ICH, achieves the top at 2–5 day, and continues at least 2 weeks [91–95]. BBB permeability detected by Evans blue extravasation was aggravated by AQP4 deletion following ICH [91, 96, 97]. The up-regulated expression of AQP4 led to BBB integrity after ICH by morphologically suppressing tight junction opening and endothelial cell swelling [91, 98]. AQP4 knockout reduced expression of tight junction proteins including occludin, ZO-1, and claudin-5 through activation of JNK and p38-MAPK pathways [98]. Cerebrolysin reduced the ipsilateral brain edema and Evans blue dye extravasation in ICH via decreasing the expression of AQP4 and increasing a higher expression of tight junction proteins [99].

5.4.6 *Other Mediators Following ICH*

In addition, other mediators implicated in regulating the function of BBB after ICH have been recently revealed in the animal models. Thrombin, an essential component in the coagulation cascade, involves in the pathogenesis of ICH through BBB disruption. Thrombin stimulated protease-activated receptor 1 on brain pericytes to induce BBB damage by releasing MMP-9. Brain pericytes and their specific signaling pathways provide novel therapeutic targets for ICH induced by thrombin [100]. Heme oxygenase-1, an antioxidant protein, is induced by oxidative stress via Nrf2-regulated transcriptional activation. Overexpressing heme oxygenase-1 in astrocytes preserves BBB in mice ICH model [101]. The metalloprotease ADAMTS 13 treatment significantly reduced microglia activation and neutrophil recruitment following ICH. The anti-inflammatory effect of recombinant ADAMTS 13 was accompanied by promoted pericyte coverage of brain microvessels and reduced BBB permeability [102].

In summary, many factors can alter BBB disruption after ICH, and there has been a progress in elucidating the mechanisms that underlie BBB breakdown at the molecular, cellular, and tissue levels.

5.5 Oxidative Stress and ICH

Oxidative stress is defined as a condition in which prooxidant-antioxidant balance is disturbed in the cell; cellular biomolecules undergo severe oxidative damage, ultimately leading to cell viability [103]. Oxidative stress is known for the

association with neurodegenerative diseases of the central nervous system and stroke [104, 105]. Three major types of reactive oxygen species (ROS), including the superoxide radical ($O_2^{\bullet -}$), the hydroxyl radical (•OH), and the hydrogen peroxide (H_2O_2), have been reported [106, 107]. Reactive nitrogen species (RNS) are another major type of free radicals, which mainly consist of nitric oxide and its derivatives. Nitric oxide is produced in various cells by three isoforms of nitric oxide synthase, including endothelial nitric oxide synthase, neuronal nitric oxide synthase, and inducible nitric oxide synthase [108–110]. Increasing evidence demonstrates that oxidative stress plays a prominent role in secondary brain injury after ICH [17, 111]. A variety of pathways can be responsible for the generation of free radicals after ICH, of which there are two major pathways: firstly, hemoglobin metabolic products such as iron, heme, and hemoglobin [112, 113], and secondly, inflammatory cells, such as microglia and neutrophils, that can generate free radicals [114].

5.5.1 Oxidative Stress and Inflammation Following ICH

Oxidative stress and inflammation are closely related. Neuroinflammation is induced by intracerebral blood including the activation of resident microglia, extracellular proteases, and ROS [115]. ROS can evoke the expression of acute pro-inflammatory cytokines such as TNF-α and IL-10, and an optimal balance between them is of crucial importance in mitigating both inflammation and oxidative stress. Similarly, pro-inflammatory cytokines can promote the production of ROS [116]. In a collagenase-induced ICH model, the HMGB1, an important pro-inflammatory molecule, was linked to the microglial activation, cytokine expression, and BBB disruption. Anti-HMGB1 mAb reduced the oxidative stress and the release of inflammatory molecule, improving the behavioral performance of rats [52]. In the case of ICH, released hemoglobin from ruptured erythrocytes and subsequent by-products including ROS and RNS is dramatically increased [117, 118]. Oxidative stress or superoxide triggers activation of MMP-9 after intracerebral injection of hemoglobin into rat striatum [117]. Ding et al. suggested that ONOO− has a vital role in hemoglobin-induced neurovascular dysfunction and subsequent cell death as well as the neurological deficits. What's more, their results indicated that scavenging ONOO− by FeTPPS, a 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrin iron III chloride peroxynitrite decomposition catalyst, can attenuate the pathophysiological process through inhibiting the activation of MMP-9. Reducing ONOO− accumulation may be a new therapeutic target for secondary injury after ICH [119].

Additional studies have shown that prostaglandin mediated inflammatory mechanisms and involved in secondary brain damage after ICH. Prostaglandin E2 receptor 1 expressed primarily in neurons and axons but not in astrocytes and microglia after ICH induced by collagenase. In middle-aged male mice subjected to collagenase-induced ICH, inhibition of prostaglandin E2 receptor 1 mitigated brain injury, brain edema, cell death, neuronal degeneration, neuroinflammation, and neu-

robehavioral deficits, whereas its activation exacerbated these outcomes. The inhibition effect of prostaglandin E2 receptor 1 is mainly through the Scr kinases and MMP-9 signaling pathway, thus attenuating oxidative stress and white matter damage [120].

In summary, oxidative stress, inflammatory damage, iron overload, and cytotoxic injury form a complex cascade of reactions, in which oxidative stress and inflammation may play major roles in secondary brain injury after ICH. However, the relationship between oxidative stress and inflammation is complicated and needs further study.

5.5.2 Oxidative Stress and Apoptosis Following ICH

Apoptosis is observed in various neurological and neurodegenerative disorders, such as Alzheimer's, ischemic stroke, and ICH [121]. The processes leading to neuronal apoptosis include lack of neurotrophic factor, over-activation of excitatory neurotransmitter glutamate receptors, and an increased oxidative stress. In fact, the brain is quite sensitive to oxidative stress attributing to three major causes, such as the highest metabolic rate than any other organs of the body, high levels of iron, and an increased amount of unsaturated fatty acids [122]. ROS cause neuronal apoptosis through a variety of pathways. Overloaded free radicals can induce the peroxidation of lipid, nucleic acid, and protein by means of direct and indirect pathways, as a result of apoptosis [123]. Hydrogen peroxide and nitric oxide can act synergistically to induce neuronal death through apoptosis, in which activation of p38 MAPK and caspase-3 is involved [124, 125]. Additionally, nitric oxide synthase activation and peroxynitrite ion overproduction can induce apoptosis of hippocampal and dopamine neurons [126]. Hydrogen peroxide can induce apoptosis through disrupting mitochondrial function and promoting pro-apoptosis gene expression [127, 128]. Lu et al. found that rats pretreated with pyrroloquinoline quinone, an amine oxidase, effectively improved the locomotor functions, alleviated the hematoma volumes, and reduced the expansion of brain edema after ICH. Moreover, activated caspase-3, the apoptotic executor, showed coincident alleviation in pyrroloquinoline quinone-treated rats after ICH [129].

In summary, the intrinsic and extrinsic pathways of apoptosis are interactional of each other; some of the factors in both types of pathway may have a synergistic effect in regulating the apoptosis process, activated by a single stimulator.

5.5.3 Oxidative Stress and NADPH Oxidase Following ICH

NADPH oxidase (NOX) is a multi-subunit enzyme complex, which is mainly composed of five subunits, including gp91phox and gp22phox subunit in the plasma membrane and p47phox, p67phox, and p40phox subunits in the cytoplasm [130].

NADPH oxidases are major sources involved in the production of ROS in the vasculature [131]. Numerous evidences have demonstrated that ROS are essential in the pathogenesis of ICH [132]. There are seven NOX isoforms, including NOX1, NOX2, NOX3, NOX4, NOX5, and dual oxidases 1 and 2. All of them have been identified, and NOX2 (gp91phox) is abundant in the brain [133]. Oxidative stress origin from activation of NOX2 contributes to the severity of ICH and causes brain injury in gp91phox knockout mice model. The gp91phox knockout mice with ICH model showed lower levels of oxidative product, brain water content, ICH volume, and neurological deficit [134]. Yang et al. found that NOX2 was up-regulated in the ICH animal model. C1q/tumor necrosis factor-related proteins reduced NOX2 expression and decreased oxidative stress, improved functional outcomes, and reduced brain edema [135]. In patients with ICH, the A930G polymorphism of the p22phox subunit of NADPH oxidase gene affects the susceptibility to ICH, and certain haplotypes of the gene may be associated with a higher susceptibility to ICH [136].

Severe hemorrhagic transformation after mechanical thrombectomy predicts poor clinical outcome in acute ischemic stroke. To better understand the mechanism of hemorrhagic transformation, we recently investigated the role of NOX in hemorrhagic transformation after reperfusion during acute ischemic stroke and whether NOX inhibitor VAS2870 reduces reperfusion-induced hemorrhagic transformation after mechanical recanalization. Hemorrhagic transformation and NOX2 and NOX4 up-regulation were observed in hyperglycemic rats after cerebral ischemia reperfusion. VAS2870 reduced infarct volume, brain edema, BBB breakdown, and reperfusion-induced hemorrhagic transformation after cerebral ischemia, resulting in improved neurological outcome and reduced mortality. VAS2870 exerted its protective effect via suppressing oxidative stress, neuronal apoptosis, and NOX2/NOX4 induction. Altered levels of microRNA-29a, microRNA-29c, microRNA-126a, and microRNA-132 were identified after VAS2870 treatment suggesting their regulatory roles in hemorrhagic transformation after mechanical reperfusion [43, 137].

Our findings suggest that NOX2 and NOX4 could exacerbate hemorrhagic transformation in stroke rats after mechanical reperfusion. Infusion of NOX inhibitor VAS2870 before mechanical thrombectomy represents a novel adjunctive therapeutic strategy to prevent reperfusion-induced hemorrhagic transformation and improve outcome of acute stroke treatment. Several microRNAs potentially targeting NOX2 and NOX4 genes displayed altered levels in hyperglycemic rats with cerebral ischemia reperfusion, suggesting their regulatory roles and targeting potentials for acute ischemic stroke treatment. Targeting specific microRNAs may represent a novel intervention opportunity to improve outcome and reduce hemorrhagic transformation after mechanical reperfusion for acute ischemic stroke.

In summary, oxidative stress can cause brain damage after stroke and NADPH oxidases are the important source of ROS. The expression of several NOX subtypes significantly increases in injured brain tissue after ICH. Inhibition of NADPH oxidases, especially NOX2 and NOX4, reduces brain tissue damage and improves neu-

rological outcome following ICH. Therefore, the development of novel drugs focusing on the NOX enzymes might be meaningful for patients with ICH.

5.6 Conclusions

New evidence of inflammation, BBB dysfunction, and oxidative stress mechanisms for ICH-induced brain injury has been shown in the recent studies. Some molecules and signaling pathways are attractive as potential therapeutic targets to ameliorate the ICH-induced brain injury. These new mechanisms involved in ICH pathogenesis need to be further researched for the identification of novel therapeutic targets and promising therapeutic approaches to patients with ICH.

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Chapter 6
Brain Imaging for Stroke

Jin Cao and Min Lou

Abstract Brain imaging (neuroradiology) is a subspecialty of radiology. Benefiting from the newly emerging radiological techniques, neuroradiology has greatly broadened our understanding on diagnosis, characterization, and mechanism of central nervous diseases, especially stroke. Brain reperfusion therapy is the most convincing treatment in acute ischemic stroke. It is of great importance to prudently select patients potentially benefiting from reperfusion therapy and minimize reperfusion injuries, not only in clinical practice but also in demanding insights from basic research. Neuroradiology is thereby a bridge translating basic research results to clinical applications, and clinical neuroradiological findings could in turn help to apprehend underlying disease mechanism.

In this chapter, we are going to discuss the main neuroradiological advances on translational research in stroke. Firstly, we introduce basic radiological methods and techniques for stroke research briefly. Then, we discuss the core concept in stroke imaging: penumbra and collateral vessel imaging, which are important characteristics in patient selection for reperfusion therapy. Finally, we talk about the reperfusion injury and introduce some useful radiological markers.

Keywords Blood-brain barrier • Cerebral blood flow • Collateral vessel imaging • Computed tomography • Diffusion • Functional imaging • Glucose metabolism • Hemorrhagic transformation • Magnetic resonance imaging • Neuroradiology • No-flow effect • Penumbra • Perfusion • Positron emission tomography • Postischemic hyper-perfusion • Probe • Reperfusion injury • Structural imaging • Tracer

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Abbreviations

ADC	Apparent diffusion coefficient
AIF	Arterial input function
AIS	Acute ischemic stroke
ASITN/SIR	American Society of Interventional and Therapeutic Neuroradiology/ Society of Interventional Radiology
ASL	Arterial spin labeling
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
CAT	Computerized axial tomography
CBF	Cerebral blood flow
CBV	Cerebral blood volume
CMBG	Cerebral metabolic rate of glucose
$CMRO_2$	Cerebral metabolic rate of oxygen
CPP	Cerebral perfusion pressure
CT	Computed tomography
CTA	CT angiography
CTP	CT perfusion
CTV	CT venography
DCE	Dynamic contrast enhanced
DEFUSE	Diffusion and Perfusion Imaging Evaluation for Understanding Stroke Evolution
DEFUSE2	Diffusion and Perfusion Imaging Evaluation for Understanding Stroke Evolution 2
DSA	Digital subtraction angiography
DSC	Dynamic susceptibility contrast
DTI	Diffusion tensor imaging
DWI	Diffusion-weighted imaging
ECASS-4	European Cooperative Acute Stroke Study-4
EEG	Electroencephalogram
EPITHET	Echoplanar Imaging Thrombolytic Evaluation Trial
FDG	Fluorodeoxyglucose
FLAIR	Fluid-attenuated inversion recovery
GRE	Gradient echo
HARDI	High-angular resolution diffusion imaging
HARM	Hyper-intense acute reperfusion marker
HI	Hemorrhagic infarction
HT	Hemorrhagic transformation
HU	Hounsfield unit
ICP	Intracranial pressure
MAP	Mean arterial pressure
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion

MMP	Matrix metalloproteinase
MR	Magnetic resonance
MRA	MR angiography
MRI	Magnetic resonance imaging
MRP	MR perfusion
MRS	MR spectroscopy
MRV	MR venography
MTT	Mean transit time
NAA	N-Acetylaspartate
NCCT	Non-contrast CT
NIHSS	National Institutes of Health Stroke Scale
OEF	Oxygen extraction fraction
PET	Positron emission tomography
PH	Parenchymal hemorrhage
PWI	Perfusion-weighted imaging
QMRA	Quantitative MRA
rt-PA	Recombinant tissue plasminogen activator
SPECT	Single-photon emission computed tomography
SWI	Susceptibility weighted imaging
TOF	Time of flight
TTP	Time to peak
VLCBV	Very low CBV

6.1 Radiological Methods and Techniques for Stroke Research

Modern radiological techniques to visualize brain can be categorized as structural imaging and functional imaging. The former visualizes the brain structures obviously, while the latter can provide brain physiological and pathological information.

6.1.1 Structure Imaging

Structural imaging can show the normal cranial structure such as the bone, the meninges, the brain tissue, the vessels, calcification, and the cerebrospinal fluids, as well as abnormal structures like foreign matters, hemorrhage, edema, tumors, vascular malformations, etc. We can use *contrasts* to enhance the visualization of structures.

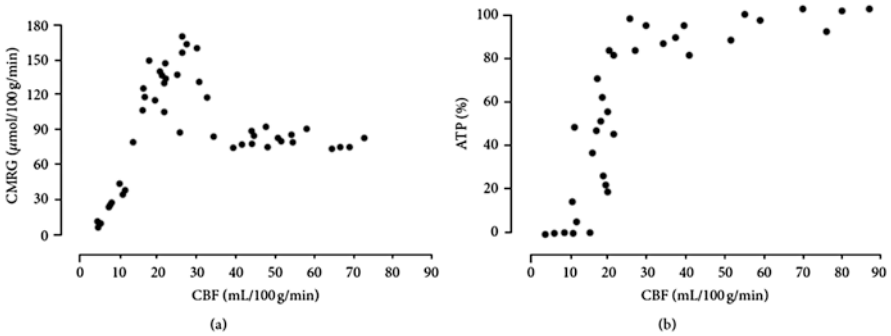

Fig. 6.1 (**a**) The association of cerebral blood flow (CBF) with cerebral metabolic rate of glucose (CMRG) and (**b**) ATP content

The most commonly used techniques are computed tomography (CT, also called computerized axial tomography, CAT) and magnetic resonance imaging (MRI or MR).

6.1.2 Functional Imaging

Functional imaging, or physiological imaging, can detect or measure the changes in blood flow, metabolism, and regional chemical composition. It is mainly based on tracing specific molecules like water or tracer, to reveal the physiological and pathological process in the brain. *Tracers* and *probes* are artificial compounds that can be easily visualized in radiological techniques and are natural substance analogs to mimic metabolism. Functional imaging and structural imaging are often overlapped.

6.1.2.1 Glucose Metabolism

Glucose metabolism is important for the nervous system, as neurons mostly use glucose as fuel and have to maintain high degree of ion channel activities for resting and action potential. Radiologically, we can quantitatively measure the amount of glucose entering neurons by positron emission tomography (PET) using glucose analog ^{18}F-fluorodeoxyglucose (FDG) as tracer. During acute phase of ischemic stroke, cerebral blood supply decreased. FDG-PET demonstrates (Fig. 6.1) that along with the decrease of cerebral blood flow (CBF), cerebral metabolic rate of glucose (CMBG) first goes up and exceeds the normal level, and the ATP concentration is compensated. As the CBF continues to decrease, CMBG decreases, and neurons would destined to die. Some studies have found that the CMBG exceeding area corresponds with CT perfusion (CTP)/MR perfusion (MRP) penumbra area, and the

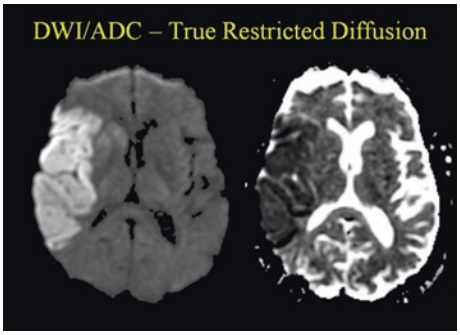

Fig. 6.2 MRI from a patient with acute ischemic stroke. *Left*: diffusion-weighted imaging (DWI) image shows hyper-intensity (diffusion restricted) area in the right cerebral middle artery territory, *right*: apparent diffusion coefficient (ADC) image shows hypo-intensity in the same area

decreased CMBG area is corresponding with the core area. (For penumbra and core concept, see Sect. 6.2 in this chapter.)

The potential cellular mechanisms accounting for increased FDG uptake in the peri-infarct areas are activation of glucose transporters, hexokinase, and neuroinflammation.

Spectrum MRI is another method to reveal lactate in a specific area and the level of lactate is increased during ischemia.

6.1.2.2 Diffusion

Diffusion-weighted magnetic resonance imaging (MRI-DWI), firstly described by Le Bihan in 1985, is a radiological technique to display tissue water movement [1]. In free space, water molecule movement is a Brownian motion, which travels randomly to any direction. However, in biological tissue, water molecule movement is not truly random due to hindering of membranes, macromolecules, cytoskeleton, and other obstacles. Tissue cellularity and cellular swelling may be the underlying pathology used for DWI. During acute phase of ischemic stroke, cytotoxic edema restrained the pathway for water movement, which is called restricted diffusion. However, during subacute or chronic phase, brain cell structures are destroyed and water moves freely again or even faster than normal tissue, leading to normal diffusion. Thus, DWI can clearly demonstrate infarct area during hyperacute stage within less than 2 h from symptom onset and easily differentiate them from chronic lesions. It should be noted that other pathological processes could also present restricted diffusion, like cellularity in lymphoma and abscess, hematoma, and other causes of cell necrosis such as infections, tumors, and acute demyelination. Apparent diffusion coefficient (ADC) is a measure of the magnitude of diffusion within tissue and is commonly calculated using DWI in clinical settings. ADC value is thought to have less artifacts than DWI (Fig. 6.2).

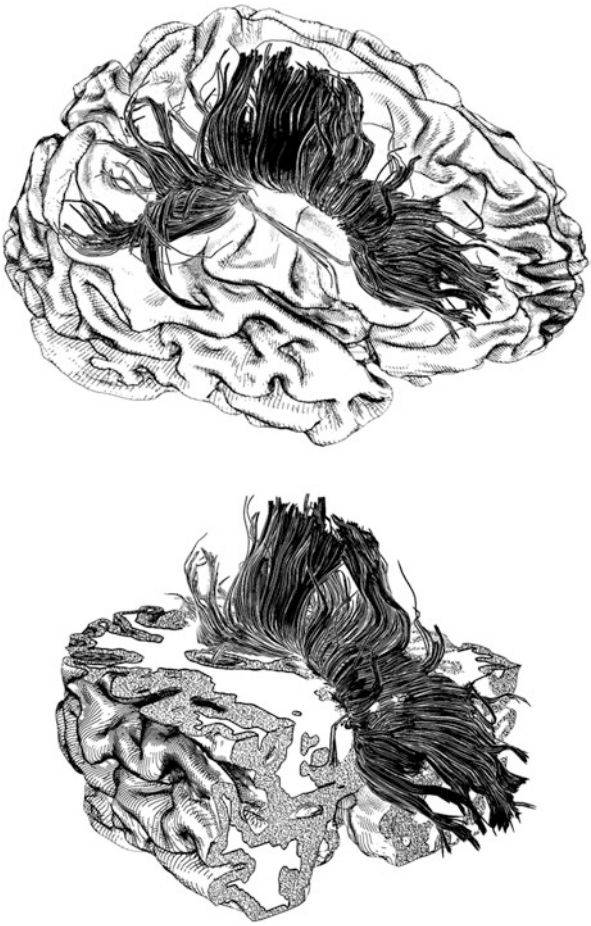

Fig. 6.3 Typical MRI diffusion tensor imaging (DTI) maps show white matter fibers

Water molecule diffusion direction is another prospect. In white matter, axons are aligned mainly in the same direction within the very tiny area. As a result, water movement is anisotropic, which means to be restricted to one direction. When axon structure is impaired, the orientation of this arrangement would be destroyed. Diffusion tensor imaging (DTI) is such an imaging technique to monitor the rate of diffusion and a preferred direction of diffusion and thus reveals lesions apparently by visualizing the whole neural pathway. However, it was recently estimated that more than 30% of the voxels had at least two directions because neuronal fiber tracts converged together leading to a flaw of this technique. David Tuch was the first to describe a complicated working solution called high-angular resolution diffusion imaging (HARDI) [2]. However, it takes lots of time for computation, making it hard to implement clinically. Q-Ball method may be an alternative in the future, which is simple and helps to investigate newly developing neuronal fiber tracts during early recovery of stroke, as their directions are in chaos [3] (Fig. 6.3).

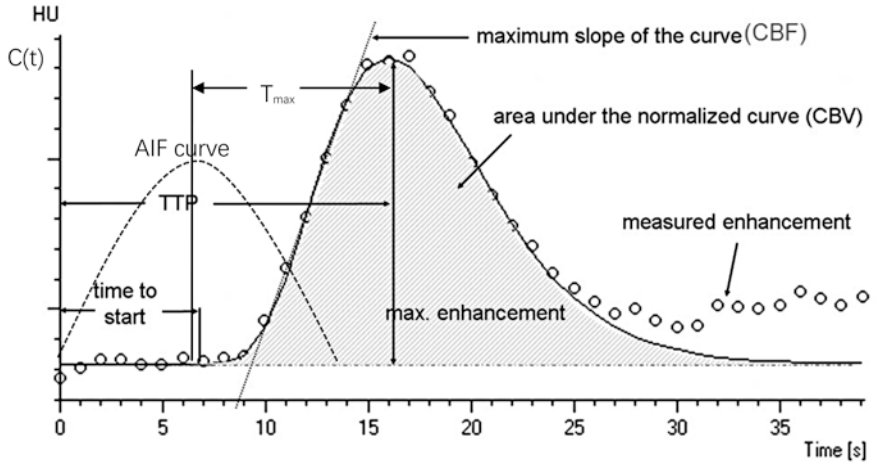

Fig. 6.4 Curves illustrating contrast concentration in CT perfusion to explain the methodology of perfusion image. The horizontal coordination represents the time from contrast injection to enhancement of arteries and brain tissue, while the vertical coordination represents the CT density which is proportion to contrast concentration. The *left dotted curve* represents the artery concentration curve, and the *right real line* represents the brain tissue concentration curve. Perfusion dynamic parameters could be calculated from the curves. *TTP* time to peak, *HU* Hounsfield unit

6.1.2.3 Perfusion

Perfusion is defined as the passage of fluid through the blood vessels to an organ or a tissue, and perfusion scan is the process to observe, record, and quantify this perfusion, which provides us hemodynamic information. It can tell us how blood enters, infiltrates, and leaves a specific tissue, which are greatly useful in stroke research. Perfusion methods including CT perfusion, MR perfusion, MR arterial spin labeling (ASL), and single-photon emission computed tomography (SPECT) are discussed later in detail.

A few hemodynamic parameters could describe brain perfusion (Fig. 6.4). Here we use contrast tracer method as an example. When a bolus of contrast tracer is injected to the vessel, tracer bolus appears in the input artery at first. The contrast concentration in the input artery is defined as arterial input function, abbreviated as AIF or AIF(t). The contrast bolus is then distributed in the given brain tissue and eventually leaves. The tissue contrast agent concentration is called C(t). Cerebral blood volume (CBV) is measured as milliliters of blood per 100 g of brain tissue (ml/100 g), which reflects the viability of brain tissue (see in Sect. 6.2.1). Cerebral blood flow (CBF) is the blood volume flowing through a given mass of brain in a certain time (ml/100 g/min) and can be interpreted as the maximum slope of the C(t) curve. Mean transit time (MTT) is the time from wash in and out of blood. It can be calculated according to the central volume principle as MTT = CBV/CBF. Time to peak (TTP) is the time to peak of the curve. Tmax is defined as the time from AIF peak to C(t) peak or time to reach the maximum of tissue residue function, which is

calculated and called deconvolution. TTP reflects the time it takes until the bolus of blood reaches the tissue, while Tmax represents the time blood flows from input artery to tissue. Both the two parameters are markers for cerebral ischemia.

6.1.2.4 Blood-Brain Barrier Permeability

Blood-brain barrier (BBB) is a complex structural and functional barrier that regulates the brain's extracellular environment, maintains stable concentrations of ions, controls the transport of required nutrients (e.g., glucose and amino acids), and keeps macromolecules and neurotoxins from entering the brain [4].

6.1.3 Radiological Methods

6.1.3.1 CT Scan

CT scan is based on computer processing of X-ray images taken from different angles, in order to produce cross-sectional images of a scanned object and unveil the inner structure of the object. The density of CT image is called radiodensity, which is referred to as the relative inability of X-ray to pass through a particular material using the Hounsfield unit (HU) scale. In approximation, the denser the material is, the whiter it appears on the scan. CT scan is well available and fast, performed in just 10–30 s. CT is good at viewing high-density materials, such as bones, calcification, hemorrhage, and metals. The disadvantages of CT include unsatisfied display of posterior fossa structures and relatively lower resolution than MRI.

Computed tomography angiography (CT angiography or CTA) is a CT technique used to display arterial and venous vessels, while CTV specifically refers to the technique for venous visualization. CTA can reveal the morphology of the inner lumen of a vessel; thus, it is a good tool for arterial stenosis and occlusion. It can also show the relationship between vessels and bones, which helps to localize the lesions. But CTA cannot tell the velocity and direction of blood flow.

CT perfusion (CTP) is an updated version of CTA. It cannot only obtain all brain perfusion parameters but also trace the contrast flow in and out the tissue. It can obtain the time information of blood flow with repeated scans in multiple phases. After processing with specialized software, we could even see the blood flow dynamically like a video from any angles, which is also called four-dimensional CTA.

Table 6.1 Summary of useful neuroradiological methods and techniques

Methods	Techniques	Usages
CT	CT	Cranial structure and lesions
	CTA	Vessels structure
	CTP	Brain perfusion, blood flow, and vessel structure
MRI	MRI	Cranial structure and lesions
	MRI-DWI	Cytotoxic edema, especially infarction
	MRI-DTI	Neuronal fiber tracts integrity
	MRI-PWI	Brain perfusion
	MRI-spectrum	Some specific molecules like lactate
DSA	Digital subtracted angiography	Brain perfusion, blood flow, and vessel structure
SPECT	SPECT brain perfusion	Brain perfusion
PET	PET-FDG	Brain metabolism, especially infarction

6.1.3.2 MRI

MRI technique is far more absurd than CT, but can provide a vast amount of detailed tissue information for stroke assessment. Basic MRI includes T1-weighted and T2-weighted sequences. T1-weighted sequence is good at displaying normal structures, while T2-weighted sequence is good at displaying water-rich lesions, such as infarction, edema, demyelination, and tumors. Fluid-attenuated inversion recovery (FLAIR) sequence provides almost the same images as T2-weighted sequence, except the suppression of free water. MR perfusion-weighted image (PWI) or MRP is similar as CTP in CT scan. Two main MRP approaches have been developed: those with and without the use of exogenous contrast agent. The first technique includes dynamic susceptibility contrast (DSC) MRI and dynamic contrast-enhanced (DCE) MRI, and the second is called arterial-spin labeling (ASL). ASL is completely noninvasive as it needs no exogenous contrast but magnetically labeled as arterial blood water itself. MR spectroscopy (MRS) provides measurement of brain chemistry and is best suitable for some molecule detection, such as N-acetylaspartate (NAA) and lactate, which can help to observe neuronal function and hypoxia in identified regions. MR angiography (MRA) is MR methods to display brain arteries including time-of-flight (TOF) method and contrast-enhanced method. A new technique called quantitative MRA (QMRA) can also measure the blood flow velocity. Venous can be visualized using MR venography (MRV). Because MRI is based on electromagnetic character of objects, it is particularly good at displaying substances with iron. Gradient echo (GRE) sequences and susceptibility-weighted imaging (SWI) are sensitive techniques for venous blood, hemorrhage, and iron storage (Table 6.1).

6.2 Penumbra and Collateral Vessel Imaging

6.2.1 Pathology of Penumbra

6.2.1.1 Cortical Neuronal Function and Survival Are Related with CBF

It is obvious that blood flow is important for neuronal function and viability. Decreased blood flow, namely, oligemia, causes decreased oxygen and nutrients delivered to the brain tissue, contributing to brain dysfunction or even irreversible damage. Researchers figured out their relationship in quantity mainly through primate studies and human carotid endarterectomy (a surgical procedure for carotid stenosis). During this operation, the carotid artery needed to be clamped, which causes a temporary decline of blood flow in the ipsilateral brain cortex. Electroencephalogram (EEG) is a good and noninvasive tool to investigate the cortical neuronal function. In 1973, Frank W. Sharbrough et al. found that there was no EEG change with cerebral flow above 30 ml/100 g/min but invariably occurred with a flow below 17 ml/100 g/min using continuous EEG detecting in carotid endarterectomy [5].

Another series of experiments conducted by Neil M. Branston et al. from 1974 to 1977 showed that cortical evoked potential was inversely related with local cortical flow following acute middle cerebral artery occlusion in the baboon [6]. They measured the somatosensory evoked potential at various sites on the exposed postcentral gyrus of the anesthetized baboon, and cortical blood flow was assessed in the region of the evoked potential electrode by the highly focal method of hydrogen clearance. Following occlusion of the artery, the amplitude of the evoked potential typically diminished steadily at a rate depending on the level of residual local blood flow. This strongly suggested a threshold-type relationship between the amplitude of the evoked potential and the local blood flow: If the blood flow was above ~16 ml/100 g/min, the evoked potential was not affected; while less than ~12 ml/100 g/min, the evoked potential was abolished. They had done a subsequent experiment showing that the cortical evoked potential could be recovered after reperfusion [7].

In 1977, Jens Astrup, along with Neil M. Branston, Lindsay Symon, *and* Niels A. Lassen, published an important paper in modern stroke research, finding brain area mismatch of electrical dysfunction and ischemia [8]. They used microelectrode techniques to measure blood flow, extracellular activity of K^+ and H^+, as well as evoked potential in the baboon neocortex during middle cerebral artery occlusion. Extracellular K^+ was thought to represent the neuronal membrane Na^+-K^+ pump activity. When the cellular ATP was depleted, the Na + -K+ pump activity fails inducing extracellular K+ to raise. The experiment showed that after the onset of focal ischemia, electrical activity ceased but no K+ increased, suggesting that neurons in this area were not yet dead. In central areas of the stroke, blood flow deficits were severe and cells died rapidly. But in peripheral areas of the stroke, blood flow deficits were mild and the neuronal ability to firing action potential was lost, while

Fig. 6.5 Collateral arteries between extracranial and intracranial vessels, shown are (*a*) facial artery, (*b*) maxillary artery, middle meningeal to (*c*) ophthalmic artery and (*d*) dural anastomoses, and occipital artery through the (*e*) mastoid foramen and (*f*) parietal foramen

they still retained enough energy to sustain resting membrane potentials. When the researchers raised the systemic blood pressure and improved collateral blood flow, these areas recovered and action potentials were transiently restored. But after long-time ischemia, these neurons died. They called this region penumbra, a presumably astronomical term indicating the areas of half-light and half-shadow in eclipse. The center of the penumbra region, where cells were inevitably destined to die, was called the core. Penumbra region cells are alive and could return to normal after reflow makes reperfusion therapy a potential strategy for acute ischemic stroke diagnosis.

6.2.1.2 Collateral Circulation

Collateral vessels are assessor vessel networks that stabilize cerebral blood flow when principle conduits fail. Arterial insufficiency, no matter due to thromboembolism, hemodynamic compromise, or combination, could lead to recruitment of collateral vessels. Collateral circulations are variable among ethnics, ages, and individual health conditions like smoking, diabetes, and hypertension. Collateral circulations are different in each brain region, as there are more collateral vessels in the cortex and scarce than that in deep regions like basal ganglia. It is believed that penumbra zone is supplied with blood from collateral arteries anastomosing with branches of the occluded vascular tree. However, cells in this region will die if reperfusion is not established during early hours since collateral circulation is inadequate to maintain the neuronal demand for oxygen and glucose indefinitely.

The arterial source of collateral vessels includes extracranial sources and intracranial sources (see Figs. 6.5 and 6.6). Extracranial sources are facial, maxillary and middle meningeal arteries to the ophthalmic artery, dural anterior anastomoses from

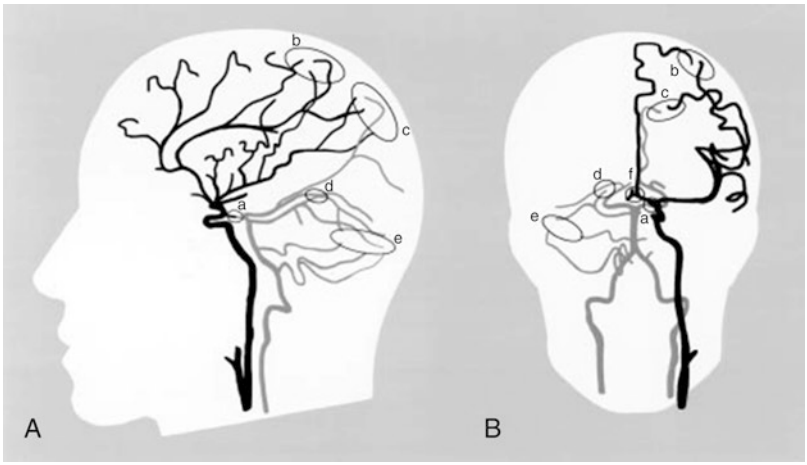

Fig. 6.6 Intracranial arterial collateral circulation in lateral (A) and frontal (B) views. Shown are (*a*) posterior communicating artery, leptomeningeal anastomoses (*b*) between anterior and middle cerebral arteries and (*c*) between posterior and middle cerebral arteries, tectal plexus (*d*) between posterior cerebral and superior cerebellar arteries, anastomoses of (*e*) distal cerebellar arteries, and (*f*) anterior communicating artery

the middle meningeal artery, and occipital artery to the temporal region and posterior fossa. Intracranial sources are cerebral intrinsic arteries discussed in detail later. Based on their diameter and function, collateral arteries are commonly subdivided into primary, secondary, and tertiary collateral pathways. Primary collaterals include the segments of the circle of Willis, that is, the bilateral proximal anterior cerebral arteries, anterior communicating artery, bilateral posterior communicating arteries, and bilateral proximal posterior cerebral arteries, where major cerebral arteries meet together and share blood flow from each hemisphere and between the anterior and posterior circulation arteries. The circle of Willis is frequently asymmetric, and an ideal configuration is seen only in a minority of cases. Up to 30% of people are absent or hypoplasic of either posterior communicating artery. Secondary lateral vessels include anastomoses between distal segments of the major cerebral arteries. Leptomeningeal and dural arteriolar anastomosing with cortical vessels also belongs to secondary lateral vessels. Tertiary collateral vessels are newly generated vessels at the periphery of ischemic region after stroke onset. Because angiogenesis is not a rapid process, only the primary and secondary collateral arteries contribute to the formation of penumbra.

When a major cerebral artery blood flow decreases, primary collaterals provide immediate diversion of cerebral blood flow to ischemic regions through the preexisting anastomoses. Secondary collaterals may anatomically be present, but time is required to develop blood flow. The opening of collaterals depends on hemodynamic, metabolic, and neural mechanisms. Hypertension decelerates the development of collaterals in rat experiment.

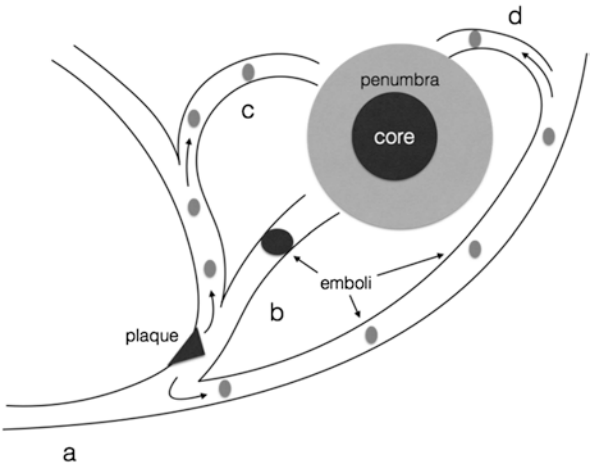

Fig. 6.7 Illustration to demonstrate one hypothesis of collaterals failure. *Black triangle* represents an unstable plaque located at the distal portion of a trunk artery (*a*), one big embolus detaches from the plaque which occludes at the artery (*b*) and causes a core and penumbra illustrated as *black* and *gray* concentric rounds, respectively. More emboli detach from the plaque, travel to collateral artery (*c*), and (*d*), and occlude them. Finally cause collaterals failure and penumbra dying

Table 6.2 Summary of minimal functional and survival cerebral blood flow thresholds from human and animal experiments

Experiment	Object	Method	Change threshold (ml/100 g/min)	Abolished threshold (ml/100 g/min)
Carotid endarterectomy	Human	EEG	17	NA
Middle cerebral artery occlusion	Baboon	Cortical evoked potential	16	12

The development of collaterals does not guarantee their persistence, which is called collateral vessel failure (Fig. 6.7). Hemodynamic fluctuations may influence the endurance of collaterals. Moreover, thrombus in the proximal arteries may also go anterograde or retrograde to distal narrow collateral branches. Given an example in the figure, an arterial plaque is located on the vessel (a) and a large fragment of the plaque occludes the vessel (b), causing an ischemic area with core and penumbra. The penumbra is maintained by collateral vessel (c) and (d). Fragments may also be detached and flow anterograde to vessel (c) and retrograde to vessel (d) making these collateral vessels fail to maintain, and the penumbra would then turn to infarction.

Chronic or recurrent hypo-perfusion may promote collateral development, such as primary and secondary collateral broadening and tertiary collateral angiogenesis, making the affected region less vulnerable to ischemia (Table 6.2).

6.2.1.2.1 Hemodynamic Parameters for Penumbra

Cerebral perfusion pressure (CPP), which equals to mean arterial pressure (MAP) minus intracranial pressure (ICP), represents the net pressure gradient pressing cerebral blood flow (CBF) to the brain. Cerebral autoregulation is an important process to maintain adequate and stable cerebral blood flow. This regulation of cerebral blood flow is achieved primarily by small vessels, which either dilate or contract under complex control systems, and change the tissue cerebral blood volume (CBV). In ischemic stroke, when the artery is under occlusion or stenosis, regional CPP decreases, CBF is maintained by increasing CBV, and cell function and viability are not changed. When CPP continues to drop and exceeds the autoregulation threshold, CBF decreases, along with delayed MTT. At this point, more oxygen is extracted from blood toward tissue or, in other words, by increasing oxygen extraction fraction (OEF) to maintain cerebral metabolic rate of oxygen ($CMRO_2$) and cellular functions. As OEF reaches peak and no other compensation could help, $CMRO_2$ decreases, causing neuron and vascular cell dysfunction, and CBV turns to drop. When CBF decreases to the critical point, cells would irreversibly die.

Schematically, we could divide the brain into four areas depending on their hemodynamic and metabolic status discussed above. Area 1 is the unaffected area, and 1′ within it is the compensated area due to vessel autoregulation. Area 2 is the benign oligemia, area 3 is the ischemic penumbra, and area 4 is the ischemic core (see Figs. 6.8 and 6.9). Time-to-peak map with a delay of 4 s or more might be more specific than typical MTT maps to estimate the volume of tissue at risk, because the latter might include benign oligemia area, which does not progress to infarction regardless of reperfusion [9].

6.2.1.3 Radiological Method for Penumbra and Collateral Vessels

There are several radiological methods/combinations to elucidate penumbra. DWI plus PWI is the most reliable and feasible method. DWI can well clarify the core and PWI can show penumbra region clearly. In adding MRA for viewing the vessels and MRI SWI for microbleeds, MRI is a versatile technology providing clinically useful information. The foibles are also obvious, and MRI consumes longer scanning time, which may be difficult for some severe or incorporative patients.

Non-contrast CT (NCCT) only shows infarct core until 6 h after symptom onsets. CTP can get hemodynamic parameters and is easy to visualize perfusion defect territory, but core boundary could only deduce from these hemodynamic parameters. Based on research data in Sect. 6.2.1.1 and comparison with MRI [10], parameter thresholds for defining acute infarct core and perfusion lesion were developed.

Digital subtraction angiography (DSA) is the gold standard technique to visualize the status of collaterals, because it is capable to show small arteries and dynamically see how bloods enter and leave the brain tissue. DSA-based collateral grade could be determined using the American Society of Interventional and Therapeutic Neuroradiology/Society of Interventional Radiology (ASITN/SIR) collateral flow

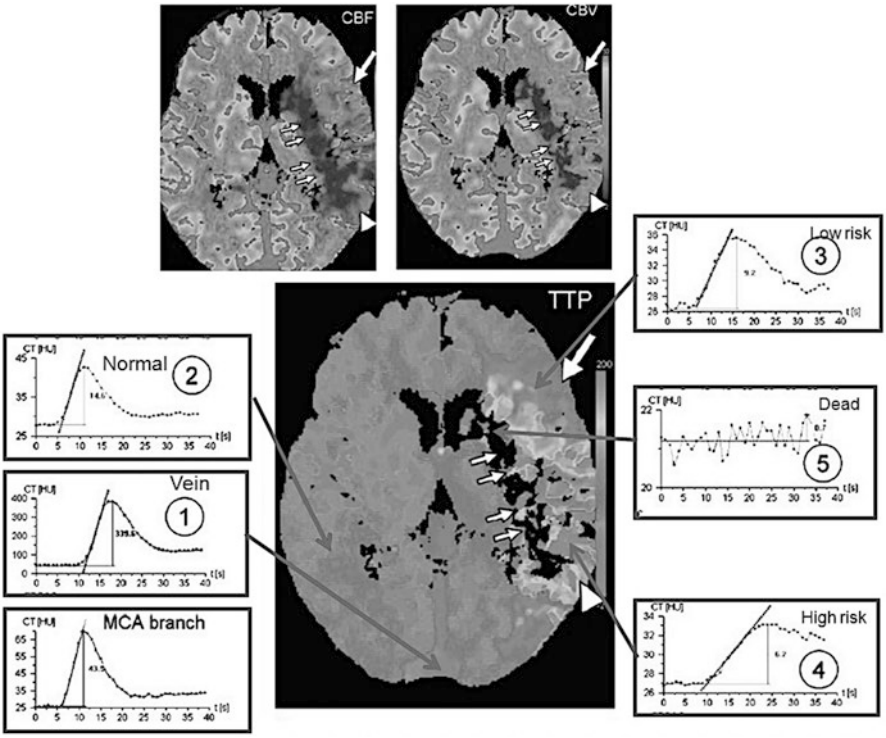

Fig. 6.8 Different tissue contrast concentration curves in different brain tissue status from normal, oligemia (low risk), penumbra (high risk), and infarct core (dead)

grading system. This scale assigns grade 0 to patients with no visible collaterals to the ischemic site, grade 1 with slow collaterals to the periphery of the ischemic site with persistence of some of the defect, grade 2 with rapid collaterals but persistence of some of the defect, grade 3 with slow collaterals but complete blood flow to the entire ischemic vascular bed, and finally grade 4 to patients with complete and rapid collateral blood flow in the entire ischemic territory by retrograde perfusion. Suk Jae Kim et al. developed a novel MRP approach to collateral flow image [11]. They merged temporally consecutive images derived from MRP source data to generate collateral flow maps and matched them with arterial, capillary, venous, and late venous phases of DSA image in the same patient and graded the MR collateral flow with the idea of DSA ASITN/SIR grading system. They found that there was a good correlation between MRI-based and DSA-based collateral grades. They found that the collateral status and early reperfusion after therapy were two main determinants of favorable functional outcome and neurological improvement.

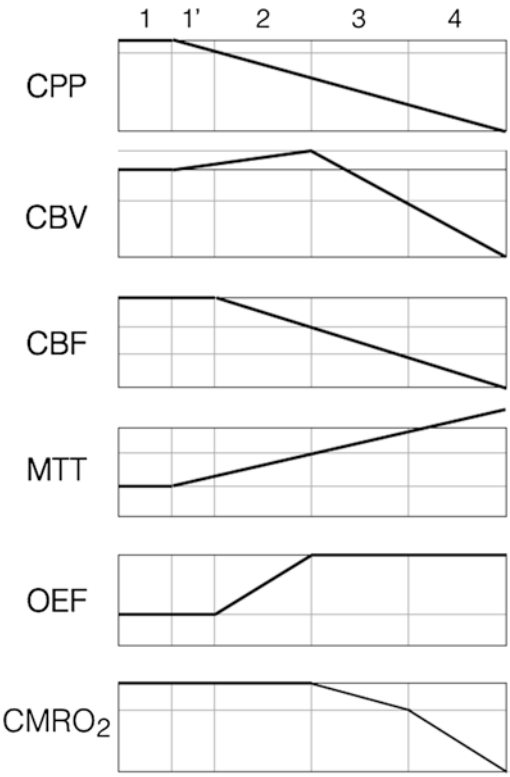

Fig. 6.9 Schematic illustration of four areas depending on their hemodynamic and metabolic status. Area 1 is the unaffected area; area 1′ within area 1 is the autoregulated part. Area 2 is the benign oligemia, area 3 is the ischemic penumbra, and area 4 is the ischemic core. *CPP* cerebral perfusion pressure, *CBV* cerebral blood volume, *CBF* cerebral blood flow, *MTT* mean transient time, *OEF* oxygen extraction fraction, $CMRO_2$ cerebral metabolic rate of oxygen

6.2.1.4 Clinical Significance of Penumbra

Currently, the only proven treatment for acute ischemic stroke (AIS) is reperfusion therapy, including intravenous recombinant tissue plasminogen activator (t-PA or rt-PA) thrombolysis and intra-arterial thrombectomy. Time is the key for better prognosis in these treatments. The lesser the time from stroke onset, the larger the penumbra area, which would be salvaged after reperfusion therapy. But the area of penumbra and the duration of penumbra are variable due to complicated stroke etiologies and compensatory mechanisms. Reperfusion therapy complications are also well related with penumbra characteristics. Plain CT or MRI could not visualize penumbra.

For a clinically suspected AIS patient administered to hospital within 4.5 h from stoke onset, intravenous thrombolysis improves outcome based on plain CT imaging [12, 13]. For patient admitted to hospital after the time window of 4.5 h, intravenous thrombolysis is not recommended routinely by current guideline. However, penumbra may maintain up to 48 h. Darby et al. reported that the proportion of all patients with DWI/PWI mismatch declined steadily over the time of stroke onset from around 100% to about 50% at 24 h [14]. Using PET, Heiss et al. showed that mismatch tissue was present at about 48 h [15]. Would patients with radiological

identified penumbra (perfusion/core mismatch) respond to reperfusion therapy at delayed times?

The Diffusion and Perfusion Imaging Evaluation for Understanding Stroke Evolution (DEFUSE) study [16] recruited 74 consecutive stroke patients, and an MRI scan was performed immediately before and 3–6 h after treatment with intravenous t-PA. Baseline MRI profiles were used to categorize patients into subgroups. This study found that early reperfusion was significantly associated with increased odds of achieving a favorable clinical response in patients with a perfusion/diffusion mismatch and an even more favorable response in patients with the target mismatch profile. Patients with the no-mismatch profile did not appear to benefit from early reperfusion. Early reperfusion was associated with fatal intracranial hemorrhage in patients with malignant profile.

Effects of alteplase beyond 3 h after stroke in the Echoplanar Imaging Thrombolytic Evaluation Trial (EPITHET) is a randomized, placebo-controlled clinical trial published in 2008 [17]. The aim was to test whether alteplase, given 3–6 h after onset, promoted reperfusion and attenuated infarct growth in patients who had a mismatch in PWI and DWI. Although this trial did not show significant association with lower infarct growth, it revealed an association between increased reperfusion and mismatch. High rate of reperfusion, less infarct growth, and better neurological outcome were found in patients with alteplase than those with no reperfusion. Because reperfusion was associated with improved clinical outcomes and failure of EPITHET to show clinical improvement might be due to limited sample, the authors claimed for larger trials.

The European Cooperative Acute Stroke Study-4 (ECASS-4) is an investigator-driven, phase 3, randomized, multicenter, double-blinded, placebo-controlled study (clinical trial no: ISRCTN71616222, http://www.isrctn.com/ISRCTN71616222) [18]. Ischemic stroke patients presenting within 4.5 and 9 h of stroke onset, who fulfill clinical requirements and pre-stroke modified Rankin scale 0–1, will undergo MRI, and patients who meet imaging criteria of existence of penumbra (infarct core volume <100 ml, perfusion lesion: infarct core mismatch ratio >12, and perfusion lesion minimum volume of 20 ml) will be randomized to either rt-PA or placebo treatment. The primary outcome measure will be the categorical shift in the clinical independency at day 90. This trial is estimated to end at August 31, 2016, and may tell us more about the efficacy of penumbra imaging on guidance to intravenous thrombolysis.

6.3 Reperfusion Injury in Stroke and Imaging

Reperfusion is characterized by initial restriction of blood supply followed by subsequent vascular restoration and concomitant reoxygenation of downstream tissue. During ischemia, both vascular and neuronal parenchymal cells undergo ischemic injuries as well as gene translational changes to adapt hypoxia, which reacts along with the reperfused blood cells to induce inflammation. Leukocytes in the vascular

bed may cause the "no-reflow effect," leading to the failure of recanalization and progression of the infarction. Inflammation responses subsequently break down blood-brain barrier (BBB) and induce cellular injuries, brain edema, infarct expansion, and hemorrhagic transformation (HT). On the other hand, postischemic reperfusion could induce hyper-perfusion, mostly due to increased systemic blood pressure and impaired vascular autoregulation. Reperfusion therapy may further aggravate reperfusion injury. Here we thus discuss the possible mechanism and radiological findings.

6.3.1 Blood-Brain Barrier Changes

The BBB is crucial in cerebral ischemia and reperfusion. The BBB is composed of vascular endothelial cells, pericytes, astrocytes, and extracellular matrix. BBB endothelial cells have tight junctions and minimal pinocytotic activity and express lots of enzymes to degrade harmful molecules as well as drugs. BBB changes happen throughout all the process during cerebral ischemia and reperfusion and itself modulates the ischemic and reperfusion process.

During cerebral ischemia, vascular endothelial cells undergo hypoxia attack. Energy failure leads to decrease adenosine triphosphate (ATP) production and ion channel activity, lactic acidosis, release of extracellular glutamate, and eventually necrosis, resulting in BBB disruption. Endothelial swelling may occur within minutes to hours of ischemic onset, leading to the narrowing of the internal diameter of the blood vessel. Lactic acidosis also contributes to swelling of endothelial cells, neurons, and astrocytes. Furthermore, proteases and inflammatory cytokines such as matrix metalloproteinases (MMP) could contribute to BBB extracellular matrix degradation and increase BBB permeability.

It is proposed that there are three stages of paracellular permeability after reperfusion. Stage 1 is hyperemia, as a result of impaired cerebral autoregulation, which increased BBB permeability, systemic hypertension, and elevated regional blood flow. In this stage, parenchymal edema is mainly caused by cytotoxic edema, BBB permeability is mildly elevated, and only small molecules are allowed to pass through. Stage 2 is hypo-perfusion (no-reflow effect), which occurs immediately after stage 1, and is attributed to microvascular obstruction caused by cellular swelling and neutrophil adhesion. Stage 3 is a complete opening of the BBB, which is subdivided into two phases. In phase 1, about 3–8 h post-reperfusion, it is attributed to increased inflammation along with enzymatic degradation of extracellular matrix. In phase 2, at 18–96 h post-reperfusion, it coincides with increased vasogenic edema and angiogenesis, finally resulting in increased permeability to macromolecules from moving intravascular to extracellular spaces.

Imaging contrast and recombinant tissue plasminogen activator (rt-PA) could disrupt the BBB functions. Several mechanisms are postulated for contrast extravasation and hemorrhage related to contrast agents. Contrast is toxic to basal lamina, which results in extravasation of blood elements and that contrast itself goes through

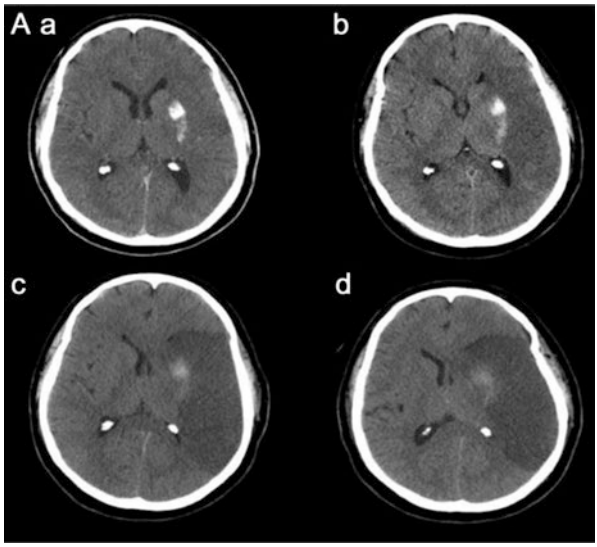

Fig. 6.10 Sign of contrast extravasation. A patient with left-sided ischemic stroke underwent intra-arterial thrombolysis. Immediately post-therapy head CT showed a new area of hyper-intensity (*a*) and 14 h post-procedure head CT showed persistent hyper-intensity and CT early infarct sign of left MCA territory infarct (*b*). Hyper-intensity was persistent at 36 h (**c**) and 60 h (**d**)

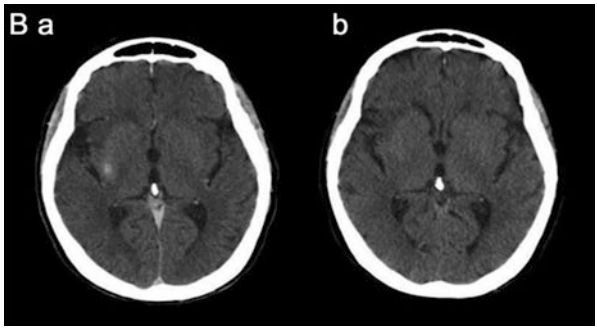

Fig. 6.11 Sign of contrast enhancement. Head CT immediately post-intra-arterial thrombolysis showed a hyper-intensity (**a**), which was resolved completely on a repeat head CT performed 16 h post-procedure

the barrier is called contrast extravasation. Contrast extravasation has been shown to have a strong association with HT and poor prognosis. BBB disruption can be easily detected using radiology protocols of BBB permeability, such as contrast-enhanced CT/MR and CT/MR permeability protocols. Contrast extravasation, showed as hyper-density (Hounsfield unit >90) in CT, can be found in parenchymal tissue at 24 h and disappears in a few hours (Figs. 6.10 and 6.11).

Latour et al. used MRI to characterize (Fig. 6.12) early BBB disruption in human focal brain ischemia and examined its association with reperfusion, HT, and poor outcome in 144 patients, including 38 intravenous thrombolyses and 3 intra-arterial thrombolyses [19]. Sixty-three percent of the patients had evidence of reperfusion within 1 week. BBB disruption was more common in patients with reperfusion (45%) than those without reperfusion (18%). Both HT and BBB disruption were more common in patients undergoing rt-PA thrombolysis (31–55%) than those without treatment (14–25%). In the reperfused group, patients with BBB disruption were more likely to have poor outcome (63%) than those without BBB disruption

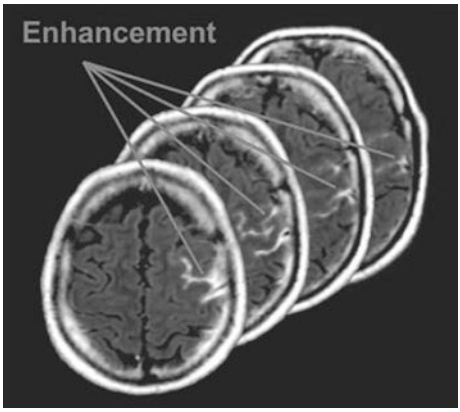

Fig. 6.12 MRI exhibiting evidence of early BBB disruption. Hyper-intense signal in the left central and precentral sulci is caused by extravasation of contrast agent into the cerebral spinal fluid

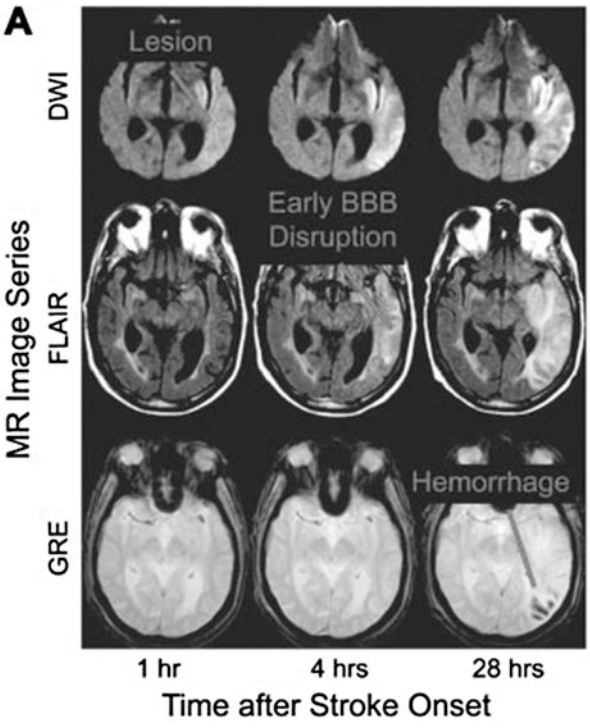

Fig. 6.13 MR images from a patient undergoing HT. From *top* to *below* are DWI images, FLAIR images, and GRE images at three different times after symptom onset. DWI at 1 h after onset showed hyper-intensity, FLAIR acquired 4 h after onset exhibited evidence of BBB disruption, and GRE at 28 h showed HT

(25%). Moreover, early BBB disruption was an independent predictor of HT (Fig. 6.13).

Hyper-intense acute reperfusion marker (HARM), the post-contrast enhancement of cerebrospinal fluid in FLAIR, was also used to characterize BBB disruption (Fig. 6.14). Warach et al. used this marker to investigate 213 patients undergoing rt-PA thrombolysis [20]. BBB disruption was more common in patients with

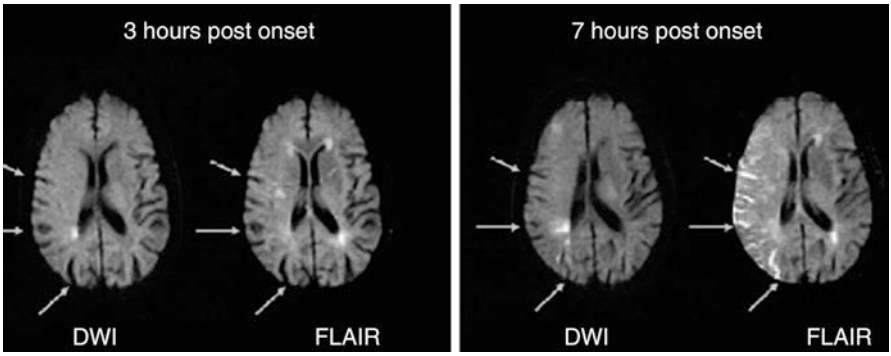

Fig. 6.14 A typical case of HARM (hyper-intense acute reperfusion marker). The initial MRI obtained 3 h after onset of symptoms shows an early evolving lesion (hyper-intensity); FLAIR showed negligible cerebrospinal fluid signal. At 7 h after onset (4 h after gadolinium contrast injection), enhancement is observed in the cerebrospinal fluid spaces throughout the right cerebral artery territory

reperfusion (45%) than those without reperfusion. Reperfusion was the strongest independent predictor of early BBB disruption. HARM was associated with HT and worse outcomes.

6.3.2 Postischemic Hyper-perfusion

Postischemic hyper-perfusion is a frequently recognized phenomenon in stroke. Several animal studies suggested that hyper-perfusion is consistent with restoration of blood flow. Hyper-perfusion could contribute to brain edema, BBB disruption, or hemorrhage, causing reperfusion injury.

Heiss et al. used a temporary middle cerebral artery (MCA) occlusion of 30, 60, and 120 min in a cat model and found that reactive hyper-perfusion is transient, and all cats survived without cerebral injury on histological study in the 30-min group [21]. In the 60-min and 120-min group, the degree of hyper-perfusion was significant, reaching up to 300% compared to pre-occlusion status, and some animals died. Postischemic hyper-perfusion was severe in the dead cats; larger infarct volume and increased intracranial pressure were thought to be the cause of death. In survived cats, postischemic hyper-perfusion was mild and had less severe and lower degree of intracranial pressure. Tamaru et al. found that the degree of hyper-perfusion was significantly correlated with cerebral petechial hemorrhages in the damaged cortex [22].

MRP is a good tool to evaluate postischemic hyper-perfusion. Kidwell et al. investigated 11 patients with vessel occlusion who were recanalized following intra-arterial thrombolysis within several hours and performed MRP at the time after recanalization and at day 7 (Fig. 6.15) [23]. They found that postischemic

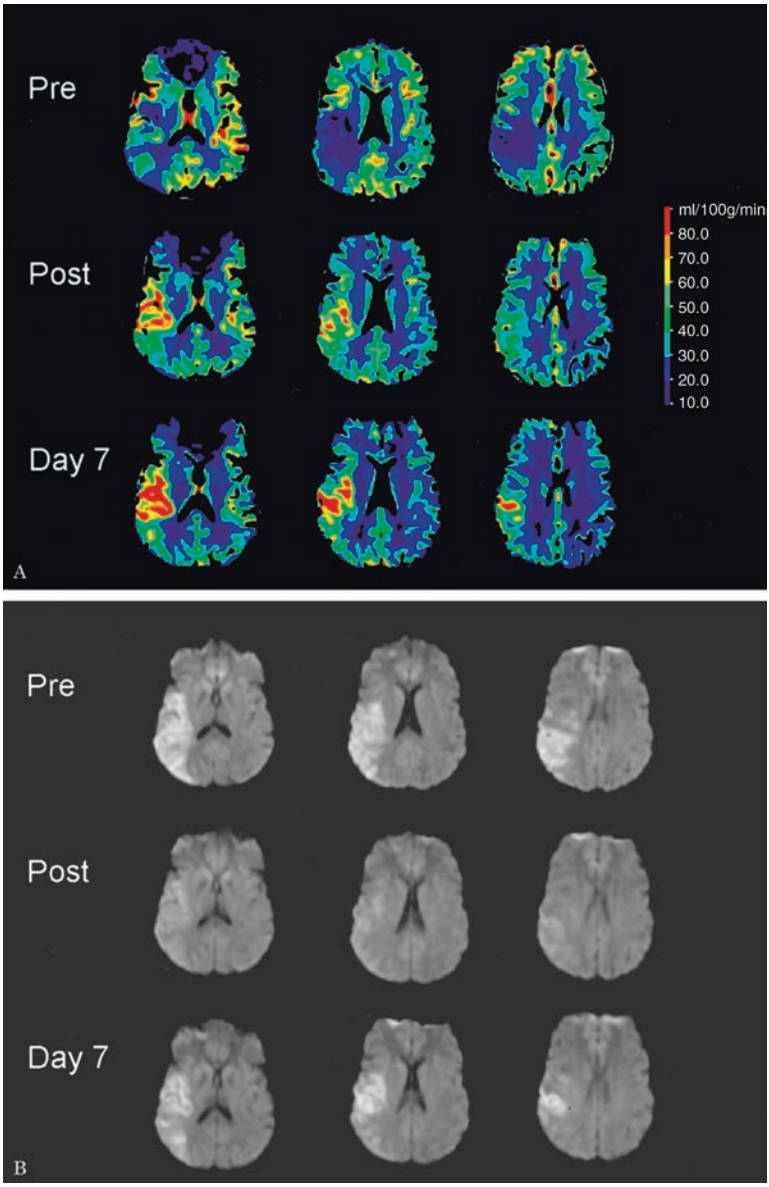

Fig. 6.15 MR perfusion (*above*) and diffusion-weighted imaging (DWI, *below*) map acquired from acute ischemic stroke patients before, just after intra-arterial thrombectomy, and 7 days after procedure. MR perfusion demonstrated postischemic hyper-perfusion following recanalization. Tissue with postischemic hyper-perfusion developed into infarction

hyper-perfusion occurred in about 40% of patients within hours and in about 50% of patients at day 7. Compared with other abnormal regions, tissues that developed postischemic hyper-perfusion had greater bioenergetic compromise in pretreatment apparent diffusion coefficient values and greater impairment in pretreatment blood flow measures. Hyper-perfusion occurred mainly in regions that developed into infarction.

6.3.3 No-Reflow Effect

Leukocytes play an important role in the development of cerebral reperfusion injury. Activated leukocytes interact with vascular endothelial cells and plug capillaries and disrupt blood-brain barrier through neutrophil-derived oxidants and proteolytic enzymes, and then toxic substances extravasate from capillaries and infiltrate the brain tissue further promoting the release of cytokines and inflammation response. The inflammatory cascades result in the deterioration of salvageable penumbra.

In an animal study, Zhang et al. use nylon monofilament to occlude MCA [24]. Two groups of rats were investigated: those with permanent MCA occlusion and those having the arterial occlusion released after 2 h. Experiments were conducted at different hours after the onset of ischemia to investigate the temporal evolution. Initially, the cortical lesion was smaller in rats subjected to transient MCA occlusion than that in permanent MCA occlusions. Six hours after restoration, neutrophils accumulated at the site of neuronal injury. The neutrophil accumulation occurred earlier and in a greater extent in transient occlusion group than permanent occlusion group. The infarct volume increased dramatically between 6 and 24 h following reperfusion, and the time course of maximal infarct expansion was corre-

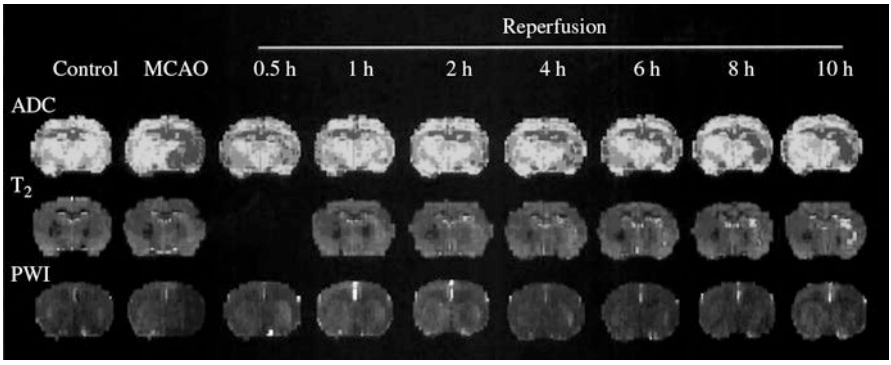

Fig. 6.16 Dynamic MR change in a rat model with middle cerebral artery occlusion (MCAO) and release within 1 h. Compared with control, ADC and PWI value dropped abruptly just at the time of MCAO and recovered after occlusion release. Along with the reperfusion time, ADC and PWI gradually drop again, and finally infarction change in T_2 emerged. *ADC* apparent diffusion coefficient, *PWI* perfusion-weighted imaging

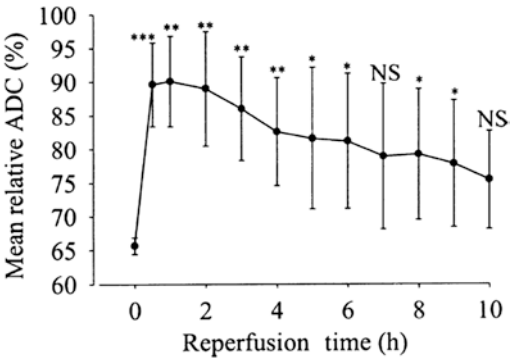

Fig. 6.17 Curve to quantitatively demonstrate ADC change in Fig. 6.16

lated with that of neutrophil infiltration. Another animal study, using anti-neutrophil antiserum in a rabbit model of transient ischemia, showed that [25] the regional CBF of iatrogenic neutropenic rabbits recovered from less than 5 ml/100 g per minute to 20–30 ml/100 g per minute after reperfusion, while the control group CBF remained at less than 10 ml/100 g per minute. Correspondingly, the infarct size was significantly smaller in the neutropenic animals.

Olah et al. investigated the dynamics of MRI change including ADC, PWI, and T_2 in a rat ischemic model. Results showed that PWI value and ADC value in the corresponding territory dropped after MCA occlusion, which was a manifestation of vessel occlusion and cellular injury. Upon reperfusion, PWI and ADC recovered at once, but gradually re-decreased and eventually went in parallel with T_2 [26].

The first drop of PWI and ADC value corresponded to the direct injury caused by abrupt hypoxia, and T_2 value was used for measuring irreversible injury. After vascular recanalization, PWI value recovered due to the blood reflow, and ADC value would recover if the injury was reversible. Later secondary drop of PWI was associated with the decrease of CBF due to reperfusion injury, ADC dropped along with the gradually injured tissue, and finally T_2 change was seen as irreversible infarct. Cells could undergo apoptosis after initial injury, but cellular apoptosis could not be detected because apoptotic cells went shrinkage, and would not alter free water diffusion and ADC values.

There are other studies using different animals and different occlusion time with different characteristics. First, the time course of the secondary reduction of ADC is different, ranging from 2 to 72 h after reperfusion. This is probably due to the different duration of ischemia and different animal models. Second, the secondary deterioration of ADC could be predicted by early changes of T_2 during reperfusion, that is, an increase in T_2 value early in the ischemic period predicts a secondary deterioration in ADC after reperfusion. In other words, the more severe the initial injury is, the more severe the reperfusion injury is. It can help to assess the likelihood of benefiting from reperfusion therapy (Fig. 6.17).

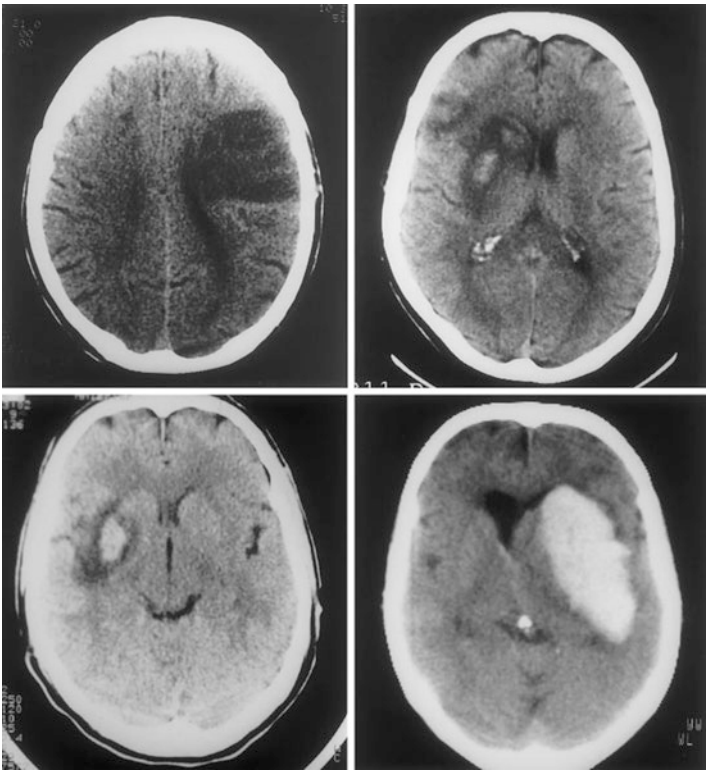

Fig. 6.18 Subtypes of hemorrhagic transformation: HI1 (*top left*), HI2 (*top right*), PH1 (*bottom left*), and PH2 (*bottom right*)

Table 6.3 Summary of neuroimaging factor associated with hemorrhagic transformation

Factors	Possible mechanism	Effect	References
Large infarct size/DWI infarct volume	Delayed reperfusion	Increase	[29, 30]
Early infarct sign	Delayed reperfusion	Increase	[31–33]
Dense cerebral artery sign	Delayed reperfusion	Increase	[32]
Leukoaraiosis	Parenchymal factor	Increase	[31]
MRI enhancement pattern	BBB disruption	Increase	[34]
Increased BBB permeability	BBB disruption	Increase	[35]
HARM	BBB disruption	Increase	[23]
Decreased ADC value	Delayed perfusion	Increase	[36]
Good collateral flow	Delayed perfusion	Decrease	[37]
Good CBF of CBV	Delayed perfusion	Decrease	[38]

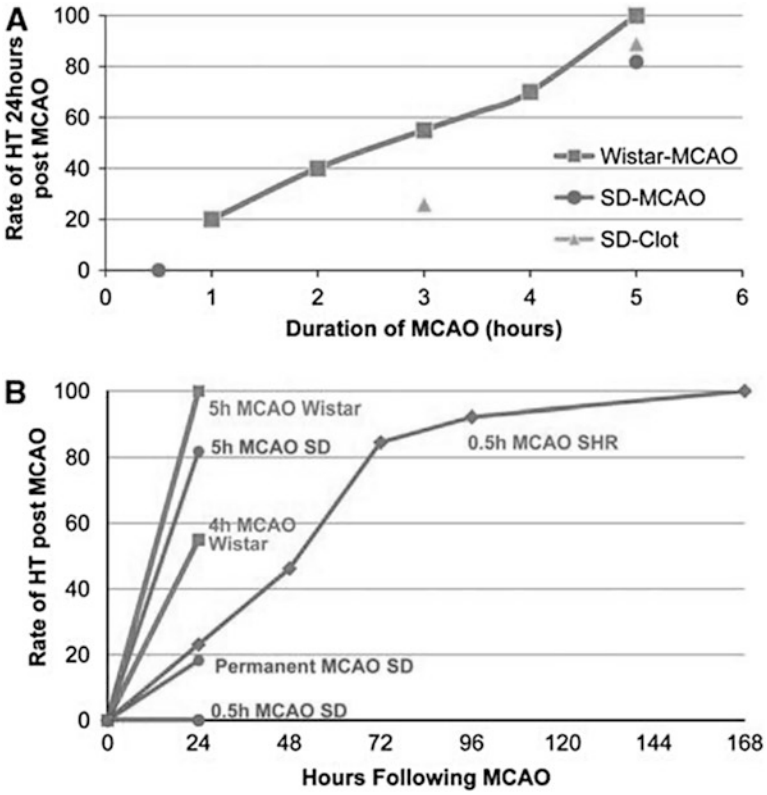

Fig. 6.19 (**a**) The rate of hemorrhagic transformation (HT) increases with longer duration of cerebral ischemia followed by reperfusion. (**b**) The rate of HT as a function of middle cerebral artery occlusion (MCAO) duration. Increased duration of MCAO increases the rate of HT in several rat models

6.3.4 Hemorrhagic Transformation

Hemorrhagic transformation (HT) occurs in 10–40% of patients with ischemic stroke and is associated with increased morbidity and mortality. It is also the major complication of rt-PA treatment. The presentation of HT varies from microscopic bleeding to large hemorrhages. Clinical studies usually divide HT into four groups based on ECASS I criteria: small petechial hemorrhagic infarction (HI1), confluent petechial hemorrhagic infarction (HI2), small parenchymal hemorrhage (PH1) (<30% of infarct, mild mass effect), and large parenchymal hemorrhage (PH2) (>30% of infarct, marked mass effect [27]. HT can be symptomatic, defined as an increase of more than four points in the National Institutes of Health Stroke Scale (NIHSS), within the first 36 h (Fig. 6.18).

Early HT (<18–24 h) is associated with leukocyte-related inflammation and cerebral edema, and the delayed HT (>18–24 h) is associated with BBB repair,

vascular remodeling, and neuroinflammation. Neuroimaging researches clarified enormous amount of imaging signs associated with HT (Table 6.3). These findings could broaden our knowledge of HT mechanism and help better treatment in clinical practice.

In rats, the rate of HT increased depending on the duration of time from stroke onset to reperfusion of ischemic tissue (Fig. 6.19). In Sprague-Dawley rats with middle cerebral artery occlusions (MCAO), the HT rate was 81.8% in reperfusion rats, while only 18.2% in permanent MCAO rats. Mortality rates were also increased in reperfused group compared with permanent MCAO group (54.5% vs 18.1%). Thus, delayed reperfusion after prolonged ischemia increased the rate of HT and worsened stroke outcome. In humans, increased time from ischemia onset to reperfusion is also associated with an increase in HT risk in both rt-PA-treated and untreated patients. Vascular recanalization beyond 6 h after stroke onset is an independent predictor of HT in human stroke [28]. Thus, time duration from stroke onset to reperfusion is a key factor in determining the rate of HT, with longer duration of ischemia increasing the likelihood of HT when blood flow is restored.

DEFUSE2 trial (the Diffusion and Perfusion Imaging Evaluation for Understanding Stroke Evolution 2) was a prospective observational study of patients who were treated with endovascular therapy [39]. Patients with or without previous

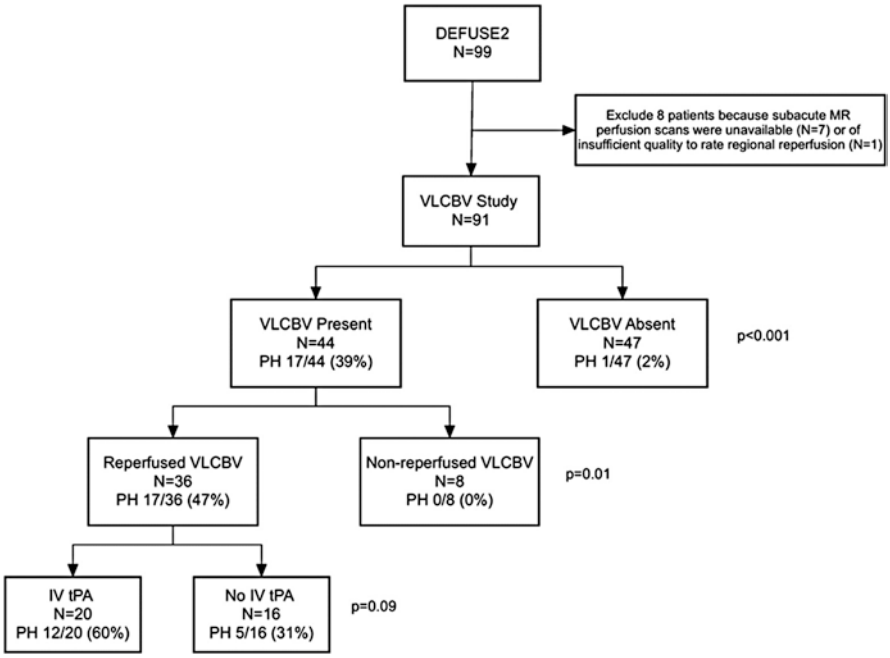

Fig. 6.20 Flow diagram illustrates the risk of parenchymal hemorrhage stratified by the presence of very low cerebral blood volume (VLCBV), regional reperfusion, and pretreatment with intravenous rt-PA in DEFUSE2 trial. Patients with VLCBV, reperfusion after therapy, and previous IV rt-PA had the greatest possibility to develop HT

rt-PA thrombolysis were included in the trial and then underwent intra-arterial therapy after baseline MRI scanning. They found that the presence of very low CB (VLCBV) was an independent predictor of HT and poor functional outcome at 90 days, which is consistent with the previous animal finding that the longer time from occlusion to perfusion, the larger chance to HT, as VLCBV was signified as the presence of infarction core and long time from disease onset (Fig. 6.20).

Collateral circulation is also associated with HT in patients with ischemic stroke. The extent and rate of pial artery backfilling has been used as an indirect assessment of collateral circulation. Poor collaterals are a predictor of HT in patients. In a study of 105 ischemic stroke patients, HT occurred more frequently in those with poor pial collaterals than those with good collaterals (25% vs 2.8%). In a study of 222 intra-arterial-treated patients, symptomatic HT was more common in patients with poor collaterals than those with good collaterals (30.2% vs 14.3%) after recanalization, which suggested that the extent of reperfusion in infarct tissue was a critical factor for HT, and poor collaterals were likely to increase the size and severity of cerebral infarct thus promoting HT.

Further researches are needed to identify suitable patients for reperfusion therapy and explore possible interventions to minimize reperfusion injury.

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Chapter 7
Unstable Carotid Artery Plaque Evaluation by Ultrasound

Noelia Rodriguez-Villatoro and David Rodriguez-Luna

Abstract Although evaluation of carotid stenosis has been traditionally focused on degree of stenosis, there is a growing evidence about the importance of the evaluation of morphological features of carotid plaques. Unstable plaques can lead to stroke regardless of the degree of stenosis they cause. Ultrasound is a useful, easy-to-use, and widely available technique to assess both degree of stenosis and morphological characteristics. Unstable plaques are classically described by ultrasound as irregular and hypoechoic. Further, new ultrasound techniques have been developed to detect plaque neovascularization, one of the most important features which confer instability to atheroma plaques. Besides carotid ultrasound, other image techniques, such as computed tomography angiography, magnetic resonance imaging, and positron emission tomography, have proven their usefulness in the evaluation of unstable carotid plaques in several studies. Due to the growing knowledge of unstable carotid plaques and the improvements achieved in their evaluation, a yield of investigation is emerging: non-stenotic but unstable carotid plaques as a source of cryptogenic strokes.

Keywords Carotid artery • Plaque • Atheroma • Instability • Echogenicity • Neovascularization

Abbreviations

18-FDG	18-Fluorodeoxyglucose
ACA	Anterior cerebral artery
CCA	Common carotid artery

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CEUS	Contrast-enhanced ultrasound sonography
CTA	Computed tomography angiography
ECA	External carotid artery
ECST	European Carotid Surgery Trial
EDV	End-diastolic velocity
GSM	Gray-scale median
HITS	High-intensity transient signals
ICA	Internal carotid artery
MCA	Middle cerebral artery
MDCT	Multidetector-row CT
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
NASCET	North American Symptomatic Carotid Endarterectomy Trial
PCA	Posterior cerebral artery
PET	Positron emission tomography
PSV	Peak systolic velocity
RI	Resistance index
TOAST	Trial of Org 10172 in Acute Stroke Treatment

7.1 Introduction

Ischemic stroke is one of the leading causes of morbidity and mortality worldwide. Carotid atherosclerosis is responsible for 10–20% of ischemic strokes [1], being carotid ultrasound one of the most important techniques in its diagnosis and follow-up. Currently used stroke classification schemes, such as Trial of Org 10172 in Acute Stroke Treatment (TOAST) [2], consider atherosclerotic lesions of the carotid bifurcation as responsible for stroke only if they cause a significant luminal narrowing. Similarly, patients included in randomized controlled trials that compared medical and surgical carotid stenosis treatment, such as the North American Symptomatic Carotid Endarterectomy Trial (NASCET) [3] or European Carotid Surgery Trial (ECST) [4], were selected according only to stenosis grade. However, many patients with high-degree extracranial internal carotid artery (ICA) stenosis have stable plaques, with low risk of rupture and cerebral embolization [5]. Conversely, the evidence about the importance of plaque instability and their risk of cerebral embolization is growing faster, regardless of the degree of luminal stenosis [6].

It is known that approximately 60% of myocardial infarcts occur in coronary arteries with less than 50% degree of stenosis [7]. Some studies have shown non-stenotic unstable ICA plaques according to their characteristics at magnetic resonance imaging (MRI) in patients diagnosed with cryptogenic stroke [8]. Further, it is estimated that for every clinical stroke diagnosis related to carotid stenosis, there are around five asymptomatic strokes, which can cause cognitive impairment [9]. Thus, a paradigm shift including not only evaluation of degree of stenosis but also

characterization of plaque morphology has been developed over the last years to help in the risk of embolization stratification of these patients.

Carotid ultrasound is a safe and fast technique that can assess both degree of stenosis and plaque morphology. Classically, unstable plaques are associated with thin fibrous caps, large lipid cores, intra-plaque hemorrhage, and inflammation [10–13], which are traduced in ultrasound as echolucency, heterogeneous echotexture, irregular surface, and ulceration. Additionally, one of the most important characteristics of unstable plaques is neovascularization. Neovascularization of carotid plaques, which can also be easily evaluated by ultrasound, is related to the risk of cerebral embolization from carotid plaques.

We will review current knowledge about features of unstable extracranial carotid plaques and their evaluation, basically, by ultrasound examination. In addition, we will highlight some of the current evidence of non-stenotic unstable carotid plaques as a cause of cryptogenic stroke.

7.2 Carotid Ultrasound Examination

7.2.1 Technical Considerations

In order to perform an accurate and complete examination of the carotid system, several technical considerations have to be contemplated [14]. First, a linear-array transducer with an operating frequency of 7.0–7.5 MHz is needed for imaging cervical vessels. Second, the patient has to lie in a supine position, the throat should be slightly extended, and the head turned to the contralateral side. Longitudinal (Fig. 7.1) and transversal planes of the cervical vessels should to be obtained. Beginning the examination with a transversal plane to identify the common carotid artery (CCA) and its bifurcation in ICA and external carotid artery (ECA) is recommended. The exact position of the bifurcation can be delimited by sliding the transducer caudad and cephalad. Once an anatomical idea of the disposition of vessels is obtained, a longitudinal plane proceeds. Before the bifurcation, the transducer is turned by 90° so that the vessel appears in an anterior or anterolateral longitudinal plane. By angling the transducer medially, the origin of the ECA can be examined (Fig. 7.2). By angling the transducer laterally, we can explore the origin of the ICA (Fig. 7.3). It is highly recommended to identify both the ICA and the ECA without the aid of the Doppler spectrum, in order to avoid mistakes when a flow obstruction or another flow disturbance is present. The ICA differs from the ECA in that its lumen is wider at its origin (carotid bulb) and, opposite to ECA, does not have branches along the neck.

When observing the color-coded imaging of the carotid system, real-time blood flow hemodynamic processes can be examined. The color coding depends on the direction of flow with respect to the transducer (flow toward the transducer is coded as red color, and flow away from the transducer is coded as blue color). It is remark-

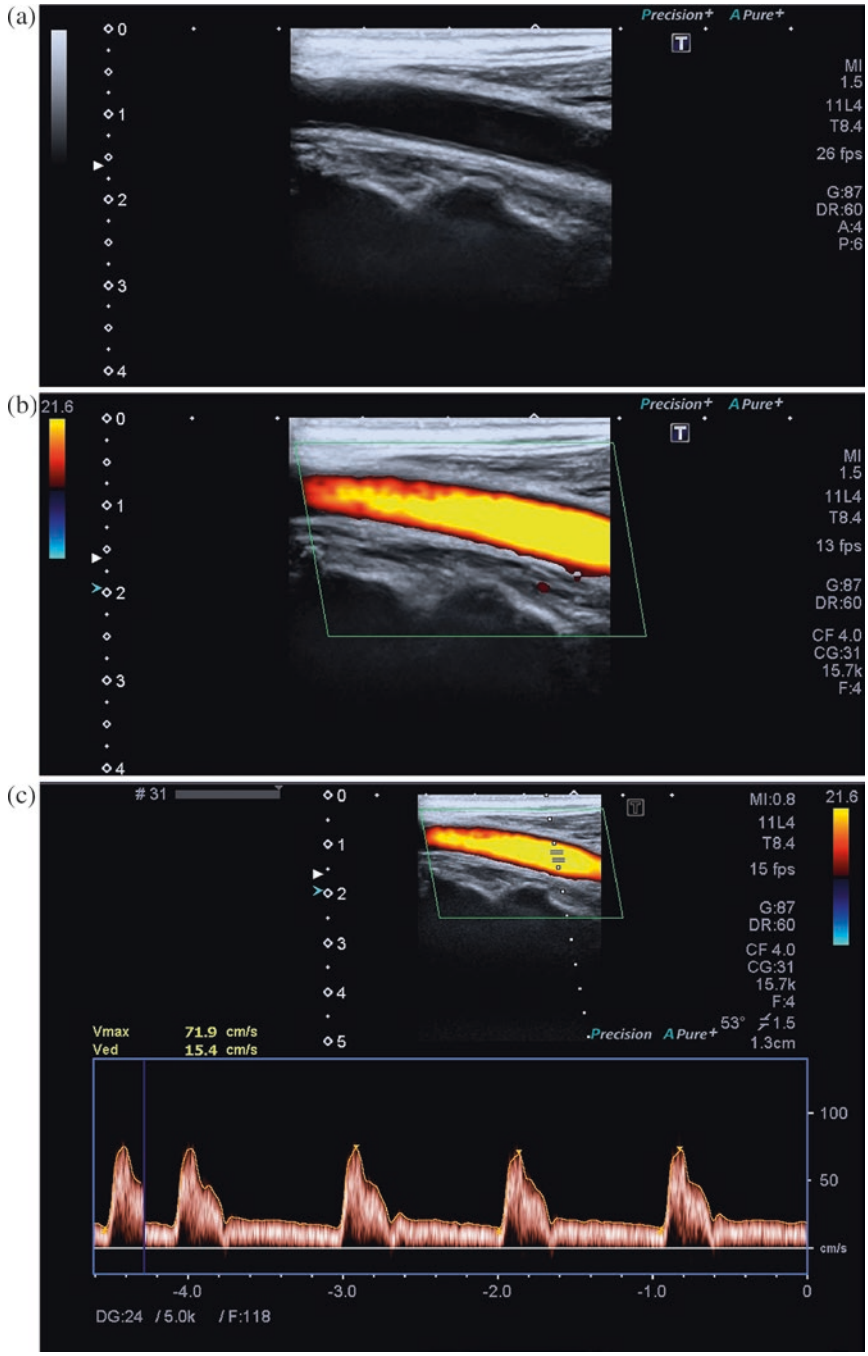

Fig. 7.1 Common carotid artery imaged in longitudinal plane in (**a**) B-mode ultrasound, (**b**) color-coded ultrasound, and (**c**) Doppler spectra

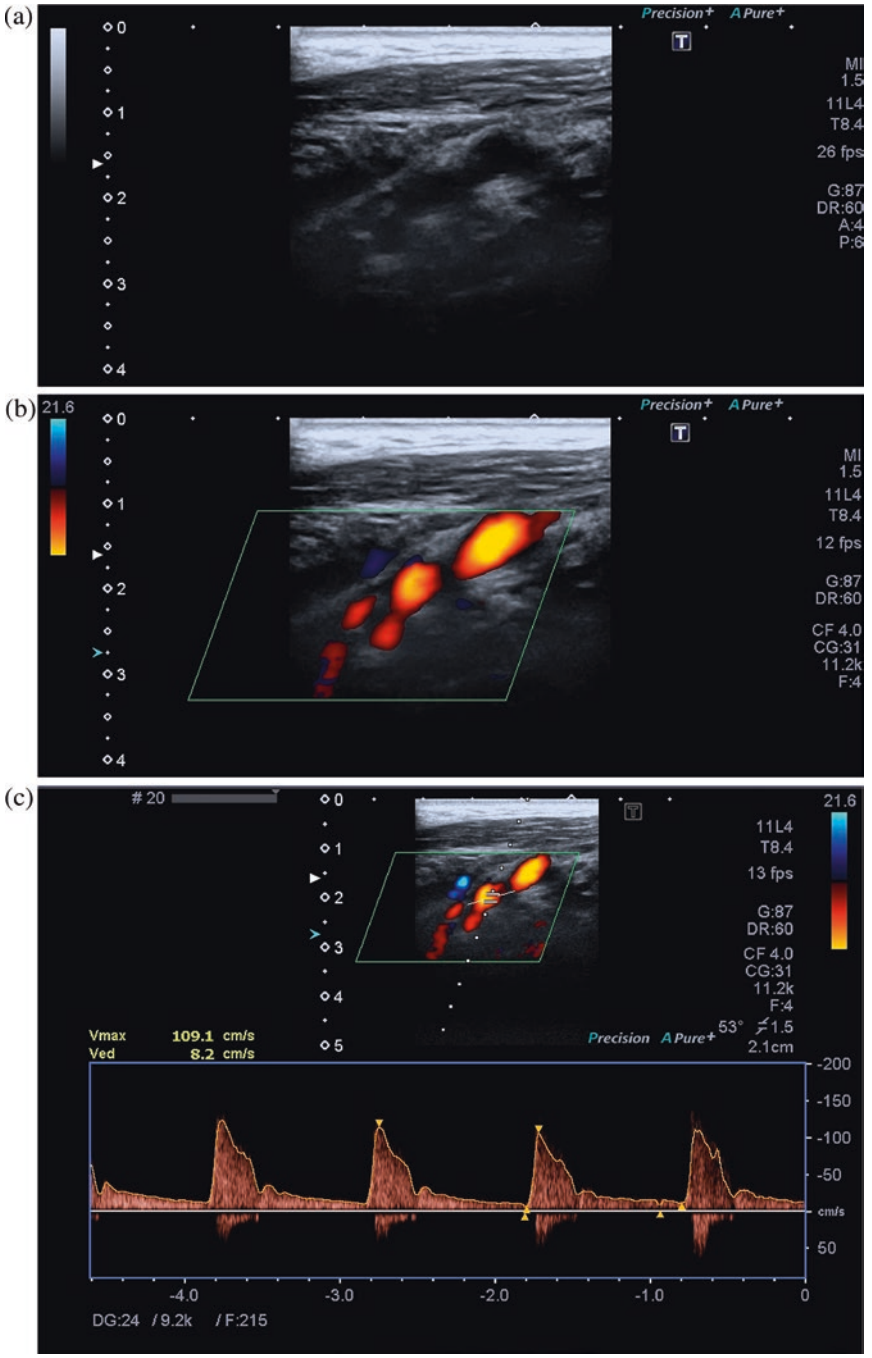

Fig. 7.2 External carotid artery in (**a**) B-mode ultrasonography, (**b**) color-coded ultrasonography, and (**c**) Doppler spectra waveform

able that a blue-coded area is possible to be seen in the carotid bulb in addition to the red-coded laminar flow due to the helical course of counter-rotating flow components inherent in this area.

Once neck vessels have been identified, Doppler spectra of each one must be obtained to complete the examination. The Doppler sample volume must be placed in the vessel lumen under visual control, and all the vessel length must be explored carefully. To obtain a more exact measure of flow velocity, an angle correction must be used by manually positioning the cursor of the sample volume as parallel as possible to the vessel wall. As the ICA supplies the brain parenchyma, a territory with low peripheral resistance, the typical spectral waveform of this artery has a high diastolic component (Fig. 7.3d). Conversely, as ECA supplies territories with high peripheral resistance, its spectral waveform has a low diastolic component (Fig. 7.2c). CCA has a relatively constant anatomical course, with a systolic velocity flow which is usually higher than the internal carotid artery and a diastolic component which lies between the ICA and ECA diastolic component (Fig. 7.1c).

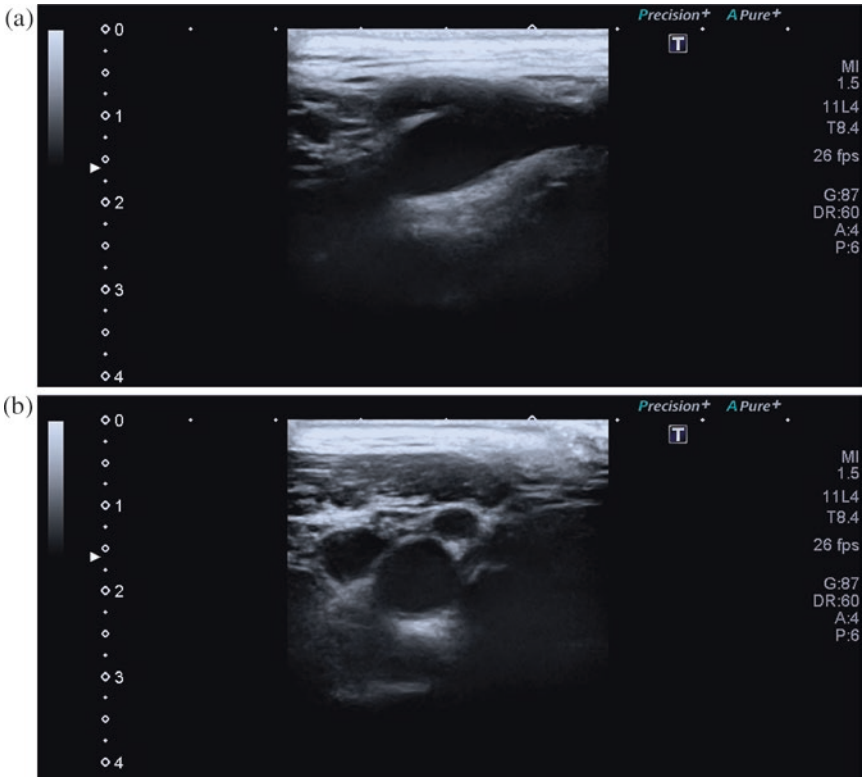

Fig. 7.3 Carotid bifurcation. Internal carotid artery (ICA) origin is wider than the origin of the external carotid artery, usually called carotid bulb in (**a**) B-mode longitudinal plane, (**b**) B-mode transversal plane, (**c**) color-coded longitudinal ICA, and (**d**) ICA Doppler spectra

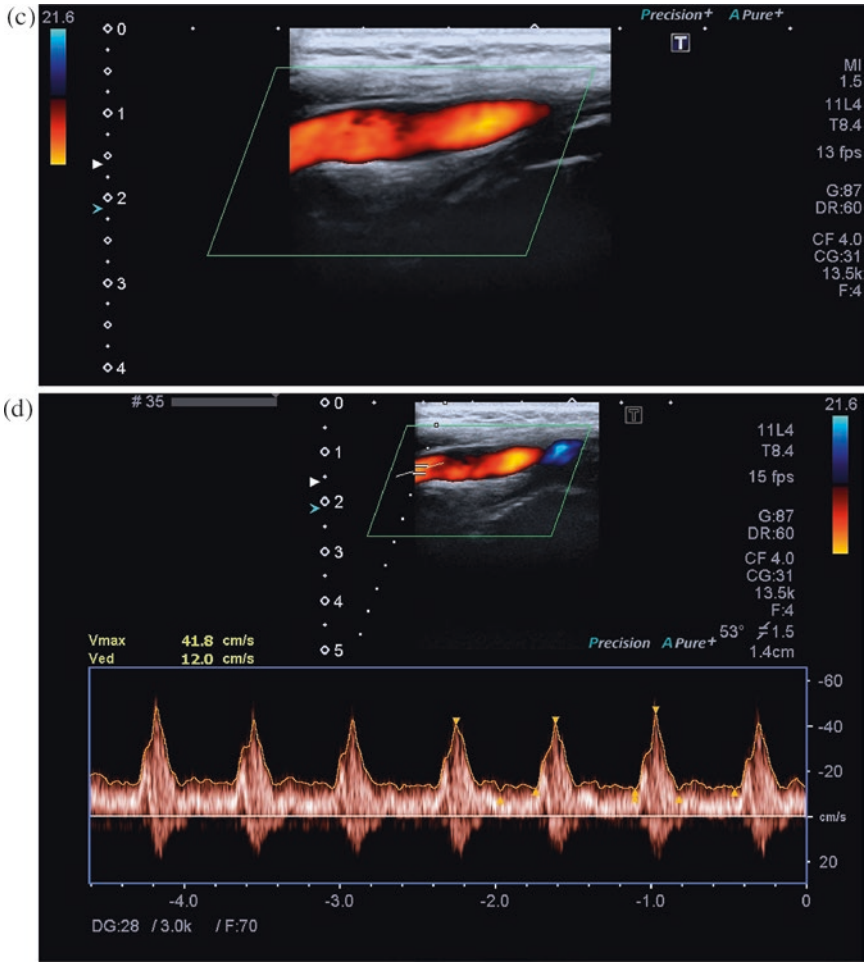

Fig. 7.3 (continued)

7.2.2 Unstable Plaques: Ultrasonographic Features

Plaque morphology could be assessed by different techniques, including computed tomography angiography (CTA), MRI, or positron emission tomography (PET). However, one of the most useful and used techniques is ultrasound examination. B-mode sonography provides important information for the physician such as location and number of plaques, size, morphology, and constitution. Structure, surface, and echogenicity can also be assessed.

Atherosclerotic lesions are a dynamic process as a result from a variety of pathogenic processes, including macrophage foam cells formation and death, accumulation of extracellular lipid, displacement and reduction of extracellular matrix and

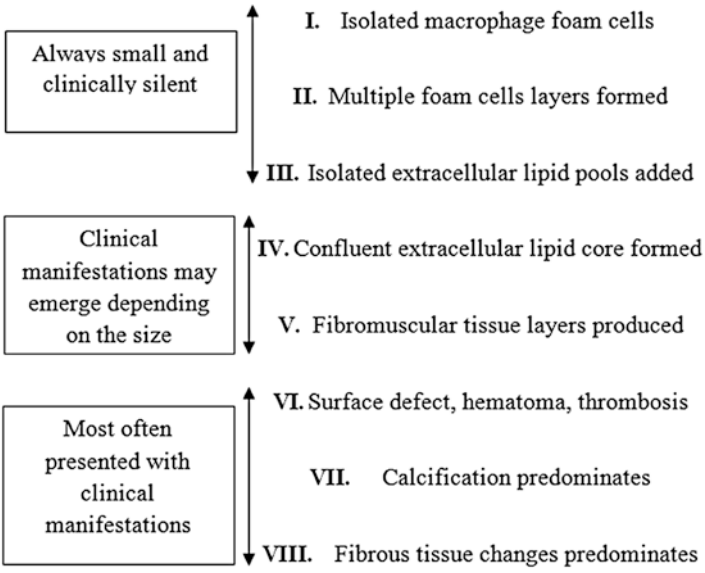

Fig. 7.4 Classification of atherosclerotic lesions of the American Heart Association [15]

smooth muscle cells, generation of mineral deposits, chronic inflammation, neovascularization, disruption of the lesion surface, and formation and transformation of hematoma and thrombus to fibromuscular tissue. The American Heart Association developed a numerical histological classification of the natural development of atherosclerotic lesions, ranging from I to VIII (Fig. 7.4) [15]. Lesions I, II, and III are always small and clinically silent. Conversely, there is no certain correlation between the composition of the plaque and size or the degree of lumen narrowing and clinical manifestations in lesion IV to VIII. Type IV, V, and VI lesions might reduce the lumen vessel to the point of producing a clinical event, or lesions of the same histologies might exist without significant obstruction of the lumen. Despite this, numerous studies indicate that clinical manifestations and fatal outcomes are more often related to processes included from type VI lesion (although, even such processes may remain asymptomatic). Both type IV and V lesions contain a lipid core, but they differ from each other in the nature of the fibromuscular layer that faces the lumen above this core. While in type IV lesions the cap is constituted by preexisting intima, in type V lesions, the cap is formed by tissue disrupted by accumulated lipid and hematoma or organized thrombotic deposits. In type VI lesions, a surface defect, hematoma, or thrombotic deposits are present. Finally, regression or change in lipids in type IV to VI lesions may result in types VII or VIII lesions, in which calcification (type VII) or fibrous tissue changes (type VIII) predominate.

Given the great interest unstable plaques are generating in the last years, ultrasonographic features of these plaques, such as surface, texture, echogenicity, and neovascularity, are detailed below.

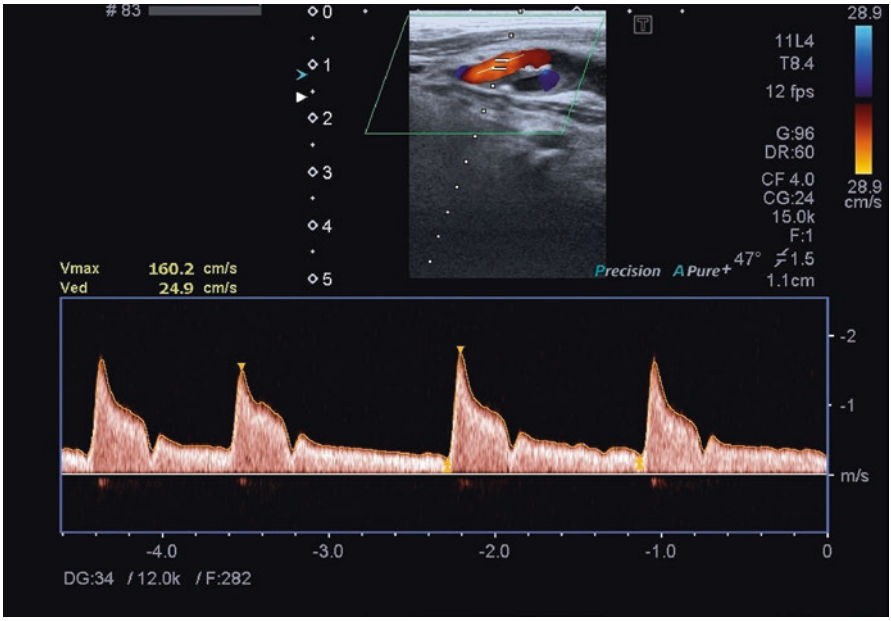

Fig. 7.5 Plaque surface ulceration at the origin of the internal carotid artery

7.2.2.1 Plaque Surface

Among the features of unstable plaques that can be assessed by ultrasound, plaque surface structure is an interesting potential candidate to be included in stroke risk models. The appearance of the plaque surface can be defined as (1) smooth and regular; (2) mildly regular, when height variations between 0.4 and 2 mm are seen along the contour of the lesion; and (3) ulcerated, when flow vortices with reversal flow in the color-coded examination and recesses with an extent of at least 2 mm in depth and 2 mm in length and a well-defined back at its base are present (Fig. 7.5) [16]. While irregular plaques could lead to more disturbed blood flow patterns with high- and low-velocity regions and subsequent increase in plaque stress and in situ thrombosis, ulcerated plaques or with its surface integrity compromised are thought to be vulnerable and prone to embolization.

There are some problems to be considered when assessing plaque surface [17]. An irregular surface does not necessary mean an ulcerated plaque surface, as ulceration can be mistaken for cul-de-sac or pits in fibrotic tissue. Further, small ulcerations or surface defects may not be seen if they are smaller than the resolution of the ultrasound imaging system. Thus, quantitative analyses of plaque surface have been developed to standardize plaque surface evaluation, like plaque surface irregularity index. The plaque surface irregularity index has been found to be greater for plaques with ipsilateral strokes or transitory ischemic attack than for asymptomatic plaques and could predict the presence of ipsilateral hemispheric cerebrovascular symptoms with an accuracy of 66% on its own or 83% in combination with the degree of stenosis [18].

7.2.2.2 Texture

This parameter has several aspects, from homogeneous and mixed forms to completely heterogeneous. The more heterogeneous the plaque, the more complex the structure is and, consequently, the more difficult to analyze. Changes in plaque texture, a good predictor of ipsilateral vascular events [19], are possible to be monitored through carotid ultrasound.

7.2.2.3 Echogenicity

The most studied and validated feature in the evaluation of carotid plaques is echogenicity. This characteristic varies from anechoic forms to mixed and hyperechoic forms. To classify plaques in different grades according to its echogenicity, blood flow is the reference for anechoic plaques, sternocleidomastoid muscle for isoechoic plaques, and cervical vertebrae bone for hyperechoic plaques. To standardize echogenicity evaluation, an analysis known as gray-scale median (GSM) was developed. In this analysis, B-mode ultrasound images are digitized, processed, and normalized with specialized software to calculate GSM value [20, 21]. A good agreement with ultrasound GSM values predicting the type of tissue present in plaques of patients who underwent carotid endarterectomy has been observed, with higher amount of blood and lipid associated with symptomatic plaques [20].

Gray-scale findings that have been associated with unstable plaques are (1) low GSM values [21–24], (2) juxtaluminal black area size [25, 26], (3) discrete white area presence [21], and (4) large plaque area [13, 21]. While a higher GSM value has been associated with a higher percent of calcium in plaque specimen, the presence of discrete white area has been related to a higher hemosiderin grade and inflammation on histopathology examination and juxtaluminal black area near the surface to a higher surgical ulceration score [27].

Echolucent plaques are more commonly found in symptomatic carotid stenosis [28, 29]. A systematic review and meta-analysis showed that, even in asymptomatic patients, predominantly echolucent plaques had approximately 2.3-fold higher risk of future ipsilateral stroke than those with predominantly echogenic plaques [30]. Moreover, patients with >50% carotid stenosis and predominantly echolucent carotid plaques had a 2.6-fold higher risk of ipsilateral stroke compared to those with echogenic plaques.

Combining both texture and echogenicity, a classification of atherosclerotic plaques was performed according to the criteria established by the Consensus Conference concerning the morphology and the risk of carotid plaques [31]. According to this classification, carotid plaques can be divided in the following groups:

– Class I: Uniformly anechoic plaques. These plaques contain material that has low-level echoes close to that of flowing blood (Fig. 7.6).

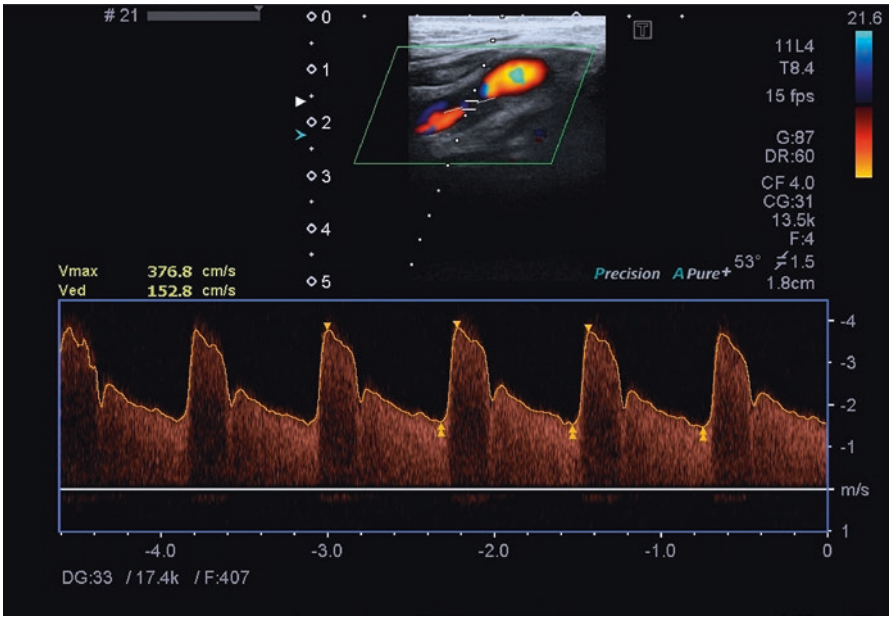

Fig. 7.6 Uniformly anechoic plaque (class I)

- Class II: Predominantly hypoechoic or anechoic plaques. They contain hypoechoic signals within an area corresponding to ≥50% of the cross-sectional area (Fig. 7.7).
- Class III: Predominantly echoic or isoechoic plaques. They exhibit hypoechoic zones occupying <50% of their total cross-sectional area (Fig. 7.8).
- Class IV: Uniformly isoechoic or hyperechoic plaques. These plaques can involve small calcifications. They are found to contain mainly fibrous tissue (Fig. 7.9).
- Class V: Unclassified calcified plaques. Calcified plaques have zones of acoustic shadowing that are difficult or prevent the evaluation of plaque features. Those portions of the plaque that are not obscured by the zone of calcification can still be classified according to the current classification (Fig. 7.10).

7.2.2.4 Neovascularization

Neovascularization originates from vascular tunnels formed by endothelial cells. As they lacked the support from connective tissue and basement membrane, blood vessels become fragile and have a high tendency for vessel rupture and hemorrhage. Neovascularization might promote intra-plaque hemorrhage, rapid growth of the plaque, plaque rupture, and embolization to cerebral territory. Thus, plaque neovascularization has been consistently associated with higher stroke rates [32].

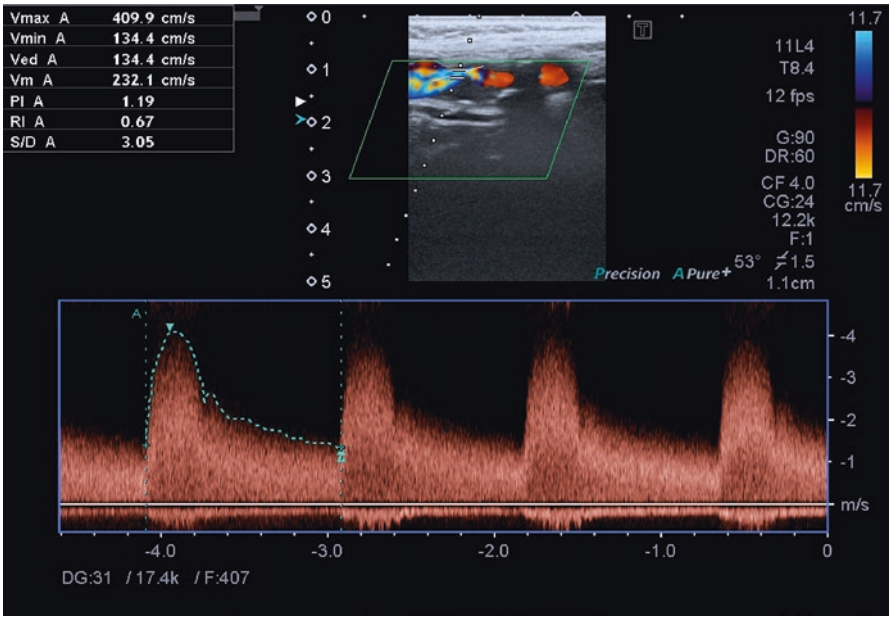

Fig. 7.7 Predominantly hypoechoic or anechoic plaque (class II)

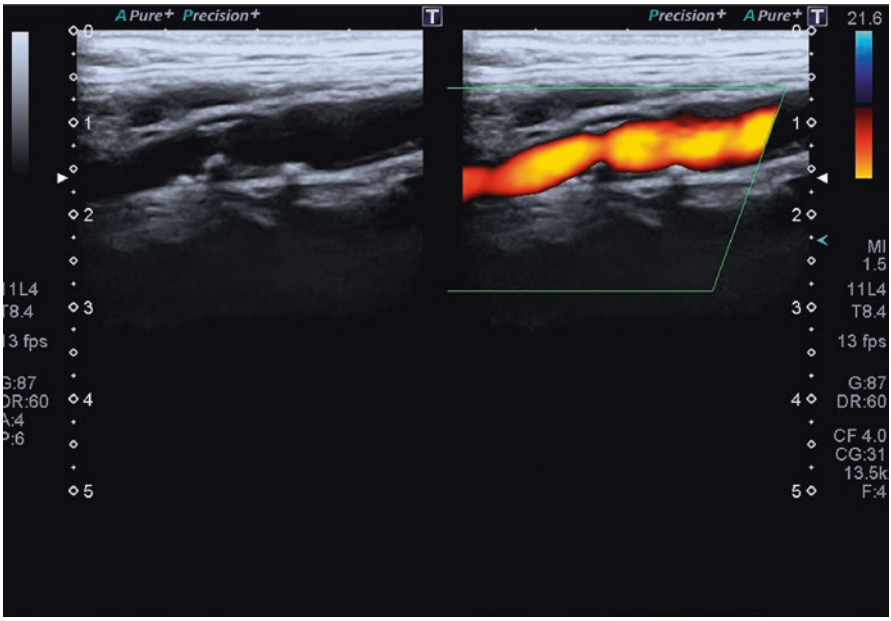

Fig. 7.8 Predominantly echoic or isoechoic plaque (class III)

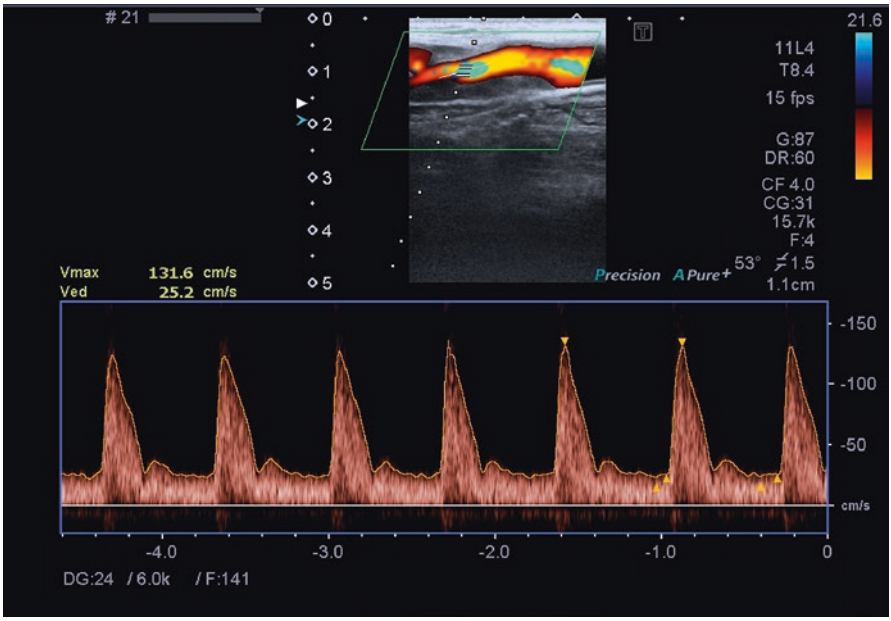

Fig. 7.9 Uniformly isoechoic or hyperechoic plaque (class IV)

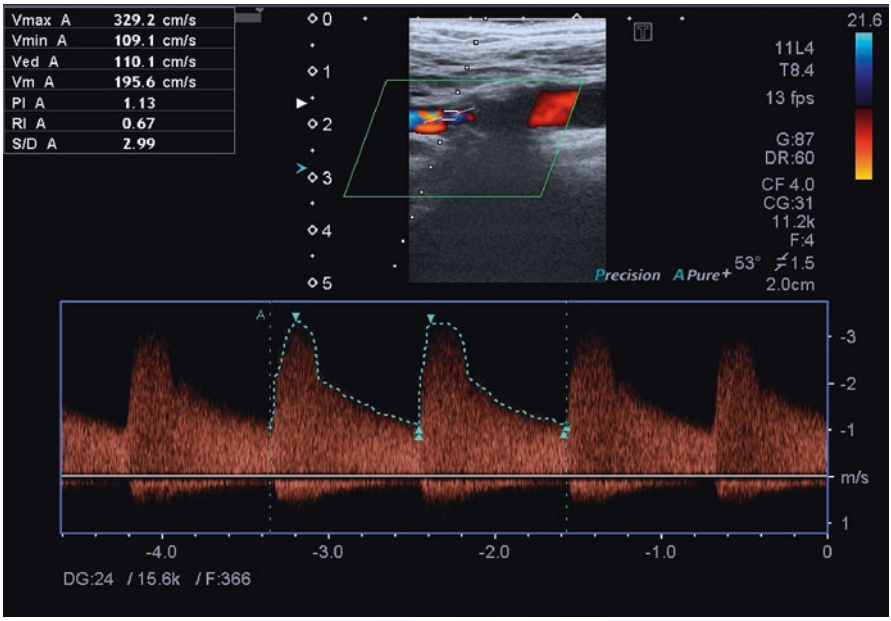

Fig. 7.10 Carotid plaque with acoustic shadow (class V)

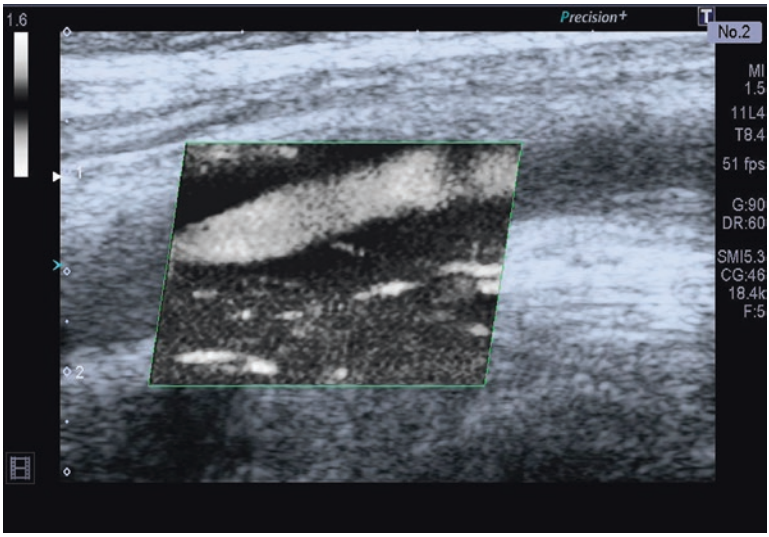

Fig. 7.11 Neovessel through carotid plaque evaluated by Superb Microvascular Imaging®

Neovascularization can be evaluated by means of ultrasound using contrast-enhanced ultrasound sonography (CEUS). CEUS can generate real-time images of microbubbles as intravascular tracers that penetrate the plaque from the vessel lumen or advential side through neovessels. A relationship between contrast enhancement, neovascularization, and inflammation found in anatomopathological studies of plaques after carotid endarterectomy has been described [33]. Thus, CEUS is currently considered the gold standard method to evaluate plaque neovascularization.

New methods to evaluate neovascularization without the need of contrast agents are being developed, such as Superb Microvascular Imaging® technology (Fig. 7.11). Using this technology, it is possible to evaluate neovascularization in real time, yet its correlation is not well established to CEUS.

7.2.2.5 Evidence of Embolic Activity as High-Intensity Transient Signals

The presence of plaque surface irregularities has been related to detection of high-intensity transient signals (HITS) in transcranial Doppler sonography [34, 35], supporting the hypothesis that these lesions might be the source of cerebral embolisms. Under such conditions of plaque irregularities or ulceration, thrombi can be directly exposed to blood flow in carotid artery stenosis. Then, thrombi might be dislodged, either spontaneously or during manipulation, such as revascularization procedure, causing cerebral embolism, which can be detected as HITS on transcranial Doppler monitoring.

Neovascularization by CEUS has also been related to the presence of HITS during endarterectomy [36]. Conversely, plaque echogenicity evaluated by means of GSM does not seem to accurately predict the development of HITS during carotid endarterectomy, possibly due to the overlap among different components of carotid plaques [37, 38].

7.2.2.6 Change in Plaque Appearance over Time

As atherosclerosis is a dynamic process, ultrasound should be repeatedly performed once a carotid artery plaque is diagnosed, evaluating not only changes in degree of stenosis but also in plaque features. Progression of stenosis, defined as a decrease >15% in the lumen cross-sectional area, has been associated with a greater risk of stroke [39, 40]. Further, change in plaque appearance, especially toward a less echogenic structure with anechoic areas, seems to be related to an increased morbidity [41].

7.2.3 Quantification of Degree of Stenosis

Patients included in randomized controlled trials comparing medical and surgical treatment were selected according to the degree of stenosis. Although each laboratory that routinely performs carotid ultrasound should use its reference values previously validated for its population, some recommendations are needed to evaluate properly the degree of stenosis of carotid plaque: (1) insonation angle must be as parallel as possible to blood flow direction, without exceeding 60°; (2) all stenosed area must be examined carefully to find the zone of maximum velocity; (3) peak systolic velocity (PSV) and end-diastolic velocity (EDV) must be recorded at ICA and CCA; (4) stenosis degree should be expressed as an interval, not as an absolute value; (5) recommended intervals are no stenosis, <50%, 50–69%, ≥70% (can be subdivided in 70–79%, 80–89%, ≥90%), and occlusion; (6) stenosis degree is measured according to hemodynamic parameters and not based on the decrease of diameter or the area of the arterial lumen on a B-mode ultrasound; and (7) systolic index (PSV ICA/CCA) is useful especially when a contralateral extracranial ICA high-degree lesion is present [42].

Changes in flow velocity in the area of maximum narrowing of the artery are the most commonly used parameters to grade carotid stenosis, known as direct signs. Conversely, indirect signs include the hemodynamic changes resulting from carotid stenosis that can be observed at CCA, post-stenotic extracranial segment of the ICA, and intracranial circulation. These indirect signs indicate that a hemodynamically significant stenosis or occlusion is present in the extracranial ICA. Both direct and indirect signs are shown in Table 7.1.

Table 7.1 Hemodynamic criteria for establishing the degree of carotid stenosis (adapted from [42])

	Degree of arterial stenosis				
Criteria	<50%	50–69%	70–79%	80–89%	>90%
Direct signs					
PSV	<125	125–230	>230	>300	Variable
EDV	<40	40–100	≥100	Variable	Variable
Indirect signs					
Post-stenotic PSV in ICA	Normal	Normal	≥50	<50	<30
Collateral flow in CW	No	No	No/present	Present	Present

PSV peak systolic velocity, *EDV* end-diastolic velocity, *ICA* internal carotid artery, *CW* circle of Willis

7.2.3.1 Internal Carotid Stenosis of Less Than 50%

This degree of stenosis does not imply hemodynamic repercussion. Although carotid artery plaques might be present, PSV is less than 125 cm/s. Plaques located in the carotid bulb may cause a turbulent pattern of flow instead of typical laminar flow of this area, but with velocities in normal range. Besides, stenosis less than 50% does not result in a decrease of cerebral blood flow and, therefore, no indirect findings will be found.

7.2.3.2 Internal Carotid Stenosis of 50–69%

Carotid stenosis 50–69% will show initial hemodynamic repercussion. Flow velocity will increase at the point of maximum narrowing of the artery, with a higher risk of plaque rupture and cerebral embolism. These levels of stenosis imply a moderate increase of flow velocity, ranging from 125 to 230 cm/s. Indirect findings at CCA, extracranial post-stenotic segment of ICA, or intracranial circulation are not still present since ICA stenosis <70% does not provoke decrease in the cerebral blood flow.

7.2.3.3 Internal Carotid Stenosis of 70–79%

The most clinically relevant cutoff point for stenosis is 70%, since it is the most used threshold for carotid revascularization. The PSV rises above 230 cm/s. To maintain a constant cerebral blood flow, EDV rises above 100 cm/s. Indirect signs include decrease in the flow velocity at the ipsilateral CCA and extracranial ICA post-stenotic flow velocity ≥50 cm/s. A 70–79% degree of stenosis is not associated with a significant decrease in the cerebral blood flow, thanks to an increase in both systolic and diastolic blood flow velocity components. The typical intracranial hemodynamic finding is a discrete decrease of the pulsatility index at the ipsilateral middle cerebral artery (MCA).

7.2.3.4 Internal Carotid Stenosis of 80–89%

The most relevant finding is an increase over 300 cm/s of the PSV. In contrast, EDV tends to decrease as narrowing degree increases. Indirect findings include a decrease in both PSV and EDV, more evident at EDV (CCA externalization). At extracranial post-stenotic ICA segment, PSV is less than 50 cm/s. If the direct findings are inconclusive, a diagnosis of stenosis ≥80% can also be made based on indirect findings if at least two of the following are present: (1) resistance index RI (RI= PSV-EDV/PSV) >0.15, (2) blood flow inversion in the ipsilateral ophthalmic artery, or (3) inversion in the first segment of the anterior cerebral artery (ACA).

As stenosis exceeding 80% is associated with a drop in cerebral perfusion pressure. The MCA ipsilateral to the degree of stenosis will be dampened with a decrease in the pulsatility index. The total cerebral blood flow in the MCA may be normal or abnormal, depending on the quality of collateral intracranial circulation through the anterior and posterior cerebral arteries.

In case the anterior collateral circulation system is activated, we will find an inverse flow at the first segment of the ACA, with a contralateral orthodromic and accelerated ACA. When the posterior collateral circulation system is activated, we will find an asymmetry between both first segments of posteriors cerebral arteries (PCA), due to an increased flow in the first PCA segment ipsilateral to extracranial stenosis. Finding increased blood flow in the second segment of ipsilateral PCA implies the presence of collateral circulation through the long circumferential arteries. The contralateral MCA might show an increased velocity, pointing at collateral flow through the long circumferential arteries. Findings of the ophthalmic artery vary, showing a reduced to null or inversed flow.

7.2.3.5 Internal Carotid Stenosis Greater Than 90%

Increases in velocity in carotid stenosis are proportional to the degree of stenosis as long as stenosis remains below about 90%. At the point of maximum artery narrowing, the flow resistance is so high that the velocity decreases, so it is not a reliable parameter in this case, being indirect signs more useful in the diagnosis. Proximal to the stenosis, a clear increase in the pulsatility index is usually observed in the CCA, and the blood flow in the extracranial ICA segment beyond the point of maximal stenosis is less than 30 cm/s. Indirect findings of the intracranial cerebral circulation are similar to those described for stenosis degree >80%.

Sometimes, it is difficult to differentiate between a stenosis >90% and an occlusion of the artery. It is advisable to obtain multiple longitudinal and axial sections of the artery, and to use the power Doppler mode, which is able to detect slow blood flow more accurately than color mode. Moreover, the pulse repetition frequency must be as lowest as possible, in order to detect slow-moving flows. Although using these technical considerations, it is advisable to use another image technique, such as CTA or MR angiography (MRA) to confirm artery occlusion, regarding clinical implications of the diagnosis.

7.2.3.6 Internal Carotid Artery Occlusion

Internal carotid artery occlusion might be mistaken for a normal exploration if we are not careful. It is necessary to identify both carotid arteries, the ICA and the ECA in the B-mode examination, before beginning the spectral waveform analysis. In case of ICA occlusion, regarding alone spectral waveform, an internalized ECA might be confused for a normal ICA. As it is said previously, another exploration is recommended in order to confirm artery occlusion.

7.3 Other Techniques of Carotid Plaque Evaluation

The development of different techniques has allowed improving the evaluation of unstable plaques, such as CTA, MRI, and PET, as described below.

7.3.1 Computed Tomography Angiography (CTA)

Some studies have shown that CT Hounsfield density can be used to distinguish between lipid-rich necrotic core, connective tissue, intra-plaque hemorrhage, and calcifications. Calcifications are easily identified because of their high density, yet there is an overlap between connective tissue, lipid-rich necrotic core, and intra-plaque hemorrhage. As a general rule, and similarly to ultrasound examination, the lower the density of the plaque, the more likely it is to be vulnerable.

Multidetector-row CT (MDCT) allows for multiplanar reconstruction in the axial, sagittal, and coronal planes. This technique is also effective at detecting plaque neovascularization and ulceration [43, 44]. Plaque contrast enhancement has also been shown to correlate to both plaque neovascularization and ulceration [45, 46]. Further, plaque contrast enhancement evaluated by MDCT is correlated with plaque symptomatology [47]. It is useful to use delayed phase images to distinguish between stable and unstable plaques. While stable plaques tend to have progressive enhancement of the plaque in delayed phases, unstable plaques have more washout, possibly due to the presence of an increased neovascularization, leading to increase washout of contrast [5]. This technique might be also used to monitor progression of carotid artery plaques and its response to medical treatment such as statins [48].

7.3.2 Magnetic Resonance Imaging (MRI)

MRI is possibly the most well-established technique in the evaluation of components of carotid plaques. There is a huge variety of sequences that can be used in plaque characterization [49], such as fast spin echo, which allows for an extremely

high spatial resolution, and gradient echo, useful to detect intra-plaque hemorrhage and lipid-rich necrotic core. On the other hand, fat suppression, used in all sequences to suppress the signal from subcutaneous tissue, results in an improved contrast among plaque components, as well as carotid wall and surrounding tissues. Contrast-enhanced images with gadolinium are needed to better differentiate necrotic core from fibrous tissue and to evaluate plaque neovascularity. The lipid-rich necrotic core and the intra-plaque hemorrhage do not enhance as they are avascular components of the plaque, so this sequence is not useful to distinguish between both components of plaques.

Some studies that compared MRI findings to histopathology demonstrated that MRI can accurately differentiate plaque calcification, fibrous cap, intra-plaque hemorrhage, and lipid-rich necrotic core [50]. Further, MRA is extremely sensitive in detecting plaque ulceration, due to the fibrous cap that appears as a dark band between the bright lumen and the gray plaque, so the absence of that dark band indicates ulceration.

Plaque characterization with MRI is a valuable and reliable tool in predicting ischemic events due to rupture and embolism of the plaque. A systematic review of patients with carotid artery plaques characterized by MRI showed that intra-plaque hemorrhage, lipid-rich necrotic core, and thinning or rupture of the fibrous cap are associated with a higher risk of transient ischemic attack or stroke [8]. Furthermore, MRI plaque characterization has been shown to have a stronger association with symptomatic patients than the degree of stenosis [51]. Intra-plaque hemorrhage is another finding that has been correlated with symptomatic events, independently of the degree of narrowing of the artery [52, 53]. Similarly, plaque enhancement is also associated with ipsilateral ischemic events regardless of the degree of stenosis [54, 55].

Several studies have shown the usefulness of MRI in monitoring the evolution and progression of carotid plaques and the response to different medical strategies, such as statins [56, 57].

7.3.3 Positron Emission Tomography (PET-CT) with 18F-Fluorodeoxyglucose (FDG)

18F-FDG is partially metabolized through glycolysis within the atherosclerotic plaque, so it serves as a marker of plaque inflammation and hypoxia. Several studies have shown the correlation between findings of 18F-FDG PET/CT and histopathological examination. Specially, it has been proven to be useful as a marker of inflammation (macrophage infiltration) [58]. Conversely, the relationship between plaque neovascularization and FDG uptake is considered weak, and, therefore, it is not a good image modality to assess plaque vulnerability and progression [48].

Increased FDG uptake is known to be associated with symptomatic patients and future ischemic events [59–61]. A promising alternative to 18F-FDG PET/CT alone

is its combination with another image modality that adds anatomical resolution to the analysis of atherosclerotic plaque, such as MRI. Some investigations have examined the combination of 18F-FDG PET/CT to detect inflammation and MRI to better describe morphological features and characterization of unstable carotid plaques [62].

As the other techniques previously described, 18F-FDG PET/CT has been shown to be useful in monitoring the response to medical therapy [63]. However, its expensive cost limits are cost-effectiveness.

7.4 Non-stenotic Carotid Artery Plaques as a Potential Source of Cryptogenic Strokes

Since secondary prevention of ischemic stroke varies according to the etiology of the primary event, it is mandatory to try to find its cause with a complete large artery study and cardiologic investigation. However, in up to 25% of cases standard investigations fail to identify the source [64]. Regarding TOAST classification [2], to attribute a stroke to carotid stenosis it has to cause at least 50% degree of lumen narrowing. Despite this, more recent stroke etiology schemes include the carotid plaques with less than 50% of lumen narrowing as a potential cause of stroke [65]. It has been observed that large (≥3 mm thickness) but non-stenotic carotid artery plaques measured by CTA are more commonly found ipsilateral to cryptogenic stroke than contralateral [66]. Other authors have recommended endarterectomy for patients with recurrent cryptogenic stroke and large, non-stenotic carotid artery plaques [67], even if previous clinical controlled trials did not demonstrate benefits of endarterectomy for non-stenotic plaques.

High-resolution carotid plaque MRI enables a detailed analysis of vulnerable plaque features [8], such as intra-plaque hemorrhage, lipid-rich necrotic core, and disrupted fibrous cap. A study that included patients with cryptogenic stroke found that high-risk imaging features, indicative of type VI plaques according to the American Heart Association classification (Fig. 7.4), were more frequently located ipsilateral to the side of the stroke than contralateral [68]. Other studies using MRI have shown an association between intra-plaque hemorrhage (evaluated by 3D T1-weighted [69] and time-of-flight [70] pulse sequences) and ipsilateral cryptogenic ischemic events in patients with less than 50% of lumen narrowing in the carotid artery.

Although carotid ultrasound is a well-recognized, noninvasive, and widely used technique to assess carotid plaque features, data investigating the role of ultrasound for determining the cause of cryptogenic strokes is lacking.

7.5 Summary

Carotid artery plaques are a common and well-recognized cause of ischemic stroke worldwide. Ultrasound is a safe, easy-to-use, and widely available technique that allows a complete evaluation of atherosclerotic plaques. On the one hand, it allows assessing the degree of stenosis according to well-established intervals regarding velocimetry parameters of the Doppler spectra waveform. On the other hand, analysis using B-mode of the different components of carotid plaque is also available and correlated to histopathological examinations. As a general rule, the more hypoechogenic, the more vulnerable and fragile the plaque is. By adding the use of contrast agents, the plaque neovascularization, an important marker of plaque instability with a growing evidence of its usefulness, can be evaluated.

Other techniques such as CTA, MRI, and 18F-FDG PET/CT have proven their correlation to histopathological studies in the evaluation of plaque instability and, in case of CTA and MRI, their utility in monitoring the progression of plaques and response to different medical therapies. It is foreseeable that the increasing knowledge about pathophysiological involvement of unstable plaques, even those which do not cause a significant narrowing of the lumen, makes us rethink about current stroke classification and therapeutic strategies.

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Chapter 8
Hypertension and Stroke

Jun Huang and Pingjin Gao

Abstract Hypertension is considered as the most important contributor to stroke patients among all the risk factors. Over 70% of stroke patients accompany with chronic hypertension. Hypertension could induce the brain vascular system change, and these changes likely impair brain blood flow autoregulation and contribute to the increased infarct size after vessel occlusion. As the preclinical researches developed, a better understanding is emerging of how hypertension induces damage of the cerebrovascular dysfunction, raising the possibility of protecting the brain from the ravages of hypertension. The mechanisms that high blood pressure increased incidence and severity of stroke in hypertensive patients remain poorly understand. The appropriate blood control will promote function and potentially repair ischemia damaged of stroke patients. By examining hypertension and antihypertensive treatments in hypertensive animal stroke models, we may develop a better understanding of how to manage hypertension in the acute stroke setting. And with our constantly broaden understanding of hypertensive mechanisms, scientist now have the opportunity to develop new strategies to control blood pressure in the acute stroke therapy or chronic blood pressure management after stroke, which could help recovery or prevent stroke recurrence.

Keywords Antihypertensive treatment • Animal research • Autoregulation • Blood pressure management • Brain • Cerebrovascular • Clinical trial • Drug discovery • Hypertension • Stroke • SHR

Abbreviations

AHA	American Heart Association
AngII	Angiotensin II
AS	Arterial stiffening

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BBB	Blood-brain barrier
BP	Blood pressure
DBP	Diastolic blood pressure
EPCs	Endothelial progenitor cells
L-NAME	L-nitroarginine methyl ester
MCAO	Middle cerebral artery occlusion
MSCs	Mesenchymal stem cells
RHRSP	Stroke-prone renovascular hypertensive rats
SBP	Systolic blood pressure
SHRs	Spontaneously hypertensive rats
SHRSP	Stroke-prone spontaneously hypertensive rats
WKY	Wistar-Kyoto rat

8.1 Introduction

Hypertension, defined as elevation in blood pressure (BP) above 140 mmHg systolic or 90 mmHg diastolic, is the most prevalent of all noncommunicable chronic disease throughout the world [1]. It afflicts 25% of the general population and is the premier risk factor for serious diseases affecting the brain, heart, and kidneys [1]. The brain is a major target of the deleterious effects of hypertension and is responsible for a large portion of the related mortality and morbidity [2]. Several studies have shown that the elevation of blood pressure could dramatically increase the risk of stroke. In clinical trials, antihypertensive therapy has been shown associated with reductions in stroke incidence, with an average 41% reduction in stroke risk with SBP (systolic blood pressure) reductions of 10 mmHg [3]. It is estimated 52% of all strokes are attributable to hypertension [4].

8.2 The Relationship of Hypertension and Stroke

8.2.1 Hypertension and Brain Vascular Damage

The intracranial cerebral arteries take off from the circle of Willis at the base of the brain and branch out into smaller vessels, which penetrate into the substance of the brain. The brain vascular has the adaptive mechanisms that assure that the brain receives an adequate amount of blood at all times. Hypertension could disrupt the adaptive mechanisms and induces both structural and functional brain vascular alterations.

8.2.2 Impairment of Cerebrovascular Autoregulation

Cerebrovascular autoregulation maintains cerebral blood flow stability during variations in arterial pressure within a certain range, 60–150 mmHg mean arterial pressure [2, 5]. Arterial pressure varies markedly lead to potentially dangerous increases or decreases in cerebral blood flow. Cerebral arterioles adjust their resistance according to intravascular pressure; they constrict when the pressure increases and relax when the pressure decreases. Autoregulation is related to the ability of arterial myocytes to constrict when intravascular pressure rises [6].

Hypertension impairs autoregulation by reducing the vascular lumen and increasing cerebrovascular resistance [7, 8]. Alterations in autoregulation increase the susceptibility of the brain to cerebral ischemia when blood pressure drops because cerebral blood vessels fail to compensate for the reduction in perfusion pressure [9]. Impaired autoregulation also leads to more severe ischemia after arterial occlusion. That is one of the major reasons that spontaneously hypertensive rats lead to larger infarcts than normotensive rats when middle cerebral artery occlusion [10].

8.2.3 Endothelial Dysfunction

Cerebral endothelial cells can release vasodilators and vasoconstrictors to keep the vascular function [11]. Endothelium-derived vasoactive factors participate in maintenance of the vasodilation, while hypertension alters endothelium-dependent relaxation of cerebral blood vessels [11]. The increase in cerebral blood flow produced by neocortical application of endothelium-dependent vasodilators is attenuated in spontaneously hypertensive rats or in wild-type mice infused systemically with AngII [12–14].

8.2.4 Hypertension Alters the Structure of Cerebral Blood Vessels

Hypertension induces the formation of atherosclerotic plaques in cerebral arteries and arterioles, which may lead to arterial occlusions and ischemic injury [2]. In addition, hypertension induces fibrinoid necrosis (lipohyalinosis) of penetrating arteries and arterioles supplying the white matter, which could result in small white matter infarcts (lacunar infarction) or brain hemorrhage [15].

8.2.5 Hypertrophy, Remodeling, and Stiffening

Hypertension induces adaptive changes in systemic and cerebral arteries known as hypertrophic and eutrophic remodeling. In hypertrophic remodeling process, hypertension increases the artery wall thickness and reduces the lumen of the vessel [8].

This process could reduce stress on the vessel wall and protect downstream microvessels from the effect of high blood pressure [8, 16]. Failure of this protective mechanism leads to BBB alterations, cerebral edema, and cerebrovascular pathology. Several factors contribute to hypertrophy, including sympathetic perivascular innervations [17], growth factors, oxidative stress, and nitric oxide (NO) [18, 19].

Angiotensin II (AngII) is a key factor in the mechanisms of cerebrovascular remodeling [20]. It is reported that treatment of spontaneously hypertensive rats with angiotensin-converting enzyme inhibitors attenuates remodeling independently of effects on blood pressure [21]. AngII-induced hypertension is associated with activation of MMP and breakdown of matrix proteins [22]. In AngII-treated mice, the increase in cerebral blood flow induced by activation of the somatosensory cortex by stimulation of the facial whiskers is markedly attenuated [13].

Vascular stiffening is also induced by hypertension, which increases collagen content and rigidity of the vessel wall [8]. The stiffening leads to increase in pulse pressure, which is a good predictor of stroke [23].

The intracranial artery stiffening is common in hypertension patients with stroke. It is reported that the mean SBPs during the day were significantly associated with intracranial arterial stenosis [24]. The increased arterial stiffness was independently associated with intracranial arterial stenosis, and blood pressure might be useful to identify those more likely to have intracranial artery disease among hypertensive patients [25].

Briefly, hypertension alters the functional hyperemia, autoregulation, and endothelial function. The structural alterations of cerebral blood vessels reduce the compensatory capacity of the cerebral circulation and increase the susceptibility of brain vascular insufficiency.

8.2.6 Hypertension Increases the Risk of Stroke

There are ten known attributable risk factors of ischemic stroke, including hypertension, current smoking, obesity, poor diet, inactivity, diabetes mellitus, high alcohol intake, psychosocial stress and depression, cardiac causes, and lipid abnormalities [4]. And 90% of the stroke population is due to these risk factors. Of all these risk factors, hypertension is considered as the most important contributor to overall risk and has been reported in 77% of patients experiencing their first stroke [26]. As reported previously, there is a linear relationship existing between blood pressure and stroke mortality, and in patients with treated hypertension, a 1 mmHg increase in systolic blood pressure increases stroke deaths by 2% [27]. Another report showed the link between BP and the risk of a first stroke is observed at a low threshold of 115/75 mmHg; among the aged 40–69 years, each increase of 20/10 mmHg doubles the death rate from stroke [28]. A meta-analysis of 19 prospective cohort studies found that prehypertension is associated with incident stroke. The risk is particularly noted in those with BP values in the higher prehypertension range [29]. The benefit of BP-lowering therapy for stroke prevention depends positively on the magnitude of the reduction [30, 31].

In young ischemic stroke patients, high acute phase BP levels are independently associated with a high risk of recurrent strokes [32]. Several studies have shown significantly lower rates of recurrent stroke with lower BP. Most recently, the BP reduction component of the clinical trial showed that targeting a systolic BP <130 mmHg was likely to reduce recurrent stroke by about 20% ($P = 0.08$) [33]. Further support for the importance of hypertension as a stroke risk factor is antihypertensive medication clinic trail research.

8.3 Blood Pressure Management in the Stroke

8.3.1 BP Control in Acute Ischemic Stroke

The benefit of BP control is obvious in stroke therapy, while limited data are available elaborating on immediate BP management in acute ischemic stroke. How to control high blood pressure within the first 24–48 h after stroke and how the presence of hypertension affects the efficacy of neuroprotective treatments administered within this period are still less understood.

Some researches show that high baseline and high variability of blood pressure during the first 24 h were associated with higher numbers of early adverse events and early deaths. A larger decline in blood pressure and the use of blood pressure-lowering treatment during the first 24 h were associated with a reduced risk of poor outcome at 6 months. There are some other reports showing that approximately 70% have elevated SBP ≥140 mmHg in the acute period [34]. The elevated SBP can help to rapidly increase cerebral blood flow, oxyhemoglobin, and cerebral metabolic rate of oxygen in both the core and penumbra, thus preventing the expansion of cerebral blood flow deficit in the acute ischemic stroke [35, 36], and decreases spontaneously over the next few days [37].

Though conflicts exist, most guidelines recommend over progressive measures. Therapeutic intervention, indicated for established ≥140/90 mmHg or preexisting hypertension, is suggested to start beyond the first several days from stroke onset [38] or at least after the first 24 h [39]. Treatment within the first 24 h is warranted only for patients eligible for fibrinolytic therapy (to maintain BP <185/110 mmHg) or with SBP >220 mmHg or DBP >120 mmHg [38, 39]. However, the benefits of treating hypertension in the acute setting of ischemic stroke remain controversial [39], especially for those with SBP/DBP <140/90 mmHg. Furthermore, an optimal target is not specified in most guidelines, but instead, individualized BP management based on the patients' condition may be a good choice [39, 40].

8.3.2 Chronic Blood Pressure Management After Stroke

To prevent stroke recurrence, long-term management of hypertension and numerous interventions are needed [41]. In a statement for healthcare professionals from the AHA, patients with stroke were considered to be at high risk of further coronary and

cardiovascular events [42]. The association between hypertension and risk of second ischemic stroke is well established [38]. It is reported that antihypertensive treatment contributes to secondary stroke prevention and risk reduction for cardiac events in patients with previous stroke [43], while controversy still remains about the timing of optimal BP reduction and the target or ideal antihypertensive management for recurrent stroke prevention [44].

The best time to restart long-term antihypertensive therapy is not well established but after the initial 24 h is considered reasonable [39, 41]. The newest 2014 AHA stroke guidelines recommend that an individualized therapeutic BP goal is <140/90 mmHg. Chronic blood pressure management should be more aggressive for patients with a recent lacunar stroke [38]. At the same time, lifestyle modifications are highly recommended, including salt restriction, weight loss, healthy diet, regular aerobic physical activity, and limited alcohol consumption [41]. In addition, considering specific patient characteristics for specific drugs and goals, determination is highly recommended [41].

8.4 Hypertension and Experimental Stroke Therapies

Although recent studies on stroke are improving, the mechanisms underlying the protection should be more clearly recognized in basic research. In order to improve the translatability of preclinical stroke research to the stroke patient, animal models with comorbid disease need evaluation of interventions in animals [45]. Various models of experimental hypertension have been primarily developed to mimic hypertensive responses observed in humans [46]. These models are beneficial in the pharmacological screening of potential antihypertensive drugs, in addition allowing researchers to have a better understanding of the etiology, development, and progression of stroke with hypertension [47].

8.4.1 Hypertensive Animal Models in Stroke

Animal models of hypertension in the stroke studies research are not widely used from published reports. There is a report showing that only 10% were tested in hypertensive animals of preclinical evaluation of 502 experimental therapies for acute focal ischemic stroke [48]. Analyzing studies conducted in hypertensive animals using middle cerebral artery occlusion (MCAO—the most widely used model of ischemic stroke), more than half of the studies were done using SHRs. This is followed by stroke-prone spontaneously hypertensive rats (SHRSP) and stroke-prone renovascular hypertensive rats (RHRSP) [48].

8.4.1.1 SHR

Spontaneously hypertensive rats (SHRs), as a genetic model of hypertension, have been proposed to model essential hypertension in humans and are one of the most important animal models for stroke research [49]. It has been more than 50 years since the SHRs were established [50]. They were initially obtained by selective in breeding from the Wistar-Kyoto rats (WKY) with the highest blood pressure. SHRs were sent to the National Institute of Health (NIH) at the F13 generation in 1966 [51].

The blood pressure levels of SHR could be classified into three time points: (1) the prehypertensive period (4 weeks), (2) the period of rapidly rising blood pressure (8 and 12 weeks), and (3) sustained hypertension (16–18 weeks) [52]. Blood pressure of SHR rises between 1 and 5 months, and blood pressure and body weight remain constant thereafter [53].

There are at least three advantages of using SHR in contrast to normotensive rats. First, SHRs present with comorbidity. Second, the SHRs after middle cerebral artery occlusion (MCAO) are reproducible and have the adequate-sized infarction. Furthermore, SHRs have similar therapeutic time window and cerebral blood flow threshold for infarction to normotensive rats [54–56].

There are disadvantages of using SHR, including (1) SHRs are more expensive compared to normal rats, (2) high mortality in aged SHR, and (3) resistance to some therapy. Another confounding problem with using SHR in stroke research is that SHR and WKY from different sources are genetically heterogeneous [56–58].

The mostly used models of MCAO in SHR are distal MCAO and the intraluminal suture model. According to a recent review, distal MCAO is the predominant method of focal ischemia in SHR, including models of distal MCAO, distal MCAO combined with ipsilateral common carotid artery occlusion, and photo thrombotic distal MCAO; they were all based on the occlusion of distal portion of MCAO. The intraluminal suture model was the second frequently used MCAO model [48].

8.4.1.2 SHRSP

The stroke-prone SHRs (SHRSP) were established from SHR with stroke phenotype [59]. They have a high incidence of spontaneous stroke though the tendency of SHRSP to develop spontaneous strokes is unclear. SHRSP can also be modulated by the type of diet and degree of salt loading, which with salt-loading SHRSP show higher BP levels than SHR [60]. SHRSP develop not only cortical infarcts and hemorrhage; SHRSP may also mimic small vessel disease and lacunar stroke. Interestingly, SHRSP also show BBB leakage even before the development of full hypertension and vessel damage, though whether these changes are directly linked to the high blood pressure is unclear [61]. As they develop spontaneous stroke, SHRSP are often used in primary stroke prevention studies [58]. However, they are also used in post-stroke intervention studies through induction of MCAO.

8.4.1.3 Other Models

Other models of hypertension that develop spontaneous stroke include the Dahl salt-sensitive rats. These rats were developed by Lewis K Dahl through selective breeding of rats that showed a pronounced hypertensive response upon salt ingestion. These salt-sensitive rats developed moderate hypertension on normal salt diet [62]. They can develop blood-brain barrier disruption, stroke lesions, and intracerebral hemorrhage as early as 5 weeks when they were on a high-salt diet (8% NaCl) [63, 64]. Further increases of BP of hypertensive mice or transient acute increase of BP on top of chronic hypertension has also been described to cause spontaneous intracerebral hemorrhage. Double transgenic mice (overexpress both human renin and human angiotensinogen) develop further increase in BP and intracerebral hemorrhage upon treatment with high salt + L-NAME (L-nitroarginine methyl ester, an inhibitor of nitric oxide synthase) [65]. Similarly, AngII infusion + L-NAME-induced chronically hypertensive mice develop intracerebral hemorrhage upon injection of angiotensin II or norepinephrine [66].

8.5 Treatment Research in Hypertensive Animal Models

8.5.1 Antihypertensive Treatment

The category of antihypertensive treatment in the hypertensive stroke research includes (1) angiotensin-converting enzyme inhibitors, (2) beta (adrenergic) blockers, (3) calcium channel blockers, (4) vasodilators, (5) AngII antagonists, (6) diuretics, (7) anti-adrenergic agents, (8) aldosterone antagonists, and (9) all other treatment types. Usually treatments were ascribed to a particular antihypertensive treatment class if the therapy had been used clinically—not just experimentally—for this purpose. The "all other treatment" category included experimental therapies, which may also have effects related to hypertension (such as stem cell therapy) but which have not been used clinically for this purpose. Therapeutic effects of all these antihypertensive treatments have shown success in the stroke treatment.

8.5.2 Stem Cell Therapy After Hypertension Stroke

Current treatments for acute ischemic stroke mainly on vascular recanalization, including intravenous thrombolysis and interventional treatments, have a narrow therapeutic time window after onset. Endothelial progenitor cells (EPCs) participate in endothelial repair and angiogenesis. EPCs have the ability to differentiate into endothelial cells and to secrete protective cytokines and growth factors [67, 68]. Studies on ischemic brain, heart, and limbs indicate that transfusion of EPCs is able to reduce tissue injury and promotes angiogenic repair and function recovery. These

positive results provide a good rationale for using EPCs to treat ischemic stroke in hypertension.

Several studies have shown that the blood pressure values have a close relationship with the impaired EPC function and number. On the basis of epidemiological and pathophysiological significance of hypertension, many authors have focused on the relationship between this condition and EPCs, but with conflicting results. It is reported that EPC migration was inhibited in hypertension patients [69]. A strong correlation between the number of circulating EPCs and the patient's combined Framingham risk factor score were observed [70]. Similarly, another report showed the correlation between low EPC count and high blood pressure values [71]. The number of circulating EPCs was significantly reduced in refractory hypertension as compared to healthy subjects [72]. Consistently, preliminary data about renal denervation in refractory hypertension patients showed that after the procedure, the improvement of blood pressure control was accompanied by the increase of peripheral $CD34^+$ cell number [73]. Even in prehypertensive adults (SBP greater than 130 mmHg but lower than 139 mmHg), the ability of EPCs to form colonies is impaired compared to normotensive subjects [74]. Otherwise, in essential hypertensive patients, reports showed that the EPC number was not statistically different from that found in control subjects [75]. When hypertensive patients were divided into two groups according to the presence of isolated arterial stiffening (AS) or AS and both carotid intima-media thickening and left ventricular hypertrophy, studies found that hypertension with more advanced vascular and cardiac involvement had fewer circulating $CD34^+$ cells than hypertension with earlier vascular lesions but more than normotensive controls [76]. Even more, it is suggested that different EPC phenotypes may exist in different subsets of hypertensive patients [77, 78]. Different phenotypes have been used by different authors; accordingly, a straightforward comparison among the studies remains difficult to be performed. So how to explore the EPC function after stroke in hypertension patients is a potential therapy target.

Bow marrow-derived mesenchymal stem cells (MSCs) are another popular stem cells used in stroke therapy [79, 80]. MSCs are believed to be promising for cell administration therapy after ischemic stroke, because of their advantageous characteristics, such as ability of differentiation into neurovascular lineages and avoidance of immunological problems. MSC administration is also considered as a potential therapy in hypertensive stroke research.

8.6 Limitations and Perspective of Translational Research of Hypertension and Stroke

8.6.1 Limitations

As one of the leading comorbidities in stroke patients, hypertension could be research in several animal models in stroke. While, from the perspective of translational medicine, the results of animal and human therapeutic efficacy are not always

inconsistent, the major strengths and weaknesses of the most commonly used animal models should be highlights. There are several limitations causing the discrepancy between the basic science research and clinical trial.

First, human ischemic stroke with hypertension is complex, and no single preclinical animal model can mimic all the variables of the blood pressure and stroke injury and recovery. Though SHR is regard as the best animal model to mimic the progress, the genes involved in human essential hypertension may not be the same as those involved in SHR, and the similarities have been a subject of debate.

Second, different mechanisms could be involved in the development of hypertension in humans versus SHR. SHRs have neural and cerebrovascular changes that are different from normotensive rats and may mimic the changes in the cerebravasculature of people with genetic hypertension [81].

Third, depending on different age of the animals and environmental conditions, mean arterial blood pressure can vary from 120 to 200 mmHg [58]. Stroke induction leads to further increases in BP, which may affect the BP data collection at the early stage of stroke [82].

Forth, BP is greatly affected by the depth of anesthesia, and therefore, the degree of anesthesia and duration of surgery should be controlled [58].

Fifth, age and sex may be the factors that accounted for the discrepancy between the preclinical animal experiments and the clinical trial in aged patients. The use of predominantly young adult animals in experimental stroke research has created a barrier for translation of findings to patients.

Among the mentioned limitations are the inevitable differences between animal experiments and human stroke, for example, the animal model could not totally mimic human hypertension. Some limitations should be controlled in the experiments, such as animal age, sex, and the depth of anesthesia. Also, in both animal and human studies, the limitations with respect to the mechanistic aspects of the therapeutic strategy should be considered, since different mechanisms of protection between experimental and clinical settings may lead to failure in translational research.

8.6.2 Perspective

To translate the results in animal models into human stroke, common mechanisms underlying the ischemic injury or protection by therapy need to be elucidated both in the animal model and human stroke.

In preclinical stroke research conditions, diverse brain ischemia models and hypertension models are being incorporated into the research design. Such as SHR, which have comorbidity and are suitable for stroke research, only 11% of animal experiments on neuroprotection involved testing in hypertensive rats [83]. There are still lots of development space in the hypertensive stroke research, especially in the preclinical research.

1. Special attention to details such as mean blood pressure in hypertension, mean blood glucose, lipid profile, and body weight needs to be clearly reported. This detail information is useful for identifying the disease duration and severity.
2. Mortality is often higher in stroke models with preexisting hypertension. Reduction in stroke-related mortality can often be achieved with reducing the duration of occlusion or reducing the infarct size. Distal MCAO are highly common in hypertension, for distal MCAO could make small, cortical, survivable infarcts and subcortical white matter strokes, which are desirable for long-term recovery studies. If the large artery stroke model is needed, for comparisons for therapeutic interventions, mortality rate, which is usually doubled in comorbid disease states, should be reported.
3. When comparing stroke outcomes between controls and animals with hypertension, either age or weight must be matched, because hypertensive animals do not gain weight at the same rate as normal healthy animals.
4. After the effect research of a therapeutic intervention on stroke compared in adult male control and hypertension animals, studies in female and aged animals could be followed.

In summary, we have got the great success in research hypertension and stroke treatment in the last 50 years. The current effort to reduce the influence factors of animal model mimics the patient hypertension, and the dialogue over optimal treatment targets offers an opportunity to sustain and accelerate the positive trends in stroke research. The challenge remains for the clinicians and basic science researchers for further advancement. As we broaden the knowledge of hypertension and stroke, we can continue to move in the right direction toward protecting the brain from the ravages of hypertension.

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Chapter 9
Diabetics and Stroke

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Abstract Diabetes mellitus is one of the largest global health emergencies threating public health worldwide. Chronic elevation of blood glucose level can lead to injury of blood vessels resulting in chronic diabetic complication, that is, diabetic vasculopathy. With respect to this vascular dysfunction in brain vasculature, diabetes mellitus confers an increased risk of ischemic stroke. Strokes in patients with diabetes are usually associated with a worse outcome. The cerebrovascular protection is not only limited to prevention of diabetes but also diabetes-induced detrimental changes in vascular structure and function before the occurrence of stroke. Effective and acute prevention of vascular dysfunction and blood–brain barrier disruption following acute ischemic stroke is important in diabetic stroke treatment.

Keywords Diabetes • Brain • Stroke • Epidemiology • Atherosclerosis • Vasculopathy • Remodeling • Biomarker • Oxidation • Inflammation • Blood–brain barrier • Hemorrhagic transformation • White matter • Recovery • Imaging • Prevention • Stem cells

Abbreviations

ACCORD	Action to Control Cardiovascular Risk in Diabetes
ADA	American Diabetes Association
ADEPS	Amrita Diabetes and Endocrine Population Survey

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AGEs	Advanced glycation end-products
BBB	Blood–brain barrier
BDNF	Brain-derived neurotrophic factor
BMSCs	Bone marrow stromal cells
BOLD	Blood-oxygen-level dependent
BP	Blood pressure
CRP	C-reactive protein
CURES	Chennai Urban Rural Epidemiology Study
CVD	Cardiovascular disease
DM	Diabetes mellitus
DTI	Diffusion tensor imaging
ECs	Endothelial cells
eNOS	Endothelial nitric oxide synthase
ENSANUT 2012	2012 National Health and Nutrition Survey
EPC	Endothelial progenitor cell
fMRI	Functional MRI
GDM	Gestational DM
GIST-UK	Glucose–potassium–insulin infusion in the management of poststroke hyperglycemia in UK
GK	Goto-Kakizaki
GRASP	Glucose regulation in acute stroke patients
HbA1c	Glycated hemoglobin A1c
HT	Hemorrhagic transformation
HUCBCs	Human umbilical cord blood cells
IADPSG	International Association of Diabetes in Pregnancy Study Groups
ICAM-1	Intercellular adhesion molecule-1
ICH	Intracerebral hemorrhage
IDF	International diabetes federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL-6	Interleukin-6
IUGR	Intrauterine growth retardation
LDL	Low-density lipoprotein
MCAO	Middle cerebral artery occlusion
miRs	Micro-ribonucleic acids
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
NIDD	Non-insulin-dependent diabetes
NINDS	National Institute of Neurological Disorders and Stroke
NO	Nitric oxide
ODP	Overt diabetes in pregnancy
OGTT	Oral glucose tolerance testing
OLETF	Otsuka Long-Evans Tokushima Fatty
OPCs	Oligodendrocyte progenitor cells

PAI-1	Plasminogen activator inhibitor-1
PCA	Post cerebral artery
PC-MRA	Phase-contrast magnetic resonance angiography
PET	Positron emission tomography
PKC	Protein kinase C
RAGE	Receptor for advanced glycation end-products
ROS	Reactive oxygen species
SCI	Subcortical infarctions
SDT	Spontaneously diabetic Tori
SMC	Smooth muscle cell
SSS	Scandinavian stroke scale
STZ	Streptozotocin
SWI	Susceptibility-weighted imaging
T1DM	Type 1 DM
T2DM	Type 2 DM
TCD	Transcranial Doppler
THIS	Treatment of hyperglycemia in ischemic stroke
TM	Thrombomodulin
TNF-α	Tumor necrosis factor-α
t-PA	Tissue-type plasminogen activator
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor
ZDF	Zucker diabetic fatty

9.1 Diabetes Mellitus

9.1.1 Epidemiology of Diabetes Mellitus

9.1.1.1 Prevalence of Diabetes Mellitus: A Growing Noncommunicable Disease Epidemic

Diabetes mellitus (DM), a group of metabolic diseases marked by high levels of blood glucose, is one of the largest global health emergencies threating public health worldwide. WHO has reported an increasing prevalence of DM over the past few decades in different parts of the world. According to the International Diabetes Federation, there are 415 million people aged 20–79 years living with diabetes worldwide in 2015; this number is expected to rise to 642 million by 2040 [1]. Despite the high prevalence of diagnosed DM, half of diabetic patients are unaware of their disease, which indicates a lack of awareness of the disease. In Chennai Urban Rural Epidemiology Study (CURES), the prevalence of known diabetes was 6.1%, which of undiagnosed diabetes was 9.1% [2]. Similarly, in the Amrita Diabetes and Endocrine Population Survey (ADEPS), the prevalence of diagnosed and undiagnosed DM was 9.0% and 10.5% [3]. In summary, DM is becoming one of the most important public health challenges to all nations.

There are three main types of DM: type 1 DM (T1DM), type 2 DM (T2DM), and gestational DM (GDM).

T1DM accounts for ~5% of all diagnosed cases of DM; it is a kind of chronic autoimmune disorders typically manifests in early childhood and adolescence. There are about 40,000 people who are diagnosed with T1DM each year in the USA according to Juvenile Diabetes Research Foundation [4]. GDM is defined as any degree of glucose intolerance with onset or first recognition during pregnancy [5]. According to American Diabetes Association, approximately 7% of all pregnancies are complicated by GDM. The prevalence may range from 1% to 14% of all pregnancies, which depends on the studied population and the employed diagnostic tests. In addition to the immediate perinatal risk, the US Preventive Task Force has concluded that GDM treatment significantly reduces the risks of preeclampsia, macrosomia, and shoulder dystocia in 2013 [6]. GDM carries an increased risk of metabolic disease in the mother and child. GDM increases the substantial future risk of developing T2DM. Women who are suffering from GDM have a sevenfold higher risk of progression to T2DM and an incidence estimates of 35–60% in the two decades following delivery [7–10]. Intrauterine exposure to diabetes conveys a high risk for the development of diabetes and obesity in offspring, which is more than of risk attributable to genetic factors alone [11].

T2DM accounts for ~90–95% of all diagnosed cases of DM. Trend in prevalence of T2DM has been rapidly growing during the past few decades. T2DM was relatively rare in developing countries some decades ago. However, because of rapid economic development, urbanization, and nutrition transition over a relatively short period of time [12], the epidemic of T2DM has now emerged in Asia. In addition, the Gulf regions in the Middle East [13] and Africa [14] are other "hot spots" for diabetes mellitus. Close to 80% of the 415 million cases of diabetes mellitus worldwide live in less developed countries and areas [13]. T2DM is traditionally considered as a metabolic disorder exclusively for adults. Results from the 2012 National Health and Nutrition Survey (ENSANUT 2012) of Mexico showed that the highest prevalence was found in adults aged 60–69 years (26.3%) [15]. However, early-onset T2DM (defined as onset before 40 years of age) has increased in recent years. T2DM and prediabetes are increasingly observed among younger adults and even adolescents and children. For example, the crude prevalence of T2DM among North American youth aged 10–19 years in 2001 was estimated to be 42 cases per 100,000 youth [16, 17]. The prevalence and incidence of T2DM in youth vary dramatically between different ethnic groups: Native American, Australian Indigenous, African American, Hispanic, Pacific Islander, and Asian populations, which are of higher risk [17, 18]. The prevalence of prediabetes is even higher in young ones who have risk factors such as obesity, hyperinsulinemia, or a family history of DM [19–21]. Youths with T2DM represent a population at increased risk of development of early and higher lifelong chronic complications owing to the long duration of the disease.

9.1.1.2 The Burden of Diabetes

The excess global mortality in the year 2000 was attributable to diabetes, most of which, T2DM, was estimated to be 2.9 million deaths (5.2% of all deaths). In people aged 35–64 years, 6–27% of deaths were attributable to diabetes [22]. People died of diabetes mainly because of its complications. Cardiovascular death accounts for 44% of deaths in T1DM and 52% of deaths in T2DM [23]. Heart disease and stroke account for 68% and 16% of diabetes-related death certificates in the USA in 2004, respectively. Diabetes is responsible for around 44% of end-stage renal failure and 60% of nontraumatic lower-limb amputations in the USA. It is also the leading cause of blindness among adults aged 20–74 years. According to the Centers for Disease Control and Prevention in 2011, Asian population has a different pattern of complications, with more deaths attributed to strokes and renal failure [12].

The countries with the largest number of people with diabetes are and will be India, China, and the USA in the year 2025. By the year 2025, >75% of people with diabetes will reside in developing countries, and the majority are in the age range of 45–64 years, which is the working age [15, 24]. Most patients with T2DM require drug therapy for glycemic control and for preventing vascular risk factors. In the USA, the cost of outpatient medications for DM was estimated to be $5.5 billion in 2002, excluding insulin and oral hypoglycemic agents, which is just 6% of all DM-related healthcare expenditure and hospital inpatient care, and medication to complications is the larger components of the expenditures [25]. The US national economic burden of prediabetes and diabetes reached $218 billion in 2007. This burden represents a cost of approximately $700 annually for each American regardless of diabetes or not [26]. In 2015, the International Diabetes Federation (IDF) estimated that most countries devote 5–20 % of total healthcare expenditures to diabetes. Global spending to treat diabetes and its complications was estimated to be $673 billion in 2015 [27]. The economic burden DM imposes on healthcare system is and will be enormous. Prevention and control of diabetes should be a top public health priority.

9.1.2 Diagnosis

Recommendations for diagnostic strategies and criteria for hyperglycemic disorders have been revised and issued by the WHO, the American Diabetes Association (ADA), and the International Association of Diabetes in Pregnancy Study Groups (IADPSG) [28, 29]. The criteria are shown in Table 9.1.

Prediabetes includes impaired glucose tolerance (IGT), impaired fasting glucose (IFG), or both. It is associated with increased incidence of diabetes (~34% increase in risk in 7.5 years) and cardiovascular disease (~11% in 10 years) [30].

Table 9.1 Diagnostic criteria for diabetes, IFG, IGT, prediabetes, and gestational diabetes [31]

	Diabetes	IFG and IGT	Prediabetes	GDM and ODP
HbA_{1c} (%)	≥6.5%	NA	≥5.7% and <6.5%	ODP ≥6.5%
Fasting plasma glucose (mmol/L)	≥7.0	IFG ≥6.1 and <7.0	≥5.6 and <7.0	GDM ≥5.1, ODP ≥7.0
75 g OGTT post-load plasma glucose (mmol/L)	2 h, ≥11.1	IGT 2 h, ≥7.8 and <11.1	2 h, ≥7.8 and <11.1	GDM 1 h, ≥10.1 GDM 2 h, ≥8.5
Random glucose (mmol/L)	≥11.1 with classic symptoms	NA	NA	ODP ≥11.1

HbA_{1c} glycated hemoglobin A_{1c}, *OGTT* oral glucose tolerance testing, *IFG* impaired fasting glucose, *IGT* impaired glucose tolerance, *GDM* gestational diabetes mellitus, *ODP* overt diabetes in pregnancy, *NA* not applicable

9.1.3 Pathophysiology of Diabetic Vasculopathy

9.1.3.1 Pathophysiology of DM

Diabetes development is due to inadequate islet β-cell and adipose tissue responses to chronic fuel excess, which results in the so-called nutrient spillover, insulin resistance, and metabolic stress. T1DM is due to a progressive destruction and dysfunction of β-cell, usually leading to absolute insulin deficiency. It requires vigilant replacement for homeostatic maintenance and prevention of life-threatening ketoacidosis. T2DM is due to a progressive insulin secretory defect on the background of insulin resistance leading to hyperglycemia; the condition is termed non-insulin-dependent diabetes (NIDD). Chronic fuel surfeit is the primary pathogenic event that drives the development of T2DM in genetically and epigenetically susceptible people [32]. The metabolic defects that contribute to the development of T2DM are as follows: peripheral insulin resistance, inability of islet β-cells compensating for the fuel surfeit, increased glucagon secretion and incretin response reduction, impaired expansion of subcutaneous adipose tissue and hypoadiponectinemia, inflammation of adipose tissue, and increased endogenous glucose production [32–37].

9.1.3.2 Diabetic Vasculopathy

Chronic elevation of blood glucose level can lead to injury of blood vessels resulting in chronic diabetic complication, that is, diabetic vasculopathy. It becomes the major cause of morbidity and mortality in DM patients. Diabetic vasculopathy is generally divided into two major types: macrovascular and microvascular complications. Diabetes-associated microangiopathy includes diabetic cardiomyopathy, diabetic nephropathy, diabetic neuropathy, and diabetic retinopathy, whereas

diabetes-associated macrovasculopathy includes coronary artery disease, peripheral vascular disease, and stroke, in which the accelerated atherosclerosis is thought of as a crucial mechanism [38]. Atherosclerotic lesion formation is initiated by endothelial cell damage leading to endothelial dysfunction [39]. In addition to the accelerated atherosclerosis, diabetic blood vessels show enhanced proliferation of vascular smooth muscle and production of extracellular matrix after injury. For instance, exaggerated restenosis often accompanies angioplasty revascularization procedures in diabetic patients [40]. In summary, vascular complications of diabetes are characterized by vascular/endothelial dysfunction and pathological remodeling; diabetes-related endothelial dysfunction precedes morphologic and structural vascular changes.

9.1.3.2.1 Vascular/Endothelial Dysfunction Development of Atherosclerosis

The endothelium is an important locus controlling vascular functions in several aspects. It can actively regulate vascular tone and permeability and keep the balance between coagulation and fibrinolysis. It also affects the composition of the subendothelial matrix, the extravasation of leukocytes, and the proliferation of vascular smooth muscle cells [41]. To carry out these functions, the endothelium produces components of the extracellular matrix and a variety of regulatory mediators, such as nitric oxide (NO), prostanoids, endothelin, angiotensin II, tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1), von Willebrand factor (vWF), adhesion molecules, and other cytokines [42].

Normally, the endothelium can adapt to temporal and local requirements. Endothelial dysfunction differs along with the injury type and depends on its intrinsic properties. Exposed to high plasma glucose environment, the rate of intracellular glucose transport will decrease, so the majority of cells can maintain a relatively homeostatic intracellular glucose level. However, endothelial cells cannot effectively regulate this process; consequently, vascular endothelial cells are very susceptible to elevated plasma glucose levels [43]. The elevated glucose levels can induce endothelial dysfunction through the following mechanisms:

1. Excess intracellular glucose results in oxidative stress, which is of vital importance for the mechanism of glucose-induced damage [44–46]. The mechanism includes the depletion of NADPH, impaired clearance of reactive oxygen species (ROS), and increased production of ROS [47]:
2. Elevated intracellular glucose can lead to impaired endothelial-dependent vasodilatation due to inhibition of endothelial nitric oxide synthase (eNOS) and upregulation of endothelin-1 by activation of the PKC pathway [48].
3. With suppressed NO and prostacyclin synthesis, combined with platelet resistance, excess glucose leads to glycation of clotting factors and the loss of control over platelet activation, creating a prethrombotic condition [49]. There is an increase in the number of coagulation factors such as PAI-1, vWF, fibrinogen,

factor VII, and thrombin–antithrombin complexes in association with poor glycemic control [50, 51].
4. Hyperglycemia enhances the formation of advanced glycation end-products (AGEs) which can be found in plasma and vessel walls. Sustained accumulation of AGEs and pro-inflammatory cytokines, together with engagement of receptor for advanced glycation end-products (RAGE) by its pro-inflammatory ligands, forms a chronic cycle of vascular inflammation. This cycle contributes to the accelerated atherosclerosis [52].
5. High glucose concentration causes the release of mediators (e.g., cytokines) by adipocytes in the presence of insulin resistance. Enhancement leukocyte–endothelial interaction and glycation of lipoproteins or apolipoproteins also contribute to endothelial dysfunction [53].

9.1.3.2.2 Pathological Remodeling: Alteration of Vasculature

In diabetes, remodeling extends to capillaries, microvascular beds, and arteries of different caliber. Prolonged glycemic condition can affect microvascular conditions by reducing perfusion rates, thickening capillary walls, and causing abnormal proliferation of endothelial cells with increased vascular permeability. Characteristics of the remodeling are accelerated disappearance of capillary endothelium [54], weakening of intercellular junctions, altered protein synthesis, and expression of adhesion glycoproteins on endothelial cells [55], promoting attachment and transendothelial migration of monocytes and leukocytes [56].

Hyperglycemia enhances matrix production of endothelial cells. It upregulates collagen synthesis enzymes and specifically enhances collagen IV and fibronectin synthesis [57], which may contribute to basement membrane thickening [57]. Moreover, hyperglycemia also stimulates the atherogenic activity of vascular smooth muscle cell (SMC). It alters SMC function in ways that promote atherosclerotic lesion formation and plaque instability. This thickening alters vessel function, including arterioles in the glomeruli, retina, myocardium, skin, and muscle, which results in the classic diabetic microangiopathy.

In summary, a dysfunctional endothelium allows macrophages and low-density lipoprotein (LDL) to penetrate the medial layer of arteries heralding the formation of foam cells. Then, a macrophage-rich fatty streak is formed. Then, vascular SMCs in the medial layer of the arteries migrate into this nascent intimal lesion and replicate, which indicate the formation of advanced atherosclerosis and change in the vasculature. During this process, the metabolic, humoral, and hemodynamic factors responsible for characteristic dysfunction of diabetic vasculopathy are interrelated: hyperglycemia-induced oxidative stress promotes both the formation of AGE and protein kinase C (PKC) activation. Hyperglycemia, nonenzymatic glycation, lipid modulation, alteration of vasculature, expression of cytokines, and growth factor activation are thought to contribute to the development of diabetic vasculopathy [58].

9.1.3.3 Related Biomarkers of Endothelia Injury

9.1.3.3.1 Coagulation-Related Biomarkers

DM is associated with alterations in the balance of prothrombotic and antifibrinolytic state. vWF is a representative marker of endothelial activation; it plays an important role in platelet adhesion and aggregation [59]. Framingham Offspring Study reported that vWF was an independent risk factor for DM (relative risk, 1.37; CI, 1.07–1.75) [60] and vWF was a product of the acute phase response associated with the risk of DM complications [61]. Thrombomodulin (TM) is the major substance of the protein C anticoagulant system; plasma TM was a marker of endothelial damage and elevated in T2DM patients with incipient nephropathy [62]. PAI-1 concentration increases in individuals with T2DM which belongs to typical signs of thrombophilia in T2DM pathogenesis [59]. Elevated plasma levels of E-selectin can predict development of T2DM in initially nondiabetic women [63].

9.1.3.3.2 Inflammatory Biomarkers

The inflammatory biomarkers that have been proved to provide prognostic information on the outcome and progression of the disease in diabetic patients are C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) [61]. Increased CRP levels are independent predictors of T2DM in healthy people. CRP can also predict the outcomes in DM patients with cardiovascular disease [64]. Pattern of circulating inflammatory cytokines modifies the risk for T2DM; combined elevation of IL-1β and IL-6 was independently associated with increased risk of T2DM [65]. ICAM-1 and VCAM-1 are important mediators for the adhesion of leukocytes to the endothelial surface and predict development of DM and significantly relate to the risk of DM complications [63].

9.1.3.3.3 Oxidative Stress Biomarkers

Increased oxidative stress is a major contributor of endothelial dysfunction in DM. It has been demonstrated that isoprostanes and antibodies against oxidized low-density lipoprotein (oxLDL) were increased in humans with diabetes. There is a strong correlation between endothelial nitric oxide synthase (eNOS) activity and glomerular endothelium function in patients with diabetic nephropathy [66].

9.1.3.3.4 Other Biomarkers

Micro-ribonucleic acids (miRs), a class of approximately 22 nucleotide noncoding ribonucleic acids, are able to regulate gene expression and are considered as significant modulators of several processes nowadays [67]. In newly diagnosed DM patients, the levels of miR-9, miR-29a, miR-30d, miR-34a, miR-146, and others were significantly higher compared with normal people [68]. Downregulation of numerous miRs (miR-20b, miR-21, miR-126, and others) has also been found in plasma of DM patients. Overexpression of miR-21 mitigates endothelial cell apoptosis, which may serve as a therapeutic target for DM vasculopathy [69].

9.1.3.4 Experimental DM Models

It is important to replicate the clinical findings in the laboratory for better understanding of the cellular and molecular mechanisms in basic science study.

These rodent models can be classified into two broad categories:

Genetically induced spontaneous diabetes models: they spontaneously develop diabetes, insulin resistance, and reduced β-cell mass [70], but are comparatively expensive and do not absolutely mimic the pathogenesis observed in human. Examples are *db/db* mice, Goto-Kakizaki (GK) rats, Zucker diabetic fatty (ZDF) rat, Otsuka Long-Evans Tokushima Fatty (OLETF) rats, spontaneously diabetic Tori (SDT) rats [71]. *db/db* mice is leptin receptor deficient and serves as a rodent model for obesity and T2DM. Diabetic features follow an age-dependent progression, with early insulin resistance followed by an insulin secretory defect resulting in profound hyperglycemia. They are mainly utilized to assess the cardiac consequences of diabetes.

Experimentally induced nonspontaneous diabetes models: the models are induced by high-fat diets, streptozotocin (STZ) or alloxan injection at neonatal or adult age, partial pancreatectomy, intrauterine growth retardation (IUGR), or combinations of these [71]. So, they are of comparatively lower cost, ease of maintenance, and wider availability. One prevalent T1DM animal model is generated by administrating streptozotocin (STZ), which can selectively destroy pancreatic β-cells via an oxidative stress mechanism. It can render hyperglycemia leading to sequelae that mimic T1DM [72]. This model is popular for routine pharmacological screening. However, it will not result in a state of insulin resistance. Low dose of STZ in combination with a high-fat diet can be used to mimic T2DM, which takes a very long period of time. Without dietary manipulation, nicotinamide, a drug with antidiabetic properties, could be used in combination with STZ to develop a model resembling the pathogenesis of T2DM in relatively short time.

9.2 Diabetic Stroke

With respect to vascular dysfunction of the brain, both T1DM and T2DM confer an increased risk of ischemic stroke [73]. T1DM is a major risk factor for ischemic cerebrovascular disease that increases morbidity and mortality worldwide [74, 75]. Stroke occurs fivefold more often in patients with T1DM and results in intense consequences compared with those in patients without diabetes [76]. And the incidence of stroke increased two- to sixfold among T2DM patients. The Nurses' Health Study revealed that the incidence of total stroke was fourfold higher in women with T1DM (relative risk, 4.7; 95% CI, 3.3–6.6) and twofold higher among women T2DM (relative risk, 1.8; 95% CI, 1.7–2.0) than nondiabetic women [77]. Approximately 30–40% of ischemic stroke patients present with hyperglycemia on admission either as a result of DM or acute stress response [78]. Meanwhile, stroke patients with diabetes have a worsen outcome. The risk of death from cardiovascular causes increases two to six times in T2DM than nondiabetic patients [79]. Mortality rate is increased in diabetic stroke patients at 1 week, 1 month, and 3 months after stroke than nondiabetic patients. Diabetic stroke survivors recovered badly, and they suffered from more profound neurological deficits and disability [80]. Abnormalities in glucose metabolism and vascular hemodynamics may play important roles in the pathogenic progress of stroke in diabetic patients [81].

9.2.1 Clinical Manifestations and Prognosis of DM Stroke Patients

9.2.1.1 Clinical Manifestations

There are many differences in clinical presentations of stroke in patients with diabetes compared to those without. Diabetes increases the risk of ischemic stroke more than hemorrhagic stroke; the increasing rate of ischemic to hemorrhagic stroke is approximately from 5:1 to 10:1 [82]. Moreover, diabetic individuals had a lower relative prevalence of intracerebral hemorrhage (ICH); deep hemorrhages were more frequent in those with DM, whereas lobar hemorrhages were more frequent in non-DM patients [83]. Based on the topography of ischemic stroke, subcortical infarctions (SCI), including "lacunar" infarctions, are more common to be seen in patients with diabetes [84]. According to a case control study, lacunar infarct had a higher prevalence than all other subtypes in TOAST classification [85]. An MRI study found that 41% of type II DM patients are initially free of "lacunes" and developed them after 5 years. In addition, people with DM are more likely to develop "silent" lacunar cerebral infarcts compared to the general population, while less likely to develop transient ischemic attack [86]. The involvement of the post cerebral artery (PCA) territory was more frequent in DM patients [83]. There has

preponderance for ischemic stroke in diabetes to affect the brainstem, cerebellar, or midbrain region (infratentorial infarcts) compared with normal population [87].

9.2.1.2 Prognosis

Strokes in patients with diabetes are usually associated with a worse outcome [88]. Diabetes and hyperglycemia predict early neurologic deterioration during the acute phase of ischemic stroke [89]. Diabetes is clearly associated with a higher Scandinavian Stroke Scale (SSS), a kind of neurological deficit score, on hospital admission [85]. Case fatality rate during hospitalization was higher in diabetic than nondiabetic patients [82]. A clinical study reported that persistent poststroke hyperglycemia was independently associated with expansion of the infarct and worse clinical outcome [90]. Moreover, there has a higher incidence of hemorrhage following early reperfusion with t-PA in hyperglycemic patients [91]. Furthermore, clinical results have indicated that diabetes increases stroke recurrence and long-term mortality and worsens the neurological outcomes after stroke [81, 92, 93].

Patients who died during hospitalization had significantly higher blood glucose concentrations on admission compared with patients who survived. The effect of high admission blood glucose levels after stroke may play the key role rather than diabetes history independently of age, stroke type, and severity [94]. The National Institute of Neurological Disorders and Stroke (NINDS) rt-TPA stroke trial reported that higher admission blood glucose levels were associated with worse outcomes even after adjusting for diabetes as a confounding factor [95]. A meta-analysis showed the relative risk of in-hospital and 30-day mortality was higher only in hyperglycemic nondiabetic patients (relative risk, 3.28; 95% CI, 2.32–4.64) [78]. These findings suggest that acute elevations of blood glucose may be more detrimental in stroke pathophysiology than chronic hyperglycemia.

9.2.2 The Mechanisms of Severe Damage Induced by Stroke in Diabetes

The severe damage of cerebral ischemic injury in experimental diabetic models has been traditionally evaluated by measuring infarct size which is an indicator of neuronal damage. On the other hand, blood–brain barrier (BBB) damage and exacerbated secondary hemorrhagic transformation (HT) can also reflect the ischemic vascular damage and influence stroke outcome [96, 97]. They could be consistent to prolonged ischemia or follow by reperfusion.

9.2.2.1 Endothelial Injury and BBB Disruption

BBB is characterized by a well-defined basement membrane, presence of tight junctions between endothelial cells (ECs), and absence of fenestrations, involving astrocytes' end feet and pericytes. These unique characteristics confer distinct properties that differentiate the BBB from peripheral capillaries [98]. Hyperglycemia and DM increase the BBB permeability and infarct volume [99, 100] after stroke in rats. The possible mechanisms are as follows.

According to pathophysiology of DM, vascular remodeling is a complex phenomenon associated with restructuring of the vessel wall as a consequence of vascular dysfunction. The vascular wall is mainly composed of ECs and mural cells (pericytes and SMCs). The thickening of the cerebral microvascular basement membrane impairs the integrity of adjacent structure including astrocytes and pericytes [43, 101, 102]. The decreased mural cell recruitment of T2DM mice may contribute to cerebral pathological vasculogenesis and also disrupt the BBB integrity.

Angiopoietins and their receptor Tie2 play a role in vascular integrity and neovascularization in DM. Hyperglycemia may suppress Ang1-induced vascular protection and provoke endothelial dysfunction [103]. Tie2 expression was significantly attenuated, whereas Ang2, who can destabilize pericytes, was increased in *db/db* mice subjected to myocardial ischemia [104]. The same expression pattern of Ang1/Ang2/Tie2 could also be observed in the cerebral vascular walls in the ischemic brain and arteries. Therefore, targeting the Ang1/Ang2/Tie2 signaling pathways may have a beneficial effect on decreasing abnormal angiogenic activity and vascular permeability in the DM patients [105].

Matrix metalloproteinases (MMPs) are associated with degradation of tight junction proteins (such as occludin and ZO-1) and are a known mediator of BBB compromise [106–108]. Expression and activity of the gelatinases MMP-2 and MMP-9 are increased by hyperglycemia in vitro and elevated in patients with type 1 and type 2 diabetes [109–111]. Experimental diabetes is associated with increased gelatinolytic (MMP-2 and/or MMP-9) activity in the blood and increased BBB permeability, which suggests that circulating MMPs are a potential target for prevention of complications in diabetes [112].

Diabetes is associated with abnormal cerebral blood flow in surviving brain regions and greater disruption of the blood–brain barrier. Genetically or pharmacologically inhibited vascular endothelial growth factor (VEGF) signaling can mitigate vascular dysfunction and improve stroke recovery in diabetic animals [113].

9.2.2.2 Hemorrhagic Transformation

If ischemia is prolonged or reperfusion involved, bleeding into the brain parenchyma known as HT ensues and worsens stroke outcome [114]. In experimental transient middle cerebral artery occlusion (MCAO) models, acute hyperglycemia induced by glucose administration augments ischemic injury and increases HT.

The remodeling response may contribute to increased hemorrhage in diabetes. Cerebrovascular tortuosity index was increased in the diabetic rats, which indicates changes in vascular architecture, increases the risk for HT, and exacerbates the neurovascular damage [96]. Pial vessels of diabetic animals were dilated and tortuous; increased arteriovenous shunting can be observed [115]. Whether increased tortuosity is because of remodeling of existing vessels or neovascularization remains to be determined. Other factors related to HT are MMP activity and t-PA activity. Chronic glycemic control confers vascular protection and reduces the risk of HT after ischemic reperfusion injury in DM [116]. Chronic glycemic control with metformin or MMP inhibition with minocycline decreased hemorrhage and improved functional outcomes.

9.2.2.3 White Matter Damage and Cognitive Impairment

Hyperglycemia can lead to the aberrant metabolite accumulation, inflammation, and oxidative stress, which results in neuronal dysfunction and apoptosis [117]. Chronic hyperglycemia can affect cerebral vasculature and alter cerebral blood flow [118]. Moreover, long-term uncontrolled hyperglycemia can elicit a progressive impairment of neuronal function. Insulin dysregulation in DM could interfere with the metabolism of amyloid-β and tau, which is important in AD pathogenesis [119]. Due to the deleterious effect of diabetes on the cerebral vasculature and associated metabolic changes, DM may increase the likelihood of developing dementia. The Rotterdam Study indicates that DM almost doubled the risk of dementia (relative risk, 1.9; CI, 1.3–2.8) and AD (relative risk, 1.9; CI, 1.2–3.1) [120]. So, T2DM is considered to be a predisposing factor for cognitive impairment, including vascular dementia and Alzheimer's disease [121]. Individuals with T2D showed microstructural abnormalities in white matter in diffusion tensor imaging (DTI) and autopsy [122, 123]. Damages to white matter tracts can lead to a slowing information processing speed and cognitive function deficits.

The white matter injury induced by diabetes has effects on stroke severity and prognosis. The specific diabetically damaged white matter (like corticospinal tract and superior longitudinal fasciculus) beyond stroke lesions is critically relevant to the recovery [124]. Ischemic white matter lesion was exacerbated in *db/db* mice in chronic cerebral hypoperfusion mice model. Proliferation and mobilization of oligodendrocyte progenitor cells (OPCs), as well as survival and maturation of OPCs, are reduced in ischemic white matter lesion in diabetes which may due to glial inflammation [125]. Also, hyperglycemia inhibits proliferation and migration of Schwann cell and restricts axons regeneration, which indicates a hamper to white matter repair [126].

9.2.2.4 Poststroke Recovery

Clinical evidence suggests that diabetes mellitus hampers recovery after stroke [127–129]. STZ-treated mice with 2–4 weeks chronic hyperglycemia showed worse neurological deficits after both focal ischemic and embolic strokes [93, 130].

Neurological recovery is directly correlated to the degree of angiogenesis, microvascular density, and restoration of blood flow after stroke [131, 132]. T2DM mice subjected to ischemia showed decreased number of microvessels after stroke in the ischemic hemisphere and a reduced rate of angiogenesis 7 days after stroke [133, 134]. DM impairs poststroke reparative neovascularization and impedes the recovery. Glycemic control using metformin after stroke can improve neurovascular repair and motor and cognitive recovery [135].

The brain function restoration after stroke depends not only on size of the incurred lesion but also on reorganization of the surviving tissues by cortical plasticity, mainly the peri-infarct region [136]. Diabetes causes maladaptive changes in cerebrovascular system that ultimately limits neuronal circuit rewiring and the recovery of functions after stroke [113]. Hyperglycemic mice show very little improvement in cortical responses to forepaw mechanical touch at 7–100 days post stroke by adopting voltage-sensitive dye imaging, while this response can reemerge in nondiabetic ones due to cortical plasticity. Furthermore, treatment with insulin shows little benefit in improving cortical responsiveness, which means diabetes leads to persistent impairments of cortical plasticity and functional recovery after stroke [137]. Diabetes can also affect the reorganization of large-scale cortical networks by exacerbating the loss of synaptic connections. Researchers observed a significantly lower density of intracortical axons (labeled by axonal neurofilament markers) at peri-infarct cortex in T1DM rats than their normoglycemic controls [138]. Diabetic mice exhibit greater loss of cortical apical dendritic spines and reduced expression of synaptophysin on layer V neurons in peri-infarct cortex at 1 week after stroke, which also indicate impairments in cortical plasticity [139].

9.2.3 Imaging Changes of DM Stroke

Compared to normal people, T2DM patients show more global brain atrophy, which increases gradually over time compared with same age counterparts [140, 141]. Hippocampal atrophy also occurs in patients with T2DM [142]. The type of infarct lesions of diabetic patients is more often lacunar infarcts, which are focal hyperintensities on T2-weighted images with corresponding hypointense lesions on T1 or FLAIR imaging.

A relationship between T2DM and degree of white matter hyper-intensities was reported in a case control study [143]. White matter changes after stroke can be measured by diffusion magnetic resonance imaging. Entropy of diffusion anisotropy was most effective to identify microstructures and low-density axonal fibers after middle cerebral artery occlusion in T2DM rats, which is superior to the

fractional anisotropy metric that only provided measures related to organization of neuronal fiber bundles [144]. Diffusion fractional anisotropy demonstrated lower axonal density in T2DM rats; this result suggested that the white matter reorganization involving the corpus callosum after stroke is hampered by T2DM [145].

The dynamic and chronic cerebrovascular changes after stroke in T2D were revealed by employing magnetic resonance imaging (MRI). Longitudinal measurements using CE-T1WI with Gd-DTPA showed that T2DM rats exhibited significantly larger volumes of BBB disruption compared with WT rats. Using susceptibility-weighted imaging (SWI), hemorrhagic transformation was measured to be significantly larger in the T2DM rats [145].

With functional MRI (fMRI) and positron emission tomography (PET), it is possible to show changes in regional brain activity. By adopting the blood-oxygen-level-dependent (BOLD) contrast of fMRI, neurovascular coupling might be altered in T2DM [146]. Within T2DM patients, insulin resistance defined by HOMA-IR and HbA1c levels was correlated with functional connectivity in several brain regions [147, 148]. Decreased glucose metabolism in multiple brain areas in patients with T2DM could be investigated by FDG-PET. T2DM has been related to diminished cerebral blood flow and cerebrovascular reactivity by phase-contrast magnetic resonance angiography (PC-MRA) and transcranial Doppler (TCD) [149–151].

9.3 Prevention and Treatment for Diabetic Stroke

9.3.1 Prevention and Treatment of DM

The pandemic of DM, along with its high economic costs, calls for approaches to prevent, slow the progression, and limit the consequences of this disease.

Mothers with gestational diabetes are at high risk of permanent diabetes after pregnancy [9, 152, 153]. Women are frequently not diagnosed and do not receive interventions for gestational diabetes until around 28 weeks' gestation. To encourage women to adopt healthy lifestyles before and during pregnancy, health checks, advice, and public health programs would be useful preventive approaches.

Fetal and neonatal programming via epigenetic phenomena might contribute to susceptibility to obesity, β-cell and adipose tissue dysfunctions, and the metabolic syndrome. Appropriate diet and exercise, good-quality medical care, and breastfeeding should be supported during gestation and early childhood; this period could be crucial in the determination of later metabolic health [154, 155].

Effective management of prediabetes can prevent or delay the onset of disorders and complications. Lifestyle interventions such as healthy diet and appropriate exercise can decrease the risk of incident diabetes by 28–59% [156–158]. Due to the recommendation of ADA's Standards of Medical Care in Diabetes [159], patients should adopt intensive diet, and physical activity behavioral counseling program should target a loss of 7% of body weight and increase moderate-intensity physical

activity to at least 150 min/week. Pharmacotherapy with α-glucosidase inhibitors, metformin, and thiazolidinediones can also effectively decrease the risk of incident diabetes [157, 160–162]. For instance, low-dose combination therapy with rosiglitazone and metformin was highly effective in prevention of T2DM in patients with impaired glucose tolerance, which of little clinically relevant adverse events [162]. Metformin has demonstrated long-term safety as pharmacological therapy for diabetes prevention. Combination of voglibose and lifestyle modification can reduce the development of T2DM in high-risk Japanese individuals with impaired glucose tolerance [161].

The foundations of established diabetic healthcare are diabetes self-education, medical nutrition therapy, physical activity, smoking cessation, psychosocial assessment and care, and immunization [159]. Glycemic control should be conducted under careful monitoring and assessment according to ADA's recommendation; insulin therapy, metformin, and GLP-1 mimic and other drugs are involved [159]. The desired goal of glycemic control should not only achieve target HbA_{1c} but also reduce chronic fuel surfeit, prevent islet β-cells and adipose tissue dysfunction, restore normal regulation of endogenous glucose production, etc. The clinical management of established diabetes also involves optimum control of factors that cause complications. It includes control of blood glucose, lipid concentrations, blood pressure, bodyweight, and smoking. Moreover, regular screening and appropriate management of microvascular (nephropathy, retinopathy, neuropathy, and diabetic foot) and macrovascular (coronary, cerebral, and peripheral) complications are also important.

9.3.2 Prevention of Diabetic Stroke

The cerebrovascular protection is not only limited to prevention of diabetes but also prevention of diabetes-induced detrimental changes in vascular structure and function before the occurrence of stroke. Efficacy of controlling individual cardiovascular risk factors in preventing or slowing cardiovascular disease (CVD) in diabetic patient is proven. According to comprehensive risk assessment, primary prevention for CVD in diabetic patient focused on lifestyle management, blood pressure (BP) control, lipid control, blood glucose control, antiplatelet agent use, and tobacco use cessation [163]. Annually assessing risk factors of CVD (dyslipidemia, hypertension, smoking, family history of premature coronary disease, and albuminuria) in DM patients is necessary.

Glucose control is crucial for diabetic patients. After adjustment for other CVD risk factors, an increase of 1% in HbA_{1c} was associated with an increased risk of 18% in CVD events [164] and 37% in retinopathy or renal failure [165]. The impact of elevated BP is obvious on micro- and macrovascular complications of DM, but the target systolic and diastolic BP goals are on dispute. The Appropriate Blood Pressure Control in Diabetes trial indicated that an intensive BP control (mean BP:

128/75 mmHg) diminished the incidence of stroke and also slowed the progression to diabetic nephropathy and retinopathy [166]. However, Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial challenged this concept; this trial reported that T2DM patients had no significant cardiovascular benefit and experienced more drug side effects at a lower systolic pressure [167]. Major guidelines published after ACCORD raised the blood pressure target in diabetic patients to less than 140/90 mmHg [168–170]. Studies also showed that a 33–40% reduction in LDL cholesterol is associated with a 31–37% reduction in combined cardiovascular end points [171, 172]. Smoking cessation demonstrated a reduction in mortality rate with a trend toward reduction of CVD deaths [173].

The use of aspirin for the primary prevention of CVD events in patients with DM remains controversial; meta-analysis found that aspirin was associated with a 10% decrease in the risk of stroke (RR, 0.90; 95% CI, 0.71–1.13) but not statistically significant [174]. However, low-dose aspirin (75–162 mg/day) for primary prevention is reasonable for most men over age 50 years and most women over age 60 years with one or more major risk factors.

9.3.3 Treatment of Diabetic Stroke

Effective and acute prevention of vascular dysfunction and BBB disruption is important in diabetic stroke treatment in acute ischemic stroke [175].

Clinical trials testing the efficacy and safety of aggressive glycemic control on improving acute ischemic stroke outcomes were conducted, but the result is debatable. Glycemic control has been an effective treatment for prevention of microvascular complications. However, in Treatment of Hyperglycemia in Ischemic Stroke (THIS) study [176], assessment of outcomes at 90 days after aggressive glycemic control is not significantly different in diabetic compared to nondiabetic groups even after adjusting for confounders. Glucose–potassium–insulin infusion in the management of poststroke hyperglycemia in UK (GIST-UK) study included hyperglycemic patients mostly without a diabetes history [177]; 90-day mortality has no significant difference between study groups after aggressive glycemic control during the initial 24 h of stroke onset. The Glucose Regulation in Acute Stroke Patients (GRASP) Trial only reported that insulin infusion for patients with acute ischemic stroke is feasible and safe, but did not assess efficacy [178]. However, the INSULINFARCT trial used intensive insulin therapy to improve glucose control in the first 24 h of stroke but was associated with larger infarct growths [179].

Acute atorvastatin treatment at the beginning of reperfusion almost abolished hemorrhagic transformation in diabetic murine stroke model [180]. A single injection of valproic acid following permanent ischemia in diabetic rats ameliorates neurological deficits and reduces neuronal degeneration by inhibiting inflammation [181]. Many other drugs or compounds possess protection against diabetic stroke, such as sitagliptin (a dipeptidyl peptidase-IV inhibitor) [182], etanercept (a recombinant TNF receptor (p75)-Fc fusion protein) [183], exendin-4 (glucagon-like

receptor 1 agonist) [184], berberine (natural isoquinoline alkaloid) [185], and neamine (aminoglycoside antibiotic that blocks nuclear translocation of angiogenin) [186]. They can prevent exacerbation of cerebral ischemic injury in the diabetic state by reducing inflammation or oxidative stress. This finding suggests that acute interventions can be beneficial in reducing neurovascular injury after diabetic stroke.

9.3.4 The Mechanism and Prospect of Stem Cell Therapy for Diabetic Stroke

Development of stem cell therapies may ultimately lead to viable options for treatment of stroke. It has been proved that autologous intravenous stem cell (mononuclear and mesenchymal cell) therapy in stroke patients is safe and feasible [187]. The mechanism of stem cell therapy is either to replace infarcted brain tissue or to provide trophic support for tissue at risk or help promote survival, migration, or differentiation of endogenous stem cells [188]. Stem cell therapy could benefit diabetic stroke patients.

High glucose and AGEs can damage mesenchymal stem cells (MSCs), which contribute to tissue regeneration, differentiation, and immunomodulation [189]. Intravenous administration of rat MSCs to diabetic rats 1 week before whole-body hyperthermia could slightly rescue BBB breakdown and brain edema and reduce brain pathology, whereas TiO2-nanowired MSC treatment can induce almost complete protection. Modification of MSCs delivery may have better therapeutic effects in diabetic rats, pointing to novel clinical perspectives. [190].

Circulating stem/progenitor cells play an important role in endothelial cell regeneration [191]. The number of circulating endothelial progenitor cells (EPC) is sharply reduced, and the function is impaired in patients with diabetes through the p38 MAPK signaling pathway [192–194]. EPCs are damaged in patients with diabetes, and their ability to home to damaged areas is limited, leading to an abnormal repair process [43, 45]. Combination of EPC transplantation and p38 mitogen-activated protein kinase inhibitor administration can promote angiogenesis, neurogenesis, and the white matter remodeling, which might be due to increased VEGF and brain-derived neurotrophic factor (BDNF) [195]. Transfusion of CXCR4-modified EPCs via tail vein reduces cerebral ischemic damage and promotes repair in *db/db* diabetic mice.

Bone marrow stromal cell (BMSC) treatment via tail vein injection at 3 days after brain ischemia of T2DM rats significantly ($p < 0.05$) decreased white matter injury compared with the control rats [144], enhanced brain remodeling, and improved neurological function [196]. BMSCs also induced reduction of HMGB1 and RAGE, attenuated BBB leakage, and improved functional outcome after stroke in T2DM-MCAO rats [197]. However there is controversy; BMSC therapy starting in 24 h in T1DM stroke rats does not improve functional outcome; on the contrary, it exacerbates BBB leakage, cerebral artery neointimal formation, and arteriosclerosis

[198]. Luckily, cotreatment with Niaspan can rescue this malignant effect accompanied with decreased angiogenin and MMP-9 [199].

Human umbilical cord blood cell (HUCBC) treatment significantly increased vascular and white matter axonal remodeling as well as decreased neuroinflammation at 3 days after stroke, which contribute to the HUCBC-induced beneficial effects in T2DM stroke rats [200]. HUCBCs also have neurorestorative effects after stroke in T1DM rats, and the underlying mechanism may be attributed to the increasing angiopoietin-1 and decreasing RAGE expression [201]. MiR-126 may contribute to HUCBC-induced neurorestorative effects [202].

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Part II
Stroke Mechanisms

Chapter 10
Mitochondrial Dysfunction in Ischemic Stroke

Qiang Li and Shane Gao

Abstract Mitochondrion is the powerhouse of the cell, which is essential for cell survival after cerebral ischemia/reperfusion. Mitochondrion is a sensitive organelle susceptible to brain ischemia/reperfusion injury. Mitochondrial dysfunction is one of the foremost events involved in brain ischemia/reperfusion process and then induces further damage to brain cells. It influences not only the fate of neural cells but also blood-brain barrier permeability after ischemic stroke. The underlying mechanism of mitochondria dysfunction in determining cell survival and cell death involves in many cell signaling pathways including apoptosis, autophagy, and mitochondrial biogenesis. Mitochondria apoptosis pathway was extensively explored in the past. Many apoptosis-related regulator families were involved in mitochondria apoptosis pathway, like Bcl-2 family, caspase family, p53 gene family, and so on. On the other hand, ROS injury, Ca^{2+} overload, and mPTP opening are also detrimental to mitochondrial function after cerebral ischemia/reperfusion. Recent interests were focused on the important role of mitophagy and mitochondrial biogenesis on cell survival after cerebral ischemia/reperfusion, which are thought to be endogenous protective mechanisms of mitochondrial dysfunction. Therefore, under ischemia/reperfusion conditions, promoting endogenous protective mechanisms and inhibiting exogenous damage mechanisms are both important therapeutic strategies. In summary, mitochondrial dysfunction is not simply the result of ischemia/reperfusion injury but also the cause of cascading damage. So, protecting dysfunctional mitochondria is pivotal to cell survival after ischemic stroke.

Keywords Apoptosis • Autophagy • Blood-brain barrier permeability • Cerebral ischemia/reperfusion • Drug discovery • Mitochondrial dysfunction • Reactive oxygen species

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Abbreviations

AD	Alzheimer's disease
AIF	Apoptosis-inducing factor
ALS	Amyotrophic lateral sclerosis
Apaf-1	Apoptotic protease-activating factor-1
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BER	Base excision repair
BH	Bcl-2 homology
CoA	Coenzyme A
CsA	Cyclosporine A
DETC-MeSO	S-Methyl-N, N-diethyldithiocarbamate sulfoxide
ER	Endoplasmic reticulum
ETC	Electron transport chain
ETF	Electron transfer flavoprotein
GPxs	Glutathione peroxidases
H_2O_2	Hydrogen peroxide
IAP	Inhibitor of apoptosis protein
IMM	Inner mitochondrial membrane
IR	Ischemia/reperfusion
IRI	Ischemia/reperfusion injury
MA	Malibatol A
MB	Methylene blue
mCa^{2+}	Mitochondrial Ca2+
MCAO	Middle cerebral artery occlusion
MOMP	Mitochondrial outer membrane permeabilization
mPTP	Mitochondrial permeability transition pore
NO	Nitric oxide
O^{2-}	Superoxide radical anions
OGD-R	Oxygen-glucose deprivation and reoxygenation
OH-	Hydroxyl radical
OMM	Outer mitochondrial membrane
ONOO–	Peroxynitrite species
OS	Oxidative stress
OXPHOS	Oxidative phosphorylation
PARP-1	Poly(ADP-ribose) polymerase-1
PBR	Peripheral benzodiazepine receptor
PD	Parkinson's disease
PhSe	Diphenyl diselenide
PPAR	Peroxisome proliferator-activated receptor
Prxs	Peroxiredoxins
ROS	Reactive oxygen species

Smac	Second mitochondrial-derived activator of caspases
SOD	Superoxide dismutases
THC	Tetrahydrocannabinol
TNF	Tumor necrosis factor

10.1 Introduction

Mitochondrion is the powerhouse of the cell. The primary physiological function of mitochondria is to generate adenosine triphosphate (ATP) which is essential for cell survival. Various structures and compartments of mitochondria, such as the electron transport chain (ETC) complexes I–IV, inner mitochondrial membrane (IMM), and ATP synthase, are involved in the process. These compartments together are able to work in harmony to generate ATP in a complex multistep process [1, 2]. ATP plays an important role in ionic homeostasis, cell proliferation, and gene regulation. Several studies have shown that brain ischemia sustained longer than a few minutes exacerbated impairments of mitochondrial function [3]. After 2 h of focal ischemia, mitochondrial respiratory chain activity was reduced by 45–60% in focal tissue and by 15–40% in perifocal tissue [4–6]. Ischemia/reperfusion, a pathological aggravation phenomenon due to a certain time interval of ischemia followed by reperfusion, is a complicated and multifaceted event. At the same time, it incurs various dramatic metabolic changes including failure of energy outputs, cytoplasm organelle dysfunction, overproduction of reactive oxidative species (ROS), various cell signaling networks adjustments, even cell apoptosis, necrosis, and cell death. Brain tissue takes up only 2% of the total body weight but consumes 20% of total energy and 25% of total glucose in human beings. Under ischemia/reperfusion condition, the brain neuron cells are the foremost targeted cells. To restore their normal function, exploring exogenous therapeutic targets of mitochondria ETC and ATP synthesis system becomes important.

10.2 Mitochondrion Is a Sensitive Organelle Susceptible to Brain Ischemia/Reperfusion

Ischemia/reperfusion firstly destroys the ultra-microstructure of mitochondria. Then the mitochondrial ETC system is damaged, which leads to the obstruction of oxidative phosphorylation and insufficiency of high-energy bond of ATP. The short supply of ATP will further induce compromised ability of sodium-potassium pump, iron, and H_2O transferring into the cytoplasm, which cause the matrix swelling and mitochondria rupture. At the early stage of ischemia/reperfusion, mitochondria always appear as swelling and ponding. They always show diluted matrix and low electronic concentration. The total number of mitochondrial cristae decreases, their

length becomes shorter, and their arrangement gets more turbulent. As the swelling becoming severe, the cristae could even disappear. Some mitochondria may rupture due to overswelling. As ischemia goes further, dramatic decrease of energy supply will induce the opening of mitochondrial permeability transition pore (mPTP). Oxidative radicals and Ca^{2+} overload will incur the structure variation of inner mitochondrial membrane in a time-dependent manner [7].

10.3 Mitochondrial Dysfunction Induces Neurovascular Unit Damage After Ischemia/Reperfusion Injury

10.3.1 Mitochondrial Dysfunction Triggers Cell Apoptosis/Necrosis After Ischemia/Reperfusion Injury

At the normal state, mitochondria generate energy by oxidizing hydrogen (derived from the dietary carbohydrates by TCA cycle and fats by β-oxidation) with oxygen to provide heat and ATP [8]. Mitochondria are the "powerhouse" of the cell, which have been established as important regulators of ischemic neuronal death for many years. Evidence shows that mitochondrial dysfunction can promote apoptotic and necrotic cell death. The underlying molecular mechanism of mitochondria in determining cell survival and cell death is their active or passive involving in many cell signaling pathways [9]. Accordingly, defective mitochondria will contribute to cancer, diabetes, and neurodegenerative diseases [10]. Mitochondrial dysfunction is one of the foremost events involved in brain ischemia/reperfusion process and then induces further damage to cerebral neural cells. Mitochondrial dysfunction, iron accumulation, and oxidative damage are conditions often observed in damaged brain areas of degenerative diseases including stroke [11]. Mitochondrion matrix swelling, outer membrane rupture, and apoptosis-related factors releasing into the intermembrane space always coordinate and trigger t cell apoptosis [7, 12].

10.3.2 Mitochondria Dysfunction and Blood-Brain Barrier (BBB) Permeability After Ischemic Stroke

The BBB, which is composed of cerebral vascular endothelial cells and astrocytes, strictly controls exchanges between blood and brain compartments. BBB disruption after stroke can worsen ischemic injury and mortality by increasing edema and causing hemorrhage [13]. As well as injury of endothelial cells, ischemia/reperfusion can also induce degradation of the extracellular matrix components in basal lamina which further increases BBB permeability. Mitochondria can provide energy in the form of ATP which promotes the varied activities in diverse cell types. Mitochondrial numbers and composition in the cerebral vascular endothelium are

affected by factors such as ischemic stress. More evidence showed the important role of mitochondrial-based mechanisms on BBB integrity. Recent data showed the activation of mitochondria by diazoxide-promoted relaxation of VSM cells in endothelium-denuded cerebral arteries via a mechanism primarily involving ROS [14]. Study demonstrated that the mitochondrial mechanisms regulated BBB integrity and permeability using oxygen-glucose deprivation and reoxygenation (OGD-R), an in vitro model of ischemic/reperfusion injury, which demonstrated that compromised mitochondria led to the disruption of tight junctions, opening of the BBB, and exacerbation of stroke outcomes [15]. As such, regulation of mitochondrial function may affect BBB openings and could be critical in limiting the pathological progression of cerebrovascular diseases.

Dysfunctional mitochondria have been shown to act as an important source of free radicals in ischemic stroke. Free radicals can cause proMMP activation, and these activated MMPs cause BBB injury through degradation of the neurovascular matrix, thereby playing a deleterious role in stroke [15, 16]. One study revealed overexpression of SOD1 can inhibit MMP activity in rodent cerebral ischemia models [17]. Another study showed degradation of tight junction proteins was more severe in $SOD2^{-/-}$ mice, which indicates dysfunctional mitochondria could damage tight junction proteins [18]. $SOD2^{-/-}$ mice also demonstrated delayed BBB breakdown and higher brain hemorrhage rates [19]. SOD maintains BBB integrity and reduces brain damage by protecting endothelial cells and inhibiting MMP activity in experimental stroke. The activation of mitochondrial mechanisms after the insult may benefit the brain by protecting BBB and restricting further neuronal and glia cell death.

10.4 The Molecular Mechanism of Mitochondrial Dysfunction After Ischemic Stroke

10.4.1 Ischemia/Reperfusion Induces Opening of the mPTP

In the unstressed state, the mPTP is closed, and the IMM is impermeable to ions. Upon oxidative stress, sudden mPTP opening causes massive ion influx that dissipates $\Delta\Psi m$ and shuts down oxidative phosphorylation and ATP production. This is the serial consequence of the opening of the mPTP [20]. When ischemia happens, ETC complex activity is depressed, H^+ leak is increased in the IMM, and therefore mitochondrial ability to maintain $\Delta\Psi m$ and to continuously supply energy for normal cell living is reduced [11]. Opening of mPTP firstly leads to impairment of ATP-dependent ion pumps which are required for maintaining ion homeostasis [9]. Intracellular acidosis occurred during ischemia quickly recovers during reperfusion, which increases intracellular Na^+ and Ca^{2+} concentration [9]. The alteration of cytosolic Na^+ and Ca^{2+} concentration eventually predisposes mitochondria turbulent of ion homeostasis and Ca^{2+} overload. A large increase in matrix Ca^{2+} and recovery of

matrix pH (less acidic) might alter structure and function of mitochondria including opening of mPTP.

10.4.2 Ca^{2+} Overload in Mitochondrial Dysfunction

Calcium is one of the most important second messengers in the cell and is crucial for cell homeostasis maintenance. A small increase in mitochondrial Ca^{2+} (m Ca^{2+}) during increased workload is thought to be necessary for activity of TCA cycle enzymes to furnish the reducing equivalents. Then they can match energy demand with supply. Mitochondria are involved in buffering cytosolic Ca^{2+}. Under ischemia/reperfusion conditions, the overloaded Ca^{2+} in mitochondrial matrix results in disruption of bioenergetics and stimulates the mitochondrial production of ROS. And it also results in the increase of mitochondrial membrane permeability, releasing of apoptosis-related factors, functional impairment, and structural collapse [9]. Mitochondria within synapses appear to be more susceptible than nonsynaptic mitochondria to Ca^{2+} overload [21]. Mitochondria locate nearest to endoplasmic reticulum (ER), which releases lots of Ca^{2+} during ischemia/reperfusion stress. This will absolutely overload the mitochondria with Ca^{2+}. Under brain ischemia/reperfusion condition, resulting from overstimulation of glutamate receptors, Ca^{2+} overload in neurons is significant. When mitochondria become overloaded with Ca^{2+}, they undergo mPTP opening, osmotic swelling, and outer mitochondrial membrane (OMM) rupture.

10.4.3 Ischemia/Reperfusion Injury and Reactive Oxygen Species

Current evidence demonstrated the close correlation between mitochondrial dysfunction and endogenous cellular ROS [22]. ROS are generated from various sources such as the Nox family of NADPH oxidases, cytochrome P450 enzymes, and xanthine oxidase; over 90% of intracellular ROS is produced inside mitochondria. ROS, in the form of superoxide radical anions (O_2^-), are produced by incomplete reduction of oxygen or by leak of electrons from the respiratory chain, mostly complex I and III [23, 24]. Superoxide radical anions are highly reactive and can oxidize and damage the structure and function of other molecules at the toxic levels. It can also react with nitric oxide (NO) and give rise to the highly deleterious peroxynitrite species ($ONOO^-$). To prevent these harmful reactions, superoxide is rapidly converted into hydrogen peroxide (H_2O_2) by the superoxide dismutases (SOD). ROS scavenger enzymes, like peroxiredoxins (Prxs), glutathione peroxidases (GPxs), or catalase, not only function to limit ROS or reactive nitrogen species (RNS) induced by oxidative damage but also buffer cellular ROS species (especially

H_2O_2) thus reducing them to a level at which they function as signaling molecules under cell stress [25]. In excitable tissue, especially cardiac and neuronal, mitochondria represent a major source of O_2^- as the consequence of mitochondrial respiration, which generates unpaired electrons that interact with molecular O_2 to produce O_2^- [26]. O_2^-, H_2O_2, and OH^- are all active ROS [27]. ROS determines the cell fate in a concentration-dependent manner, nmol of ROS can promote cell proliferation, μmol of ROS can cause cell apoptosis, and mmol of ROS will cause cell necrosis/death [28].

Mitochondrial DNA is one of the main cellular targets of ROS-induced oxidative damage due to their lack of histone protein protection [28]. There is a feed-forward cascade hypothesis, that is, ROS induces mtDNA damage and mitochondria peptide expression variance, resulting in decreased ETC ability, lower ATP output, decreased ΔΨm, increased ROS, release of cytokine C and AIF, and ultimately cell apoptosis. On the one side, mitochondrial ROS is involved in cell signaling pathways in ischemic and pharmacological pre- and post-conditioning. On the other side, ROS is necessary for normal cell transcriptional regulation and cell proliferation; it could mediate apoptosis required for cell elimination during development and elimination of injured mitochondria or poorly performing cells through autophagy [9]. In all, ROS regulates cellular stress responses to maintain cellular homeostasis and health by redox regulation of transcription factors, phosphatases/kinases, and chromatin structure that control nuclear gene networks [22].

10.4.4 Ischemia/Reperfusion Injury and Activation of Proapoptotic Signaling Pathway

10.4.4.1 Apoptosis-Related Regulator Families

The mitochondria are stressed by overloaded ROS under the condition of ischemia/reperfusion, thus releasing many apoptotic initiators. In mammals, apoptosis regulators include the Bcl-2 family [29–31], the cysteine-containing aspartate-specific protease (caspase) family [32], mitochondrial proteins or proteins that influence mitochondria, p53 gene family, cell surface death receptors, cytochrome c, apoptosis-inducing factor (AIF), second mitochondrial-derived activator of caspases (Smac), the inhibitor of apoptosis protein (IAP) family, and HtrA2/Omi. Up to now, three caspase-related signaling pathways have been identified that can lead to apoptosis [33–35]; they form a complex regulation network with many cross-talks and feedbacks. An important one is the intrinsic mitochondria-mediated pathway controlled by Bcl-2 family proteins. Mitochondria release cytochrome c due to the elevated permeability of mitochondria membrane under ischemia/reperfusion. Cytochrome c will promote the activation of caspase-9 through Apaf-1 and then activate caspase-3 [33, 34]. Apaf-1 is a cytoplasmic protein that contains several copies of the WD-40 domain, a caspase recruitment domain, and an ATPase domain. Upon binding cytochrome c and dATP, Apaf-1 forms an oligomeric apoptosome.

The apoptosome binds and cleaves caspase-9 preproprotein (Apaf-3), thus releasing its mature, activated form. Activated caspase-9 cleaves pro-caspase-3. Another important one is the extrinsic death receptor pathway which involves the activation of cell surface death receptors, including Fas and the tumor necrosis factor (TNF) receptor, leading to the formation of the death-inducing signaling complex and caspase-8 activation.

Caspase-8 activation in turn cleaves and activates downstream caspases such as caspase-3, caspase-6, and caspase-7 [36]. Caspase-8 can also cleave Bid leading to the translocation, oligomerization, and insertion of Bax or Bak1 into the mitochondrial membrane [36]. Another apoptotic pathway involves the activation of caspase-2 by DNA damage or ER stress as a premitochondrial signal. p53-induced protein with a death domain (PIDD) is identified as a p53-inducible molecule implicated in p53-dependent apoptosis and has been considered as a primary p53 target gene [11, 37]. Recent publication showed PIDD can form a complex with caspase-2 and provide a platform for caspase-2 activation indicating PIDD involves in caspase-2 signaling pathway [37].

10.4.4.2 How Do Mitochondria Integrate into the Apoptosis Network

It was discovered that the mitochondria integrate into apoptosis network through the Bcl-2 family and release of proapoptotic molecules residing in the mitochondrial intermembrane space and activating caspases leading to internucleosomal cleavage of DNA [33, 34]. Under conditions of ischemia, neuronal apoptotic processes are initiated through the activation of proapoptotic Bcl-2 family members including Bax and Bak, then the induced opening of the mitochondrial permeability transition pore (mPTP), and the release of cytochrome c from the mitochondrion into the cytoplasm [37]. Cytochrome c interacts with apoptotic protease-activating factor-1 (Apaf-1) to form the apoptosome, and caspase-9 becomes activated and initiates a downstream caspase cascade. These caspases cleave several different substrates that include poly(ADP-ribose) polymerase-1 (PARP-1) which could lead to DNA damage. Furthermore, overactivation of PARP-1 will decrease NADH and ATP which results in energy failure and cellular necrosis.

Calcium-mediated activation of mitochondrial calpains results in the cleavage of AIF and translocation from inner membrane to nucleus facilitating caspase-independent programmed cell death. AIF is a mammalian mitochondrial protein classified as a flavoprotein oxidoreductase [35] with an N-terminal mitochondrial localization signal that is cleaved off to after import into the mitochondrial intermembrane space generating a mature protein. Under normal physiological conditions, AIF might function as a ROS scavenger targeting H_2O_2 or in redox cycling with nicotinamide adenine dinucleotide phosphate [38]. After some apoptotic stimuli, AIF is released from mitochondria and translocates to the nucleus [35]. Overexpression of AIF in cultured cells induces cardinal features of apoptosis, including chromatin condensation, high-molecular-weight DNA fragmentation, and loss of mitochondrial transmembrane potential [35]. In vitro studies showed NMDA

receptor stimulation leads to transient mitochondrial calcium elevations in cultured neurons, which resulted in delayed mitochondrial depolarization and cellular calcium dysregulation. Together, ischemia leads to mitochondrial dysfunction leading to excitotoxic neuronal death.

10.4.4.3 p53 in the Apoptosis-Related Mitochondrial Signaling Pathway

Ischemia/reperfusion stress induced mitochondrial translocation of p53, which could induce the transcription-independent apoptosis via the intrinsic mitochondrial pathway. This pathway depends on mitochondrial outer membrane permeabilization (MOMP) regulated by Bcl-2 family members. Physical interactions of mitochondrial p53 with anti- and proapoptotic members of the Bcl-2 family mitochondrial permeability regulators are central for the transcription-independent pathways [39, 40]. Previous data showed that mitochondrial p53 was highly efficient in inducing the release of soluble and insoluble apoptogenic factors such as cytochrome c and AIF by severely disrupting outer and inner mitochondrial membrane integrity. Under ischemia/reperfusion stress, p53 interacts with the proapoptotic mitochondrial membrane protein Bak, which causes oligomerization of Bak and release of cytochrome c from mitochondria. p53 can then bind and activate monomeric Bax in the cytoplasm via transient formation of a "hit-and-run" complex, causing Bax homo-oligomerization and translocation to the outer membrane [41, 42]. Some evidence shows that formation of the p53-Bak complex coincides with loss of interaction between Bak and the anti-apoptotic Bcl-2 family member Mcl1 [43, 44]. Not only directly involved in the apoptosis pathways, p53 also regulate the mitochondrial length, which manipulate the mitochondrial function in neurologic and other pathologies. p53 directly influences mitochondrial dynamics by modulating the expression and activity of fission and fusion proteins, which are dynamin-related GTPases that include Drp1 (the main fission protein) in a transcription-dependent and transcription-independent manner [34, 35, 45].

10.5 Endogenous Protective Mechanism Against Mitochondrial Dysfunction After Ischemic Stroke

10.5.1 mtDNA Self-Repair Mechanism After Ischemic Stroke

A number of mechanisms may underlie the mtDNA damages after ischemic brain injury. The primary mechanism is likely to be oxidative stress [46–48]. Endonuclease activation may also cause mtDNA damage. Nuclear DNA fragmentation mediated by endonucleases is a common feature of cells dying of apoptosis or necrosis and has been noted in the ischemic brain after cerebral ischemia/reperfusion [49, 50].

The maintenance of swift and effective mtDNA repair is therefore essential for post-mitotic cells like neurons. mtDNA self-repair ensures transcription of mRNA from intact genes. Past study showed a decrease in mtDNA content was restored to almost control levels in the rats subjected to 30-min, but not 90-min, ischemia [51]. It is therefore possible that short-term ischemia did not result in irreversible mitochondrial dysfunction, allowing oxidative mtDNA damages to be repaired or replenished. In order to adapt to an increased demand, a cell may try to increase its repair efficiency. Mitochondrial base excision repair (BER) seems to be of particular significance in cerebral ischemia and under other conditions of oxidative stress [52, 53]. During BER, DNA glycosylases excise damaged or modified bases. A general activation of DNA repair, as well as an increase in mitochondrial BER capacity following preconditioning, has been demonstrated [54]. However, DNA repair is neither inexhaustible nor infallible. Persisting mutations are the result of erroneous DNA repair, which might trigger mitochondria-induced apoptosis to avoid erroneous protein synthesis.

10.5.2 Mitophagy Mechanism After Ischemic Stroke

The roles of autophagy in the cerebral ischemia process have been widely studied. Recent reports have showed that autophagy can be induced in both in vitro and in vivo cerebral ischemia models [55, 56]. Mitophagy is a well-studied type of selective autophagy which is extremely important for maintaining mitochondria homeostasis by removing damaged mitochondria. Reperfusion following focal cerebral ischemia can promote mitochondrial dysfunction in rats [57]. Studies have also demonstrated that Parkin could be translocated to mitochondria during reperfusion and that ischemia-induced neuronal injury was aggravated after administration of mitophagy inhibitor mdivi-1 in the reperfusion phase, suggesting that mitophagy underlies the neuroprotection that occurred in the process of cerebral ischemia/reperfusion [56]. When primary cortical neurons were treated with OGD for 6 h followed by different periods of reperfusion (24, 48, 72 h), the Bnip3 protein level increased accompanied by increased delayed neuronal loss, which may due to Bnip's triggering of excessive mitophagy [58, 59]. Because of the crucial role of mitophagy in promoting cell survival, mitophagy has been considered as a clinical target for ischemic stroke. Several mitophagy-related proteins, such as Parkin and Beclin1, have been proved to be beneficial targets for ischemic brain injury treatment [58, 60]. In the future, the pathologic processes of ischemic brain injury will become increasingly clearer, and mitophagy could be a valuable therapeutic target.

10.5.3 Mitochondrial Dynamics After Ischemic Stroke

Mitochondrial biogenesis (fusion and fission) after ischemia may reflect compensatory adaptations to mitigate the damage associated with the multistage reperfusion period after the ischemic episode [61]. Mitochondrial responses to ischemic injury can be initiated by activating pertinent mitochondrial transcription factors, such as peroxisome proliferator-activated receptor (PPAR)-g coactivator 1-a (PGC1-a), PPAR, and NRF-1, leading to transcription of mitochondrial genes and mtDNA replication [62–64]. It has been shown that PGC1-a may be a major regulator of mitochondrial biogenesis [62]. The upstream induction of NRF-1 has been mostly attributed to the activity of PGC1-a. After hypoxia, increases in mitochondrial DNA and total mitochondrial number, upregulation of the mitochondrial transcription factors (NRF-1 and Tfam), and mitochondrial protein HSP60 are detected [63]. Under the condition of transient global ischemia, the PGC1-a signaling pathway is activated, which may trigger the UCP2 and SOD2 expression and promote mitochondrial biogenesis in the hippocampal CA1 subfield [64]. The replication of mtDNA and the induction of both nuclear- and mitochondrial-encoded mitochondrial genes are necessary for mitochondrial biogenesis [65].

Fission of mitochondria is another important way to degrade "weaker" mitochondria that requires motor-like processes, which involves dynamin-related protein 1 (Drp1). Drp1 executes the scission of membranes by forming an oligomeric ring-shaped structure around the membrane region and pinching off the targeted mitochondrial region. Global cerebral ischemia induces the elongation of hippocampal mitochondria distinct from swelling and altered expression of Opa1, Fis1, and phosphorylated Drp1 during an extended reperfusion period after focal ischemia [66, 67]. Additionally, knockdown of the fission protein Drp1 blocked toxicity in a glutamate-induced oxidative stress model in HT22 cells, and inhibitors of Drp1 reduced infarct in a transient focal ischemia model [68]. In view of mitochondria as an energy center which is important for cellular homeostasis, exploring the roles of mitochondrial biogenesis as an endogenous protective response to cope with ischemic insult may help us to develop a strategy to enhance this beneficial effect and counteract the ischemia-related detrimental effects.

10.6 Exogenous Therapeutic Targets to Mitochondrial Dysfunction After Ischemic Stroke

10.6.1 Restoring Mitochondrial Metabolism and ATP Synthesis

Ischemic stroke can result in oxidative damage and pro-oxidant/antioxidant imbalance; disturbances in mitochondria may compromise ATP production. Restoring mitochondrial metabolism and ATP synthesis fluent are promising therapeutic

targets to mitochondrial dysfunction after ischemic stroke [69]. As an energy substrate, the administration of glucose tends to deteriorate brain damage in various models of ischemia, by enhancing lactate production and decreasing pH. Therefore, strategies have been developed to improve energy metabolism in the brain without requiring glucose utilization. When using other energy source instead of glucose during brain ischemia, β-hydroxybutyrate has protective effects by reducing the deleterious accumulation of lactate. This inhibition of lactate production may firstly lead to acetyl-CoA increase, resulting in inhibition of pyruvate oxidation through feedback inhibition of the pyruvate dehydrogenase complex. Subsequently, it reduces the glycolytic rate via phosphofructokinase inhibition. Studies have shown that pyruvate also exerted neuroprotective effects both in vivo and in vitro [70]. However, the underlying mechanism involved in the above process remains unknown. It may include normalizing energy metabolism disturbances, a key factor in neuronal death which has been ascribed to protect against zinc toxicity. Acetyl-L-carnitine can also be used to the recovery of normal brain energy metabolism and attenuate neuronal damage in adult brain ischemia. This compound reduces neurologic injury in immature rat brain as well. Acetyl-L-carnitine reverses ischemia-induced inhibition of pyruvate dehydrogenase and allows the transferring of the acetyl group to coenzyme A (CoA) to form acetyl-CoA as the primary source of energy [71]. Some data also demonstrated that triheptanoin diet can enhance mitochondrial respiratory activity and preserve ATP levels and mitochondrial potential; thus triheptanoin can be regarded as a promising new dietary agent for neuroprotection [72].

10.6.2 Regulating mPTP Opening

mPTP mediates the lethal permeability changes of the mitochondrial membranes leading to mitochondria-mediated cell death. It has also been implicated in providing protection against cellular injury by transient opening. This dual role of the mPTP in the survival and death of the cell is therefore critical in selective targeting of the pore for therapeutic interventions. Therefore, pharmacological agents or any other maneuvers that regulate calcium flux and mPTP opening could influence the extent of permanent damage after ischemia stroke. Liu et al. [73] found that the activation of the mPTP is an early reversible event during brain ischemia that could trigger delayed cell death. Cyclosporine A (CsA) provides neuroprotection in various in vivo models of ischemia/reperfusion injury, such as focal ischemia, whether given several days before ischemia induction or immediately after ischemia [74]. CsA inhibits the phosphoprotein phosphatase calcineurin and can therefore interfere with many intracellular processes and block the formation of mPTP. CsA binds to cyclophilin D, preventing this last compound from binding to the adenine nucleotide translocator in IMM and presumably the mPTP. N-methyl-valine-4-cyclosporine A, an analog of CsA, also provides neuroprotection against ischemic injury. It does not inhibit calcineurin but can bind cyclophilin D and block mPTP opening [74, 75].

Taken together, these results provide evidence that mPTP is induced during reperfusion following focal ischemia and contributes to the severity of the damage. They also indicate that cyclophilin D may be a good target against ischemia/reperfusion injury. Hence, obtaining non-immunosuppressive compounds specific for cyclophilin D versus other cyclophilins and having a sufficiently small molecular weight to allow penetration across the blood-brain barrier as well as the brain cell membrane become the challenge for stroke therapy [76, 77]. The amino acid taurine; S-Methyl-N, N-diethyldithiocarbamate sulfoxide (DETC-MeSO), one of N-methyl-D-aspartate receptor (NMDA receptor) partial antagonists [78]; and nortriptyline, an antidepressant drug, could exert neuroprotective effects against cerebral IR injury in part via delayed mPTP opening by resisting Ca^{2+} overload [79]. The peripheral benzodiazepine receptor (PBR) is an 18-kDa hydrophobic transmembrane protein located in the OMM. In the central nervous system, PBR is expressed chiefly in astrocytes but also in neurons and microglial cells [80, 81]. It is normally expressed at low levels in the brain but is upregulated in a number of neurological pathological conditions, including ischemia/reperfusion injury. Composed by at least three subunits, PBR is a component of mPTP and may be a target for preventing mPTP opening during ischemia [71]. Recent studies have shown that mitochondrial ROS generation induced by the binding of specific ligands to PBR in cultured neuronal cells can be inhibited by CsA treatment [82]. The OMM protein porin which is involved in mPTP opening is another new target for the mPTP strategy. Thus the F(ab) fragments of anti-porin antibodies can penetrate living cells and reduce Ca^{2+}-induced mitochondrial swelling in brain slice cultures exposed to excitotoxic and ischemic events [83]. However, further investigation is urgently needed to evaluate the therapeutic potential of anti-porin antibodies.

10.6.3 Preventing ROS-Induced Damage via Antioxidant Treatment

In ischemic stroke, a small amount of ROS produced by mitochondria can damage the ECT due to hypoxia, the release of excitatory amino acids, and calcium overload; then mitochondria can produce a large number of ROS with excessive consumption of antioxidant substances and decrease the activity of antioxidant enzyme. All these can disturb the balance between elimination and production of ROS, give rise to a substantial increase of ROS in brain cells, and ultimately cause brain cell damage. Therefore, administration of antioxidants may play an important role in protecting the mitochondria from ROS-induced damage by neutralizing ROS. But a major hurdle faced by this strategy is the diversity of ROS, which includes superoxide anion, hydrogen peroxide, hydroxyl radical, nitric oxide, and peroxynitrite. Three main strategies targeting antioxidant functions have been proposed.

Firstly, natural compounds present in cerebral tissues that exhibit antioxidant properties can be used. Vitamin E (i.e., α-tocopherol), a lipid-soluble chain-breaking

compound which is found in membranes, is one of the most extensively studied antioxidants and could scavenge lipid peroxyl radicals. Thus, administration of vitamin E ensures protection of polyunsaturated fatty acids against peroxidative damage and prevents cerebral ischemia in vivo in stroke models. Vitamin E protects mitochondria against ischemia/reperfusion damage in experimental in vivo models as well as in model of isolated mitochondria in vitro [84]. Other vitamin E isoforms, g-tocotrienol and d-tocopherol, are also potent for preventing cerebral infarction induced by middle cerebral artery occlusion [85]. Several other physiological antioxidants, such as N-acetyl cysteine, vitamin C, and melatonin, have been shown to improve mitochondrial functions during ischemia [86–88].

Secondly, natural compounds extracted from various plants are well-known antioxidants that could be used to combat ischemia/reperfusion injury. Among natural antioxidants identified in epidemiologic studies, polyphenolic compounds are one of the most important families. Resveratrol is a liposoluble molecule that can penetrate into the mitochondrial membrane and prevent lipoperoxidation of unsaturated fatty acids. Thus, resveratrol exerts neuroprotective effects during ischemic insults. It protected mitochondria from oxidative damage in a mitochondrial model of ischemia/reperfusion in vitro not only when it was added to the experimental buffer but when mitochondria were isolated from rats previously treated by resveratrol [89, 90]. Malibatol A (MA), a novel resveratrol oligomer, can restore the increased levels of ROS induced by right middle cerebral artery occlusion (MCAO) in mice [91]. Cannabis has potential therapeutic use, but tetrahydrocannabinol (THC), its main psychoactive component, appears as a risk factor for ischemic stroke in young adults. THC increases oxidative stress and induces cerebral mitochondrial dysfunction [92]. Results also showed that galangin alleviated the neurologic impairments, reduced cerebral infarct at 24 h after MCAO, and exerted a protective effect on the mitochondria with decreased production of mitochondrial ROS [93].

For the third one, synthetic antioxidants can be applied to reduce oxidative damage induced by ischemic injury. Many of these compounds are synthetic derivatives of natural antioxidants and are not merely scavengers of oxygen radicals. They are composed of the antioxidant structure of the natural compound covalently linked to one of the mitochondriotropic cationic amphiphiles, which are chemicals rapidly taken up by mitochondria in living cells [94]. Mitochondriotropic agents are amphiphilic and have a positive charge, delocalized between two or more atoms. Thus, their sufficient lipophilicity, combined with the delocalization of their positive charge that reduces the free energy change when they move from an aqueous to a hydrophobic environment, results in intramitochondrial accumulation driven by the mitochondrial potential [94]. Methyltriphenylphosphonium and Rhodamine 123 seem to be the most common ones among these mitochondriotropic molecules. Diphenyl diselenide (PhSe)2 can significantly reduce the mitochondrial damage induced by IR which may be attributed to the maintenance of mitochondrial redox balance [95].

Another antioxidant strategy to diminish ROS-induced damage during ischemia/reperfusion consists in mimicking the activity of antioxidant enzymes. Natural antioxidant enzymes such as SOD or glutathione peroxidase do not easily cross the

blood-brain barrier or cell membrane; increasing the extracellular levels of these enzymes fails to eliminate the effects of intracellular produced ROS. Thus, low-molecular-weight molecules are developed to overcome this difficulty. They show better penetration within cells and tissues while remain able to catalyze superoxide anion dismutation with similar potency to the natural enzyme. For instance, curcumin and some of its derivatives can be bound to manganese to form complexes that are powerful antioxidants with SOD-like properties and ability to cross the blood-brain barrier. Various chemical classes of SOD-mimetic agents are included, such as salen, macrocyclic complexes, and metalloporphyrin. They are protective in a wide variety of oxidative stress models, both in vivo and in vitro [71]. An alternative to mimicking SOD activity consists in enhancing glutathione peroxide activity, which converts H_2O_2 to H_2O. Ebselen mimics glutathione peroxidase activity, thereby inhibiting H_2O_2 production during ischemia/reperfusion. Ebselen also reacts with peroxynitrite and inhibits various enzymes involved in oxidative stress such as NO synthases, NADPH oxidase, lipoxygenases, and protein kinase C. Ebselen limits lipid peroxidation and reduces ischemic brain injury, most notably by protecting mitochondria from damage due to oxidative stress [71].

10.6.4 Inhibiting Mitochondrial Release of Apoptosis Signals

Under conditions of hypoxia/ischemia, neuronal apoptotic process is initiated through the actions of proapoptotic Bcl-2 family members, including Bax and Bak, to open the mPTP and enable the release of cytochrome c from the mitochondrion into the cytoplasm. Strategies described above indirectly inhibit the release of pro-apoptotic factors such as cytochrome c and AIF. As a result, the apoptotic signal released by mitochondria can be inhibited. MA could increase Bcl-2 and decrease Bax expression, thus inhibiting the release of apoptosis signals [91]. Silent information regulator 1 (SIRT1), a type of histone deacetylase, is a highly effective therapeutic target for protection against ischemia/reperfusion (IR) injury. Melatonin can preserve SIRT1 by increasing the anti-apoptotic factor, Bcl-2, and reducing the pro-apoptotic factor Bax to reduce IR-induced mitochondrial dysfunction [96]. Galangin also inhibited apoptosis in a dose-dependent manner concomitant with the upregulation of Bcl-2 expression, downregulation of Bax expression, and the Bax/Bcl-2 ratio. It also caused a reduction in cytochrome c release from the mitochondria to the cytosol and the reduced expression of activated caspase-3 and the cleavage of PARP [93].

However, pharmacological protection against events that precede caspase activation, in particular the release of mitochondrial proapoptotic proteins regulated by Bcl-2 homology (BH) proteins, may be another effective approach to inhibiting this signal. “BH3 domain-only” proteins, such as Bid and Bim, may act either by binding to and antagonizing the function of multidomain pro-survival proteins such as Bcl-2 and Bcl-XL or by activating the multidomain pro-death proteins Bax or Bak. Thus, organic compounds that mimic BH3 domain-binding properties and induce

apoptosis have been characterized. Recently, studies have shown that Bax-induced permeability changes and cytochrome c release could be inhibited by dibucaine and propranolol via a direct interaction with the lipid membrane [97, 98]. However, Bax insertion into brain mitochondrial membrane is not inhibited by dibucaine or propranolol and does not involve mPTP. The effects of these two drugs may involve cardiolipin or some of its metabolites that associate with Bid and may facilitate outer membrane discontinuity and leakage of apoptogenic factor induced by Bax [71]. Further studies on the mechanism of action of dibucaine, propranolol, and other similar drugs should contribute to a better understanding of OMM permeabilization by BH3 domain proteins such as Bax, and a new class of therapeutic drugs with anti-apoptotic and neuroprotective properties may be developed consequently.

10.6.5 Regulating Mitophagy in Cerebral Ischemia

Rapamycin is known to exhibit neuroprotective functions via the activation of autophagy, as shown in a cerebral ischemia model [99]. Our lab study reported that rapamycin could reduce brain injury after cerebral ischemia by promoting mitophagy [55]. The results demonstrated that rapamycin could significantly increase the expression of L3-II and Beclin1 level, which means increased autophagy and mitophagy [55]. This process is thought to be mediated by promoting p62 translocation to the mitochondria in response to ischemia injury, which led to reduced infarct volume and inhibition of mitochondrial dysfunction [55]. Methylene blue (MB) is a lipophilic compound and has been demonstrated to play neuroprotective roles in cerebral ischemia/reperfusion injury [100–102]. In a MCAO model, MB improved neurological function and reduced the infarct volume after acute cerebral ischemia due to augmenting mitophagy [103]. In an OGD model, they revealed MB promoted mitophagy by maintaining the MMP at a relatively high level [103].

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Chapter 11
Molecular Mechanisms of Inflammation in Stroke

Parisa Tabeshmehr, Seyed Mojtaba Hosseini, Aliashghar Karimi, and Seyyed Mohyeddin Ziaee

Abstract Stroke is one of the most common causes of death and the leading cause of disability worldwide. Pathophysiological events such as excitotoxicity, oxidative and nitrative stress, inflammation, and apoptosis play significant roles in the brain injury development following stroke. Cytotoxicity can be the first negative process in stroke that is followed by cellular death and damage. Therefore, the brain tissue triggers an ordinated chain of molecular mechanisms to reduce injuries of this cytotoxicity. Inflammation cascade as one of these responses applies the most effective role in brain tissue protection and repair. Principally, the cellular and molecular mechanisms of inflammation involved in stroke injury can be categorized in three major phases from deleterious to beneficial.

This chapter decides to introduce a new insight into molecular pathways of stroke development and other mechanisms that prevent more brain injuries. Moreover, it demonstrates particularly the association between stroke and inflammation in detail.

Keywords Inflammation • Stroke • Brain ischemia • Cerebrovascular disease • Immune system

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Abbreviations

BBB	Blood-brain barrier
BMMNCs	Bone marrow mononuclear cells
CEI	Cardio-embolic
CNS	Central nervous system
CRP	C-reactive protein
DAMPs	Danger-/damage-associated molecular patterns
HMGB1	High mobility group binding protein 1
HSP	Heat shock protein
HI	Hypoxia-ischemia
ICH	Intracerebral hemorrhage
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LAAS	Large-artery atherosclerosis
LAC	Lacunar
MCAO	Middle cerebral artery occlusion
MHC	Major histocompatibility complex
MMPs	Multiple matrix metalloproteinases
NF-KB	Nuclear factor kappa B
ODE	Other determined etiology
PMN	Polymorphonuclear leukocytes
ROS	Reactive oxygen species.
TGF-B	Transforming growth factor beta
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TOAST	Acute stroke treatment
Treg	Regulatory T-cell
UDE	Undetermined etiology

11.1 Stroke

Stroke is one of the leading causes of death and long-term or permanent disability in the world [1]. With about six million deaths each year, it is the third cause of mortality among lethal medical conditions [2]. There are generally two categories of stroke with opposite features: hemorrhage and ischemia. Occurrence of hemorrhage causes an excessive intracranial pressure due to the presence of extravascular blood in a specific region of brain tissue. Paradoxically ischemia is caused by lack of adequate blood supply to a part of the brain and, consequently, hypoxic damage to the nervous tissue [3]. Each of these categories can be divided into various subtypes with different causes, clinical pictures, clinical courses, outcomes, and

treatment strategies; that acute ischemic stroke is the most common stroke type, accounting for >80% of all stroke [4].

Generally, stroke induces a complex web of pathophysiology that may evolve over hours to days and weeks after the onset. The full spectrum of inflammatory pathways is likely to act in a tandem in stroke. Cytokines are the noticeable mediators of stroke-induced immunological/inflammatory reactions, which contribute to brain infarct progression, disease severity, and outcome. Since subtypes of stroke have specific differences in their pathophysiology, it is safe to consider that different patterns could be involved in the activation of immune-inflammatory process for these subtypes. In other words, the cerebral ischemia initiates a complex cascade of events at genomic, molecular, and cellular levels, which, in this cascade, inflammation plays important roles both in the central nervous system (CNS) and in the periphery.

The Trial of Org 10172 in Acute Stroke Treatment (TOAST) is a system for diagnosis of subtypes of ischemic stroke that was designed by Adams et al. in 1993 [5]. Thus, the types of acute ischemic stroke were defined according to this classification:

1. LAAS (Large-artery atherosclerosis): In this subtype there is a significant (>50%) stenosis or occlusion of the major brain artery or branch cortical artery, presumably due to atherosclerosis. Moreover, cortical or cerebellar lesions and the brain stem or subcortical hemispheric infracts are greater than 1.5 cm in diameter on CT or MRI.
2. CEI (Cardio-embolic): This class is the presence of a cardio-embolic source (atrial fibrillation, recent anterior MI, mechanical valve, endocarditis, etc.) in the absence of cerebrovascular disease in a patient with a non-lacunar stroke. Clinical and brain imaging findings are similar to those described for LAAS.
3. LAC (Lacunar): This group is a situation with small-artery occlusion and subcortical or brain stem infract <1.5 cm; a history of diabetes mellitus or hypertension supports the clinical diagnosis.
4. UDE (Undetermined etiology): In this subtype the cause of a stroke cannot be determined with any degree of confidence.
5. ODE (Other determined etiology): This class refers to rare causes of stroke, like non-atherosclerotic vasculopathies, hypercoagulable states, or hematologic disorders.

11.2 Association Between Stroke and Inflammation

A general systemic inflammatory response happens after both the ischemic and hemorrhagic strokes, either as part of the process of brain damage or in response to complications such as deep venous thrombosis. According to previous studies, higher levels of inflammatory markers such as C-reactive protein (CRP) and interleukin-6 (IL-6) might be the reasons of worse outcomes after both ischemic [6] and

hemorrhagic [7, 8] strokes. Several chronic systemic inflammatory conditions, such as atherosclerosis, diabetes, and obesity, are associated with the increased risk of stroke, which suggests that systemic inflammation may affect the progression of stroke in humans.

11.2.1 Intracerebral Hemorrhage (ICH) and Inflammation

Spontaneous intracerebral hemorrhage (ICH) accounts for approximately 4–14% of all strokes and is associated with a high mortality and morbidity [9]. In 2004 Castellanos et al. reported the association between high levels of inflammatory molecules within 24 h of the ICH onset and the volume of the perihematoma brain edema measured on days 3-4 after the stroke, which supports the view that edema is an indicator of the inflammatory response induced by the ICH. Also, as fibrinogen increases in response to inflammatory molecules, fibrinogen levels may reflect the activation of inflammatory mechanisms responsible for tissue damage around the hematoma [7].

11.2.2 Acute Ischemic Stroke and Inflammation

Acute ischemic stroke is the most common stroke type, accounting for >80% of all strokes [4]. One of the chief factors in pathobiology and prognosis of acute cerebral ischemia is the immune response to this situation. The initiation of immune response takes place locally and in the occluded vasculature and hypoperfused and ischemic brain parenchyma. But the inflammatory mediators generated in this area are later released in the circulation and, thus, spread throughout the body. This spillover leads to a systemic inflammatory response first, followed by immunosuppression aimed at dampening the potentially harmful pro-inflammatory milieu [10].

The ischemic cascade is represented by a complex series of interlinked molecular and cellular mechanisms that contribute to ischemic cell death via necrosis or apoptosis. Two important pathophysiologic contributors to ischemic brain damage are thrombotic and inflammatory pathways. At ischemic vascular lesions, blood platelets adhere and become activated, increasing the risk of secondary thrombotic events [11]. At the same time, cerebral ischemia elicits a strong inflammatory response involving the upregulation of cell adhesion molecules and cytokines as well as the adhesion, activation, and transmigration of several subsets of leukocytes [12]. Previous studies revealed an important link between these thrombotic and inflammatory pathways in stroke, which led to the concept of thromboinflammation in stroke pathology.

The immune system's response to disruption of tissue homeostasis is indicated as postischemic inflammation, days and weeks after the event. But the inflammatory

cascade is activated immediately after vessel occlusion has occurred [13, 14] (Fig. 11.1).

The pattern of cytokine inflammation response differs depending on the stroke type and localization. Specific regions, including the caudate body, putamen, insular ribbon, paracentral lobule, and precentral, middle, and inferior frontal gyri, are vulnerable to ischemic injury [15]. Even though regional cerebral blood flow may be restored to near normal values after middle cerebral artery occlusion (MCAO) through reperfusion [16], a reproducible cerebral infarct occurs [17].

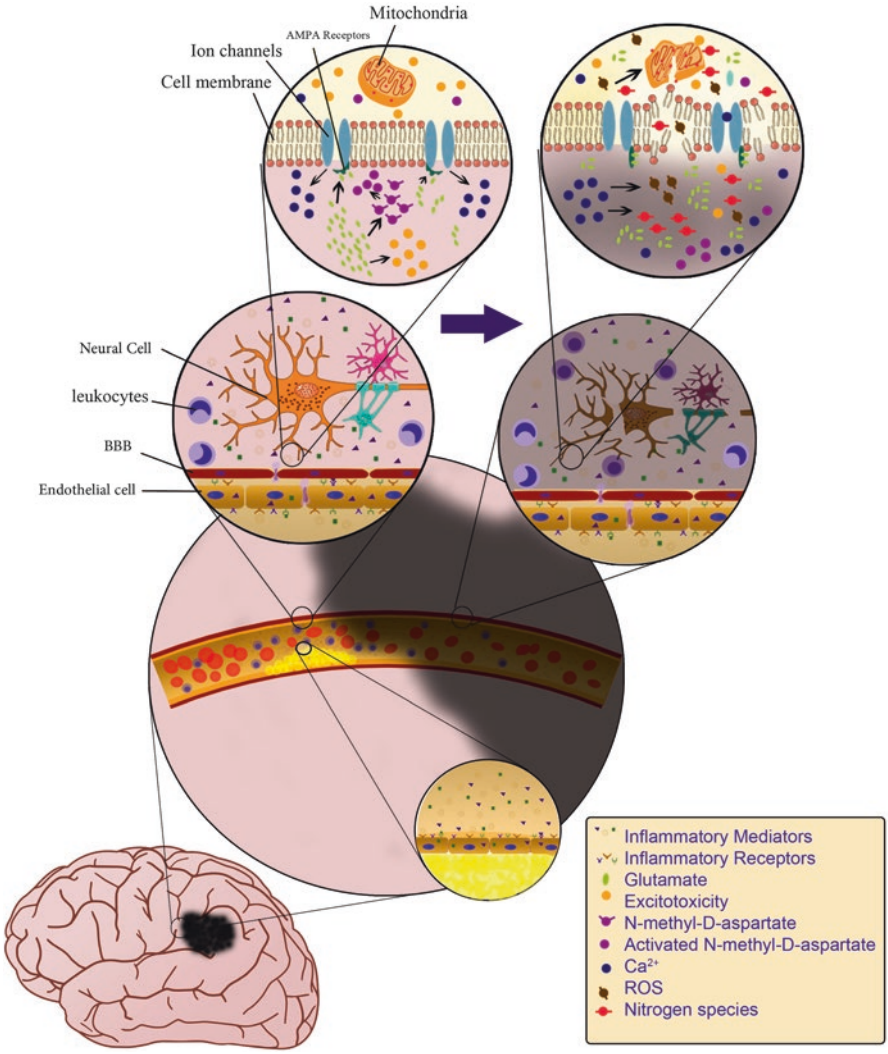

Fig. 11.1 The stroke and immune cascade demonstrating a complex series of interlinked molecular and cellular mechanisms that contribute to ischemic cell death via necrosis or apoptosis

Tumor necrosis factor (TNF) is involved in the development of ischemic brain lesions. It is rapidly expressed in the ischemic brain after the onset of the disease [18]. The upregulation of TNF also leads to the damage of the blood-brain barrier (BBB) [19], as neuronal cell death during the stroke [20]. TNF is also involved in the apoptotic death of neurons exhibiting peripherin aggregates [20]. In the nucleus, the activation of TNF-kB plays a key role [21]; it promotes gene expression and mediates transcription of many genes implicated in the inflammatory response (e.g., TNF-α, IL-1ß, IL-6, iNOS, inter alia) [22]. In 2010 Maddahi et al. explained elevated microvascular expression of TNF-α, IL-1ß, IL-6, and iNOS following focal ischemia and suggested that this expression is transcriptionally regulated via the MEK/ERK pathway [23]. Moreover, stroke triggers an intense inflammatory response that could be a consequence of Toll-like receptor (TLR) activation. TLR2 and TLR4 are associated with the outcome in stroke patients, and TLR2/TLR4 or their endogenous ligands, cFN/HSP60, could be new therapeutic targets for the ischemic stroke [24]. During the course of brain ischemia, inflammatory mechanisms both intrinsic to the brain and blood are among the important mediators of focal cerebral injury [25]. Poly(ADP-ribose) polymerase-1 activation and the activation of multiple matrix metalloproteinases (MMPs) are the other reasons for further local neuronal injury. The activation of MMPs disrupts the blood-brain barrier, alters the microvascular endothelial function, and impairs the functional integrity of the neurovascular unit [25].

The ischemic region consists of two parts: the ischemic core and the penumbra. In the core of the ischemic territory, regional cerebral blood flow <20 % leads to rapid acute neuronal death, within minutes to hours, after the arterial occlusion; energy deficit results in intracellular ionic imbalance with the abnormal influx of Na+ and efflux of K+, contributing to a widespread anoxic depolarization in the membranes of neurons and glial cells [26] and mitochondrial failure, and activation of intracellular proteases, lipases, and ribonucleases leads to fast breakdown of cellular structural elements and loss of cell integrity. However, outside the ischemic core the brain tissue is still partially perfused although at a reduced rate. This region, referred to as ischemic penumbra, is often defined by a reduced perfusion rate that is, however, greater than the one observed in the ischemic core [27]. While penumbral neurons are functionally compromised, they are salvageable if blood flow is restored [4]. However, even if blood flow is restored, neurons in the penumbra face major challenges to their survival, such as excitotoxicity and inflammation. Excitotoxicity develops as a result of uncontrolled release of the neurotransmitter glutamate from depolarizing or dying neurons. The glutamate-mediated activation of N-methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, potentiated by prolonged activation of transient receptor potential melastatin ion channels, leads to uncontrolled extracellular calcium influx and dysregulation of intracellular calcium homeostasis that results in the generation of reactive oxygen (ROS) and nitrogen species, mitochondrial dysfunction, the activation of the apoptotic cascade, and poly(adenosine diphosphate ribose) polymerase activation [4].

Contemporary with those processes, the persistence of arterial occlusion contributes to a critical reduction of pO2 and a concomitant increase in pCO2. In the case of hypercapnia, the tissue pH could fall to around 6.6 or lower if severe ischemia and tissue hypoxia occur; in the last situation, anaerobic glycolysis leads to lactic acid accumulation with signs of irreversible injury identifiable in the cell morphology [28]. The acidosis state increases necrosis and cell death via a mechanism called acidotoxicity [29]. Other deleterious effects of acidosis influence the synthesis and degradation of cellular constituents, the mitochondrial function, the cell volume control, the postischemic flow, and the stimulation of ROS production, all conditions that occur also in the ischemic penumbra [30]. Acidosis and ROS contribute to trigger a subsequent and concomitant phase represented by the activation of innate immunity and involving both resident cells (microglia) and circulating cells [31].

Inflammation and brain tissue injury are due to an organized cascade of molecular pathways. Then, this phenomenon leads to the release of pro-inflammatory mediators from ischemic endothelium and brain parenchyma. Put another way, as the initial point, stagnant blood flow and altered rheology induce shear stress on the vascular endothelium and platelets. Thus, the adhesion molecule P-selectin deploys to the cell surface. Selectins are crucial in slowing down circulating leukocytes by attracting them to the endothelial surface and interacting with P-selectin glycoprotein ligand-1 expressed on most leukocytes [14] According to the studies of Walberer et al., higher peripheral leukocyte and neutrophil counts, but not lymphocyte counts, are associated with larger infarct volumes in acute ischemic stroke [32]. Other adhesion molecules are rapidly induced at the transcriptional level after activation of endothelial pattern recognition and cytokine receptors. Among them, E-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1 play an important role in orchestrating blood leukocyte recruitment, adhesion, and transmigration [33].

The activation of coagulation cascades can generate inflammatory cues [34]. Thrombin is a chemotaxin for monocytes and neutrophils and induces expression of adhesion molecules on endothelial cells through the activation of protease-activated receptors and nuclear factor kappa B [35]. Also it directly activates both C3 and C5 components of the complement system and can disrupt endothelial barrier function [36]. Complement system activation is associated with unfavorable outcome of stroke [37]. In the setting of cerebral ischemia, the complement system might be activated intravascularly by proteases of the coagulation cascade, at the level of C3 and C5 cleavage, thus bypassing classical, alternative, and lectin pathways. It has been indicated that the activation of alternative and lectin pathways might also contribute to ischemic brain injury [38], and intravascularly generated active complement proteins might gain access to the brain parenchyma through a compromised blood-brain barrier (BBB). The mechanisms of complement-mediated neurotoxicity in brain ischemia are not fully understood. It is rather believed that anaphylatoxins (C3a, C5a) act on complement receptors found on most immune cells of the myeloid origin to increase ROS production, the secretion of pro-inflammatory cytokines, and degranulation [39]. This intravascular inflammation, BBB breakdown,

and leukocyte invasion of the ischemic tissue leads to the initiation of inflammatory processes in the brain parenchyma. It can be mentioned that injured and dying neurons may release danger-/damage-associated molecular patterns (DAMPs) as one of the immediate events. These DAMPs consist of different substances such as purines (ATP, UTP, and their catabolites), high mobility group binding protein 1 (HMGB1), heat shock proteins, peroxiredoxins, and mitochondrial-derived N-formyl peptides and activate pattern recognition receptors on microglia, brain's resident immune cells, and astrocytes [33, 40–42].

Within hours of the onset of ischemia, the altered environment causes microglia to respond by altered morphology and increased contact with neurons with excitotoxic calcium overload [42]. Thus, microglia release large intracellular multiprotein complexes called inflammasome-mediated interleukin (IL)-1β and produce tumor necrosis factor (TNF). This process induces cytokine and chemokine production in endothelial cells and astrocytes and affects inflammatory cascade. Therefore, in cerebral ischemia-induced brain injury, cytokines such as TNF-α, IL-1β, IL-6, IL-18 [43], and iNOS are produced by a variety of activated cell types including endothelial cells, neurons, platelets, monocytes, macrophages, and fibroblasts [44]. These cytokines contribute to leukocyte infiltration in the damaged tissue, and they activate the presentation of antigens between dendritic cells and T-cells [45]. T-cells lead to tissue damage by innate immunity, through interferon gamma and ROS. As the result of this process, an early downregulation of systemic cellular immune responses occurs that leads to a functional deactivating of monocytes, T-helper cells, and invariant natural killer T-cells [46].

Activation of pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS) in vessel walls after cerebral ischemia may facilitate the injury process. Thus, neuroinflammation is in principle a defense mechanism designed to neutralize an insult and to restore structure and function of the brain after an insult. Basically, neuroinflammation can be viewed as a protective mechanism that isolates the damaged brain tissue from uninjured areas, destroys affected cells, and repairs the extracellular matrix [47]. All cells in the brain participate in these inflammatory responses, including microglia, macrophages, astrocytes, neurons, and oligodendrocytes. The main mediators of neuroinflammation are glial cells, constituting 70% of the total cell population in the central nervous system. Thus, microglial cells show a rapid response involving cell migration, proliferation, and release of cytokines, chemokines, and trophic factors. These affected cells are considered as "immunologically silent" cells owing to very low levels of MHC antigen expression [48]. In addition, there is some recruitment of polymorphonuclear leukocytes (PMN) from the circulation. PMN migration involves chemotaxis, adhesion to endothelial cells, penetration of tight junctions, and migration through the extracellular matrix [49].

Early activation of the immune system is superseded by a state of systemic immune depression that predisposes to poststroke infections [50, 51]. This process is characterized by the increased apoptosis of lymphocytes, the suppression of peripheral cytokine release, and finally the inhibition of T-helper-1 cells and alteration of the T-helper-1/T-helper-2 ratio [52]. Accordingly, complication from

pulmonary or urinary tract infections [53], prolonged peripheral lymphopenia, and reduced T-cell responsiveness [54] have been reported in patients with stroke. This immunosuppression results in increased susceptibility to nosocomial bacterial infection and to higher mortality rate [51]. The sympathetic nervous system is fundamentally involved in the decrease of lymphocytes and increased splenocyte apoptosis, spleen atrophy, and regulatory T-cell (Treg) expansion [51, 55]. Additionally, as the immunosuppressive effects of ischemic brain injury on the bone marrow, tyrosine hydroxylase and norepinephrine levels increase 1 day after stroke [56]. Thus, a response in mesenchymal stromal cells might be initiated through activation of β3-adrenergic receptors. This event results in the reduction of homeostatic and cell retention factors such as IL-7, C-X-C motif chemokine 12 (stromal cell-derived factor 1), vascular cell adhesion protein 1, stem cell factor, and angiopoietin-1. Downregulation of these factors increases hematopoietic stem cell proliferation. This proliferative response, however, does not involve all arms of blood cell lineages equally, and the hematopoietic system becomes skewed toward the myeloid lineage [56]. This switch is accomplished by increased expression of transcription factors associated with myeloid lineage progression such as the ETS-domain transcription factor PU.1 and CCAAT/enhancer-binding protein. Collectively, the data pose that the injured brain exerts a strong influence on the peripheral immune system by regulating development and homeostasis of splenic and bone marrow immune cell populations and that increased sympathetic output after stroke is the main efferent branch responsible for these effects.

The termination of inflammation triggers structural and functional reorganization of the injured brain. The first step involved in this phase is the removal of dead cells, which is performed by microglia and infiltrating macrophages, mainly comprising phagocytes [57, 58]. The purines released from injured cells and chemokines are the main factors for improving this process. Immunoglobulins directed against antigens of the central nervous system may also promote the release of IL-10 and TGF-B, which contribute to suppressing the immune process and to inhibiting the expression of adhesion molecules and the production of proinflammatory cytokines [59]. Tissue repair can be facilitated after promoting the resolution of inflammation by these immunoregulatory cytokines. Thus, cytoprotective effects can be exerted on the surviving cells in the ischemic area [60]. Concomitant growth factors released by inflammatory cells, neurons, and astrocytes [61] support cell sprouting, neurogenesis, and angiogenesis as well as matrix reorganization after ischemic injury. Insulin-like growth factor-1 is a critical factor in the sprouting of neurons after cerebral ischemia, while the reactivity of astrocytes is mandatory for the functional recovery of damaged tissue [62]. Concomitant actions of vascular endothelial growth factor and neutrophil MMPs are required in angiogenesis, supporting the need for the combined activity of inflammatory cells and astrocytes [63].

11.3 Regenerative Medical Experiments as Stroke Therapeutic Approaches

Brain regeneration, rehabilitation, and functional recovery can be facilitated by different approaches including pharmacological and cell therapies. Cell-based therapies are increasingly explored in preclinical and clinical settings for a variety of acute CNS injuries, especially for stroke [64]. Consequently, stem cell therapy with the aim of protecting subacutely ischemic and surrounding brain by suppressing inflammation and apoptosis can be a good therapeutic approach for repairing and regenerating chronically damaged brain by stimulating growth factor secretion, cell replacement, and biobridge formation.

In 2006, Vendrame et al. indicated that human umbilical cord blood (HUCB) transplantation can be a promising treatment for perinatal and adult ischemic brain injury. According to their results, in HUCB-treated MCAO rats, TNF-α and IFN-γ levels were significantly depressed. Additionally, HUCB transplantation increased the levels of IL-10, the regulatory cytokine that plays an important role in maintaining the anti-inflammatory environment within the CNS. HUCB transplantation significantly reduces the number of activated microglia and blocks the infiltration of CD11b-positive amoeboid-shaped immune cells in the brain following hypoxia-ischemia (HI) injury. It should be considered that HUCB may modulate the beneficial or harmful signals of microglia [65]. In 2006, Newcomb et al. suggested that the therapeutic mechanism of HUCB transplantation preceded and ameliorated the massive infiltration of pro-inflammatory cells [66]. Therefore, transplanted HUCB cells act through anti-inflammatory mechanisms that reduce the damage caused by the HI-induced immune responses.

In 2014, Prasad et al. posed new evidences that supported the feasibility, tolerability, and safety of intravenous transplantation of autologous bone marrow mononuclear cells in patients with subacute (7–30 days) stroke [67]. Another stereotactic intracerebral transplantation of human neural stem cells in patients with chronic (6–60 months) stroke was accomplished by Kalladka et al. in 2016 [68].

In 2016, Farhaan et al. revealed that bone marrow mononuclear cells (BMMNCs) can confer protection in traumatic brain injury and stroke [69]. Another recent study found that BMMNCs depleted of myeloid cells lost their protective capacity in a model of transient MCAO in mice by elusive mechanisms, while lymphocyte or erythroid depletion had no effect. The results illustrated that delivery of BMMNCs 24 h after transient MCAO increased serum IL-10 and IFN-γ levels, while IL-1b, IL-6, and TNF were reduced [70, 71].

Despite the bright pathway of different therapeutic approaches for stroke, scientists perceive that stem cell therapy can be a potent field as a regenerative method for repairing the injured brain tissue.

11.4 Conclusion

Stroke is one of the most common causes of death and the leading cause of disability worldwide. Brain injury following stroke results from a complex series of pathophysiological events including excitotoxicity, oxidative and nitrative stress, inflammation, and apoptosis. Experimental discoveries have begun to define the cellular and molecular mechanisms of inflammation involved in stroke injury that can be categorized in three major phases from deleterious to beneficial. The first negative reaction is the response of brain cells to cytotoxicity that is followed by cellular death and damage. Hence, the brain tissue starts a preconditioning status to preserve itself from more injuries after an insult. In this condition inflammation exerts its protective effects. Finally, due to this pathological process should be changed into repairing the injured parenchyma; other inflammatory mediators play their roles. Undoubtedly, further observations will contribute to a better understanding of stroke for development of more effective therapeutic approaches.

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Chapter 12
Immunology of Ischemic Stroke: Impact, Mechanisms, and Immunomodulatory Therapies

Jia Jia and Jian Cheng

Abstract It is increasingly recognized that ischemic stroke is not only a brain disease characterized by brain cell death and damage but is also marked by the dysfunction of immune organs, as evidenced by systematic immune dysregulation prior to or after cerebral ischemia. For instance, with the prevalence of comorbidities such as diabetes, obesity, and hypertension, pre-existing chronic systematic inflammation has been considered an essential contributor that exacerbates stroke pathology. Conversely, mild immune responses induced by preconditioning have also been shown to protect against cerebral ischemic injury. Moreover, once stroke occurs, the injured brain evokes immune responses both in the brain and in the peripheral by communicating with the immune system via danger-associated molecular patterns or antigens, cytokines, and chemokines as well as via specific neural circuits, such as the sympathetic and parasympathetic nervous system. This bidirectional communication between the injured brain and the immune system determines the progression of acute infarct damage, long-term tissue repair, as well as postischemic systematic immunosuppression. In summary, the pre-existing immune responses as well as the immune responses evoked by cerebral ischemia play essential roles in shaping stroke outcomes. The therapies that target the immune responses, especially the immunomodulatory therapies, are promising treatments for ischemic stroke.

Keywords Alternative activation • Cytokines • Dendritic cells • Immunology • Immune responses • Glia • Leukocytes • Microglia • T lymphocytes • Neutrophils • Sympathetic nervous system • Parasympathetic nervous system • Danger-associated molecular patterns • Chemokines • Peripheral immune system • Immunomodulatory therapies

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Abbreviations

ANS	Autonomic nervous system
APC	Antigen processed cells
ApoE	Apolipoprotein E
BBB	Blood–brain barrier
CARDs	Caspase activation and recruitment domains
CCL2	Monocyte chemotactic protein-1
CCL3	Macrophage inflammatory protein-α
CCL5	Chemokine (C-C motif) ligand 5
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CRH	Corticotrophin-releasing hormone
CX3CR1	C-X3-C motif chemokine receptor
DCs	Dendritic cells
HDAC	Histone deacetylase
HIFs	Hypoxia inducible factors
HMGB1	High-mobility group box-1
HPA	Hypothalamic–pituitary–adrenal
ICAM	Intercellular adhesion molecule
IL	Interleukin
iNKTs	Invariant natural killer T cells
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
MAP 2	microtubule-associated protein 2
MBP	Myelin basic protein
MCP-1	Monocyte chemoattractant protein-1
MMP	Metalloproteinase
MOG	Myelin oligodendrocyte glycoprotein
nACH	Nicotinic acetylcholine receptors
NLRs	NOD-like receptors
NLRP1	NLR family pyrin domain containing 1
NPCs	Neural progenitor cells
OGD	Oxygen-glucose deprivation
OPCs	Oligodendrocyte precursor cells
PET	Positron emission tomography
PNSN	Parasympathetic nervous system
PVN	Paraventricular nucleus
Rag1	Recombination-activating gene 1
RAGE	Receptor for advanced glycation end products
RANTES	Regulated on activation, normal T cell expressed and secreted
S1P	Sphingosine-1-phosphate

SCID	Severe combined immunodeficiency mice
SDF1	Stromal cell-derived factor 1
siRNA	Small interfering RNA
SNS	Sympathetic nervous system
Th	T helper
TLR	Toll-like receptor
tPA	Tissue plasminogen activator
Tregs	Regulatory T cells
VCAM	Vascular adhesion molecule
vMIP-II	Virus-derived macrophage inflammatory protein-II

12.1 Introduction

For the past decade, it has become increasingly clear that ischemic stroke is not only a brain disease characterized by neuronal and glial cell death but is also marked by the dysfunction of multiple organs, including immune organs, as evidenced by systematic immune dysregulation before or after stroke [1–3]. Although great efforts have been afforded to investigate how stroke-evoked immune responses contribute to stroke pathology, it should be emphasized that immune activation prior to stroke also exerts a great impact on stroke incidence and outcomes [1]. As a heterogeneous and multifactorial disease, comorbidities, such as diabetes, hypertension, obesity, and atherosclerosis, are commonly present in stroke patients. Moreover, infections have also been reported as a triggering factor for cerebral ischemia [4]. Since all of these risk factors elevate pro-inflammatory profiles, pre-existing systemic immune activation is increasingly recognized as a customary characteristic of stroke pathophysiology. Paradoxically, pre-existing systemic immune activation also exerts beneficial effects on stroke outcomes, as evidenced by the mild systematic inflammation induced by preconditioning [5]. Thus, the impact of pre-existing systemic immune responses on stroke outcomes is complex and multifaceted.

The immune response evoked following ischemic stroke also displays multifaceted and multiphasic effects on stroke outcomes. Once cerebral ischemia occurs, the central nervous system (CNS) and the immune system impact each other specifically and profoundly. A reduction in blood supply to the brain depletes local energy and oxygen, ultimately leading to cell death within the affected areas. The dying neurons and other types of cells within the lesion secrete numerous mediators, including damage-associated molecular patterns (DAMPs) and brain antigens, which, in turn, initiate the activation of innate and adaptive immune cells both centrally and peripherally [2]. On the other hand, the injured brain also communicates with the peripheral immune system via specific neural circuits, such as the sympathetic and parasympathetic branches of the autonomous nervous system and the

hypothalamic–pituitary–adrenal (HPA) axis, which, in turn, profoundly modulates peripheral immune functions [6]. In contrast to the traditional view on the detrimental role of immune responses evoked by stroke, the postischemic, bidirectional communication between the CNS and the immune system presumably evolves to clear the brain of cellular debris. Moreover, emerging evidence suggests that poststroke immune responses also play pivotal roles in long-term recovery and repair following cerebral ischemia [7, 8]. Thus, cerebral ischemia induces immune activation to protect the brain from necrotic contents and to support brain remodeling and repair.

Unfortunately, in most cases, stroke induces over-activation of the immune system, exacerbating tissue damage and impeding brain repair. Not surprisingly, the research on immune responses following stroke has mainly focused on how secondary neuroinflammation impact the progression of brain infarcts for decades. However, it has been established that stroke-induced inflammation is one of the key pathological mechanisms that promotes cerebral infarct growth, and it can be targeted to confer neuroprotection to a large portion of stroke patients. As mentioned above, recent evidence is also accumulating to suggest an essential role of the poststroke immune response in long-term brain remodeling and neuroregeneration following stroke [7, 8]. Thus, harnessing the endogenous immune system to propel poststroke tissue repair and functional recovery holds promise as a novel therapeutic strategy for ischemic stroke [7].

Not only immune responses shape stroke outcomes. On the other hand, stroke also greatly impacts immune functions. Particularly, it has been shown that stroke not only triggers excessive neuroinflammation in the brain but also paradoxically results in peripheral immunosuppression [6, 9]. Poststroke immunosuppression has long been discovered both experimentally and clinically [10, 11]. It is increasingly clear that poststroke immunosuppression represents an independent risk factor for fatal secondary infection, which mainly occurs in the respiratory system and the unitary tract [12]. Recent studies begin to shed light on how stroke impacts immune function and suggest that targeting cerebral ischemia-induced immunosuppression is a promising strategy to treat ischemic stroke [10, 11, 13].

To conclude, the role of immune responses in stroke is multifaceted and complex, which leads to excessive inflammation and immunosuppression as well as neuroprotection and brain repair. Therefore, communication between the injured brain and the immune system can be targeted for developing effective immunomodulatory therapies to dampen the detrimental neuroimmune responses and enhance the beneficial neuroimmune responses. We discuss in the following sections (1) how pre-existing systematic immune activation influences stroke incidence and shapes stroke outcomes; (2) how immune responses are activated by cerebral ischemia and how stroke-evoked immune responses contribute to neuroinflammatory injury, as well as brain tissue repair; (3) how ischemic stroke impacts immune responses, with a focus on cerebral ischemia-induced peripheral immunosuppression; and (4), finally, the potential therapies that aimed at the immune response to treat ischemic stroke.

12.2 Impacts of Pre-existing Systemic Immune Activation on Stroke Incidence and Outcomes

12.2.1 Clinical and Experimental Evidence

With the prevalence of comorbidities, such as atherosclerosis, it is increasingly recognized that pre-existing systemic immune activation is a customary characteristic of stroke pathophysiology [1]. Thromboembolism resulting from atherosclerosis plaque rupture is a major cause of ischemic stroke. Inflammation plays a vital role in atherosclerosis evolution as well as in the transformation of benign plaques into unstable plaques [14]. Not surprisingly, atherosclerosis lesion development is significantly hampered in mice with deletion of the pro-inflammatory cytokines interleukin (IL)-1ß in bone marrow-derived cells [15]. Consistently, mice deficient in IL-1 receptor 1 also displayed reduced atherosclerosis when fed with high-fat diet [16]. Moreover, pharmacologically targeting the pro-inflammatory processes by neutralizing IL-1ß and tumor necrosis factor (TNF)-α or by antagonizing regulated on activation, normal T cell expressed and secreted (RANTES) also delays atherosclerosis progression. Since atherosclerosis is a major trigger for ischemic stroke logically the pre-existing inflammatory status will significantly modify stroke susceptibility by affecting atherosclerosis progression.

Diabetes, obesity, and hyperglycemia are also powerful, modifiable stroke risk factors, influencing both stroke incidence and outcomes. As shown by retrospective and perspective studies, stroke patients with diabetes displayed higher mortality, more severe neurological deficits, and delayed recovery from ischemic insults [17, 18]. Obesity is associated with higher stroke incidence and poorer prognosis following ischemic stroke [19]. Experimental studies show that elevated pro-inflammatory responses, as evidenced by enhanced production of pro-inflammatory cytokines, account for exacerbated infarct damage and edema in animal models of diabetes and obesity [18, 20].

The comorbidities not only enhance stroke incidence and exacerbate stroke outcomes individually but also display synergistic effects when they coexist. The synergistic effects of these comorbidities on stroke incidence are likely to be mediated by the shared inflammatory cascades. For instance, aged, diabetic, and hypertensive rats displayed increased mortality, more severe neurological deficits, and larger infarction than wild type [21]. Consistently, aged, corpulent rats have exaggerated inflammatory responses and spontaneously develop obesity, hyperlipidemia, insulin resistance, and atherosclerosis [22]. More relevantly, elevated neuroinflammation in the brain of corpulent rats can be paralleled with the positron emission tomography (PET) studies of patients with multiple risk factors [23]. Experimentally, peripheral administration of IL-1 receptor antagonist at reperfusion reduced infarct volumes and alleviated microglial activation and neutrophil infiltration in comorbidity animals [22].

In addition to comorbidities, infection also impacts stroke incidence and outcomes. While it is essential for the resolution of pathogenic insults, infection-induced inflammation contributes to stroke pathogenesis. It has been long recognized that infection may be an important risk factor for ischemic stroke [24]. According to epidemiology studies, up to one-third of ischemic strokes are preceded by infection. Two large clinical studies performed in the UK show that the risk of stroke peaks at 3 days after infection onset and that an increased stroke risk persistently remains for up to 3 months following infection [4, 25]. The stroke risk following infection is independent of other risk factors, such as age, other comorbidities, and fever [4]. Moreover, the increased stroke risk is most frequently associated with infection in the urinary and respiratory tracts. Both bacterial infection by *Streptococcus* and viral infection by influenza have been implicated to enhance stroke incidence [26]. Chronic infection has also been shown to elevate stroke risk [27]. Infection does not only increase stroke incidence; multiple clinical studies suggest that infection also exacerbates stroke outcomes, as evidenced by more severe neurological deficits in stroke patients with infection [28].

Compared to clinical evidence, experimental data on the link between infection and stroke incidence and outcome are relatively scarce. An experimental study suggests that stroke is not spontaneously induced in comorbid mice infected with *S. pneumoniae.* However, *S. pneumoniae* infection accelerates atherosclerosis formation in mice fed with high-fat diet and exacerbates brain injury in healthy animals [1]. A commonly used animal model of systematic inflammation and infection is the peripheral administration of lipopolysaccharide (LPS). It has been shown that peripherally administered LPS exacerbates brain damage and neurological deficits following experimental stroke by enhancing the systematic levels of IL-1 and promoting neutrophil infiltration into the ischemic brain [29]. Moreover, infection with human influenza increases brain infarcts in mice subject to experimental stroke; this is likely to be attributed to enhanced neutrophil infiltration into the brain parenchyma and exacerbated disruption of the blood–brain barrier [30]. Mice with a chronic infection of *Trichuris*, a model of gut infection, display chronic Th1-polarized immune responses and more severe ischemic brain injury and neurological deficits following experimental stroke. In contrast, infarct damage is not enlarged in mice that develop a T helper (Th)-2-polarized immune response [31]. Taken together, experimental data strongly implicate that it is not the presence of infectious pathogens themselves, but the activation of a systematic immune response induced by infection that contributes to stroke pathogenesis.

Collectively, experimental and clinical evidence exists that the well-established comorbidities for stroke and infections are associated with elevated systematic inflammation, which likely represents a common mechanism underlying the detrimental effects of comorbidity and infections on stroke incidence and outcomes. Targeting the pre-existing systematic pro-inflammatory pathways promises potential therapies to dampen stroke incidence and protect the brain from cerebral ischemia.

12.2.2 Mechanisms Underlying the Influence of Pre-existing Inflammation on Stroke Incidence and Outcomes

12.2.2.1 Activation and Mobilization of Leukocytes

Abundant literature suggests that comorbidity-induced activation of leukocytes prior to cerebral ischemia likely represents an important mechanism through which comorbidities increase stroke incidence and exacerbate stroke outcomes. High levels of leukocyte infiltration prior to ischemia result in the instability of atherosclerosis plaques, thus increasing stroke incidence [32]. Leukocyte mobilization and infiltration into the brain parenchyma can be detected in atherosclerotic mice with comorbidities prior to ischemic stroke [23]. Consistently, diabetic and obese Zucker rats display remarkably larger infarct volumes, elevated edema, and worsened neurological functions, which correlate with a twofold increase in the number and activation of peripheral leukocytes before stroke [33]. Clinically, it has been observed that increased stroke susceptibility is associated with a polymorphism in the regulatory region of the monocyte chemoattractant protein-1 (MCP-1) gene, an essential mediator implicated in orchestrating cell infiltration into the perivascular space [34]. Collectively, available studies indicate that the pre-existing leukocyte activation and mobilization induced by comorbidities enhances stroke incidence and exacerbates ischemic damage.

12.2.2.2 Glial Priming

Pre-existing systematic inflammation and infection have been shown to enhance stroke susceptibility and damage by priming brain-resident glial cells, including astrocytes and microglia. For instance, pre-existing systematic inflammation induced by the peripheral administration of IL-1 prior to ischemic stroke results in brain pro-inflammatory response and exacerbates infarct damage, mortality, and edema [35]. Chronic systematic inflammation in atherosclerotic mice as well as in atherosclerotic, obese, and corpulent rats also leads to elevated cerebral inflammation, which is associated with microglial activation in the brain prior to ischemic stroke. These experimental data are consistent with the clinical observation that neuroinflammation is elevated in patients at risk of stroke using PET [23]. In addition, astrocyte priming also contributes to stroke pathogenesis. In salt-sensitive, hypertensive rats, neuronal death is enhanced due to metabolism and the neurotropic dysfunction of astrocytes following hypoxia and reoxygenation [36]. Collectively, there is evidence suggesting that glial priming is a mechanism by which comorbidities or infection contributes to enhanced neurological disorder following stroke.

12.2.2.3 Vascular Inflammation and Damage

Pre-existing risk factors, including infection and chronic inflammation induced by comorbidities, are able to alter vascular structure and reactivity [37]. Vascular inflammation can lead to the upregulation of adhesion molecules, such as intercellular adhesion molecule (ICAM) and vascular adhesion molecule (VCAM). Clinically, patients with acute hypertension display elevated levels of circulating ICAM, VCAM, and E-selectins [38]. Elevated adhesion molecules increase the migration of leukocytes and initiate atherosclerosis, thus enhancing stroke incidence [39, 40]. Experimentally, enhanced levels of adhesion molecules have also been observed in the aortic wall of rats fed with cholesterol-rich diet [41]. Adhesion molecules contribute to atherosclerosis development. For instance, lymphocyte infiltration into the atherosclerosis aorta is at least partially dependent on L-selectin [42]. Pre-existing inflammation affects not only peripheral atherosclerosis development but also atherosclerosis development in the cerebral vascular system. For instance, in apolipoprotein E (ApoE) $^{-/-}$ mice, an atherogenic diet also enhances leukocyte infiltration into the choroid plexus prior to brain injury [23]. Moreover, the pro-inflammatory cytokine IL-1 has been shown to be essential for cerebrovascular and microglial inflammation and leukocyte infiltration into the brain in ApoE $^{-/-}$ mice fed with high-fat diet [16]. Collectively, these studies suggest that the pre-existing risk factors for stroke enhance atherosclerosis development and stroke incidence via vascular inflammation.

Inflammation induced by pre-existing comorbidities may also increase stroke incidence and damage by affecting cerebrovascular integrity. Obesity is a well-known risk factor for stroke [43]. The elevated susceptibility observed in obese patients has been suggested to be linked with microvascular damage induced by chronic inflammation [44]. Following cerebral ischemia, obese mice are more prone to hemorrhagic transformation, which is also associated with enhanced activity of microvascular metalloproteinase (MMP)-9 and blood–brain barrier disruption [45]. Moreover, the exacerbating effects of preceding influenza infection on stroke outcomes are dependent on MMP-9-induced blood–brain barrier (BBB) disruption [30]. The BBB disruption in patients recently recovering from infection exerts multiple detrimental effects on stroke outcomes, including enhanced leukocyte infiltration and the intravascular accumulation of platelets. The contributing role of adherent leukocytes and platelets in stroke pathogenesis has been confirmed by a recent study using obese mice [46].

12.2.3 Protection Conferred by Preconditioning-Induced Mild Immune Activation

Although there is ample evidence that pre-existing inflammation enhances stroke incidence and exacerbates stroke outcomes, immune activation prior to stroke has also been shown to be protective, as reported by numerous studies on

preconditioning. For instance, preconditioning with toll-like receptor ligands, such as bacterial endotoxin LPS and unmethylated CpG oligodeoxynucleotide, induces mild immune activation and confers protection against ischemic brain damage via interferon regulatory factor-dependent signaling [47, 48]. Furthermore, preconditioning with LPS has also been suggested to confer protection against cerebral ischemia by stimulating the production of anti-inflammatory cytokines and decoys [49, 50]. Interestingly, the inflammatory cytokines that exert detrimental effects on stroke outcomes, such as IL-6 and TNF-α, have been paradoxically implicated to be essential for the neuroprotection conferred by LPS preconditioning [5, 51]. Preconditioning with LPS also reduces infarction and improves cerebral blood flow through the mechanisms of neurovascular coupling and endothelium-mediated vasodilation [52]. Moreover, preconditioning induced by sublethal transient ischemia has also been shown to act through the inflammatory mechanism to render the brain resistant to ischemic damage. For instance, ischemic preconditioning improves the postischemic functions of capillaries, which is attributed to the reduction in postischemic interactions between leukocytes and endothelial cells [53]. Remote limb ischemia also preconditions the brain against ischemic damage, and the underlying mechanisms involve inhibition of the inflammatory galectin-9/Tim-3 pathway [54]. In conclusion, the preconditioning conferred by multiple approaches, especially using toll-like receptor (TLR) ligands, induces immune activation prior to stroke and confers neuroprotection against ischemic brain damage, which contrasts with the exacerbating effects of pre-existing immune activation that is induced by comorbidities and infection. The striking difference may be attributed to time-dependent effects and the extent of immune activation.

12.3 Ischemia-Induced Immune Responses Contribute to Stroke Pathology and Tissue Repair

12.3.1 Initiation of Immune Responses Following Ischemic Stroke

Traditionally, it is thought that the brain is immune privileged and insulated from the immune system due to the BBB. However, a wide number of studies have confirmed that the brain and the immune system communicate under pathological conditions, including stroke. It is becoming increasingly clear that ischemia-induced immune responses not only contribute to ischemic brain damage but they also mediate tissue repair following cerebral ischemia. Stroke reduces cerebral blood flow and results in a localized depletion of energy and oxygen, ultimately leading to cell death in the lesion areas. The dying cells secrete danger signals, such as damage-associated molecular patterns (DAMPs) and brain-derived antigens, to activate the immune system [2]. In contrast to pathogen-associated molecular patterns (PAMPs) that initiate infectious inflammatory responses, DAMPs initiate responses to tissue

damage. In addition, some brain-specific antigens, such as myelin basic protein (MBP), have also been implicated to induce adaptive immune response following cerebral ischemia [55].

12.3.1.1 Innate Immune Responses Triggered by DAMPs

There are variety of DAMPs, including molecular determinants derived from cell debris, nuclear DNA/RNA, and cellular proteins/enzymes released from dying cells [56]. Some DAMPs, such as high-mobility group box-1 (HMGB1), ATP, and S100, have been extensively investigated as the critical initiators of postischemic immune responses. HMGB1, a nuclear and DNA-binding protein, is widely expressed by neurons and oligodendrocytes in the brain. In addition to regulating gene transcription, HMGB1 functions as a DAMP to activate microglia/macrophages when released from injured cells following ischemic stroke [57]. In vivo and in vitro evidence suggest that injured neurons rapidly secrete HMGB1 following ischemia or oxygen–glucose deprivation [58, 59]. Extracellular HMGB1 binds to several receptors expressed on microglia/macrophages, including toll-like receptors (TLRs) and the receptor for advanced glycation end products (RAGE). It has been shown that RAGE expression by glial cells is indispensable for the neurotoxicity of HMGB1 in neuron–glia cocultures [57]. Poststroke cerebral inflammation and damage are reduced in chimeric mice that were generated by transplanting the bone marrow cells derived from RAGE $^{-/-}$ mice into wild-type mice, indicating that RAGE expression by infiltrated macrophages plays a critical role in mediating neuroinflammation following cerebral ischemia [57]. In addition, the TLR4 expressed by infiltrating macrophages also remarkably contributes to infarction development, implicating that HMGB1 and TLRs may link neuron necrosis to immune responses after stroke [60]. Consistently, blocking HMGB1 effects with either small interfering RNA (siRNA) or antibodies suppresses cerebral inflammation and protects the brain following stroke, providing further evidence that HMGB1 is an important mediator of neuron–glia crosstalk and the detrimental inflammation induced following cerebral ischemia [59]. Although HMGB1 has been extensively investigated as an essential mediator of neuroinflammation that exacerbates acute infarct damage, emerging evidence also suggests that HMGB1 may be an essential mediator of poststroke recovery and tissue repair.

Following cerebral ischemia, neurons undergo energy failure, leading to an intracellular depletion of ATP and an outflow of ATP into the intercellular space. In a rat model of cerebral ischemia, extracellular ATP levels remain elevated in the striatum for 220 min following ischemia [61]. In addition to neurons, vascular cells and blood cells may also serve as important cellular sources for ATP release [62]. ATP acts on purinergic receptors, such as P2X7 and P2X4, to activate immune cells. Following cerebral ischemia, microglial expression of P2X7 is upregulated, presumably leading to enhanced microglial activation [63]. P2X7 also acts on pannexin to activate neuron and astrocyte-mediated inflammation [64]. In a rat model of preterm hypoxia–ischemia, enhanced P2X4 expression is associated with elevated

microglial activation marker Iba1 [65]. P2X4 expression is also elevated in the hippocampus of gerbils subject to bilateral common carotid artery occlusion [66]. In support of the notion that ATP released from injured cells activates neuroinflammation via purinergic receptors, P2X7 antagonists reduce cerebral inflammation and confer protection following transient global cerebral ischemia [67]. Minocycline, the potent inhibitor of microglial activation, reduces P2X4 expression following hypoxia–ischemia [65]. Moreover, a selective P2X1-7 inhibitor, 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate, suppresses cerebral ischemia-induced expression of IL-1β and TNF-α in the brain [68]. Conversely, systematic administration of ATP exacerbates stroke outcomes [69]. Taken together, these results indicate that the extracellular ATP released from dying cells acts as a DAMP to activate neuroinflammation following cerebral ischemia.

S100, a protein expressed in astrocytes, is another DAMP released from the injured brain following ischemic stroke. In acute stroke interventional trials, S100 has been used as a surrogate marker for long-term outcomes and infarct volumes [70] to predict functional outcomes and poststroke depression [71]. Experimentally, transgenic mice overexpressing human S100 display an increase in gliosis and brain infarct sizes following permanent cerebral ischemia [72]. RAGE is essential for the inflammatory cascades induced by S100. S100-stimulated microglial production of the inflammatory mediator nitric oxide is dependent on RAGE [73]. Moreover, S100-engaged RAGE enhances the expression of IL-1ß and TNF-α, which is accompanied by the increased transcriptional activity of NF-κB and AP-1 [74]. The engagement of S100 with RAGE is also important for microglia migration and microglial production of chemokines and chemokine receptors [75].

In summary, the injured brain immediately releases numerous DAMPs following cerebral ischemia. DAMPs serve as early alarms that send tissue damage signals from the brain to the immune system and trigger postischemic immune responses. There is ample evidence showing that DAMPs act as the mediators of neuroinflammation by exacerbating acute infarct damage. However, the effects of these DAMPs seem to be biphasic since emerging evidence also suggests that DAMPs may also beneficially contribute to poststroke recovery and tissue repair [76].

12.3.1.2 TLRs and NLRs as the Receptors Detecting DAMPs and Activating Innate Immune Responses

Toll-like receptors (TLR) are transmembrane proteins that contain a cytoplasmic toll/interleukin 1 domain responsible for transmitting extracellular signals into cells. TLRs are evolutionarily conserved from plants to humans. Current studies implicate that a role of TLRs is recognizing DAMPs and activating innate immune responses following cerebral ischemia. HMGB1 is an essential DAMP that contributes to poststroke neuroinflammation. TLR2 and TLR4 have been identified to mediate the inflammatory effects of HMGB1 [77]. TLRs have been shown to be expressed on brain cells. Particularly, TLR2 and TLR4 are upregulated in response to energy deprivation [78]. TLRs have been shown to play a detrimental role

following cerebral ischemia, while deletion or inhibition of TLRs confers neuroprotection against cerebral ischemic injury [78, 79]. Clinically, gene expression array research on human peripheral blood monocytes has shown that the TLR signaling cascade is one of the significantly differentially expressed cascades in patients suffering acute cerebral ischemia [80]. Moreover, TLR4 displays upregulated expression on peripheral blood monocytes that correlates with stroke severity in patients with acute ischemic stroke [81].

In addition to TLRs, intracellular pattern recognition receptors can also activate innate immune responses by detecting DAMPs following cerebral ischemia. One class of these receptors is NOD-like receptors (NLRs) that contain NACHT, PYRIN, leucine-rich repeats, and caspase activation and recruitment domains (CARDs). NLRs can detect their ligands, such as ATP, resulting in inflammasome activation. The NLR family pyrin domain containing 1 (NLRP1) gene, a member of NLR family, is predominantly expressed in the brain, and inhibition of NLRP1 has been shown to reduce inflammation following cerebral ischemia [82]. Moreover, NLRP3/NALP3 has been shown to mediate inflammatory responses in mice following experimental stroke [83]. Recently, it has been proposed that TLR-mediated signaling and inflammasome activation coordinate to induce neuroinflammation following cerebral ischemia. In the model, the first step is the induction of innate immune responses via TLR detection of DAMPs released from dying cells, which is followed by inflammasome activation by ATP or other endogenous ligands. The first step leads to the expression of immature pro-form IL-1ß that is then processed into mature IL-1ß by activated caspases following inflammasome activation [84].

12.3.1.3 Adaptive Immune Responses Induced by Brain-Specific Antigens

Following ischemic stroke, the interaction between CNS antigens with immune cells occurs both peripherally and in the ischemic brain. Cerebral ischemia leads to BBB disruption, which allows brain-specific antigens to encounter and activate the peripheral immune system. Brain-specific antigens, such as microtubule-associated protein 2 (MAP 2), N-methyl-D-aspartic acid receptor subunit NR-2A, and myelin-derived antigens, including myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP), can be detected in the tonsil and cervical lymph nodes of stroke patients [55]. Moreover, ischemia-induced activation of the adaptive immune response can also be clinically detected in stroke patients. For instance, elevated titers of circulating antibodies against neurofilaments and N-methyl-D-aspartic acid receptors are observed in patients with stroke history [85, 86]. In addition, the brain antigens processed by the antigen processed cells (APC), such as macrophages, induce autoreactive T cell responses. For instance, the number of cluster of differentiation (CD) 69 + T cells is increased in stroke patients [55]. Taken together, in addition to DAMPs, which mainly induce innate immune responses, brain antigens released from the ischemic brain induce adaptive immune responses following cerebral ischemia.

12.3.2 Cytokines, Chemokines, and Adhesion Molecules That Contribute to the Postischemic Immune Response

12.3.2.1 Cytokines

Following cerebral ischemia, microglia are the first cells to sense signals from injured neurons and other cells in the ischemic brain. Soluble DAMPs, such as purine nucleotides, are able to trigger the rapid release of pro-inflammatory cytokines from the classically activated microglia, also termed M1-polarized microglia, following cerebral ischemia [87]. IL-1 is one of the pro-inflammatory cytokines that contributes to postischemic inflammation. The pro-IL-1ß that is secreted by microglia has to be cleaved by matrix metalloproteinases or caspases to exert pro-inflammatory effects. There is controversy about the relative importance of IL-1ß vs. IL-1α in poststroke neuroinflammation. It has been shown that IL-1ß, but not IL-1α, is rapidly induced, and this induction remains in the ischemic brain for 16–24 h after ischemia [88]. Recent studies suggest that IL-1α (rather than IL-1ß) is rapidly induced following stroke and may be the major IL-1 contributing to poststroke neuroinflammation [89]. Results obtained from gene knockout mice show that the single gene deletion of IL-1α or IL-1ß has no effect on brain infarction, while the double deletion of IL-1α and IL-1ß remarkably reduces infarct volumes following cerebral ischemia [90]. These results suggest that IL-1s are important pro-inflammatory cytokines, contributing to stroke pathogenesis, and that IL-1α and IL-1ß may synergistically affect poststroke inflammation. In addition to acting as a pro-inflammatory mediator, IL-1ß also induces the expression of pro-inflammatory IL-6 and TNF-α, which are secreted by active microglia into the circulation. Elevated levels of IL-1ß, IL-6, and TNF-α can activate peripheral immune cells and, consequently, trigger inflammatory cascades. Clinically, elevated levels of IL-1ß and TNF-α have been detected in the blood of stroke patients [91]. Moreover, plasma concentrations of IL-6 are positively correlated with infarct sizes, neurological deficits, and mortality following stroke.

12.3.2.2 Chemokines

Chemokines are small chemotactic molecules that recruit immune cells to the site of inflammation. Based on the first cysteines at the N-terminal, chemokines are divided into four groups (CC, CXC, XC, and CX3C, with C representing cysteine). Following cerebral ischemia, injured CNS cells secrete lots of chemokines, including macrophage inflammatory protein-α (CCL3), monocyte chemotactic protein-1 (CCL2), and chemokine (C-C motif) ligand 5 (CCL5). In addition, immune cells are also cellular sources of chemokines. For example, CCL5 is secreted by T helper cells type I in chronic systematic infection and is deposited on cerebral vascular structures, which exacerbates stroke outcomes by recruiting lymphocytes [31]. In different animal models, chemokines have different cellular sources. For instance,

CCL2 is predominantly secreted from astrocytes in adult animals following cerebral ischemia, while it is mainly produced by injured neurons following neonatal hypoxia/ischemia [92].

CCL2/C-C chemokine receptor (CCR) type 2 interaction is important for the recruitment of monocytes and macrophages following cerebral ischemia. Clinically, CCL2 levels are increased in the cerebrospinal fluid and serum during the early hours after ischemic stroke [93]. In experimental models, CCL2 mRNA is also rapidly enhanced in the ischemic cortex during the early phase, peaking at 2 days and declining at 5 days after cerebral ischemia [94]. The time profile of poststroke induction of CCL2 suggests that the CCL2/CCR2 axis is essential for macrophage infiltration following ischemic stroke. In support of this notion, CCR2-deleted mice display smaller infarction and reduced infiltration of immune cells, while CCL2 overexpression is associated with enlarged infarction [95, 96].

By acting as a pro-inflammatory chemokine through its interactions with CCR1 or CCR5, CCL3 plays an essential role in monocyte accumulation and microglial activation in the ischemic brain. In addition, CCL3 is a potent mediator of neutrophil infiltration in mice and humans. CCL3 induction has been detected in neonatal animals following HI and in adult animals following cerebral ischemia [97, 98]. Intracerebral administration of CCL3 enlarges infarction, whereas administration of the broad spectrum chemokine antagonist vMIP-II, a virus-derived macrophage inflammatory protein, reduces infarct damage in a dose-dependent manner [99]. However, it is currently unclear how CCL3 exacerbates stroke outcomes.

CCL5 is a potent chemokine involved in ischemic brain injury, and its pro-inflammatory effects following stroke are likely mediated by leukocyte recruitment and platelet adhesion to the cerebral vasculature. CCL5 can bind to CCR1, CCR3, and CCR5. CCL5 knockout mice display reduced infarction and improved BBB integrity following cerebral ischemia. Consistently, transplanting bone marrow obtained from CCL5 knockout mice into wild-type mice reduces infarct damage [100]. During systematic inflammation, CCL5 is released from peripheral leukocytes, and it may further exacerbate BBB disruption and injury following ischemic stroke [93].

Other chemokines and their respective receptors have also been implicated in inflammatory injury following cerebral ischemia. For instance, C-X-C motif chemokine (CXCL) 12 is rapidly induced and remains elevated in ischemic penumbra following cerebral ischemia, particularly in association with astrocytes in the perivascular areas. Bone marrow cells can be recruited to the CXCL12 positive vessels, and they exhibit the characteristics of active microglia [101]. In vitro studies suggest that CCR4 expression is enhanced by hypoxia that is dependent on hypoxia inducible factors (HIFs), resulting in microglial accumulation toward CXCL12 [102]. Thus, CXCL12 likely contributes to the recruitment of monocyte-derived and local microglia following cerebral ischemia. C-X3-C motif chemokine receptor 1 (CX3CR1) knockout mice exhibit reduced neuroinflammation and neural damage following cerebral ischemia [103]. CXCL8 is an important chemotactant for neutrophils. The CXCL8 receptor antagonist reduces neuroinflammation and neurological

deficits [104]. These results suggest that multiple chemokines propel inflammatory cascades via the recruitment of peripheral and local immune cells.

12.3.2.3 Adhesion Molecules: Selectins, Immunoglobulin Superfamily, and Integrins

Following cerebral ischemia, cerebrovascular endothelial cells upregulate the expression of adhesion molecules on the cell surface to promote the recruitment of leukocytes and platelets into the ischemic areas. P-selectin, E-selectin, the members of immunoglobulin superfamily, and integrins have been implicated in the mediation of adhesion and the trans-endothelial migration of leukocytes, and they therefore contribute to inflammatory cascades in the ischemic brain.

12.3.2.3.1 Selectins

Selectins, which are type I transmembrane glycoproteins, initiate the recruitment, homing, margination, rolling, and low-affinity binding of leukocytes to capillary endothelium following ischemic stroke. P-selectin is stored in Weibel–Palade bodies in endothelial cells and in alpha granules in resting platelets. Thus, following experimental stroke, P-selectin is released and very rapidly presents on the cell surface [105]. Clinically, elevated levels of P-selectin have been correlated with stroke severity [106]. The deletion of P-selectin protects the brain against cerebral ischemic injury and reduces mortality, which is associated with decreased leukocyte accumulation in the ischemic brain [107]. Moreover, a recent study shows that the deletion of P-selectin results in reduced BBB disruption and leukocyte recruitment, although infarct damage is not decreased in P-selectin knockout mice [108]. Following focal cerebral ischemia, inhibiting P-selectin with antibodies decreases infarct damage, reduces leukocyte and platelet recruitment, and ameliorates the "no-flow" phenomenon following reperfusion [107, 109]. However, the acute inhibition of P-selectin with antibodies increases the mortality of animals subjected to global ischemia [110]. The discrepancy may be attributed to the different roles that P-selecting plays in focal vs. global ischemia or to differences in the functional inhibition of P-selectin prior to vs. following cerebral ischemia.

E-selectin is only expressed by activated endothelium, and it binds to the receptors on leukocytes. Since E-selectin is synthesized de novo, it is sequentially expressed on the surface of endothelial cells after P-selectin following cerebral ischemia. E-selectin is upregulated within a few to several hours following experimental focal cerebral ischemia. However, the upregulation of E-selectin is not detected in the serum of stroke patients [111]. In experimental stroke, transnasal administration of E-selectin prior to cerebral ischemia confers tolerance to subsequent ischemia damage [112]. L-selectin is expressed by both activated endothelium and leukocytes, and it plays a role in neutrophil margination and rolling along the activated endothelium. However, the acute inhibition of L-selectin with antibodies does not

protect the brain from experimental stroke in rabbits [113]. Moreover, L-selectin levels were not found to be elevated following human stroke [114].

12.3.2.3.2 Immunoglobulin Superfamily

Intercellular cell adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin superfamily and binds to lymphocyte function-associated antigen-s (LFA-1) on the surface of polymorphonuclear leukocytes. Compared to selectins, ICAM-1 mediates stronger binding of leukocytes to the activated endothelium following cerebral ischemia. ICAM-1 is constitutively expressed on endothelium. ICAM-1 is remarkably upregulated following cerebral ischemia [115]. ICAM-1 deletion or the acute inhibition of ICAM-1 with antibodies confers protection following experimental stroke [116, 117]. Clinically, serum levels of soluble ICAM-1 are acutely elevated in stroke patients [118]. Postmortem research also shows that stroke patients display increased expression of ICAM-1 in ischemic brain tissue [119]. Despite these studies, blocking ICAM-1 with antibodies fails to protect patients from ischemic stroke in clinical trials [120]. Vascular cell adhesion molecule-1 (VACM-1), another member of the immunoglobulin superfamily, binds to very late antigen-4. VCAM-1 expression is increased following focal cerebral ischemia in rats [121]. However, acute inhibition of VCAM-1 with antibodies also fails to confer brain protection in a model of ischemia/reperfusion [122]. Collectively, the current results suggest that the role of the members of immunoglobulin superfamily, such as ICAM-1 and VCAM-1, is complex in ischemic stroke. An incomplete understanding of the possible plurality of the role of these adhesion molecules may account for inconsistencies in the abovementioned results.

12.3.2.3.3 Integrins

Integrins are transmembrane glycoproteins that mediate the interaction between cells and extracellular matrix as well as cell-to-cell interactions. Integrins consist of α- and ß-subunits, and they mediate the strong adherence of leukocytes to the endothelium. Integrins that are expressed by leukocytes have a common ß-chain (CD18) and different α-chains, such as 11a, 11b, and 11c. CD18/CD11a (LFA-1) and CD18/CD11b (macrophage-1 antigen, Mac-1) are the most extensively studied integrins in ischemic stroke. LFA-1 is expressed on the surface of all leukocytes and mediates the high-affinity binding of leukocytes to vascular endothelium by binding to endothelial ICAM-1 or ICAM-2. Mac-1 is expressed and stored in the granules of neutrophils, monocytes, and natural killer cells. The expression of CD11a and CD18 on peripheral leukocytes is upregulated in stroke patients [123]. A chronic deficiency of LFA-1 or Mac-1 and the acute inhibition of Mac-1 with antibodies administered following cerebral ischemia reduce infarction and the accumulation of polymorphonuclear cells [124, 125]. Taken together, adhesion molecules play important roles in poststroke inflammatory cascades. However, further investigation of the mode and

timing of the functional roles of these adhesion molecules is needed in order to translate the experimental results into clinical practice.

12.3.3 Impact of Ischemia-Induced Immune Responses on Stroke Outcomes

Following cerebral ischemia, the injured brain sends tissue damage signals to elicit immune responses. It has been well-established that ischemia-evoked immune responses exert remarkable and profound effects on stroke outcomes, including the progression of infarction, tissue repair, and functional recovery. In the following section, we will discuss how ischemia-induced innate and adaptive immune responses influence stroke pathogenesis, with an emphasis on the effects of different immune cells on stroke outcomes.

12.3.3.1 Innate Immune Cells That Contribute to Stroke Pathogenesis

12.3.3.1.1 Microglia/Macrophages

Brain-resident microglia and peripherally infiltrated macrophages are the important innate inflammatory cells that rapidly respond to ischemic brain injury. Microglia normally assume a ramified morphology, but they assume an amoeboid morphology after activation. Microglial activation is rapidly apparent within a few minutes, peaks at 48–72 h, and persists for several weeks following cerebral ischemia. The expression of microglial surface markers, such as Iba-1, is enhanced following activation. However, microglia are indistinguishable from the infiltrating macrophage in terms of morphology and surface markers following cerebral ischemia. Using chimeric mice generated by transplanting GFP+ bone marrow into wild-type mice and subjecting them to irradiation and an in vivo imaging technique, recent studies suggest that microglial activation precedes the presence of activated macrophages in the ischemic brain [126, 127]. Activated macrophages are present in the ischemic brain 3–7 days following transient cerebral ischemia and decline thereafter [128]. In terms of distribution, it is found that brain-derived microglia are the major phagocytes located in the infarct's core area, while blood-derived macrophages are mainly located in the penumbral area [127].

Following cerebral ischemia, two distinctly polarized populations of microglia/macrophages have been reported: "classically activated" M1 microglia/macrophages and "alternatively activated" M2 microglia/macrophages [129]. M1 microglia/macrophages typically release pro-inflammatory mediators and cytokines, such as IL-1, TNF-α, and MMP-9, while M2-polarized microglia/macrophages produce anti-inflammatory/trophic factors and clear cell debris [129]. Cerebral ischemia induces the transient M2 polarization of microglia/macrophages, followed by the

persistent M1 polarization of microglia/macrophages in the ischemic brain [129]. In vitro studies show that the conditioned medium collected from neurons treated with oxygen–glucose deprivation (OGD) primes microglia toward M1 polarization. Moreover, M1-polarized microglia exacerbate the neuronal death induced by OGD. Conversely, M2-polarized microglia protect neurons against OGD. These results suggest that persistent M1 polarization of microglia/macrophages in the ischemic brain likely represents an important inflammatory mechanism via which the innate microglia/macrophages exacerbate infarct damage and impair poststroke tissue repair and brain remodeling [129]. Thus, stroke therapies that target postischemic immune responses mediated by microglia/microphages should focus on adjusting the polarization of microglia/microphages rather than broadly suppressing their functions. Indeed, experimental data are available to suggest that enhancing the M2 polarization of microglia/macrophage improves outcomes following cerebral ischemia [130–132].

12.3.3.1.2 Neutrophils

Both experimental and clinical studies show that neutrophils are among the first peripheral immune cells that infiltrate into the ischemic brain following stroke. In animal models, infiltration of neutrophils into the injured brain is observed within few hours after ischemia and peaks at 1–3 days after cerebral ischemia [133], which is followed by the infiltration of peripheral macrophages. Clinically, neutrophils are recruited into the brain within 24 h after stroke onset [134]. Postischemic endothelial expression of ICAM-1 and P-selectin seems to be essential for the transendothelial migration of neutrophils into the injured brain following cerebral ischemia. Mice deficient in ICAM-1 or P-selectin display reduced neutrophil infiltration as well as smaller infarct sizes [107, 117]. Consistently, clinical evidence suggests that neutrophil accumulation in the parenchyma correlates with infarction expansion and poor outcomes following human stroke [134, 135]. Deletion of neutrophils with anti-neutrophil monoclonal antibodies remarkably reduces infarction volumes and edema following experimental stroke. Taken together, these results suggest that the postischemic infiltration of neutrophils into the brain exacerbates stroke outcomes. Mechanistically, it has been implied that neutrophils exert deleterious effects on stroke outcomes probably via releasing the toxic mediator nitric oxide [2]. Nitric oxide contributes to stroke pathogenesis by activating MMP-9, which, consequently, leads to the disruption of BBB. Although neutrophils are traditionally recognized as the major pro-inflammatory cells, recent studies show that neutrophils can also be shifted to the N2 phenotype, which is similar to M2 microglia/macrophages. In an experimental stroke model, N2 polarization and the accumulation of neutrophil in the ischemic core induced by rosiglitazone, an activator of peroxisome proliferator-activated receptor-γ, confer neuroprotection against brain ischemic injury [136]. Thus, stroke therapies that target postischemic immune responses mediated by neutrophils should also focus on adjusting polarization rather than broadly suppressing the functions of neutrophils.

12.3.3.1.3 Dendritic Cells (DCs)

Among the cellular members of the innate immune system, DCs are phagocytes that quickly respond to injury. As phagocytic cells, DCs take up antigens and subsequently present the antigens to T cells. DCs are essential for the induction of adaptive immune responses against antigens that have not been previously encountered by the immune systems, such as CNS antigens. Thus, DCs link innate and adaptive immune responses. The number of DCs in the brain is increased in animals following both permanent focal cerebral ischemia and cerebral ischemia/reperfusion [137, 138]. The increase in DC infiltration is apparent at 1 day after reperfusion and is followed by a 20-fold increase at 3 days and 12-fold increase at 7 days after reperfusion [138]. Radiation chimeric mice have been used to differentiate infiltrating DCs from brain-resident DCs. Using these mice, it is found that DC infiltration starts at 1 day after experimental stroke. Moreover, the study suggests that brain-resident DCs play an essential role in mediating poststroke neuroinflammation [139]. However, the roles of DCs in postischemic innate immune responses as well as adaptive immune responses have not been sufficiently investigated and remain an intriguing area for stroke research.

12.3.3.2 Adaptive Immune Cells That Contribute to Stroke Pathogenesis

12.3.3.2.1 T Lymphocytes

T lymphocytes are an important cellular component of adaptive immunity. Emerging evidence suggests that T lymphocytes contribute to postischemic neuroinflammation and ischemic brain damage. Previous studies show that T lymphocyte infiltration into the injured brain follows macrophage and neutrophil infiltration, which occurs at 3–4 days following stroke [140]. However, new studies suggest that the infiltration of T lymphocytes may occur within 24 h after cerebral ischemia [141]. Blocking T lymphocyte infiltration into the ischemic brain with anti-α4-integrin antibodies decreases infarct sizes [142]. Mice deficient in CCL5, a potent mediator of T lymphocyte recruitment and activation, also display reduced infarct damage [142]. Moreover, reduced infarct damage and improved neurological outcomes are observed in severe combined immunodeficiency mice (SCID) and recombination-activating gene 1 (Rag1) −/− mice that lack functional T and B lymphocytes [143, 144]. Conversely, transplanting wild-type T lymphocytes into Rag −/− mice almost completely abolishes the beneficial effects of the $Rag1^{-/-}$ deletion on stroke outcomes [144]. The study suggests that T lymphocytes essentially contribute to stroke pathogenesis.

Based on the expression profiles of cellular markers, T lymphocytes can be categorized into several subtypes, including $CD3^+$ T lymphocytes that consist of $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells. In addition, regulatory T cells (Tregs) are identified based on their expression of CD25 and the transcriptional factor FoxP3. Accumulating evidence suggests that different subtypes of T lymphocytes exert dif-

ferential effects on stroke outcomes. Notably, not all subtypes of T lymphocytes exacerbate stroke outcomes. $CD8^+$ cytotoxic T cells induce the death of pathogen-infected cells and cancer cells via the release of cytotoxins or via cell–cell communication. $CD8^+$ cytotoxic T cells also produce pro-inflammatory cytokines to promote inflammatory cascades. $CD4^+$ helper T cells help to activate other immune cells, such as $CD8^+$ cytotoxic T cells. Following cerebral ischemia, both $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells are recruited to the ischemic brain [138]. Selective deletion of $CD4^+$ helper T cells or $CD8^+$ cytotoxic T cells with antibodies significantly decreases infarct damage following permanent cerebral ischemia [145]. Consistently, infarction sizes are reduced in mice lacking $CD4^+$ helper T cells or in $CD8^+$ cytotoxic T cells vs. wild-type mice [146]. To conclude, these results support that $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells contribute to the progression of brain injury following cerebral ischemia.

Tregs are a subtype of $CD4^+$ helper T cells, accounting for 10 % of $CD4^+$ helper T cells. Tregs exert immunosuppressive effects via multiple mechanisms, including the secretion of anti-inflammatory cytokines, such as IL-10, and the expression of inhibitory molecules, such as CTLA-4, on the cell surface. A recent study shows that experimental cerebral ischemia enhances bone marrow regulatory T cells in mice, and CXCR4-/stromal cell-derived factor 1 (SDF1) axis facilitates the mobilization of Tregs into the peripheral blood [147]. Boosting the number or function of Tregs with a CD28 super agonist or a histone deacetylase (HDAC) inhibitor [148] reduces brain damage after ischemic stroke in mice [149]. Moreover, transplantation of Tregs after experimental stroke remarkably reduces brain damage and improves long-term neurological functions [150]. The depletion of Tregs profoundly increased delayed brain damage and deteriorated functional outcomes [151].

The studies on the protection conferred by tolerance to MBP or E-selection provide more evidence suggesting that Tregs play a protective role following cerebral ischemia. MBP-tolerized mice have better performance on behavioral tests and are more likely to develop a regulatory response, but not a Th1 response, than ovalbumin-tolerized mice following experimental stroke [152]. Hypertensive rats tolerized to mucosal E-selectin are protected from infarct damage, and the protection conferred by tolerance to mucosal E-selectin is abolished by the deletion of endogenous Tregs [153]. However, there is controversy about the role of Tregs in cerebral ischemia. For instance, it is reported that Tregs either have no effect on acute infarction or their depletion reduces infarction, suggesting that Tregs may even play a detrimental role in cerebral ischemia [154, 155]. Collectively, these results suggest that research on the role of Tregs is still in its infancy and more research is needed to advance the field.

12.3.3.2.2 B Lymphocytes

There are some studies suggesting that B lymphocytes likely play a detrimental role in cerebral ischemia. For instance, it is reported that infarct sizes and neurological damage are remarkably reduced in SCID and Rag−/− mice that are deficient in both

T and B lymphocytes [143, 146]. However, controversial evidence exists, including the evidence that mice only lacking B lymphocytes are not protected from cerebral ischemia [146]. Moreover, there is evidence showing that B cell deficiency even exacerbates histology and the neurological outcomes following cerebral ischemia [156]. Consistently, reconstitution of B cells in deficient mice ameliorates infarct damage and neurological deficits. Further studies suggest that the protective effects of B cells can be attributed to B regulatory cells secreting Il-10 [156]. Although current evidence suggests that B regulatory cells play a protective role following cerebral ischemia, more research is warranted to advance the understanding about the role of B lymphocytes in cerebral ischemia.

12.3.3.3 Impacts of Ischemia-Induced Immune Responses on Tissue Repair

The injured brain activates brain-resident and peripheral immune cells, presumably confining the damaged tissue and fostering the clearance of cellular debris during the acute phase following cerebral ischemia. Unfortunately, in most cases, stroke induces over-activation of the immune system, ultimately exacerbating tissue damage during the early phase of ischemic stroke. On the other hand, emerging evidence suggests that sustained immune activation also exerts robust effects on poststroke tissue repair and brain remodeling. In the next section, we will discuss the role and function of innate and adaptive immune responses following stroke influence repair and remodeling following stroke.

12.3.3.3.1 Impacts on Postischemic Neurogenesis

Neurogenesis has an important role in recovery following experimental cerebral ischemia. Two brain regions, the subventricular zone and the subgranular zone in the hippocampus, have been found to be responsible for neurogenesis following CNS injury. Following cerebral ischemia, neural progenitor cells (NPCs) are continuously generated in the two areas for 4 months after stroke onset [157]. However, overall neurogenesis is insufficient following cerebral ischemia in terms of the survival of newly generated neurons and their migration into the injured regions. Almost 80 % of newly generated neurons die within 2 weeks after generation [158]. Ischemia-induced microglial over-activation is the major detrimental mechanism underlying the poor survival of NPCs in the ischemic environment [159]. The inhibition of microglia-mediated inflammation using chronic treatment with minocycline attenuates the deleterious effects of microglia on poststroke neurogenesis and promotes functional recovery [160]. Moreover, shifting the ischemia-induced proinflammatory polarization of microglia/macrophages toward alternative M2 polarization with the AMP-activated protein kinase (AMPK) activator metformin also increases poststroke neurogenesis and promotes functional recovery [130]. In addition to modulating NPC survival, emerging evidence implicates that postischemic

immune responses may also play a role in the mobilization of newly generated neuroblasts. For instance, murine NPCs express both the complement C3a and C5a receptors. The effects of C3a on basal neurogenesis are dependent on SDF-1α, which is remarkably upregulated in the perilesional area following stroke [161]. It is reported that ischemia-activated microglia in the ischemic regions facilitate the mobilization of newborn neurons via the secretion of SDF-1 [162].

12.3.3.3.2 Impact on Postischemic Angiogenesis

In the regenerating brain, newly generated neurons and neuroblasts proliferate and migrate in chain along the blood vessels that provide trophic factors. Thus, neovascularization and neurogenesis are interdependent, and angiogenesis is also critical for poststroke brain repair. Immune cells also play important roles in regulating the complex angiogenic responses following ischemic stroke. Angiogenesis can be initiated with a large number of inflammation inducers, such as CCL12, VEGF, and MMP-9. For instance, activated microglia/macrophages secrete TGF-ß, galectin-3, and VEGF to enhance angiogenesis in the ischemic regions [163]. Among the multiple inflammation-associated factors that promote angiogenesis, VEGF is the most important mitogen in the processes of angiogenesis following ischemic stroke [164]. As evidenced by the increased density of microvessels, VEFG treatment significantly enhances angiogenesis in ischemic penumbra following experimental stroke [165]. In addition, VEGF has been shown to promote postischemic tissue repair via neurogenesis, likely reflecting the interaction between neurogenesis and angiogenesis during the postischemic repair processes. Mechanistically, VEGF acts through PI3K/Akt and mitogen-activated protein kinase cascades to promote the survival, proliferation, and migration of microvascular endothelial cells. Neutrophils are first to infiltrate the ischemic region, which contributes to postischemic angiogenesis by the proteolysis of extracellular matrix and the increase of tissue-bound VEGF [166]. The infiltrating eosinophils and mast cells can also secrete VEGF [167] to facilitate angiogenesis. Moreover, enhancing the M2 polarization of microglia/macrophages following cerebral ischemia promotes angiogenesis in the ischemic brain [130]. There is also evidence that adaptive immune cells, such as Th1, Th2, and Th17 cells, are involved in poststroke angiogenic cascades [2]. However, the effects of these cells on poststroke angiogenesis and the underlying mechanisms have not been investigated in stroke models. Thus, further research is warranted to advance our understanding of how the immune system modulates angiogenesis following cerebral ischemia.

12.3.3.3.3 Impact on Oligodendrogenesis and Remyelination

Oligodendrocytes and oligodendrocyte precursor cells (OPCs) are sensitive to inflammation, and thus postischemic immune responses have great impacts on oligodendrogenesis and remyelination following cerebral ischemia. For instance,

decreasing neuroinflammation with D-4F, an apolipoprotein A-I mimetic peptide, has been shown to ameliorate white matter damage and improve functional outcomes after experimental stroke [168]. Particularly, recent evidence is accumulating to suggest that microglia/macrophages can act through OPCs to positively or negatively affect poststroke oligodendrogenesis and remyelination based on their polarization. In a mouse model of white matter injury, it is found that the switch from an M1- to an M2-dominant polarization of microglia and peripherally infiltrated macrophages is accompanied by the onset of remyelination. M2 cell-conditioned media is elevated in vitro oligodendrocyte differentiation, while in vivo remyelination is impaired following the depletion of intralesional M2 cells. These results suggest that microglia/macrophages assuming M2 polarization promote oligodendrogenesis and remyelination following CNS injury, while M1-polarized microglia/macrophages impair these processes [169]. Aged mice displayed significantly more severe neurological deficits than young adults following experimental stroke. Remarkably, this ischemia-induced deterioration of neurological outcomes is linked to white matter damage and a reduction in M2 microglia/macrophage polarization in aged mice [170]. Transplantation of gal-1-secreting neural stem cells shifts microglia/macrophage polarization toward the beneficial M2 phenotype and ameliorates white matter injury in vivo following transient focal cerebral ischemia [171]. Moreover, HDAC inhibitors possibly attenuate white matter injury via the induction of M2 polarization following traumatic brain injury or cerebral ischemia [172, 173]. Collectively, current evidence suggests that the poststroke polarization of microglia/macrophages greatly affects oligodendrogenesis and remyelination and that modulating the polarization of microglia/macrophages is a promising therapy to promote oligodendrogenesis and remyelination following ischemic stroke [7].

12.4 Postischemic Dysregulation of the Peripheral Immune System

Following cerebral ischemia, the injured brain and immune system impact each other specifically and profoundly. The contribution of ischemia-induced immune responses to stroke outcomes has been extensively studied and well-established, as discussed above. On the other hand, accumulating evidence suggests that cerebral ischemia in the brain also profoundly affects the immune system, specifically resulting in the dysfunction of peripheral immune organs. Cerebral ischemia-induced dysfunctions of the peripheral immune system have greater clinical consequences on stroke outcomes than previously thought. Indeed, stroke-induced peripheral immunosuppression represents an independent factor for poststroke infection that was previously assumed to result from a pre-existing comorbidity or the mismanagement of patient care [12]. On the other hand, the alteration of peripheral immune responses also contributes to brain ischemic damage and tissue repair. In the following section, we will discuss how the injured brain affects the peripheral immune system and how peripheral immune responses contribute to stroke outcomes.

12.4.1 Postischemic Immunodeficiency Mediated by the HPA Axis

The injured brain profoundly affects the peripheral immune system following cerebral ischemia, as evidenced by the fact that ischemic stroke results in severe peripheral immunodeficiency. Stroke-induced peripheral immunodeficiency includes a decreased number of peripheral lymphocytes, impaired activation, mitogen-induced proliferation, and cytokine production of T lymphocytes and natural killer cells (NKs). One signaling mechanism underlying the crosstalk between the brain and the peripheral immune system is the hypothalamic–pituitary–adrenal (HPA) axis. Cerebral ischemia causes an imbalance in the homeostasis of the system, resulting in the activation of the HPA axis [174]. It is thought that cytokines that are well known to be significantly induced in the ischemic brain, such as IL-1, can stimulate neurons in the paraventricular nucleus (PVN), resulting in the release of corticotrophin-releasing hormone (CRH) into the hypophyseal portal blood supply [175]. CRH, in turn, stimulates the anterior pituitary gland to secrete adrenocorticotropic hormone, which circulates to the adrenal gland and increases the adrenal production of cortisol. Glucocortids act on the receptors expressed on the immune cells to depress inflammation and the immune response. Thus, via cortisol release, cerebral-activated HPA axis leads to lymphopenia and lymphocyte dysfunction, which substantially contribute to poststroke immunodeficiency and consequent infection [176].

12.4.2 Postischemic Immunodeficiency Mediated by the Sympathetic Nervous System

The autonomic nervous system (ANS) consists of the parasympathetic nervous system (PNSN) and the sympathetic nervous system (SNS). Up to 98 % of the nerve fibers that innervate the spleen are sympathetic. Following cerebral ischemia, SNS is activated immediately [177], resulting in spleen contraction and shrinkage and the dysfunction of invariant natural killer T cells (iNKTs). All these changes mediated by poststroke SNS activation have significant contributions to immunosuppression following ischemic stroke.

The spleen is the largest storage facility for immune cells. Upon ischemia onset in the brain, SNS activation, which leads to activation of the α1 adrenergic receptor expressed on smooth muscle cells, is a major mechanism responsible for stroke-induced splenic contraction and shrinkage [177]. Blocking the α1 adrenergic receptor with the antagonist prazosin or a pan-adrenergic receptor antagonist carvedilol, but not splenic denervation, ameliorates stroke-induced spleen shrinkage and reduces infarct volumes [177]. Moreover, splenectomy prior to stroke significantly decreases neutrophil infiltration and infarct volumes [178]. These lines of evidence suggest that signals from the ischemia-activated sympathetic fibers innervating the

spleen are important for the mobilization of immune cells from the spleen and their infiltration into the ischemic brain. However, SNS activation seems to exert biphasic effects on peripheral immune responses following stroke. During the early phase following experimental stroke, with the aid of dampened parasympathetic tone, SNS activation leads to increased adrenergic tone, stimulating the mobilization of immune cells from the spleen and initiating acute inflammatory responses in the ischemic brain and the spleen. Not surprisingly, blocking the SNS activation-induced increase in adrenergic tone is protective against acute infarct damage, as evidenced by the abovementioned studies showing that adrenergic receptor antagonists reduce infarct damage [177]. Conversely, during the later phase of ischemic stroke, when spleen-derived immune cells are exhausted, SNS activation results in spleen shrinkage and postischemic peripheral immunosuppression [179]. Moreover, the activation of ß2 adrenergic receptors expressed on innate as well as adaptive immune cells can further suppress immune functions, which likely represents an alternative mechanism that is responsible for poststroke immunosuppression. In support of the notion, the ß-receptor antagonist propranolol has been shown to attenuate stroke-induced immunosuppression and, consequently, inhibit poststroke infection and associated mortality [180, 181].

Following ischemic stroke, SNS activation can also lead to peripheral immunosuppression by affecting the functions of iNKTs through noradrenergic innervations of the liver [182], which is consistent with the long-held notion that SNS activation inhibits immune functions. Blocking ß2 adrenergic receptors with the antagonist propranolol or by depletion of the noradrenergic innervations of the liver ameliorates immunosuppression and bacterial infection, which are accompanied by an increase in IFN-γ secretion from liver iNKTs. Moreover, a local noradrenergic injection into the liver exacerbates poststroke immunosuppression through the inhibition of the normal functions of iNKTs [182]. The findings further support the notion that SNS activation is an important mechanism that is responsible for immunosuppression and consequent bacterial infection following cerebral ischemia.

12.4.3 Postischemic Immunodeficiency Mediated by the Parasympathetic Nervous System

The vagus nerve is the tenth cranial nerve of the PSNS. Recent studies suggest that action potentials arising from the vagus nerve play an essential role in modulating peripheral immune responses. Stimulating the vagus nerve remarkably reduces the macrophage release of TNF-α through the nicotinic acetylcholine receptors (nACH) expressed on TNF-α-producing macrophages following endotoxin exposure [183, 184]. This pathway is termed the cholinergic anti-inflammatory pathway or the inflammatory reflex, and it represents an important mechanism underlying brain-immune crosstalk.

Multiple types of immune cells in the spleen, including macrophages, neutrophils, and T cells, have been shown to be functionally innervated by the vagus nerve. However, the spleen nerve fibers originating from the celiac ganglion (where the vagus nerve terminates) are adrenergic, and the machinery for producing nACH is lacking in these nerve fibers. This raises a question about how an action potential arising in the vagus nerve modulates the functions of immune cells via nicotinic acetylcholine signaling. Recently, the acetylcholine-producing memory phenotype T cells are shown to be required for the inflammatory reflex [185]. Thus, action potentials arising in the vagus nerve enhance norepinephrine production in the spleen, which in turn stimulates memory phenotype T cells to produce the neurotransmitter acetylcholine, which is required to inhibit innate immune cell-mediated inflammation in the spleen. Stimulation of the vagus nerve has been found to be neuroprotective against cerebral infarct damage [186]. Moreover, vagus nerve stimulation improves cognitive functioning experimentally [187] and behavioral function clinically [188]. However, the underlying mechanisms remain to be determined. Some recent studies suggest that enhanced angiogenesis, antioxidant effects, and anti-inflammatory effects may be responsible for the beneficial effects of vagus nerve stimulation on stroke outcomes [189–191].

12.5 Therapies Targeting Postischemic Immune Responses

Stroke induces immune responses both in the brain and the peripheral nervous system. Both central and peripheral immune responses essentially contribute to stroke outcomes, as discussed above. Strong evidence exists that stroke-induced brain inflammation essentially contributes to secondary brain injury. Thus, anti-inflammatory neuroprotectants are potential drugs for treating ischemic stroke. However, there are serious concerns about the application of anti-inflammatory strategies since stroke patients suffer from long-term immunosuppression. Poststroke immunosuppression serves as a double-edged sword. On one hand, it may restrict over-activated immune responses following cerebral ischemia. On the other hand, it leads to postischemic infection that substantially exacerbates neurological outcomes and contributes to mortality and comorbidities following stroke. Thus, special attention should be paid to poststroke immunosuppression when developing stroke therapies targeting immune responses in ischemic stroke.

12.5.1 Antibiotics

Since systematic infection resulting from poststroke immunosuppression is a major predictor for poor neurological outcomes, mortality, and comorbidity, some antibiotics have been tested for their potential to prevent poststroke systematic infection in experimental stroke and in clinical trials. Doxycycline, a tetracycline antibiotic

that inhibits MMP, is a potential therapeutic antibiotic for ischemic stroke. Moreover, it confers neuroprotection against global cerebral ischemia by reducing perineuronal laminin degradation via the inhibition of MMP-9 activity [192]. In a clinical trial, administration of the antibiotic moxifloxacin reduces infections after severe non-lacunar ischemic stroke compared to a placebo [193]. The antibiotic minocycline has also been tested clinically. Patients orally administered the antibiotic minocycline displayed a reduced modified Rankin Scale vs. untreated patients over 90 days following acute ischemic stroke [194]. In stroke patients, minocycline is safe up to doses of 10 mg/kg when injected alone intravenously or in combination with tissue plasminogen activator [195]. In contrast, a new clinical trial conducted on a small sample of acute stroke patients shows that intravenous minocycline is safe, but not efficacious [196]. However, as claimed by the authors, this study is not powerful enough to reliably identify or exclude a modest, but clinically important, effect of minocycline. To conclude, larger trials are needed to test the treatment effects of antibiotics, such as minocycline.

12.5.2 Anti-inflammatory Drugs

Although immunosuppressive drugs now are not considered as ideal therapies for ischemic stroke, numerous studies have examined the effects of anti-inflammatory agents on stroke outcomes, both experimentally and clinically. These studies suggest that stroke patients may benefit from anti-inflammatory drugs. Multiple cytokines, including IL-1, TNF-α, and IL-10, and adhesion molecules, such as ICAM-1, have been associated with inflammatory responses following ischemic stroke. Blocking IL-1 receptors with the IL-1 receptor antagonist IL-1ra has been tested in clinical trials. The data suggest that recombinant human interleukin-1 receptor antagonist (rhIL-1ra) is safe and well tolerated in acute stroke. Moreover, in patients with cortical infarcts, rhIL-1ra treatment improves clinical outcomes at 3 months compared to a placebo treatment [197].

Adhesion molecules are required for leukocyte–endothelial cell adhesion and the infiltration of peripheral immune cells into the ischemic brain. Experimental data indicate that the administration of anti-ICAM-1 antibody attenuates brain infarct damage and results in a reduction in polymorphonuclear leukocytes in the ischemic brain following transient focal cerebral ischemia [198]. However, in a clinical study, anti-ICAM therapy with a monoclonal antibody (enlimomab) is not effective for ischemic stroke and may even significantly worsen stroke outcomes [120].

α4-Integrin is an adhesion molecule that is expressed on T cells. α4-Integrin plays a crucial role in the invasion of T cells into the CNS by binding to the receptors for the α4 family of integrins that are expressed on activated endothelium, such as VCAM-1. Experimental data show that blocking α4-integrin improves stroke outcomes and inhibits T cell trafficking into the ischemic brain [145]. An international consortium has coordinated a preclinical trial of natalizumab, a humanized monoclonal antibody against α4-integrin, to evaluate whether the specific inhibition

of T cell trafficking into the lesion with natalizumab improves outcomes in mice following experimental stroke [199]. Moreover, a randomized, blinded clinical evaluation of the effects of natalizumab on infarct volumes is underway.

Sphingosine-1-phosphate (S1P), an important extracellular signaling molecule that binds with S1P receptors, plays important roles in regulating cell proliferation, adhesion, and migration. The benefits of targeting S1P receptors or S1P synthetase following brain ischemia have been demonstrated experimentally [200, 201]. Fingolimod, a high-affinity agonist of S1P receptors, decreases the number of circulating lymphocytes by preventing their egress from lymph nodes. Following experimental stroke, fingolimod may suppress the early infiltration of lymphocytes into the ischemic brain. In a clinical trial, patients with anterior cerebral circulation are orally treated with fingolimod beyond 4.5 h after the onset of stroke, and treatment continues for 3 days. Patients receiving fingolimod display reduced counts of circulating lymphocytes and better functional outcomes [202]. Moreover, compared with patients who received tissue plasminogen activator (tPA) alone, patients receiving the combination of fingolimod with tPA display a reduced number of circulating lymphocytes, smaller lesion sizes, reduced hemorrhage, and improved neurological deficits [203]. Collectively, these results indicate that targeting lymphocyte trafficking is a promising therapeutic strategy for ischemic stroke.

12.5.3 Immunomodulatory Therapies

An alternative strategy to treat ischemic stroke by targeting immune responses is to modulate, rather than broadly suppress, the functions of immune cells. The feasibility of this approach has been demonstrated experimentally. For instance, selectively inducing the M2 polarization of microglia/macrophages in the ischemic brain by chronic treatment with the AMPK activator metformin enhances tissue repair and promotes long-term functional recovery in the mouse stroke model [130]. The adoptive transfer of CD4+ CD25+ Tregs remarkably reduces brain infarction and accelerates long-term behavioral recovery without enhancing postischemic systematic immunosuppression [150]. In addition, transplanting wild-type B cells, rather than IL-10-deficient B cells, reduces infarct damage and improves functional deficits in B cell-deficient mice [156].

In addition to pharmacologically modulating the functions of immune cells or transplanting immune regulatory cells, vaccinating antigens prior to stroke has also been shown to be an effective approach to modulating immune functions following ischemia. For instance, mice receiving an intranasal vaccination of the brain antigen MBP display better performance on behavioral tests than ovalbumin-tolerized mice; this result is associated with an enhanced regulatory response in MBP-tolerized mice [152]. Indeed, the adoptive transfer of CD4+ T cells from MBP-tolerized wild-type mice, but not from IL-10-deficient mice, reduces brain infarction following cerebral ischemia/reperfusion, indicating that IL-10-producing CD4+ T cells are responsible for the neuroprotection observed in MBP-tolerized animals [204].

Moreover, mucosal vaccination with MOG also reduces ischemic infarct sizes and improves behavior outcomes, and the beneficial effects conferred by MOG vaccination are mediated by IL-10-producing CD4+ T [205]. E-selectin is expressed on the endothelium within a few hours following experimental stroke. Hypertensive rats tolerized to mucosal E-selectin are protected from infarct damage, and protection is abolished by the deletion of endogenous Tregs [153].

Taken together, these results indicate that modulating immune functions with pharmacological agents, vaccination, or the transplantation of immune regulatory cells (either Tregs or Bregs) is a promising strategy for stroke intervention, although translating these experimental approaches into clinics is a long way off.

12.6 Conclusion

Currently available evidence clearly demonstrates that stroke risk and outcomes can be modified by pre-existing immune responses. Following ischemic stroke, the exquisitely coordinated crosstalk between the brain and the immune system also helps shape stroke outcomes. On one hand, cerebral ischemia-induced immune responses significantly affect acute infarct damage as well as long-term tissue repair and recovery from stroke. On the other hand, the injured brain also remarkably impacts the functions of the immune system, as evidenced by the systematic immunosuppression that represents an independent risk factor for stroke-induced infection. In addition to the traditional view that the injured brain conveys danger signals to the immune system via the secretion of DAMPs, recent studies also have uncovered several neural–endocrinal circuits, such as the HPA axis and the anti-inflammatory reflex, through which the ischemic brain robustly modulates systematic immune responses. Stroke-induced immune responses used to be considered innate and antigen-unrelated. However, recent studies suggest that adaptive immune cells, such as $CD8^+$ cytotoxic T cells, $CD4^+$ helper T cells, and T and B regulatory cells, also play vital roles in the progression of ischemic infarct and tissue repair. The poststroke activation of immune cells was used to be considered to play a detrimental role in stroke outcomes, while postischemic immunosuppression was considered beneficial. However, recent studies reveal that modulating the immune functions of immune cells, rather than broadly inhibiting their functions, may be a more promising therapeutic strategy for ischemic stroke. Particularly, alternatively activated (also known as M2-polarized) microglia/macrophages and neutrophils have been shown to decrease acute infarct damage and promote long-term tissue repair and functional recovery. M2-polarized microglia/macrophages and Tregs enhance angiogenesis, neurogenesis, and oligodendrogenesis following cerebral ischemia. Based on these findings, therapies that modulate immune responses and improve tissue repair may lie on the horizon. An immunomodulatory strategy is supposed to be superior to immunosuppressive strategy because it can harness the endogenous protective and repair powers of the body. Thus, future

research on the mechanisms through which the crosstalk between the injured brain and the immune system impact stroke outcomes is highly warranted.

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Chapter 13
Microglia Function in Stroke

Ran Xu, Adnan Ghori, Ulf C. Schneider, and Peter Vajkoczy

Abstract Microglia represent the resident immune cells in the central nervous system. They survey the central nervous system and phagocytose debris in physiological and disease conditions. After brain injury events followed by stroke, microglia undergo specific phenotypic and morphological transformations. In addition, resident microglia play a pivotal role in initiating and orchestrating inflammatory events following stroke.

The inflammatory response in ischemic stroke begins at the vascular level, triggered via hypoxia, reactive oxygen species (ROS), and shear stress. Ischemia in the brain parenchyma leads to the release of danger-associated molecular patterns (DAMPs), activating purinergic and pattern recognition receptors on microglia. In concert with the loss of immunosuppression, this induces an inflammatory milieu in the resident brain cells and infiltrating leukocytes and results in cell death. In the postischemic phase, microglia play a crucial role in the repair mechanisms by phagocytosis of dead cells and production of growth factors and cytokines that are required for tissue repair and neuronal sprouting.

In subarachnoid hemorrhage (SAH), the cascade of inflammatory events occurs in an outside-in fashion: following the extraparenchymal insult, the inflammatory cascade starts at the leptomeningeal interface as well at the endothelial surface within the cerebral microvasculature. Here, an increased accumulation of monocytic cells and neutrophil precedes microglia accumulation near the site of vascular rupture. Microglia activation culminates 9–14 days after injury, expressing pro-inflammatory cytokines, and is paralleled by an interaction with neurons within the brain parenchyma, inflicting neuronal and axonal degradation, ultimately contributing to secondary brain damage in SAH.

Understanding the role of microglia in the immune response after stroke is vital in dissecting the mechanisms involved in primary damage, secondary brain injury, and regeneration processes. Analyzing the underpinnings of extracellular signals triggering phenotypic shifts in microglia and understanding the molecular switches

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involved in such signaling may help us in finding therapeutic strategies addressing the immune response and after stroke and its clinical outcome thereafter.

Keywords Microglia • Ischemic stroke • Hemorrhagic stroke • Subarachnoid hemorrhage • Microglia phenotype • Postischemic inflammation • Microglial markers

Abbreviations

Arg1	Arginase 1
BBB	Blood-brain barrier
CCL2	C-C chemokine ligand 2
CCR2	C-C chemokine receptor type 2, monocyte chemoattractant protein 1
CD11b	Cluster of differentiation molecule, integrin alpha M
CD206	Cluster of differentiation 206, mannose receptor
CD36	Cluster of differentiation 36, platelet glycoprotein 4
CD45	Cluster of differentiation 45, protein tyrosine phosphatase receptor type C
CD68	Cluster of differentiation 68
Chi3l3	Chitinase 3-like 3
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CXCL2	Chemokine (C-X-C motif) ligand 2
DAMP	Danger-associated molecular patterns
HMGP1	High-mobility group protein 1
Iba-1	Ionized calcium-binding adaptor molecule 1
ICAM-1	Intercellular adhesion molecule 1
ICH	Intracerebral hemorrhage
IFN-γ	Interferon gamma, type II interferon
IGF-1	Insulin-like growth factor 1
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-18	Interleukin 18
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
MCAO	Middle cerebral artery occlusion
MMP-9	Matrix metallopeptidase 9
MMPs	Matrix metalloproteinases
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide

NPC	Neural precursor cells
PPARγ	Peroxisome proliferator-activated receptor γ
PSGL-1	P-selectin glycoprotein ligand 1
ROS	Reactive oxygen species
SAH	Subarachnoid hemorrhage
Sall1	Spalt-like 1
TGF-ß	Transforming growth factor beta
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
Tmem119	Transmembrane protein 119
TNFα	Tumor necrosis factor alpha
TREM2	Triggering receptor expressed on myeloid cells 2

13.1 Introduction

Microglia represent the resident immune cells in the central nervous system. They survey the central nervous system and phagocytose debris in physiological and disease conditions [1]. In the developing brain, microglia are also involved in developmental angiogenesis and synapse remodeling [2]. After brain injury events followed by stroke, microglia undergo specific phenotypic and morphological transformations. Furthermore, resident microglia play a pivotal role in initiating and orchestrating inflammatory events following stroke.

Stroke is mainly divided into two different subtypes: ischemic and hemorrhagic stroke. Ischemic stroke is the major cause of stroke and occurs due to sudden occlusion of an intracerebral vessel by a thrombus or embolism, resulting in an interruption of cerebral blood flow [3]. Hemorrhagic stroke represents about 15% of all strokes and is caused by subarachnoid hemorrhage or intracerebral hemorrhage. In both instances, unique microglial changes occur. This chapter focuses on the current role of microglia in the pathophysiology of ischemic and hemorrhagic events following stroke.

13.2 Dynamics in Microglia Phenotypes

Microglia are not only critically involved in brain injuries but also are constantly involved in physiological conditions in the central nervous system (CNS). Nimmerjahn et al. demonstrated that even in their presumed resting state, microglial cells continuously survey their microenvironment by extending and retracting their ramified processes [2]. At various stages of CNS injury and inflammatory events, microglia become polarized toward two different activation phenotypes; the M1 and M2 phenotype [4]. Historically, these phenotypical dynamics are thought to be

attributed to distinct roles in the restoration of the neurovascular network: classically activated M1 microglia release destructive pro-inflammatory mediators, whereas alternatively active M2 microglia are involved in brain repair and regeneration by phagocytosis and release of numerous protective and trophic factors.

The M1 phenotype is stimulated by interferon gamma (IFN-γ) and lipopolysaccharide [5–7]. M1 are considered as pro-inflammatory, producing high levels of pro-inflammatory cytokines and oxidative metabolites such as tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), interleukin 1 beta (IL-1β), interleukin 12 (IL-12), and nitric oxide (NO) [8]. The immune response mediated by M1 phenotypes aggravates neuronal damage, and it also plays a role in hindering axonal regeneration [4, 8]. On the contrary, the M2 phenotype microglia are considered to facilitate the recovery functions after damage, releasing several anti-inflammatory mediators, such as interleukin 4 (IL-4), interleukin 10 (IL-10), interleukin 13 (IL-13), transforming growth factor beta (TGF-ß), and insulin-like growth factor 1 (IGF-1). Microglia with M2 phenotype express specific markers including arginase 1 (Arg1), the heparin-binding lectin Ym1, mannose receptor (CD206), and CD36 [9–11]. It has been proposed that Arg1 is involved in neuroprotective functions [9], whereas Ym1 and CD206 have been found in the ischemic core region 24 h after stroke, suggesting their role in tissue repair after ischemia [12]. One of the foremost characteristics of the M2 microglia phenotype is their higher phagocytic activity as compared to M1 phenotype. Activated M2 microglia have a high capacity to remove damaged cell debris, thus preventing release of their cellular content into the extracellular space [11] and promoting the reconstruction of the extracellular matrix [13]. The release of IGF-1 is involved in reducing apoptotic activity, enhancing the proliferation and differentiation of neural precursor cells (NPCs) [10].

Recently, a new microglial phenotype has been described, the so-called dark microglia, that becomes abundant during stress, aging, and neurodegenerative processes [14]. This phenotype is characterized by a condensed, electron-dense cytoplasm and nucleoplasm, making them as “dark” as mitochondria, accompanied by remodeling of their nuclear chromatin. These dark mitochondria are thought to play a significant role in the pathological remodeling of neuronal circuits, especially at synapse level.

The concept of M1–M2 dichotomy only represents a conceptual framework describing two extreme activation states of microglia that have been mainly studied in vitro [15]. The in vivo biology of microglial phenotypic switching is far more complex and includes a spectrum of different phenotypes that may also overlap, as well as different phenotype subpopulations [16]. However, the M1 and M2 classification remains a useful concept in describing and understanding the physiological characteristics of microglia changes after CNS injury and regeneration.

13.3 Microglia Changes in Stroke

13.3.1 Microglia in Ischemic Stroke

Ischemic insult induces a robust inflammatory response initiated within a few hours, with the orderly activation of microglia, other peripheral immune cells, and astrocytes [17]. The cascade of events involved in the inflammatory response begins immediately after arterial occlusion and consecutive hypoxia and production of ROS trigger the coagulation cascade. This is followed by the activation of complement, platelet, and endothelial cells (Fig. 13.1a). Intravascular fibrin traps platelets and leukocytic cells with the result of microvascular occlusion. The adhesion molecule P-selectin translocates to the surface membrane of endothelial cells and platelets, inducing inflammatory signals [18].

Furthermore, the release of nucleotides, including ATP and UTP from injured cells, leads to activation of purinergic receptors on microglia, inducing the production of pro-inflammatory cytokines. Most of these are transcriptionally induced; some of them including IL-1β and IL-18 are processed from their propeptides by the activity of caspase 1, which is activated by P2X7 receptors on microglia [19]. In addition, ischemic cell death induces the release of DAMP molecules. These include high-mobility group protein B1 (HMGB1), heat shock protein 60, and β-amyloid. DAMPs activate Toll-like receptors (TLRs), including TLR2 and TLR4, and scavenger receptors, such as CD36, which are widely expressed on microglia, inducing pro-inflammatory gene expression through the transcription factor NF-κB (Fig. 13.1b) [20, 21]. The cytokine production and activation of complement results in enhanced leukocyte infiltration which in turn increases damage to the brain tissue and, consequently, leads to the production of more DAMPs [19].

The disruption of cell-cell interaction between microglia and neurons parallels ischemia-induced neuronal cell death. Moreover, the increase of extracellular glutamate activates metabotropic glutamate receptors on microglia and leads to its inflammatory phenotype [22]. While neuronal death evolves in the ischemic core, spreading to the penumbra, the disruption of microglial-neuronal interaction fosters the loss of immunosuppression, ultimately contributing to postischemic inflammation.

Microglia are not only involved in ischemia-induced injury events but also play a fundamental role in the phases of postischemic resolution phase and brain repair. However, the specific mechanisms governing resolution of inflammation and functional reorganization within the brain are not yet fully understood. Recent evidence suggests that inflammatory resolution is characterized by suppression of the pro-inflammatory response, including the removal of apoptotic cells, a shift toward an anti-inflammatory microenvironment, and the mediation of prosurvival factors [23]. Microglia and macrophages are the main players in phagocyting dead cells and debris, triggered by specific find-me (UTPs, ATPs) and eat-me signals (UDPs) released from injured chemokines and injured cells through P2Y2 receptors [24]. In addition, postischemic upregulation of TGF-ß and IL-10 microglia and macrophages

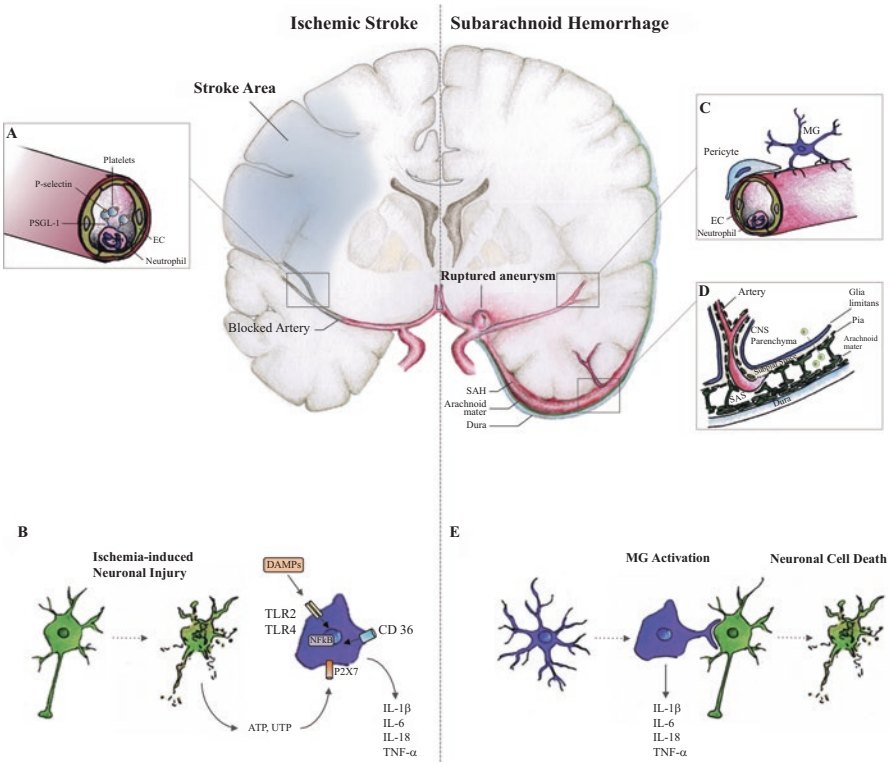

Fig. 13.1 Microglial changes in ischemic stroke and subarachnoid hemorrhage. (**a**) Ischemic insult induces a robust inflammatory response initiated within a few hours. The cascade of events involved in the inflammatory response begins immediately after arterial occlusion, and consecutive hypoxia and production of ROS trigger the coagulation cascade: intravascular fibrin traps platelets and leukocytic cells with the result of microvascular occlusion. The adhesion molecule P-selectin translocates to the surface membrane of endothelial cells and platelets, inducing inflammatory signals [18]. (**b**) The release of nucleotides, including ATP and UTP from injured cells, leads to activation of purinergic receptors on microglia, inducing the production of pro-inflammatory cytokines [19]. In addition, ischemic cell death induces the release of DAMP molecules that activate Toll-like receptors (TLRs), including TLR2 and TLR4, and scavenger receptor, such as CD36, which are widely expressed on microglia, inducing pro-inflammatory gene expression through the transcription factor NF-κB [20, 21]. While neuronal death evolves in the ischemic core, spreading to the penumbra, the disruption of microglial-neuronal interaction fosters the loss of immunosuppression, ultimately contributing to postischemic inflammation. (**c**, **d**) In contrast to other ischemic and hemorrhagic strokes, subarachnoid hemorrhage is characterized by a strictly extraparenchymal onset, occurring at the base of the brain within the basal cisterns [27]. The rupture of an intracranial aneurysm causes the spilling of blood within the subarachnoid space between pia and arachnoid mater, outside of the brain parenchyma. This is followed by a cascade of inflammatory events in an outside-in fashion [28]: within the cerebral microvasculature, an increased accumulation of neutrophil granulocytes at the endothelial surface is observed within the first 4 days after initial onset of SAH, mediated through ICAM-1 expression on endothelial cells and PSGL-1 activation on neutrophils. (**e**) Microglia accumulation and activation culminates around a week after injury and is furthermore characterized by their expression of pro-inflammatory cytokines, including IL-6, TNFα, and IL-1alpha/beta. This microglia activation is paralleled by an interaction with neurons within the brain parenchyma, inflicting neuronal and axonal degradation peaking between day 7 and day 14 after initial SAH, contributing to secondary brain damage in SAH

also facilitates neuroprotective mechanisms in the resolution of inflammation and cytoprotection on surviving cells in the ischemic areas [25]. The production of growth factors and matrix metalloproteinases (MMPs) aids in establishing a milieu required for neurogenesis, angiogenesis, and gliogenesis. Inflammatory cells are necessary to produce growth factors – among them microglia who are required for the expression of IGF-1, a crucial factor in neuronal sprouting and tissue repair [26].

In summary, the inflammatory response in ischemic stroke begins at the vascular level, triggered via hypoxia, ROS, and shear stress. Ischemia in the brain parenchyma leads to the release of DAMPs, activating purinergic and pattern recognition receptors on microglia. This, in concert with the loss of immunosuppression, induces an inflammatory milieu in the resident brain cells and infiltrating leukocytes and results in inflammation-induced cell death. In the postischemic phase, microglia play a pivotal role in the repair mechanisms by phagocytosis of dead cells and production of growth factors and cytokines that are required for tissue repair and neuronal sprouting.

13.3.2 Microglia in Hemorrhagic Stroke

13.3.2.1 Microglia in Subarachnoid Hemorrhage

Around a third of all hemorrhagic strokes are comprised of subarachnoid hemorrhage (SAH), which is caused by the rupture of an intracranial aneurysm or intracerebral hemorrhage. In contrast to other ischemic and hemorrhagic strokes, SAH is characterized by a strictly extraparenchymal onset, occurring at the base of the brain within the basal cisterns [27]. The rupture of an intracranial aneurysm causes the spilling of blood within the subarachnoid space between pia and arachnoid mater, outside of the brain parenchyma (Fig. 13.1c, d). This is followed by an increase of intracranial pressure and decrease of cerebral perfusion. Furthermore, extravasation of blood leads to early brain injury with consecutive breakdown of the blood-brain barrier and brain edema.

Current paradigm suggests that this is followed by a cascade of inflammatory events in an outside-in fashion [28]: within the cerebral microvasculature, at the leptomeningeal interface as well at the endothelial surface, an increased accumulation of monocytic cells and neutrophil granulocytes is observed within the first 4 days after initial onset of SAH. This intravascular inflammation is mediated through intercellular adhesion molecule 1 (ICAM-1) expression on endothelial cells and P-selectin glycoprotein ligand 1 (PSGL-1) activation on neutrophils (Fig. 13.1c). Experimental rodent studies have shown that around day 4 after SAH, resident microglia accumulation occurs near the site of vascular rupture [29]. Microglia accumulation and activation culminates 9–14 days after injury and is furthermore characterized by their expression of pro-inflammatory cytokines, including IL-6, TNFα, and IL-1β (Fig. 13.1e) [29]. This microglia activation is paralleled by an interaction with neurons within the brain parenchyma, inflicting neuronal and

axonal degradation peaking between day 7 and day 14 after initial SAH, both in human and murine samples. This in turn is thought to contribute to secondary brain damage in SAH.

13.3.2.2 Microglia in Intracerebral Hemorrhage

Intracerebral hemorrhage (ICH) occurs due to spontaneous rupture of an artery or small arteriole in the brain parenchyma, commonly due to hypertension [30]. Triggered by the introduction of blood components into the brain parenchyma, the activation of immune-competent microglia also plays a key role in ICH. The major roles of microglia include phagocytosing erythrocytes and debris after the hemorrhagic event as well as promoting inflammatory processes and recruiting blood-derived leukocytes [31].

Microglial activation within the perihematomal region is thought to happen as early as 1 h following the ICH event and followed by production of IL-1β within 1 day [32, 33]. Microglia accumulation peaks at day 3 in the perihematomal region with up to 40% increase in number; within 3 weeks, the microglia quantity returns to basal levels [34]. Recent studies have suggested microglia are activated by thrombin, induced by the MAPK pathway, thus promoting cell maintenance [35]. In addition, products of erythrocyte lysis, including iron and heme, activate microglial activation [36]. Similarly as in stroke and SAH, TLR4 activation is thought to lead to inflammatory processes in microglia after onset of ICH [37]. Recent studies have also indicated that HMGB1 may act as a pro-inflammatory cytokine released by microglia after ICH [38].

Experimental strategies targeting microglia after ICH include treatment with minocycline and peroxisome proliferator-activated receptor γ (PPARγ) agonists. Minocycline treatment reduces microglia accumulation after induction of ICH, as well as reduction in inflammatory cytokines, including TNFα and IL-1β [39–41]. Utilizing PPARγ agonists in ICH animal models, enhanced phagocytosis of the hematoma and less expression of IL-1β, matrix metallopeptidase 9 (MMP-9), inducible nitric oxide synthase (iNOS), and TNFα were achieved [42, 43]. Hence, targeting microglia by modulating the immune as well as the regeneration response may serve as promising treatment strategies for ICH.

13.4 Microglia: A Double-Edged Sword

Under physiological conditions, controlled neuronal-glial communication modifies microglial activities. However, under pathological conditions such as ischemia, the endogenous inhibitory signals are disabled, and the microglial activation pathway is triggered [44]. Once microglia become activated, they can exhibit a spectrum of phenotypes with both pro- and inflammatory mediators, either exacerbating ischemic injury or facilitating in neuronal recovery. These seemingly contradictory

neurotoxic and neuroprotective functions are in fact a complex and constantly shifting interplay of microglia, their mediators, and microenvironment, which need to be understood in detail to develop effective immunomodulatory treatment strategies.

Surface receptors involved in microglia-mediated inflammation include Toll-like receptors (TLRs) and purinergic receptors [45]. Activation of microglia via TLR2 and TLR4 increases brain damages after ischemia [46, 47], whereas deficiency of TLR2 or TLR4 reduces the production of TNFα, iNOS, and cyclooxygenase-2 (COX-2) production, thereby reducing the brain infarct volume and improving neurological outcome [45, 48]. Hence blocking the TLR2 and TLR4 pathways within 4–5 h after stroke has been promoted as a strategy to reduce brain injury [49]. Purinergic receptors, such as P2X4 and P2X7, are expressed on microglia and also mediate microglia activation after ischemia [50]. The activation of P2X7 triggers microglia to release pro-inflammatory cytokines including TNFα, IL-1β, NO, and chemokine (CXC motif) ligand 2 (CXCL2) [45]. The release of pro-inflammatory factors such as TNFα, IL-1β, and IL-6 can cause oxidative stress by stimulating the release of ROS and excessive production of NO [51]. On the contrary, blocking of P2X7 receptor reduced the release of TNFα, IL-1β, and IL-6 and is thought to improve the neuronal outcome after global cerebral ischemia [52]. Furthermore, cerebral ischemia leads to an upregulation of the chemokine receptor CCR2 and its ligand CCL2 in microglia [53] and is responsible for enhancing cerebral inflammation and brain edema [54].

Blood-brain barrier (BBB) disruption plays a key role in the development of the cerebral edema and is in part mediated by microglia [55]. Excessive production of ROS is a key contributor of BBB disruption. ROS can activate microglia and induce BBB damage by several mechanisms: oxidative damage, activation of MMPs, cytoskeletal reorganization, modulation of tight junction proteins, and upregulation of inflammatory mediators [56, 57]. A recent study has shown that the release of TNFα, IL-1ββ and IL-6 by microglia can upregulate aquaporin-4 expression in astrocytes and can mediate BBB disruption [58].

Microglia activation after ischemic is not just linked to poor clinical outcome in CNS diseases but is also thought to be involved in the repair processes after cerebral ischemia, exhibiting neuroprotective properties. Recently it was shown that selective eliminating microglia from the brain can enhance neuronal cell death after acute brain injury, suggesting an important neuroprotective role of microglial activation after stroke [59]. It is currently discussed that a certain ramified microglia phenotype can reduce excitotoxicity-induced neurodegeneration [60, 61]. Microglia secrete anti-inflammatory mediators such as IL-10, TGF-ß, IL-4, and IL-13 that contribute to recovery after ischemic damage [62]. IL-10 and TGF-ß are associated with debris scavenging, and they also initiate signaling pathways that result in the inhibition of pro-inflammatory cytokine production and further upregulation of anti-inflammatory cytokines [63]. IL-4, IL-5, and IL-13 are thought to enhance the expression of Arg1, which is reported to be neuroprotective, as well as CD206 and chitinase 3-like 3 (Chi3l3) which are associated with phagocytic activity and prevention of extracellular matrix degradation [11, 64]. Furthermore, recent studies have shown that activating microglia membrane protein triggering receptor

expressed on myeloid cells 2 (TREM2) promotes microglial migration and enhances phagocytic activity [65, 66]. However, further studies are needed to understand the precise role of TREM2 in cerebral ischemia.

The CX3CL1/CX3CR1 signaling pathway has been also shown to play an important role in microglial cell functions in both healthy and disease conditions, although their precise role remains controversial [67]. On the one hand, CX3CL1/CX3CR1 has been shown to play a destructive role in brain ischemic injury; lack or deficiency of either CX3CL1 or CX3CR1 contributes to the reduction of infarct size and ischemic damage as well as less inflammation [68, 69]. On the contrary, various studies have also shown that deficiency of CX3CR1 worsens the behavior impairment induced by transient global cerebral ischemia [70]. Administration of exogenous CX3CL1 reduces ischemia-induced cerebral infarct size and neurologic deficits in permanent middle cerebral artery occlusion (MCAO) [71].

The role of microglia for neurogenesis is comparably controversial. Recent studies have shown that inhibiting microglia activation by minocycline [72] or indomethacin treatment [73] enhances postischemic neurogenesis. In contrary, other studies suggested that a long-term accumulation of microglia 2–16 weeks after experimental stroke is accompanied by enhanced neurogenesis in SVZ at 2, 6, and 16 weeks, proposing that the long-term supportive effect on neurogenesis is possibly mediated by upregulation of IGF-1 [74]. Additionally, IL-4 and IFN-γ induce microglia activation, facilitating neurogenesis and oligodendrogenesis from adult NPCs [45].

Understanding the dual role of microglia in acute inflammation and postischemic tissue repair is crucial in developing efficient therapeutic approaches. The overall concern is that inhibiting the inflammatory response to ischemic injury may also have a deleterious effect on the postischemic repair and regeneration mechanisms and ultimately worsen the clinical outcome of the injury caused by stroke. At this point, there is no definitive evidence that anti-inflammatory treatment interferes with regenerative processes in the injured brain parenchyma, but recent evidence suggests that the repair mechanism is largely mediated by growth factor production and neurogenesis from immune cells. Hence, dissecting the characteristics of microglia plasticity in their pro-inflammatory and neuro-regenerative function encompasses a significant step in treatment strategies in immune modulation. Ultimately, it may be a question of timing whether the microglia response to brain injury is good or bad for tissue recovery and rehabilitation.

13.5 Research on Microglial Characteristics

13.5.1 Microglial Markers

It is difficult to distinguish microglia from other macrophages and monocyte-derived macrophages, since there is no single specific marker for microglial cells [13]. Different activated microglia phenotypes express unique cell surface proteins. Most of the identification approaches mainly rely on morphological distinctions such as ramified versus amoeboid shape; however these methods are not able to distinguish resident microglia from infiltrated microglia. One of the most commonly used microglia marker for immunohistological analysis is the ionized calcium-binding adaptor molecule 1 (Iba-1) [75]. Furthermore, CD11b and F4/80 are also used to identify microglia [76, 77]. Several studies used the hematopoietic cell surface marker CD45 to detect microglia mainly by flow cytometry [78]. Additionally, CD68 and the MHC class II are also used as markers for activated microglia [13]. Recently, different studies have proposed new tools to identify and study microglia. Transmembrane protein 119 (Tmem119) was identified as microglia-specific marker in both mouse and human CNS [79]. Tmem119 was developed for immunohistochemical analysis and FACS isolation of microglia. Spalt-like 1 (Sall1) is a zinc-finger transcription factor, which has been found to have high expression levels in adult microglia; therefore Sall1 is also proposed to define microglia identity and function [80]. Since microglial activation exhibits diverse expression of cell surface proteins and phenotypes, they can be detected via characterizing these changes. However, the similarities of microglia with other cell types still pose a hurdle in identifying and detecting them without masking effects from other cells.

13.5.2 In Vivo Models for Microglia Research in Stroke

Animal models mimic stroke and subarachnoid hemorrhage very effectively, and experimental in vivo studies have provided further insights into the role of microglia in ischemia stroke. Recently, various animal models have been developed to elucidate the beneficial and detrimental role of microglia in ischemic stroke. Among them are a number of knockout animals: some utilize an indirect approach such as depletion of gene-mediating signaling pathways; others work by knocking out receptors directly on microglia.

P2Y12 is a purinergic receptor expressed on microglia. P2Y12-deficient mice showed less microglia surrounding the areas of injury, reduced neuronal death, and reduced activation of the pro-inflammatory transcription factor NF-κB [81]. CX3CR1 mice have been effectively used to study the role of microglia after ischemia. Loss-of-microglia-function studies using CX3CR1 mice displayed reduced size and a reduction in the extravasation of contrast agent into the brain penumbra

as measured by MRI [82]. This study further indicates that inhibiting microglia activation within the first day after stroke could stabilize blood vessels in the penumbra and might be a valuable treatment for stroke patients. Another study using CX3CR1 knockout showed significantly reduced ischemic damage and inflammation in CX3CR1 mice compared to wild-type mice. The smaller infarct size is possibly associated with the decrease in IL-1β and TNFα expression [69].

TREM2 is important for the clearance of apoptotic neurons by phagocytosis. A recent study showed that phagocytosis and infarcted brain tissue resorption was reduced in TREM2 knockout mice compared with wild-type mice and as well as reduced neurological recovery in TREM2 knockout mice [83]. Another study showed that TREM2 knockout attenuates cytokine and chemokine expression, accompanied with a decrease in microglial activation 7 days after MCA; however they did not show a decrease in infarct volume [84].

The Toll-like receptors TLR4 and TLR2 on microglia are upregulated after ischemia. It has been shown that TLR4-depleted mice demonstrate reduced ischemia damage [47]. Furthermore, TLR4 signaling is implicated in ischemic brain damage through the expression of iNOS, COX-2, IRF-1, and MMP-9 [47]. Further studies have shown that TLR4-deficient mice have reduced infarct volume, improved neurological outcome, and decreased downstream NF-κB signaling post-stroke [44].

13.6 Perspective

Understanding the role of microglia in the immune response after ischemic and hemorrhagic stroke is crucial in dissecting the mechanisms involved in primary damage, secondary brain injury, and regeneration processes. The mechanisms involved in microglia activation are multifaceted and differ in certain aspects in ischemic and hemorrhagic stroke. However, in both instances, microglia are not only contributory to brain injury by releasing inflammatory mediators, but also are they involved in repair and remodeling mechanisms. Analyzing the underpinnings of extracellular signals triggering phenotypic shifts in microglia and understanding the molecular switches involved in such signaling may help us in finding therapeutic strategies addressing the immune response after stroke. Modulation of inflammatory processes may ultimately have a significant impact on stroke outcome.

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Chapter 14
Angiogenesis and Neurogenesis After Ischemic Stroke

Wanlu Li and Yongting Wang

Abstract The promotion of angiogenesis and neurogenesis is important for improving the functional injury after ischemia. In this chapter, we describe the process of angiogenesis and neurogenesis in physiological and pathological conditions and neurovascular remodeling after ischemic stroke. We summarize angiogenic factors and chemokines related with angiogenesis. We emphasize gene therapy, stem cell therapy and ischemic postconditioning that benefit recovery via angiogenesis, and gene therapy, stem cell therapy, enriched environment, and optogenetics which have potential for enhancing neurogenesis.

Keywords Angiogenesis • Gene therapy • Ischemic stroke • Neurogenesis • Neurovascular remodeling • Optogenetics • Stem cell therapy

Abbreviations

AAV	Adeno-associated virus
BBB	Blood-brain barrier
BOLD	Blood oxygen level dependent
BrdU	5-Bromo-2′-deoxyuridine-5′-monophosphate
CBF	Cerebral blood flow
CIMT	Constraint-induced movement therapy
CNS	Central nervous system
CXCL12	C-X-C motif chemokine 12
DAMP	Damage-associated molecular pattern
EC	Endothelial cell
EGFP	Enhanced green fluorescent protein

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EPC	Endothelial progenitor cell
EPO	Erythropoietin
FGF	Fibroblast growth factor
Fmri	Functional magnetic resonance imaging
G-CSF	Granulocyte colony-stimulating factor
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-induced factor-1α
HMSCs	Human bone marrow stromal cells
HMGB1	High-mobility group box 1
HRE	Hypoxia-response element
IGF-1	Insulin growth factor-1
MMPs	Matrix metalloproteases
MCAO	Middle cerebral artery occlusion
NPC	Neural progenitor cell
NSC	Neural stem cell
OGD	Oxygen glucose deprivation
PDGF	Platelet-derived growth factor
RMS	Rostral migratory stream
S1P	Sphingosine-1-phosphate
SGZ	Subgranular zone
Shh	Sonic hedgehog
SVZ	Subventricular zone
TGF-β	Transforming growth factor-beta
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

14.1 Angiogenesis After Ischemic Stroke

14.1.1 Modes of Vessel Formation in Normal Brain

In developing embryo, angioblasts, the endothelial precursors derive from mesoderm, differentiate into endothelial cells (ECs). These cells form cords and are specified to arteries or veins [1]. Angiogenesis is a process of forming new vessels from preexisting vessels; these preexisting vessels sprout to expand the vascular network. The vessels sprout, then tip cells navigate, and stalk cells proliferate. Subsequently, tip cells fuse with each other, and stalk cells realign to form lumens. Finally, vessel branches are matured by perfusion [2]. Pericytes and vascular smooth muscle cells (VSMCs) cover ECs, which keep stability and regulate perfusion [3].

14.1.2 Angiogenesis in Normal Brain and Related Angiogenesis Molecules

Firstly, stimulated by angiogenic factors, ECs become mobile following basement membrane degradation. Matrix metalloproteases (MMPs) are crucial for extracellular matrix (ECM) detachment [4]. MMPs regulate liberation of angiogenic factors and prevent secretion of antiangiogenic molecules which are unsuitable for sprouting [5, 6]. Subsequently, ECs develop into tip cells or stalk cells controlled by Notch pathway [7]. Then, tip cells migrate and form filopodia, and stalk cells also proliferate to support elongation. Attractive and repulsion cues regulate tip cell guidance. Afterward, tip cell fusion with filopodia contact and ECs forms lumen [8].

14.1.2.1 Angiogenic Factors

Vascular endothelial growth factor (VEGF). VEGF is a family of homodimeric glycoproteins; it's crucial for angiogenesis. VEGF-A just called VEGF stimulates angiogenesis by binding to VEGF receptor 2 (VEGFR-2) [9]. The co-receptors of VEGF, including neuropilin 1 (NPR1) and neuropilin 2 (NPR2), enhance stimulating effects [10]. Activating VEGFR-2 generates vascular tumor [11]. Deficiency of VEGFR-2 or lack of VEGF leads to decrease of vessel development [12]. The soluble VEGF promotes vessel expansion, while the matrix-bound VEGF induces vessel branching [10]. VEGF stimulates increase of DLL4 in tip cells, subsequently activating Notch in stalk cells and downregulating VEGFR-2 in stalk cells. It stops stalk cells responding to VEGF [13]. VEGF-B just stimulates angiogenesis in restricted position, such as the heart. VEGF-B also promotes neuronal survival and causes metabolic effects [14]. Deficiency of VEGF-B has no effects on angiogenesis [15]. VEGF-C binding to VEGFR-2 and VEGFR-3 activates tip cells [16]. In the developing embryo, VEGFR-3 is associated with angiogenesis, but in later stages, VEGFR-3 is linked with lymphangiogenesis [17]. PIGF is a VEGF homologue and acts as an angiogenic factor. The angiogenic effects are restricted in disease condition. PIGF stimulates angiogenesis via triggering bone marrow-derived endothelial progenitor cells and myeloid cells [15].

Fibroblast growth factor (FGF). FGFs are family of proteins with a variety of effects. Some of them directly stimulate receptors on EC or indirectly induce release of angiogenic factors [18]. FGF1, FGF2, and FGF4 induce proliferation of ECs through activating FGFR1 and FGFR2 [19]. FGF1, FGF2, and FGF4 increase uPA, MMPs in ECs, and uPA receptors on the surface of ECs. In the process of extracellular matrix (ECM) degradation, MMPs and uPA are the two regulators. uPA exchange plasminogen into plasmin. Plasmin degrades matrix proteins and stimulates MMPs [20]. FGF1, FGF2, FGF8b, and FGF10 promote migration of ECs by stimulating chemotaxis and/or chemokines [21–23]. FGF2 modulates adhesion of ECs via regulating integrins, including $\alpha_v\beta_3$, and cadherins [24–27]. Inhibiting FGF results in disintegration of vasculature [28]. FGF2 increases EC reorganization,

which is associated with VEGFR-1, indicating the interaction between VEGF and FGF [29].

Platelet-derived growth factor (PDGF). Angiogenic factors, such as PDGF, angiopoietins, and TGF-β, are crucial for covering by mural cell and vessel maturation. PDGF-β binding to receptor PDGFR-β recruits mural cells. PDGF-β released from ECs induces proliferation and migration of pericytes. Deactivation PDGF-β or PDGFR-β leads to decreasing of pericytes, vascular dysfunction, and microaneurysm formation, indicating the breakdown of vascular [30, 31].

Transforming growth factor-beta (TGF-β). Various cells secrete TGF-β, including ECs, pericytes, neurons, and glia. Deficiency of TGF-β receptors ALK-1, TGFR-1, TGFR-2, and ENG leads to arteriovenous malformation [32]. But the molecular basis of this pathway has not been understood. TGF-β modulates differentiation and adhesion of pericytes [33].

14.1.2.2 Chemokines

CXCL12 (also called stromal-derived factor-1) is crucial for recruiting EPCs by binding to receptor CXCR4 on the tip cells [34]. Hypoxia actives hypoxia-induced factor-1α (HIF-1α) leading to the upregulation of CXCL12. CXCL12 promotes revascularization through maintaining activity of CXCR4-positive bone marrow-derived cells. Neural progenitor cells (NPCs) express CXCR4 [35]. Peripheral neurons also express CXCL12 to regulate the neural-vessel arrangement.

Sphingosine-1-phosphate (S1P) binds to receptor S1PR to activate endothelial G protein-coupled chemokine (GPCR), which is associated with barrier function and angiogenesis. It's reported that GPCRs have crosstalk with PDGF receptors and VEGF receptors, and the process is called as GPCR-jacking [36]. GPR124 plays a role in blood-brain barrier (BBB) differentiation [37].

14.1.2.3 Ang and Tie

Ang/Tie system consists of two receptors (Tie-1, Tie-2) and three ligands (Ang-1, Ang-2, and Ang-4). Ang-1 is agonist of Tie-2, and Ang-2 is a competitive Ang-1 antagonist. Tie-1 is no ligand but plays negative effects on Tie-2 [38]. Mural and tumor cells express Ang-1, and tip cells express Ang-2. Decreasing vascular stabilization is one important step in angiogenesis [39]. Ang-1 activates Tie-2 leading to the reduction of EC permeability, the enhancement of vessel stabilization, and the suppression of inflammation [40]. In contrast, Ang-2 results in destabilization of ECs. Ang/Tie system maintains vessel in quiescence. ECs keep in quiescence maintained by Ang-1 activating Tie-2 in trans [39]. Ang-1 regulates mural cells and basement membrane sedimentation, which enhances vessel tightness and thickness. Stimulating by angiogenic factors, Ang-2 released by sprouting ECs regulates separation of mural cells, EC permeability, and EC sprouting [38]. It's reported that the

vascular of Tie-2 deficiency mice is affected, but overexpression Tie-2 mice suffer from venous malformation [41].

14.1.2.4 Ephrins

Ephrins guide angiogenesis by two different signaling. Reverse signaling in ephrin-expressing cells and forward signaling in EPH receptor-expressing cells. Ephrins-B2/EPHB4 (Ephrins-B2 receptor) regulates vessel morphogenesis [42]. The reverse signaling of Ephrins-B2 in tip cells triggers VEGFR-2 internalization, which is essential to activate tip cell filopodia extension [43]. Moreover, Ephrins-B2 facilitates recruitment of mural cells and bone marrow-derived EPCs. Ephrins-A1/EPHA2 regulates vessel growth and maturation [44].

14.1.3 Angiogenesis After Ischemic Stroke

Under normal condition, oxygen-sensing enzymes prolyl hydroxylase domain (PHD) proteins 1–3 can hydroxylate hypoxia-inducible factor 1α and 2α (HIF-1α and HIF-2α) [45]. But under the ischemia condition, hypoxia makes PHDs inactive, and HIF increases angiogenic factors (VEGF), which improve angiogenesis upregulating oxygen supply [46]. It's reported that reduced HIF-1α in mice decrease revascularization and angiogenesis in ischemic tissue [46]. HIF-1α also can indirectly activate SDF-1α to recruit bone marrow-derived cells.

Cerebral blood flow (CBF) increasing results from enhanced collateral flow in the early ischemic phase and angiogenesis in the late ischemic phase [47]. Ischemic stroke leads to endogenous angiogenesis, and methods to promote angiogenesis reduce ischemic injuries [48]. Aging is a key factor of angiogenesis. In rodent hippocampus, angiogenesis weakens with aging [49].

14.1.4 Strategies to Promote Angiogenesis After Ischemic Stroke

14.1.4.1 Gene Therapy

Recombinant human VEGFs are intravenously injected to ischemia stroke rats. Acute stage administration of VEGF (1 h after MCAO) results in increasing of blood-brain barrier (BBB) leakage, hemorrhagic transformation, and ischemic lesions. But administration of VEGF in 48 h after MCAO leads to increased angiogenesis in ischemic penumbra and improved neurological recovery. These results indicate that inhibition VEGF in acute stage and increased VEGF later can enhance angiogenesis as well as promote stroke recovery [50]. Intracerebroventricular

administration of VEGF in 1–3 days after MCAO stimulates angiogenesis in the striatum ischemic penumbra [51]. With VEGF protein continuously infusing by a mini-pump for 2 weeks, adenoviral Ang-2 are injected into caudate/putamen. VEGF protein with Ang-2 increases numbers of microvessel to promote angiogenesis after 2 weeks. However, the combination destructs BBB by increasing MMP-9 and inhibiting occludens-1 [52]. Pre-administrated with adenoviral Ang-1 before MCAO can decrease vessel leakage resulting from infusing of VEGF in ischemic mice [53]. Uncontrolled VEGF expression may cause bad effects, such as tumor growth or brain hemangioma. HIF-1 binds to hypoxia-response element (HRE) to regulate gene expression. Using a concatemer of nine copies (H9) of HRE mediates hypoxia induction. In AAVH9-VEGF-transduced ischemic mice, VEGFs express in neurons and astrocytes, but not in endothelial cells. Compared with AAVH9-lacZ ischemic mice, increased microvessels or enlarged microvessels are observed in AAVH9-VEGF-transduced ischemic mice, as well as better CBF recovery [54].

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is distributed in neurons and glia. HB-EGF is triggered by ischemia and can promote neurogenesis. Adenovirus and HB-EGF are injected into lateral ventricle of rats after MCAO 3 days. Eight days later, enhanced angiogenesis is observed in the periinfarct striatum [55].

Hepatocyte growth factor (HGF) via virus was injected into the cerebrospinal fluid of ischemic rats, which promoted angiogenesis and reduced infarcted area without cerebral edema or BBB destruction [56]. ECs with upregulated c-met (HGF receptor) expression are more responsive to HGF and have higher rate of morphogenesis. Antibodies of c-met can directly inhibit angiogenesis in vitro [57]. Transferring HGF gene into subarachnoid space of bilateral carotid arteries occlusion rats enhances angiogenesis on brain surface and reduces cerebral blood flow (CBF) impairment [58].

14.1.4.2 Stem Cell Therapy

Human bone marrow stromal cells (*h*MSCs) are intravenously injected into rats after stroke, which increases secretion of VEGF and expression of VEGFR-2 and also promotes angiogenesis in ischemic boundary zone. Mouse brain-derived endothelial cells (MBDECs) are incubated with supernatant of *h*MSCs cultured with anti-VEGFR-2, and ***h***MSC-induced capillary-like tube formation is inhibited [59]. After intravenously infuse autologous MSCs into ischemic stroke patients, serial evaluations show no adverse effects in these patients. It's suggested that transplantation of autologous MSCs provides a feasible and safe strategy to improve functional recovery for stroke patients [60]. Intracerebral infusion of peripheral blood hematopoietic stem cells ($CD34^+$) also can improve neurological function in rats after chronic cerebral ischemia. PBSCs differentiate into glial cells, neurons, and endothelial cells. In the ischemic hemisphere, increased new vessels promote local cortical blood flow after PBSC injection, and expression of neurotrophic factors (CXCL12, VEGF, and BDNF) also increases [61]. MSCs can decrease blood-brain

barrier leakage and increase Ang-1/Tie-2 in ischemic border. Coculture experiments are performed to investigate the mechanism of MSC-induced angiogenesis. Mouse brain endothelial cells cultured with supernatant of MSC show increased Ang-1/Tie-2 and Flk1. Astrocytes cultured with supernatant of MSC also secrete more VEGF and Ang-1/Tie-2. Incubating ECs with conditional medium from MSCs, MSCs cocultured with ECs, and astrocytes promote capillary tubelike formation. MSC-induced tube formation can be inhibited by the inhibition of Flk1 and Ang-1 or the knockdown of Tie-2 in EC [62].

14.1.4.3 Ischemic Postconditioning

The initial definition of ischemic postconditioning was a series of brief mechanical occlusion and reperfusions in myocardial ischemia research [63]. Postconditioning in ischemic stroke includes various stimulations, including a series of reperfusion, brief ischemia or hypoxia, and using pharmacological, neurotoxic agent [64].

It is reported that mechanical interruption of reperfusion reduces the ischemic injury. Ischemic stroke model was established by permanent distal MCAO added with transient bilateral CCAO. Two cycles of ischemic postconditioning carried after 30 s of CCAO, which result in decreased infract volume, reduced apoptosis in penumbra and superoxide products. Meanwhile, cerebral blood flow indicated that early hyperemia after reperfusion is disturbed by postconditioning [65]. Delayed postconditioning also has beneficial effects on ischemic stroke. Delayed postconditioning protected CA1 neurons in 10-min ischemia, but the protective effect missed in 15-min ischemia [66]. However, postconditioning was good at less-sensitive neurons in the cortex and striatum of 15-min ischemia [67]. The protective effects kept up to 2 months after ischemic stroke. Delayed postconditioning also improved metabolism and reduced edema and leakage of BBB [68]. As for the global cerebral ischemia, postconditioning of three cycles of 15 s release/15 s occlusion, three cycles of 30 s release/30 s occlusion, and 15 s release/15 s occlusion followed by 45 s reperfusion resulted in reduced neuronal loss and decreased deficits of spatial learning and memory [69]. Comparing ischemic postconditioning with gradual reperfusion and precondition, gradual reperfusion resulted in decreased infarction, and protection effects of postconditioning depend on numbers of cycle, duration and onset time. The effects of postconditioning were comparable to rapid preconditioning but were weaker than delayed preconditioning [70]. Experiments performing in vivo and in vitro indicated that postconditioning-induced prolonged phosphorylation of ERK, MAPK, and Akt and phosphorylation of Akt was inhibited by LY294002, an inhibitor of PI3K [71]. Wortmannin, an inhibitor of PI3K, reduced the beneficial effects of ischemic postconditioning [72]. Other papers validated Akt contributing to protection of postconditioning. EPKC (a survival-promoting pathway) activity was declined, and δPKC (death-promoting pathway) was reduced following ischemic postconditioning [73]. With organotypic hippocampal slices exposing to 30-min OGD, beneficial effects of DHPG postconditioning raised on

15 min after OGD, and there were no additive with DHPG preconditioning. This process is associated with mGlu1/mGlu5 receptor-PI3K-Akt pathway [74].

Angiogenesis naturally occurs following cerebral ischemia and can be enhanced by various interventional strategies. Nowadays survey has provided promising data that stimulating angiogenesis after ischemic stroke can improve functional recovery. The genetic therapy allows appropriately upregulated cytokine expression and/or transplanted cells that are suitable for revascularizing ischemic tissues. In addition, learning more about factors related to angiogenesis may provide the basis of developing efficient therapeutic interventions to us.

14.2 Neurogenesis After Ischemic Stroke

Neurogenesis is the process that generates neurons from neural stem cells and progenitor cells through proliferation of neural progenitors, fate specification of neural progenitors, maturation, and functional integration of neuronal progeny [63]. It was widely believed that neurogenesis just occurs in embryonic stages. However, it was recently verified that neurogenesis occurs throughout life mainly in two definite area of adult mammalian central nervous system, including subventricular zone (SVZ) of lateral ventricle and subgranular zone (SGZ) of dentate gyrus in the hippocampus [64, 75]. We have good understanding about the biology of neurogenesis. And it could be a potential neuronal replacement strategy after cerebral ischemia.

14.2.1 Neurogenesis in Normal Adult Brain

14.2.1.1 Adult Neurogenesis in the SVZ

SVZ cells include type A, B, and C cells. Type A cells are neuroblasts. Type B cells are astrocyte-separated neuroblasts with striatum and separated neuroblasts with ependymal cells. And Type C cells are proliferating cells.

Neural stem cells in the SVZ generate new interneurons in the olfactory bulb under the physiology state. Stem cells in the SVZ produce transient amplifying cells. And then they differentiate into immature neurons. These immature neurons migrate to the olfactory bulb through the chains of rostral migratory stream (RMS) ensheathed by astrocytes. Finally, these immature neurons radially migrate to outer cell layer and differentiate into granule neurons and periglomerular neurons. Stem cells in the SGZ produce transient amplifying cells.

Endogenous TGF-α are necessary for proliferation of neural progenitor cells in SVZ, and senescence results in lengthening cell cycle, which may lead to reduction of proliferation of neural progenitor cells by aging [76]. Exogenous FGF-2 and EGF increase the number of neural progenitor cells in SVZ. FGF-2 raises newborn neurons in olfactory bulb. However, EGF decreases newborn neurons and promotes

astrocytes in olfactory bulb. Meanwhile, EGF also facilitates newborn cells in the striatum through increased migration from SVZ and stimulated local neural progenitor cells [77]. Removal of Shh signaling reduces proliferation of neural progenitor cells in SVZ, which indicates that Shh is important in maintaining progenitor cells in SVZ [78].

BMP can inhibit neurogenesis by blocking generation of neurons in SVZ. Noggin is an/the antagonist of BMP. Purified Noggin enhances neurogenesis and inhibits glia differentiation. And ectopic Noggin results in increased neuronal differentiation when grafted SVZ cells into striatum. SVZ cells express BMP, but Noggin expressed in ependymal cells adjacent to SVZ can block BMP in SVZ. It creates neurogenic environment in SVZ [79].

NCAM-mutant mice, which loss PSA, result in interruption of tangential migration of interneurons in olfactory bulb, but not influence its radial migration. PSA-positive cells provide migration substrate for SVZ-derived cells [72]. Infusion of EphB2 or ephrinB2 into the lateral ventricle disrupts migration of neuroblasts and increases proliferation [9]. Anti-DCC antibodies alter protrusion direction, further change the orientation of migration, and decrease the speed [80]. Slit1/2-deficient mice lead to small altered chains of SVZ cells into corpus callosum [81].

14.2.1.2 Adult Neurogenesis in the Dentate Gyrus of the Hippocampus

Neural stem cells in the SGZ produce new granule neurons in the dentate gyrus of the hippocampus. It then differentiates to immature neurons. These immature neurons migrate into granule cell layer and extend axonal projection to the molecular layer. Lastly, new granule neurons integrate into local circuit.

Adult rat hippocampus and its neural progenitor cells both have high expression of Shh. Delivering Shh cDNA via adeno-associated viral to the hippocampus elevates the proliferation of neural progenitor cells. And its proliferation can be reduced by inhibition of Shh in vivo [82]. Astrocytes in the hippocampus instruct stem cells to neuronal fate, which indicates that astrocytes play regulatory roles other than supportive roles [83]. In the SGZ, the dividing cells are close with blood vessels and express endothelial markers. It's evident that angiogenic niche in required for neurogenesis [84]. Astrocytes in the hippocampus and dentate granule cells secrete neurogenesin-1, a factor that can induce neuronal differentiation in the hippocampus [85].

14.2.2 Neurogenesis After Ischemic Stroke

Transient global ischemia results in the loss of CA1 pyramidal neurons in the hippocampus. In adult gerbils, 1–2 weeks after bilateral common carotid artery occlusion, 5-bromo-2′-deoxyuridine-5′-monophosphate (BrdU), a mitotic indicator, increases compared with sham group in dentate gyrus, and it co-localizes with

MAP-2. They migrate to granule cell layer in 26 days. And the newborn neurons survive for 7 months. Ischemic preconditioning, a strategy to protect CA1 neurons, can't prevent the enhanced neurogenesis [86]. Newborn neurons also can migrate to CA1 pyramidal neurons [87]. The enhanced neurogenesis in the hippocampus after ischemia can be visualized by retroviral vectors expressing enhanced green fluorescent protein (EGFP) [88]. In rat, over 60% of new neurons expressed calbindin, a calcium-binding protein which is expressed in mature granule neurons after 5 weeks of transient global ischemia [89]. The proliferation of progenitor cells in SGZ decreases in older rats [90]. It's confirmed that global ischemia stimulates neurogenesis in SVZ, SGZ, and temporal neocortex in young adult macaque monkeys [91]. It's also reported that regenerated neurons can integrate into the existing local circuitry and promote functional recovery [94].

Transient MCAO also can lead to increased proliferation of neural progenitor cells in SVZ, and generated neuroblasts migrate from SVZ to periinfarct striatum. Most of newborn neurons express markers of medium-sized spiny neurons, the most striatal neurons lost after MCAO [92, 93]. The newborn GABAergic and cholinergic neurons possess synaptic structure including dendrites and spines. These new neurons can fire action potential and receive excitatory and inhibitory inputs [94]. These newborn striatal neurons form projection to substantia nigra, an output area of striatal neurons. And they express receptors of glutamate NR2 and dopamine D2L, which indicates that they can receive inputs from cortical glutamatergic neurons and nigral dopaminergic neurons same with striatal neurons [95]. Neurogenesis also can occur in the cortical of ischemic boundary zone after transient MCAO. BrdU-positive cells co-localized with MAP-2, TuJ1, and NeuN in 30 and 60 days after MCAO [96].

14.2.3 Strategies to Promote Neurogenesis After Ischemic Stroke

14.2.3.1 Gene Therapy

Intracerebroventricular injection of VEGF increases BrdU in SVZ and SGZ, and it co-localized with immature neurons, mature neurons, astrocytes, and endothelial cells. And immature neurons also co-localized with VEGFR-2/Flk1. It's implicated that VEGF is not only an angiogenesis factor but also a neurogenesis factor [97]. Intraventricular administration of FGF-2 gene enhances proliferation of progenitor cells in SVZ, SGZ, and the cortex, and some of them differentiate into neurons. But the influenced area is limited in SVZ when FGF-2 protein is infused [98]. Transgenic mice deficiency with FGF-2 gene exists reduced BrdU in the hippocampus after global ischemia. But the number of BrdU increased after intraventricular injection with FGF-2 gene. It suggests that endogenous FGF-2 is sufficient to proliferation and differentiation of neural progenitor cells in the hippocampus [99]. Intraventricular injection of EGF enhances the neuronal replacement in striatum. So it can be a

strategy to promote repair after ischemia [100]. Intracerebroventricular injection of heparin-binding epidermal growth factor-like growth factor (HB-EGF) after 1–3 days of focal cerebral ischemia decreases infarct volume and neurological deficits, increases BrdU in SVZ and SGZ, but reduces migration of neuroblasts from SVZ to striatum; the possible cause is that HB-EGF may change the attractive environment of ischemia [101]. Subcutaneous administration of granulocyte colony-stimulating factor (G-CSF) reduces infarct size and improves motor behavior of rats after MCAO. And G-CSF enhances the ability of circulating hematopoietic cells in neurogenesis [102]. Treatment with erythropoietin (EPO) after 12 h of stroke improves functional recovery. It also increases proliferation of progenitor cells in SVZ and boosts the expression of VEGF and BDNF. EPO can enhance neurogenesis in vitro, and this effect can be inhibited by anti-EPO neutralizing antibody [103].

14.2.3.2 Stem Cell Therapy

Intravenous injection of MSC and NO after 24 h of MCAO improves functional recovery, increases proliferation of neural progenitor cells in SVZ and enhances migration of neuroblasts from SVZ to ischemic boundary area [104]. Treatment in rats before 48 h of stroke with human cord blood-derived $CD34^+$ cells promotes neovascularization on ischemic area, which enhances neuronal regeneration [105].

14.2.3.3 Rehabilitation

Constraint-induced movement therapy (CIMT) indicates using affected limb along with restricting unaffected limb, which can promote behavioral recovery after stroke [106]. Restricting unaffected limb with a plaster cast after 14 days of MCAO enhances neurogenesis. BrdU-positive cells in SVZ and DG increase in the CIMT-treated group. And the protein of CXCL12 in the cortex and DG also upregulate [107]. It's also confirmed in aged rats after stroke induced by injecting endothelin-1. Forced limb use also suppresses apoptosis of neurons and increases long-term survival of newborn neurons in SVZ and the hippocampus [108]. Social interaction is a common strategy of rehabilitation. It can improve life quality and decrease mortality after stroke. Compared with isolation group, behavioral recovery increased, and mortality reduced in the mice that are caged paired with healthy partners with increased BDNF and enhanced neurogenesis [109].

14.2.3.4 Enriched Environment

Postischemic-enriched environment increases neuronal differentiation in DG and restores neuroblasts in DG; NeuN-positive cells are also enhanced in ischemic penumbra compared to the rats caged in the normal environment, which indicates that it promotes neurogenesis in DG after stroke [110]. Enriched environment increases

the proliferation of NSC/NPC in SVZ [111]. However, the increased newborn cells after stroke is not observed in rats caged in the enriched environment. Newborn astrocytes, neuroblasts, and reactive astrocytes are also reduced in SVZ. Meanwhile, enriched environment has no effects on infarct area and mortality [112]. Combining retinoic acid, which can stimulate neurogenesis in SVZ and the hippocampus, with enriched environment, neurogenesis in SVZ is enhanced. But it does not affect behavior function after stroke [113]. Further study about integration of newborn neurons in the striatum is necessary.

14.2.3.5 Optogenetics

Using optogenetics technique specifically manipulates neurons or other cells in the brain after stroke, to reveal the cell type and mechanisms of impairment and develop strategies for stroke therapy. The adult channelrhodopsin-2 (ChR2) transgenic mice are subjected to ischemia. Mice treated with photo-stimulation in the cortex show better behavior after 31 days of stroke. And optogenetics stimulation enhances neurogenesis in mice [114]. However, in the mice which receive lentivirus carrying NpHR under CaMKII promoter in striatum, photo-stimulation increases BrdU- and DCX-positive cells in SVZ and promotes nestin-positive cells in SVZ as well as periinfarct area. The NeuN-positive cells also increase in the mice received photo-stimulation. And it is consistent with the neurobehavioral test [115].

More efforts need to be devoted to explore the nature of neurogenesis in normal condition, because the limited neurogenesis after ischemic stroke. We can get more information about neurogenesis relying on new tool, such as optogenetics. More investigations should be focused on the molecular pathways and the niche of ischemia. It's the basis of increasing proliferation of neural progenitor cells, maintaining survival of newborn neurons and enhancing functional integration of newly generated neurons, which may contribute to the behavior recovery after stroke.

14.3 Neurovascular Remodeling After Ischemic Stroke

Neurovascular coupling is a term of spatial and temporal relationship between neural activity and cerebral blood flow (CBF) [116]. Revealing activation of the brain by mapping cerebrovascular changes is based on neurovascular coupling [117]. The increase of cerebral blood flow induced by neural activity is a result of coordinated effects including multiple mediators, such as neurons, glia, endothelial cells, pericytes, and smooth muscle cells. Active neurons and glia release vasoactive factors. They target to endothelial cells, pericytes, and smooth muscle cells and finally increase CBF [118].

14.3.1 Neurovascular Injuries After Ischemic Stroke

Ischemia stroke disrupts the normal circulation of cerebral blood flow. Postischemic hyperemia indicates a transient increase of flow when reperfusion, followed by a stage of postischemic hypoperfusion in which blood flow reduces. Ischemia affects normal autoregulation, and CBF evoked by neural activity is also decreased [119]. The reduction or oxidation of cytochrome a and cytochrome a3 can reveal the mitochondrial oxidative after transient cerebral ischemia. During ischemia, cytochrome a and cytochrome a3 are reduced. It also abolishes electrocortical activity. In 20 min after reperfusion, cytochrome a and cytochrome a3 become hyperoxidized and come to the original stage finally. It's faster than the recovery of electrocortical activity. But the energy required for stimulating the cortex is increased. After 30 min of reperfusion, the oxidative amplitude is increased. Lengthening the demanding time to reduce oxidative response after 2 h of reperfusion, it indicates that residual metabolic dysfunction occurs when exposing to additional work, but not in the resting stage [120].

Transient cerebral ischemia also can alter the metabolic activation in somatosensory circuit. Unilateral whisker stimulation can activate metabolism in whisker barrel circuit and alter glucose utilization in stimulated circuit and non-stimulated relay circuit. In the 30 min severe forebrain ischemia model, whisker stimulation reduces baseline metabolic rate in non-stimulated relay area after 1 day of stroke. In 2–3 days after stroke, increased glucose utilization is decreased in cortical layer, and the response of cortical to metabolic activation is also lessened. The response return to the normal level in 5 days on the activated ventrobasal thalamus and cortical layer. During 5–10 days after stroke, the abnormal laminar pattern in activated region indicates the dysfunction of intracortical circuit. It suggests that the response of CNS to peripheral activation in a reversible and long-lasting process. The functional recovery of stroke is slow and relies on the baseline of metabolic rate [118]. In patients who suffered from unilateral major cerebral artery occlusion, which appears no neurological impairment but deficit in hemodynamic reserve, the neural activity correlate with severity of cortical ischemia. The afferent signal in primary sensory area of lesioned hemisphere is positive related to regional CBF, but secondary response is negative related to it [121]. Repetitive spontaneous periinfarct spreading depolarization is one of ischemic depolarization. It can increase infarct size, which may be caused by increased metabolic demand followed by mismatch of CBF and metabolism. Anoxic depolarization and repetitive spontaneous periinfarct spreading depolarization lead to vasoconstriction and reduced CBF in ischemic cortex. Therefore, it's possible that downregulating vasoconstriction neurovascular coupling with inhibitor of cortical spreading depression is neuroprotective after ischemic stroke [119]. The neurovascular coupling is different in the various grades of global ischemia. Compared with mice who suffered unilateral carotid artery occlusion, bilateral carotid artery occlusion, bilateral carotid and right subclavian artery (SCA) occlusion, or carotid and SCA occlusion, the amplitude of functional metabolic response correlate with amplitude of somatosensory evoked potentials (SEPs)

in the same level stroke. However, with increased severity of ischemia, the reduction of blood flow is stronger than electrical response [120].

Functional magnetic resonance imaging (fMRI) is based on similar relationship of blood-oxygen-level dependent (BOLD) and neural activity. But ischemic stroke alter the BOLD signal and cerebrovascular reactivity in impaired region. BOLD signal is symmetrical in motor task and hyperventilation. However, the changed cerebrovascular reactivity indicates injury in contralateral primary sensorimotor cortex [122].

14.3.2 Strategies to Promote Neurovascular Remodeling After Ischemic Stroke

Insulin growth factor-1 (IGF-1) has neuroprotective effects against acute ischemic stroke. It has not only have neurotrophic properties but also has the ability of releasing angiogenic factors. Overexpressing IGF-1 with AAV followed by exposing mice to permanent distal MCAO improves behavior performance and reduces infarct volume. Additionally, neovessel formation is increased in the periinfarct area, and then vascular perfusion is enhanced. It also promotes neurogenesis in SVZ [121]. Postischemic administration of IGF-1 also promotes angiogenesis and neurogenesis in periinfarct area in the chronic stage if stroke [123]. So IGF-1 can enhance neurovascular remodeling and stroke recovery.

Inhibition of reactive astrocytes suppresses neurovascular remodeling and functional recovery of stroke. Inhibiting astrocytes with fluorocitrate reduces GFAP-positive cells in periinfarct cortex and worsens behavior deficits after ischemic stroke [124].

High-mobility group box 1 (HMGB1) is a member of the damage-associated molecular pattern (DAMP) family. It mediates crosstalk between reactive astrocytes and endothelial progenitor cells (EPCs) after ischemic stroke. Conditional media from reactive astrocytes increase proliferation of EPCs in vitro, but the inhibition of HMGB1 can prevent this effect. HMGB1 is increased in periinfarct area after 14 days of focal cerebral ischemia, and EPCs are accumulated associated with HMGB1. Blockage of HMGB1 can abolish the response of EPCs and reduce angiogenesis in periinfarct area, along with worsening neurological deficit. So, it is confirmed that crosstalk between reactive astrocytes and EPCs, mediating by HMGB1, can promote neurovascular remodeling and functional recovery after stroke [125].

Exosomes are small-membrane vesicles with the diameter between 30 and 100 nm. Exosomes carry proteins, functional messenger RNAs, and microRNAs (miRNAs) released by various cell types. Increasing evidence indicates that exosomes play an important role in communication between cells. Exosomes from MSC enhance neurovascular plasticity and functional recovery after ischemia stroke. Injecting proteins of exosomes generated by MSC before 2 h of MCAO can increase axonal density in ischemic boundary of the cortex and striatum. And the

number of neuroblasts and endothelial cells is increased. Therefore, exosomes provided a novel strategy for the treatment of ischemic stroke [126].

Activating CBF during neural activity is the cooperation of various cells, including neurons, glia, and vascular cells. However, it needs further study about molecular mechanism of this complex process. More improved imaging techniques provide opportunity for revealing its mechanism, for instance, two-photon confocal microscopy and fMRI. The abnormal condition of cerebral vessels may be a novel therapeutic target for ischemic stroke.

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Chapter 15
MicroRNA Biomarkers for Stroke

Xuejing Zhang, Ping Sun, and Ke-Jie Yin

Abstract Stroke is one of the leading cause of death and major cause of long-term disability in the United States. Over years, extensive efforts have been focusing on the development and improvement of diagnostic and therapeutic strategies to reduce stroke-associated neurovascular damages, such as neuronal death, blood-brain barrier (BBB) dysfunction, and brain edema. However, the only clinically approved pharmacological therapy to date for treatment of acute ischemic stroke is still thrombolysis. This is mainly due to the short therapeutic window and the activation of various pathophysiological signaling cascades triggered after ischemic stroke. MicroRNAs (miRNAs) are small sequences of non-protein-coding RNA (~19 to 25 nt) with a variety of gene regulation functions in eukaryotic cells. Ever since being first reported in the pathogenesis of stroke in 2007, miRNAs have emerged as key mediators of posttranscriptional gene silencing in the pathogenesis of ischemic stroke. Also, preclinical and clinical studies have documented miRNAs as interesting novel drug targets, which led to the development of miRNA mimics and antagomirs as miRNA-based therapies. Stroke also alters miRNA expression profiles in the circulation system, and stroke-associated miRNAs have been proposed as potential diagnostic and prognostic biomarkers in ischemia stroke. In this chapter, we summarized current knowledge about miRNAs and cerebral ischemia, focusing on the role of miRNAs as biomarkers for stroke and as targets for regulating large sets of genes in related pathways after ischemic stroke.

Keywords Angiogenesis • Apoptosis • BBB integrity • Biomarker • Brain • Cerebral ischemia • Circulating • Early detection • Gene expression • Inflammation • MCAO • MicroRNA • miRNA • Neuroprotection • OGD • Quantitative PCR • Stroke • Translational repression

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Abbreviations

AMI	Acute myocardial infarction
BBB	Blood-brain barrier
DGCR8	DiGeorge syndrome critical region gene 8
HCM	Hypertrophic cardiomyopathy
HCMV	Human cytomegalovirus
HF	Heart failure
HIF	Hypoxia-inducing factor
LNA	Locked nucleic acids
MCAO	Middle cerebral artery occlusion
miRNA	MicroRNA
MMP	Metallopeptidase
mRNA	Messenger RNA
NIHSS	National Institutes of Health Stroke Scale
OGD	Oxygen-glucose deprivation
PC	Preconditioning
PH	Pulmonary hypertension
PPAR	Peroxisome proliferator-activated receptor
Pri-miRNA	Primary miRNA
RISC	RNA-induced silencing complex
RT-qPCR	Real-time quantitative reverse transcriptase polymerase chain reaction
SS	Single-stranded
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VNS	Vagus nerve stimulation

15.1 Introduction

Ischemic stroke is caused by transient or permanent local reduction of cerebral blood flow and is characterized by a set of cellular disturbances. Although many clinical stroke trials have been conducted, the only effective treatment to date is thrombolysis [1]. There are several reasons which limit the development of effective therapies in the clinical setting: the rapid development of brain injury following ischemia, the complex interactions among signaling pathways, and the relatively short treatment window for specific targets [2]. Efforts have been focusing on exploring protein biomarkers such as C-reactive protein (CRP), interleukin-6 (IL-6), matrix metallopeptidase 9 (MMP9), vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1) as additional diagnostic tools [3]. However, their specificity and ability to distinguish acute stroke and its associated risk factors or stroke mimics are uncertain [4]. Recently, RNA-based studies

have suggested messenger RNAs (mRNAs) as promising biomarkers since changes in gene expression are reflected in the peripheral blood RNAs of stroke patients. Blood mRNA profiles could distinguish transient ischemic attack from control samples and, therefore, serve as genomic biomarkers in ischemic stroke conditions and as signature for stroke subtypes [5–7].

MicroRNAs (miRNAs) are non-protein-coding RNA molecules that negatively regulate protein expression by several mechanisms in various organisms [8–10]. MiRNAs recognize and bind to the complementary sequences on 3′ untranslated regions (3′-UTR) of target mRNAs, leading to mRNA cleavage, mRNA degradation, and translational repression in a sequence-specific manner [9, 11, 12]. It is now well accepted that miRNAs regulate expression of at least one-third of the human genome and play pivotal roles in various normal biological processes, such as cell differentiation, development, metabolism, and apoptosis [12, 13]. Due to their functions in regulating gene expression, miRNAs become fascinating candidates for disease biomarkers, since changes in their expression patterns are oftentimes detected even before the phenotypic appearance of disease [14]. The first evidence that miRNAs could be used as diagnostic biomarkers came from a lymphocytic leukemia study in year 2002 by Calin et al. [15]. Calin et al. made the connection between the frequently deleted 13q14 locus and the downregulation of the miR-15a/miR-16 cluster that is encoded within this region in chronic lymphocytic leukemia patients [15]. Ever since then, circulating miRNA expression patterns have been reported in different preclinical and clinical studies as unique disease signatures [16]. Altered miRNA expression profile has also been found in brain tissue and blood in experimental rat stroke model [17]. Besides, altered circulating miRNA expression profile has been found in stroke patients as well as in different stroke subtypes [18]. Therefore, circulating miRNAs are considered to be promising biomarkers of ischemic stroke. In this book chapter, we will summarize ischemic stroke-related miRNAs and the pathological contribution of miRNAs in cerebral ischemia and discuss their potential use as biomarkers and potential therapeutic targets.

15.2 MicroRNA Overview

MiRNAs are single-stranded (ss), non-protein-coding RNA transcripts that are approximately 19–25 nucleotides in length. They regulate gene expression through binding to the 3′-UTR of target mRNAs in a sequence-specific manner [19]. MiRNAs are involved in various biological processes, and alterations in miRNA expression profiles have been observed in numerous pathological processes in various organisms [20]. Therefore, miRNAs have become interesting study subjects given their great potential of both novel biomarkers and targets for therapeutic intervention. The first miRNA, lin-4, was identified in the nematode *Caenorhabditis elegans* by Lee et al. back in 1993 [21]. The significance of this finding became more evident several years later when a second miRNA, let-7, was also identified

[22]. It was then reported that both Lin-4 and lin-7 were highly conserved in eukaryotes, suggesting they might both play important functional roles [23, 24]. With the help of bioinformatics and molecular cloning approaches, thousands of rodent and human miRNAs have now been identified, although functional validation has not yet been established in each case.

15.2.1 Production of MicroRNA

As shown in Fig. 15.1, biogenesis of miRNAs begins with RNA polymerase II-mediated transcription of miRNA genes into primary transcripts, also known as primary miRNAs (pri-miRNAs). Pri-miRNAs have a distinctive stem-loop structure, and they may vary in length (sometimes several kilobases long). Pri-miRNAs are further cleaved into precursor miRNAs (pre-miRNAs) within the nucleus by protein complex consist of ribonuclease III (RNase III) enzyme Drosha and its cofactor DiGeorge syndrome critical region gene 8 (DGCR8). To be specific, DGCR8 binds to the base of the stem-loop structure of pri-miRNAs at a specific distance from the ssRNA-double-stranded (dsRNA) junction. Then, DGCR8 directs RNase III Drosha to the site and cleaves the pri-miRNA 11 nucleotides from the ssRNA-dsRNA junction to form a 70–100 nt pre-miRNA [25, 26]. Pre-miRNAs can be recognized by dsRNA-binding protein exportin-5 through their distinctive hairpin structure. They are exported from nucleus to cytoplasm by exportin-5 through Ran-GTP-dependent mechanism. Once pre-miRNAs are in the cytoplasm, they get further cleaved to their mature length (~22 nt) by another protein complex that consists of RNase III enzyme dicer and TAR RNA-binding protein (TRBP), which recognizes dsRNA and guides dicer to the correct cleavage site. Cleaved pre-miRNAs that form miRNA/miRNA* duplex consist of two ss miRNAs: the guide strand (miRNA) and the passenger strand (miRNA*). One of the two miRNA strands, along with argonaute (AGO) proteins, forms the RNA-induced silencing complex (RISC). The RISC-miRNA complex then binds to the 3′-UTR of its target mRNA through classical Watson-Crick base pairing. As a result, perfect sequence complementarity between miRNA and target mRNA leads to mRNA cleavage by AGO. On the other hand, imperfect pairing leads to translational repression and/or degradation of the mRNA target [27, 28].

15.2.2 Functional Mechanism of MicroRNA-Mediated Translational Repression

Mechanistic studies of miRNA-mediated translational repression have reported that miRNAs repress protein expression in four distinct ways: (1) cotranslational protein degradation, (2) inhibition of translation elongation, (3) premature termination of

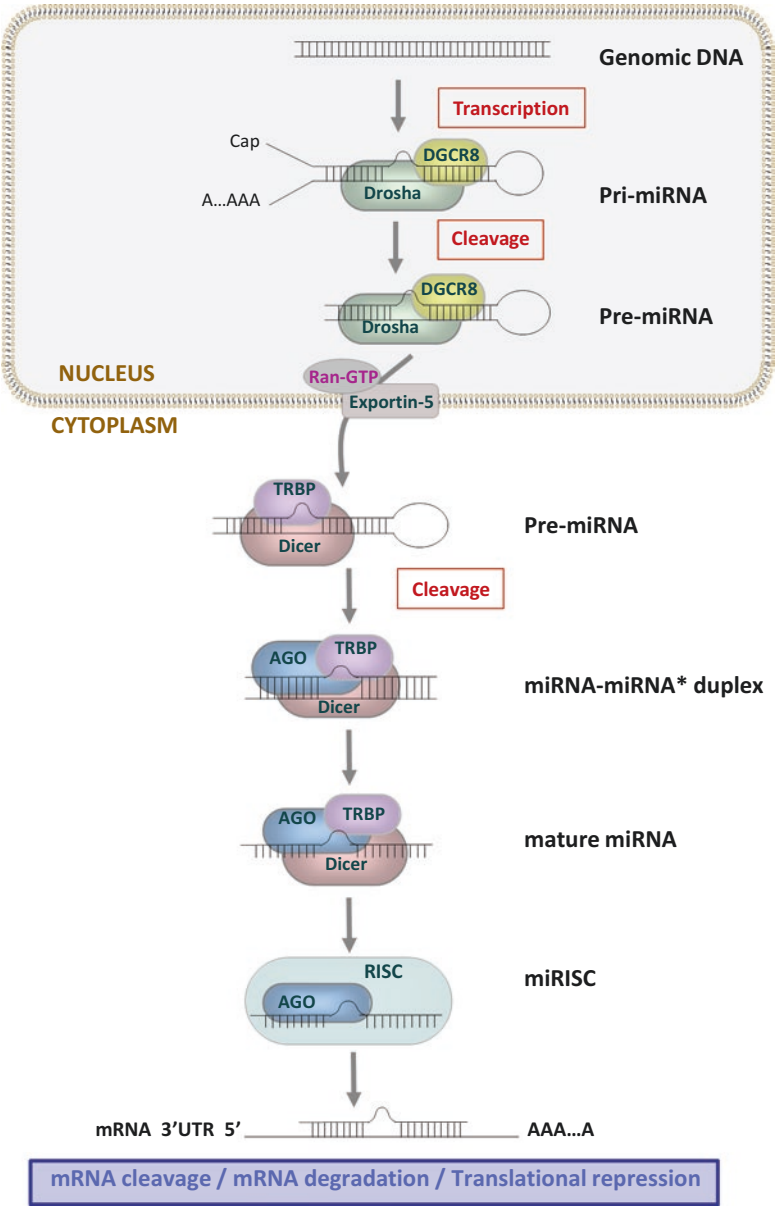

Fig. 15.1 Biogenesis and functional mechanism of microRNA
In the nucleus, miRNAs are transcribed from genomic DNA by RNA polymerase II to generate long primary transcripts (pri-miRNAs). Pri-miRNAs are subsequently cleaved by the RNase III enzyme (Drosha) and its binding partner DiGeorge syndrome chromosomal region 8 (DGCR8) to form precursor hairpin miRNAs (pre-miRNAs). Pre-miRNAs are then exported into the cytoplasm by exportin-5 through Ran-GTP-dependent mechanism. Pre-miRNAs are further cleaved to its mature length (~22 nt) by the RNase III enzyme (Dicer) in complex with the double-stranded RNA-binding protein TRBP. Argonaute (AGO) proteins unwind the miRNA duplex and help form RNA-induced silencing complexes (RISC). Then miRNAs guide RISC to complementary sites of the target mRNAs, initiating degradation or cleavage of mRNA

translation (ribosome drop-off), and (4) inhibition of translation initiation which are well summarized in [29]. The targets of miRNAs appear to be actively translated, while the corresponding protein product remains undetectable, suggesting that nascent polypeptide chain might be degraded cotranslationally [30]. Peterson et al. [31] designed a synthetic miRNA reporter containing a 3′-UTR with six identical sites partially complementary to a transfected siRNA (a miRNA mimic). When this reporter was transiently expressed, it associated with polysomes, although its expression was repressed by the siRNA. But if translation initiation was inhibited, then these ribosomes dissociated more rapidly than those associated with a control mRNA. This led to the suggestion that miRNAs cause premature ribosome dissociation or ribosome drop-off. Experiments carried out by several other laboratories indicated that miRNAs repress translation at initiation rather than post-initiation steps [32–34]. Observations that mRNAs containing an internal ribosome entry sites (IRES) or a nonfunctional cap are not effectively repressed by miRNAs, suggested that miRNAs block an early step in translation initiation, possibly involving recognition of the 5′-terminal m^7GpppN cap [35].

15.2.3 MicroRNA Mimics and Inhibitors

There are two major approaches to develop miRNA-based therapeutics: mimics to increase effective levels of miRNAs and inhibitors/antagomirs to reduce miRNA expressions. MiRNA mimics are small, chemically modified, double-stranded RNA molecules that load the active strand into the RISC which then bind the target mRNA to induce translational silencing [36]. MiRNA mimics can be used to restore the beneficial function of certain miRNAs. MiRNA inhibitors/antagomirs are modified single-stranded antisense oligonucleotides harboring the full or partial complementary sequence to the mature miRNA to reduce endogenous levels of the miRNA and increase expression of its mRNA targets. MiRNA inhibitors/antagomirs can inhibit endogenous miRNAs; therefore, they could be applied to reduce miRNAs with pathogenic function in cells or tissues. Currently locked nucleic acids (LNA) which contain modified RNA nucleotides are mostly used in clinical trials to modify miRNA antagomirs in specific disease settings [37–39].

15.3 Ischemic Stroke and MicroRNA

MiRNAs are abundantly expressed in the nervous system and regulate neural development and plasticity [40, 41]. Recently, accumulating evidence has also linked dysregulated cerebral miRNA expression profiles to a variety of neurological diseases, including Alzheimer's disease [42, 43], Parkinson's disease [44, 45], amyotrophic lateral sclerosis [46], spinal cord injury [47], traumatic brain injury [48], and

stroke [2, 17, 49, 50]. Thus, the function and potential clinically relevant intervention of unique miRNAs in these neurological disorders begin to be uncovered.

15.3.1 Ischemic Stroke-Associated MicroRNAs

Several groups have shown the involvement of miRNAs in the pathogenesis of ischemic brain injury by using miRNA profiling techniques in rodent middle cerebral artery occlusion (MCAO) model (summarized in Table 15.1) [20, 49, 51]. The first report that demonstrated the potential importance of miRNA dysfunction in the pathogenesis of stroke was published in 2007 [20]. Using brain and blood samples from rats subjected to 24–48 h reperfusion following MCAO, Jeyaseelan et al. carried out miRNA expression profiling by miRNA microarray. The results showed that approximately 106 and 82 miRNA transcripts were identified in MCAO rats' brain after 24 h and 48 h reperfusion, respectively. The miRNAs identified as highly upregulated during the ischemia reperfusion periods (24 and 48 h) included miR-292-5p, miR-290, miR-206, miR-210, miR-214, miR-215, miR-223, miR-298, miR-327, miR-494, and miR-497. Among them, miR-292-5p and miR-290 transcripts showed the highest expression after ischemic injury in the brain at 24- and 48-h reperfusion times, respectively. Several miRNAs highly expressed in the ischemic brain were also detected in blood samples. Moreover, the miRNA profiling results with DNA microarray data further showed that the expression of AQP4, MMP9, transfelin, and VSNL1 genes may be regulated by some miRNAs during the progression of cerebral ischemia.

Dharap et al. [49] employed similar miRNA microarray techniques to profile brain miRNAs in spontaneously hypertensive rats at five different reperfusion times (from 3 h to 3 days) following MCAO. Among the 238 miRNAs investigated in spontaneously hypertensive rats, 3 miRNAs (miR-140, miR-145, and miR-331) showed a significant increase and 5 miRNAs (miR-376b-5p, miR-153, miR-29c, miR-98, and miR-204) showed decreased expression at all five reperfusion time points studied in comparison with the sham group. These miRNAs may mediate inflammation, neuroprotection, receptor function, and ionic homeostasis. In addition to miRNA putative negative regulation of gene translation, the authors also provided evidence that several ischemia-induced miRNAs may bind to the promoter region of target genes to activate their transcription. Importantly, Dharap et al. identified superoxide dismutase 2 (SOD2) as the direct target of miR-145 by informatics software. Infusion of antagomiR-145 to the cerebral lateral ventricle resulted in increased SOD2 protein expression and decreased ischemic infarction area.

In a rat MCAO model, in situ hybridization analysis with LNA probes against miR-146a revealed that stroke increased miR-146a density in the corpus callosum and subventricular zone of the lateral ventricle of the ischemic hemisphere [52]. The authors further confirmed overexpression of miR-146a in neural progenitor cells significantly increased their differentiation into $O4^+$ oligodendrocyte progenitor cells. Wang et al. [53] reported that miR-30a protected neuronal death against

Table 15.1 MicroRNAs in stroke

Identified MicroRNA	Altered expression and contribution in stroke	Targeted mRNAs
Let-7f	Let7f inhibition promotes neuroprotection in rat ischemic stroke model	IGF-1
miR-15a	Inhibition results in cerebral vascular protection in vitro and in vivo	Bcl-2
miR-21	Overexpressing miR-21 protects neurons from ischemic death in vitro	FASLG
miR-23a	Sex difference in miR-23a regulates XIAP in ischemic cell death.	XIAP
miR-26a, miR-34a, miR-145, let-7b	Inhibited IFN-b expression in monocytes-derived macrophages	IFN-b
miR-29b	Increased miR-29b promotes neuronal cell death in vitro	Bcl-2
miR-30a	Prevents neuronal cell death after ischemic stroke	HSPA5
miR-98, miR-340-5p, miR-374	Regulate ischemic tolerance through Notch signaling, ubiquitin-proteasomal system, gap junction and NAD metabolism	Multiple targets
miR-124	Plasma miR-124 increased in rat following MCAO	Unknown
miR-125b	miR-125b inhibition attenuates glial cell proliferation	CDKN2A
miR-126	Decreased mirR-126 after ischemic stroke contributes to cardiact dysfunction	MCP-1
miR-132	Decreased miR-132 expression correlates with increased MeCP2 protein	MeCP2
miR-140-5p	Inhibited angiogenesis by decreasing cell proliferation, migration and tube formation	VEGFA
miR-145	Facilitates proliferation and migration of endothelial progenitor cells	JNK
miR-145	miR-145 inhibition decreases brain infarction	SOD-2
miR-146a	Mediates stroke-induced oligodendrogenesis	IRAK1
miR-181b	Electroacupuncture increases miR-18b levels in the penumbras, improves neurobehavioral function	PirB
miR-181c	Promotes apoptosis of microglia and neurons in acute ischemic stroke	Pro-/Anti-apoptotic proteins
miR-199a-5p	Mediates the neuroprotection of vitamin E α-Tocotrienol against focal cerebral ischemia	MRP1
miR-210	Higher miR-210 expression indicates better stroke outcome in patients	Unknown
miR-320a	miR-320a inhibition reduces brain infarct volume	AQP1, AQP4
miR-331, miR-885-3p	Mediates the neuroprotection of valproic acid in rat MCAO model	Multiple targets
miR-335	Plasma miR-335 was downregulated in acute ischemic stroke patients	Calmodulin

(continued)

Table 15.1 (continued)

Identified MicroRNA	Altered expression and contribution in stroke	Targeted mRNAs
miR-383	miR-383 agomir increased infarct volume and aggravated neurological damage	PPARγ
miR-497	miR-497 inhibition reduces ischemic brain infarction, and improves neurological outcomes	Bcl-2, Bcl-w

ischemic injury in mouse MCAO model. The same group also reported that reduced miR-30a in ischemic mouse increased the heat shock protein HSPA5 level and attenuated ischemic brain infarction [54]. Ma et al. [55] reported miR-181c levels were decreased in stroke patients compared to healthy individuals. MiR-181c reduced proliferation whereas increased the apoptosis of microglial cells and neuro-2a cells. Furthermore, in mouse MCAO model, miR-181c antagomir increased infarct volume, decreased microglia activation and B-cell lymphoma-2 expression, and increased the levels of proapoptotic proteins in the ischemic brain. Chen et al. [56] demonstrated that decreased miR-126 after ischemic stroke played an important role in regulating cardiac function. They found significantly decreased serum and heart miR-126 expression and increased miR-126 targeted genes, vascular cell adhesion protein-1, and MCP-1 in the heart of stroke mice compared to non-stroke mice. Endothelial cell-specific miR-126 knockout mice exhibited significantly decreased cardiac function and increased cardiomyocyte hypertrophy and inflammatory factor expression after ischemic stroke compared to miR-126 knockout control mice. Zhao et al. [57] examined plasma miR-335 expression in patients with acute ischemic stroke and healthy controls. They found that miR-335 levels were significantly lower in stroke patients compared with controls and were negatively correlated with National Institutes of Health Stroke Scale (NIHSS) scores. In another study using rat MCAO model, Deng et al. [58] reported that electroacupuncture increased miR-181b expression in the penumbras and improved neurobehavioral function rehabilitation through miR-181b-mediated regulation of PirB, RhoA, and GAP43 signaling. These findings suggest the potential of miRNAs as candidates for possible biomarkers or therapeutic targets in stroke.

Ischemic preconditioning (PC) is a short duration of ischemia that does not kill neurons; instead it induces tolerance against a subsequent damaging ischemic insult [59]. PC is also shown to alter the miRNA expression in rodent brain [59–71]. Vemuganti lab showed that alterations in many miRNAs expression levels in adult rat brain start as early as 6 h after PC, and some of the alterations can last up to 3 days, indicating their significance in promoting ischemic tolerance [59]. The studies showed upregulation of miR-374, miR-98, miR-340-5p, and miR-21 and downregulation of miR-466c, miR-292-5p, miR-328, and miR-873 following PC that modulated pathways including Notch signaling, ubiquitin-proteasomal system, gap junction proteins, and nicotinamide adenine dinucleotide (NAD) metabolism that promoted ischemic tolerance. A previous study showed differential cerebral miRNA expression profiles between PC (15 min MCAO), ischemia (60 min MCAO), and

ischemia followed by PC (15 min MCAO followed by a 60 min MCAO after 3 days) in adult mice [61]. This study showed that PC decreased the levels of miR-132 and meanwhile increased the levels of its target methyl-CpG-binding protein 2 (MeCP2) which is a transcriptional regulator. MeCP2 was thought to promote the PC-induced ischemic tolerance as MeCP2 knockout mice showed increased neuronal death compared to wild-type controls when subjected to focal ischemia following PC [61]. Oxygen-glucose deprivation (OGD)-induced PC in hippocampal neurons showed increased expression of miRNAs miR-9, miR-21, miR-29b, and miR-132 that were thought to be responsible for the ischemic tolerance [71]. This study is in contrast with the in vivo study by Lusardi et al. [61]. The authors thought that the upregulation of miR-132 mediated neuronal survival probably by targeting p250GAP, a Rho GTPase that decreased neuronal survival and by targeting MeCP2-mediated suppression of the expression of the neuroprotective brain-derived neurotrophic factor [71].

15.3.2 Contributory Role of MicroRNAs in the Pathogenesis of Ischemic Stroke

Cerebral ischemia triggers a cascade of pathological events that ultimately cause irreversible neuronal injury in stroke-affected brain tissue within minutes of stroke insult [1, 72]. The pathophysiological order of the events ranges from excitotoxicity within minutes, a robust inflammatory response within hours, to programmed cell death and tissue loss within hours and days of stroke onset. Several labs have shown that transient focal ischemia in experimental rodent models leads to changes in global expression of cerebral and vascular non-coding RNAs including miRNAs [17, 41, 49, 61, 73–76]. Bioinformatics analysis showed that altered miRNAs regulated the translation of key proteins involved in inflammation, excitotoxicity, oxidative stress, endoplasmic reticulum stress, autophagy, and apoptosis after experimental stroke [19, 49, 77].

15.3.2.1 Apoptosis

Apoptosis plays a major role in tissue loss in ischemic lesions. The therapeutic time window between the onset of stroke insult and apoptosis-induced tissue loss surrounding the ischemic site makes it a very attractive target for stroke therapy [78, 79]. Extensive studies have evaluated the role of miRNAs in poststroke neuronal apoptosis (Fig. 15.2). For example, miR-15a has been shown to contribute to the pathogenesis of ischemic vascular injury through direct inhibition of the anti-apoptotic gene bcl-2 [50]. Gain or loss of miR-15a significantly reduced or increased OGD-induced cerebral vascular endothelial cell death, respectively. Moreover, miR-15a itself was found to be transcriptionally regulated by peroxisome

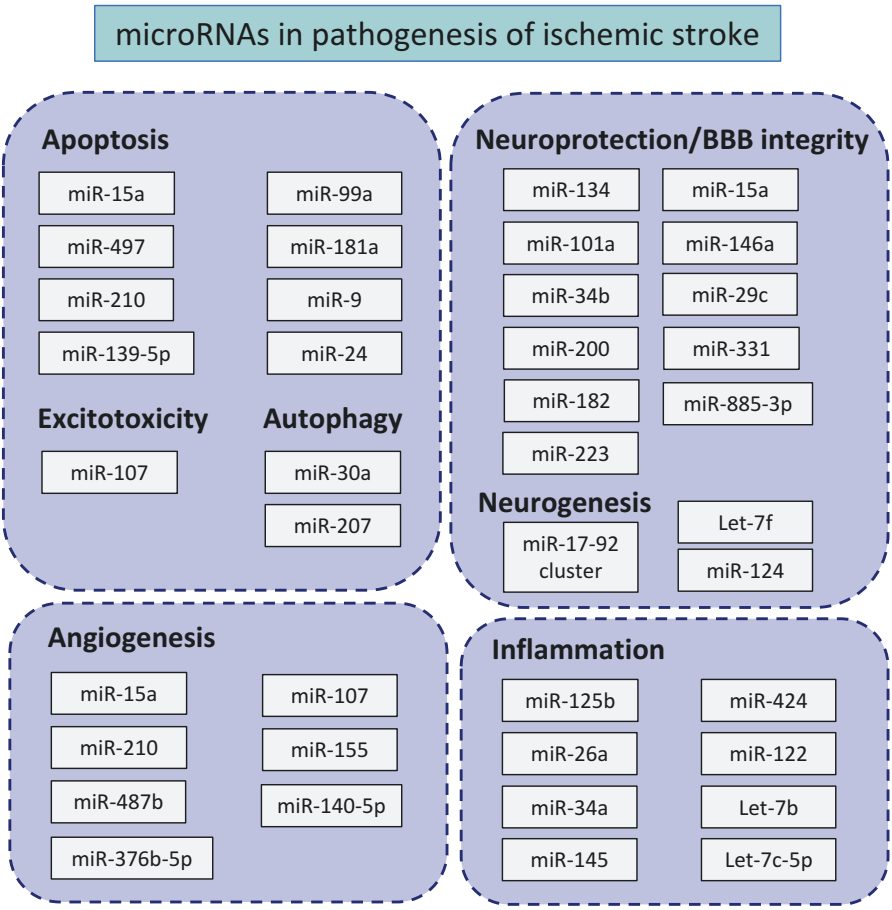

Fig. 15.2 Contributory role of microRNAs in the pathogenesis of ischemic stroke
Dysregulated miRNAs expression profile contributes to pathophysiology of ischemic stroke by mediating common pathophysiological mechanisms including apoptosis, inflammation and glutamate excitotoxicity as well as neuroprotective mechanisms such as neuroprotection, neurogenesis and angiogenesis

proliferator-activated receptor δ (PPARδ). Intracerebrovascular infusion of a specific PPARδ agonist significantly reduced ischemia-induced miR-15a expression, increased bcl-2 protein levels, and attenuated caspase-3 activity, leading to decreased blood-brain barrier (BBB) disruption and reduced cerebral infarction in mice after transient focal cerebral ischemia [50]. Another bcl-2-targeting miRNA, miR-497, was found to be induced in mouse brain after transient MCAO. MiR-497 was demonstrated to directly hybridize to the 3′-UTR target sites of bcl-2 and inhibit bcl-2 translation. In addition, in vivo repression of miR-497 by antagomirs was found to effectively decrease miR-497 levels, reduce MCAO-induced infarction, and improve

neurological deficits with a corresponding increase in bcl-2 protein [2]. MiR-210 was found to mediate neuroprotective actions of vagus nerve stimulation (VNS) in a rodent model of transient MCAO by targeting antioxidant and anti-apoptotic components [80]. In a hypoxia-ischemia (HI) rat model, miR-139-5p was shown to target human growth and transformation-dependent protein (HGTD-P) which was a promoter of neuronal apoptosis [81]. Downregulation of miR-139-5p was also observed in rat cortical neurons subjected to OGD as well as in rat brain after HI [81]. Treatment with agomiR-139-5p decreases cerebral HGTD-P expression and infarct volume in adult rats following HI [81]. In another study, Bcl2 was found to be a target of miR-181a following stroke in a rodent model of forebrain ischemia [82]. Pretreatment with antagomiR-181a led to a significant decrease in hippocampal neuronal death along with an increase in Bcl-2 levels following forebrain ischemia. Replenishing the levels of miR-9 which were downregulated in the brains of adult mice following transient MCAO was shown to prevent neuronal apoptosis by targeting proapoptotic Bcl-2-like 11 (Bcl2l11) leading to reduced behavioral deficits, smaller infarction, and decreased edema [83]. Tao et al. found a decrease in the miR-99a levels in the plasma of stroke patients compared with controls [84], whereas increasing miR-99a level decreased the secondary brain damage in a mouse MCAO model by preventing apoptosis [84]. The neuroprotective potential of miR-99a was further validated by in vitro studies that showed that miR-99a alleviated hydrogen peroxide-induced oxidative stress and apoptosis. Further studies demonstrated that miR-99a played neuroprotective roles through targeting and decreasing cyclin D1 and cyclin-dependent kinase 6 (CDK6) expression in both in vitro and in vivo ischemia conditions that attenuate cell cycle progression leading to anti-apoptotic actions [84]. Moreover, Liu et al. [85] reported that the apoptosis was significantly reduced in hippocampal neuron and cortical neuron by miR-24 inhibitor in a rat MCAO model. And miRNA-24 inhibitor prevented apoptosis in ischemic stroke by downregulating B-cell lymphoma (Bcl)-mL and heat shock protein 70 [85] (Fig. 15.2).

15.3.2.2 Autophagy

Several miRNAs were also shown to influence autophagy after stroke (Fig. 15.2). Wang et al. found that miR-30a was significantly downregulated following 1 h MCAO/24 h reperfusion in mouse cerebral cortex and in mouse N2A neuroblastoma cells after 1 h OGD and 6–48 h reoxygenation with a concomitant increase in Beclin-1 (an autophagy-signature protein expression). Treatment with antagomiR-30a decreased OGD-induced cell death in vitro and post-ischemic infarction and neurological deficits in vivo, and thus an increase in Beclin-1 expression was linked with improvement in poststroke outcome in both in vitro and in vivo conditions [53]. The miR-207 was shown to control autophagic cell death after ischemia by downregulating the expression of lysosomal-associated membrane protein 2 (LAMP2). Treatment with a miR-207 mimic decreased LAMP2 expression,

lysosome count, and infarct volume and improved the neurological function in rats after transient focal ischemia [86].

15.3.2.3 Excitotoxicity

Glutamate excitotoxicity is another major cause of ischemic brain damage [87]. Focal cerebral ischemia in adult rats increased miR-107 expression and repressed its target glutamate transporter-1 (GLT-1) expression [88]. The decrease in GLT-1 levels leads to a delay in the clearance of released glutamate from the synaptic cleft and subsequent excitotoxic neuronal death. Further studies showed that treatment with miR-107 inhibitor attenuated the downregulation of GLT-1 protein level in cultured neural cells subjected to hypoxia/reoxygenation [88] (Fig. 15.2).

15.3.2.4 Inflammation

The cascade of molecular events following focal brain ischemia transforms the cerebrovascular endothelium from a quiescent to a pro-inflammatory state. Cytokine induction of cell adhesion molecules on the vascular endothelium promotes BBB disruption and leukocyte recruitment [89]. IL-6 is a pro-inflammatory cytokine known to induce pro-inflammatory astrocytic scarring (astrogliosis) following stroke [90]. After stroke, astrogliosis is particularly localized in regions of neural cell death. Astrogliotic phenotype and miR-125b levels were found to be increased in IL-6 stressed normal human astrocytes [91]. AntagomiR-125b attenuated glial cell proliferation and increased mRNA and protein expression of its putative mRNA target cyclin-dependent kinase inhibitor 2A (CDKN2A), a negative regulator of cell growth. CDKN2A expression is known to be downregulated in chronic neurodegenerative diseases associated with astrogliosis, such as Alzheimer's disease, suggesting a role in reactive astrocyte proliferation [92]. Taken together, these findings support IL-6-mediated inflammation and miR-125b upregulation as a potential mediator of poststroke astrogliosis. Interferon-beta (IFN-β) is a regulatory cytokine with anti-inflammatory properties that has been approved for the treatment of multiple sclerosis (MS), an inflammatory disease of the central nervous system. In rodents subjected to autoimmune encephalomyelitis, a preclinical model of human MS, IFN-β therapy strongly inhibited extravasation of pro-inflammatory blood-derived monocytes into the central nervous system by preventing upregulation of vascular cell adhesion molecule 1 [93]. Several miRNAs have been biologically validated to silence IFN-β in monocytes-derived macrophages isolated from human subjects, including miR-26a, miR-34a, miR-145, and let-7b [94]. Importantly, IFN-β therapy has already shown promise in stroke. In a preclinical model of focal cerebral ischemia, systemic IFN-β delivery attenuated infiltration of neutrophils and monocytes to brain tissue and reduced stroke-induced lesion volume by 70% compared with controls [95]. Microglial activation is a widely observed response after stroke that promotes inflammation and aggravates ischemic brain damage [96]. A

recent study showed decreased levels of let-7c-5p in the plasma of stroke patients as well as in the plasma and brain of mice subjected to transient focal ischemia [97]. Overexpression of let-7c-5p decreased caspase-3 level, which led to reduced microglial activation, decreased infarct volume, and neurological deficits after focal ischemia [97]. In another study, intracerebroventricular administration of miR-424 mimic prevented microglial activation leading to decreased infarct volume and brain edema after transient MCAO in adult mice [98]. Replenishing miR-122 levels by intravenous administration of a miR-122 mimic was shown to reduce neurological deficits, infarct volume, and ICAM-1 expression together with downregulation of direct and indirect target genes and maintenance of vascular integrity in a rat model of focal ischemia by acting on blood leukocytes rather than the brain tissue directly [99] (Fig. 15.2).

15.3.2.5 Neuroprotection/BBB Integrity

Vemuganti lab reported that in neuronal PC12 cells, miR-29c directly targeted DNA methyltransferase 3a (DNMT3a) which was found to mediate neuronal cell death after subjected to OGD. Also, treatment with premiR-29c and DNMT3a siRNA decreased infarct volume after stroke insult, which indicated the importance of miR-29c-targeted DNMT3a signaling in regulating stroke-associated neuronal cell death [100]. Valproic acid is known to inhibit histone deacetylase and protect the poststroke brain [101]. Two miRNAs, miR-331 and miR-885-3p, were shown to be responsible for valproic acid-induced neuroprotection after ischemia by modulating their predicted targets associated with several networks including immune cell trafficking, neuronal cell death, synaptic depression, and branching of neurites [102]. Yin et al. [50] found that intracerebroventricular infusion of a PPAR agonist, GW501516, significantly reduced ischemia-induced miR-15a expression, leading to decreased BBB disruption and reduced cerebral infarction in mice MCAO model. Although not directly related to stroke, Duan et al. [103] showed that hyperglycemia led to decreased expression of platelet miR-223 and miR-146a, and this in turn triggered platelet dysfunction and subsequent risk for ischemic stroke onset.

Hibernation in rodents is known to provide ischemic tolerance, and the expression levels of miR-200 and miR-182 families are shown to be decreased during this process, which leads to increased expression of the small ubiquitin-like modifier (SUMO) proteins [104]. SUMO proteins belong to the ubiquitin-like protein modifier (ULM) family that promotes ischemic tolerance [104]. Furthermore, inhibition of miR-200 and miR-182 increased global protein SUMO that is thought to be critical for ischemic tolerance [104]. Many pharmacological agents including 3-nitropropionic acid, lipopolysaccharide, estrogen, resveratrol, and volatile anesthetics such as isoflurane and sevoflurane were known to induce ischemic tolerance [105–108]. MiRNAs were thought to mediate the ischemic tolerance by some of these agents. Morphine PC-induced neuroprotection in primary cortical neurons after ischemic insult was reported due to the downregulation of miR-134 [109]. Similarly, sevoflurane PC against a 6 h hypoxic injury in neuronal PC12 cells led to

altered expression of 14 miRNAs, and among them, downregulation of miR-101a and upregulation of miR-34b were linked to sevoflurane PC. Overexpression of miR-101a increased apoptosis. In contrast, overexpression of miR-34b increased cell viability, and this was further confirmed when inhibition of miR-34b increased the number of the apoptotic cells [110] (Fig. 15.2).

15.3.2.6 Neurogenesis

Preclinical and clinical studies have demonstrated that stroke promotes neurogenesis in the adult brain and miRNAs protect the brain and promote plasticity and regeneration by modulating neurogenesis [111–115]. Inhibiting let-7f with an antagomir was shown to protect the post-ischemic brain by increasing insulin-like growth factor-1 (IGF-1), a potent promoter of neurogenesis in normal and post-stroke brain [112]. The most abundant neuronal miRNA, miR-124, is expressed by neural progenitor cells in the subventricular zone of adult rodent brains [116]. It was reported by Cheng et al. that attenuation of endogenous miR-124 in neural progenitor cells can abolish neuronal differentiation, whereas overexpression of miR-124 promoted neuronal differentiation in the mouse brain [117]. Liu et al. [118] reported that overexpression of miR17-92 cluster either in cultured ischemic neural progenitor cells or in the subventricular zone of ischemic mice significantly increased cell proliferation. On the other hand, inhibition of individual members of the miR17-92 cluster, miR-18a and miR-19a, suppressed cell proliferation and increased cell death [118].

15.3.2.7 Angiogenesis

MiR-210 was found to increase poststroke angiogenesis in a rat model of transient focal ischemia by activating the Notch signaling pathway [119]. MiR-210 was upregulated following ischemic stroke, and overexpression of this miRNA in vitro increased the levels of Notch-1 and angiogenesis. The other target genes of miR-210 including ephrin A3 and hypoxia-inducing factor (HIF)-1α might also be involved in blood vessel formation [119]. A similar study indicated the role of miR-376b-5p in regulating the HIF-1α-mediated vascular endothelial growth factor (VEGF)/Notch1 signaling pathway following MCAO in rats [120]. MiR-487b that was found to be increased in the plasma of ischemic stroke patients was also a modulator of angiogenesis. Transfection of miR-487b in human umbilical vein endothelial cells (HUVECs) enhanced cell proliferation, migration, invasion, and tube formation. Furthermore, the authors demonstrated a direct binging of miR-487b to the 3′-UTR of Thrombospondin-1 (THBS-1) mRNA, which was an endogenous inhibitor of angiogenesis [121]. A similar connection between a stroke-related miRNA and angiogenesis was shown in case of miR-155. Increased stabilization of blood vessels was found to be regulated by miR-155 through its target protein Ras homolog enriched in brain in a distal MCAO mouse model [122]. Another recent

study showed that miR-107 induced in the ischemic boundary zone after permanent MCAO targets Dicer-1 leading to translational resulting in upregulation of endothelial cell-derived VEGF (VEGF165/VEGF164) that contributed to enhanced angiogenesis [123] (Fig. 15.2). Sun et al. [124] also reported the regulatory role of miR-140-5p in ischemic stroke-induced angiogenesis through targeting VEGFA. Although not directly related to stroke, Yin et al. [125] showed an anti-angiogenic effect of vascular endothelial cell enriched miR-15a in a mice hind limb ischemia model. It was suggested that pharmacological modulation of miR-15a might provide new therapeutic strategies to target angiogenesis in other pathological conditions such as stroke.

15.4 Circulating MicroRNA

MiRNAs were first found in human serum in 2008 [126, 127]. They have subsequently been detected in a wide range of bio-fluids, including urine, saliva, and cerebrospinal fluid [128]. These circulating miRNAs have several characteristics that make them attractive targets for biomedical research: they are remarkably stable in the blood, appear in concentrations measurable by current techniques, and often show tissue-specific expression patterns [129]. Furthermore, they are likely to become dysregulated before physical symptoms of disease become apparent [130]. These characters of circulating miRNAs, paired with limited availability and difficulty in obtaining human tissue samples, have made circulating miRNA studies promising in the search for miRNA-based biomarkers in human diseases.

15.4.1 Circulating MicroRNAs and Detection Methods

Despite the existence of RNases, miRNAs remain stable in serum and other body fluids [131]. Circulating miRNAs remain stable even after exposure to severe conditions, such as high temperatures, extreme pH, and prolonged storage [132, 133]. Circulating miRNAs protect themselves from degradation by several mechanisms: (1) packing in membrane vesicles such as microvesicles [134, 135], exosomes [136], and apoptotic bodies [137], (2) bounding to transporter proteins [138], and (3) inclusion in macromolecular complexes such as high density lipoproteins [139]. The existence of miRNAs in microvesicles indicates that circulating miRNAs may be a new and potential intercellular communication system which contributes to disease progression [130, 140, 141].

Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) is the most sensitive and specific method applied to quantify circulating miRNAs [142]. Detailed descriptions of this method for miRNAs quantification in serum/plasma were illustrated by Kroh et al. [143]. The challenges to quantify circulating miRNAs with sufficient sensitivity and precision are as follows: very little

amount of RNA recovered from serum or plasma and lack of appropriate endogenous controls for normalization. Because the amount of RNA extracted from plasma/serum is very little, an accurate normalization procedure is necessary. At present, it is possible to normalize the technical variability of the serum/plasma RNA extraction using the *C. elegans* spiked-in control miRNAs. During the RNA extraction, synthetic *C. elegans* miRNAs (miR-54, miR-39) and some other endogenous circulating miRNAs (such as miR-1249, miR-17-5p, U6, miR-454, 5S rRNA, and RNU6b) can be added after denaturation of serum/plasma [144]. It should be noticed that antiplatelet [145] and anticoagulation [146] drugs can affect miRNA quantification in blood samples and must be taken into account when detecting circulating miRNAs. However, real-time PCR-based detection of circulating miRNA is ligation based, which potentially creates bias and poses a challenge to quantification. Another commonly used technique is microarray screening. One of the greatest advantages of array-based techniques is that they can simultaneously assess all currently known miRNAs in the human genome (the miRNome). This was first employed by Li et al. [147] to identify a novel link between human cytomegalovirus (HCMV) and essential hypertension. By screening 1700 microRNAs in the plasma of 13 hypertensive patients and five control participants, they identified 27 microRNAs that were differentially expressed, of which 14 were validated using qPCR [147].

15.4.2 Physiological Function of Circulating MicroRNAs

Many studies of circulating miRNAs have indicated that miRNAs play a role in blood pressure regulation, usually through tissue-based studies. In 2011, Marques et al. [148] identified differential expression of miR-181a in kidneys of hypertensive and normotensive individuals. Importantly, binding of miR-181a to the 3′-UTR of the renin gene was demonstrated using luciferase reporter assays transfected into HEK293 cells. This explains how miR-181a may lead to decreased renin mRNA levels. More recently, Tomaszewski group demonstrated that in two independent cohorts, serum levels of miR-181a correlated with systolic blood pressure [149]. Interestingly, however, this association was independent of circulating renin levels, suggesting that the effects of miR-181a may be mediated by mechanisms other than renin inhibition. Indeed, they used RNA-sequencing data to explore the signature of miR-181a on the global renal transcriptome. This analysis implicated miR-181a in pathways associated with mitochondrial respiratory function, immunity, and inflammation [149].

There are other reports pointing to circulating miRNAs' ability to modulate immune cells and their ability to transfer to other cells or organs to induce more organ-specific functions [150–153]. MiR-146a in peripheral blood mononuclear cells (PBMCs) was first reported to modulate T helper cells type 1 (Th1) function in patients with acute coronary syndrome [151]. Th1 is known to be essential in the process of plaque instability and plaque rupture which is a common pathogenetic

feature in acute manifestations of atherosclerosis, such as acute coronary syndrome. It was speculated that miR-146a might contribute to the onset of acute coronary syndrome [151]. The altered expression of inflammation-related miR-155 was subsequently found to be correlated with T helper 17 cell differentiation in patients with acute coronary syndrome, indicating that it might contribute to atherosclerosis, as atherosclerosis was a chronic inflammatory disease that was upregulated by immune cells, especially T helper cells [152]. In addition, a calcium ionophore, A23187, was used to stimulate the release of miR-133a followed by exosome harvest [153]. After adding these exosomes into HEK293 cells expressing the miR-133a sensor vector, the luciferase activity was significantly reduced, indicating that circulating miRNAs might be transferred into other cells or organs to induce their specific functions [153].

15.4.3 Circulating MicroRNAs as Biomarkers for Human Diseases

Circulating miRNAs in plasma is altered both qualitatively and quantitatively in a variety of conditions, including different tumor types, cardiovascular disease, stroke, and neurodegenerative diseases. In the past few years, a number of studies [16, 129, 142, 154] have reported the use of circulating miRNAs as biomarkers for diagnosis or prognosis of cardiovascular diseases, such as myocardial infarction, heart failure, cardiomyopathy, hypertension, coronary artery disease, angiogenesis, and dyslipidemia.

15.4.3.1 Acute Myocardial Infarction

Acute myocardial infarction (AMI) is characterized by cardiac cell death after ischemia [147]. Damaged cells release various proteins into circulation, including cardiac myoglobin, creatine kinase-MB (CK-MB), cardiac troponins I (cTn I), and cardiac troponins T (cTn T), which have been extensively used as standard biomarkers for diagnosis in clinical onset [155]. However, the biggest challenge of these diagnostic assays of AMI is that these protein markers are not unique for AMI; they are increased in some other diseases, such as renal and heart failure [156, 157]. Recent studies suggest that AMI induces the cardiac-specific miRNAs released from injured cardiomyocytes into circulation. The plasma miR-208a was found significantly increased in isoproterenol-induced myocardial injury rat models and had a good correlation with cTn I [158]. To exclude the possibility that the increased plasma miR-208a was caused by nonspecific injury, it was also examined in a renal infarction model. The result showed that the plasma miR-208a remained undetectable, which indicated that the circulating miR-208a had specificity in AMI prediction [158]. In clinical trial the cardiac-specific miR-208a increased in AMI patients

with a detection sensitivity of 90.9% but was undetectable in healthy controls, which was consistent with animal studies. A parallel analysis of circulating miR-208a and cTn I showed miR-208a was increased in all AMI individuals but cTn I was detectable only in 85% of patients within 4 h of the symptoms onset [159]. Therefore, circulating miR-208a might be an alternative superior to conventional biomarkers (cTn I and cTn T) for the early detection of AMI. It was also reported that the circulating miR-1 significantly increased in AMI patients compared to non-AMI controls [160]. Additional miRNAs that were found to be increased in AMI patients include miR-133a [161], miR-133b [162, 163], miR-499 [164], miR-499-5p [165], miR-328 [166], miR-1291, and miR-663b [167], whereas miR-223 [168], miR-122, and miR-375 [169] were found to be decreased in AMI patients. In addition, a prospective research indicated that circulating miR-197 and miR-223 had negative correlations with AMI incidence. On the contrary, circulating miR-126 showed a positive correlation with AMI incidence [170].

15.4.3.2 Heart Failure

Clinical management of heart failure (HF) is facilitated by circulating biomarkers, such as brain natriuretic peptide (BNP) and N-terminal pro-brain-natriuretic peptide (NT-proBNP) [171]. However, it is still necessary to find a more reliable and objective measurement for HF diagnosis and management. It was reported that circulating miR-423-5p was used to distinguish patients recruited from a dyspnea registry between dyspnea due to HF and dyspnea without HF [172]. Circulating miR-423-5p also correlated with NT-proBNP [172]. In addition, systematic screening of 186 miRNAs that uncovered four miRNAs (miR-423-5p, miR-22, miR-320a, and miR-92b) significantly increased in the serum of HF patients. With the detection of the four miRNAs, a sensitive and specific score was defined for assessing HF patients. The miRNA-score was closely related to several important prognostic parameters, including increased serum BNP, widening QRS, and dilatation of left ventricle and atrium [171]. Another research found circulating miR-499 significantly increased in patients with acute heart failure [173].

15.4.3.3 Hypertrophic Cardiomyopathy

Several studies have demonstrated a functional role of miRNAs in cardiomyopathy. Hypertrophic cardiomyopathy (HCM) is an inherited heart disease with a prevalence of approximately 1 out of 500 among the general population [174]. In a study aimed to characterize the circulating miRNA profile of HCM, 41 HCM patients who were characterized with conventional transthoracic echocardiography and cardiac magnetic resonance were enrolled, and 41 age- and sex-matched healthy people were enrolled as control. The result showed 12 miRNAs significantly increased in plasma of HCM patients. However, only miR-29a was significantly associated with both hypertrophy and fibrosis. Therefore, miR-29a was identified as a potential

biomarker for HCM assessment [174]. Another research examined miRNA expression profiles in stable patients with isolated diastolic dysfunction, patients with stable compensated dilated cardiomyopathy (DCM), and those with decompensated congestive heart failure secondary to DCM (DCM-CHF). MiR-142-3p was found to be decreased in DCM and DCM-CHF groups and miR-124-5p only increased in DCM group [175]. Takotsubo cardiomyopathy (TTC) is an increasingly recognized acute syndrome with similar symptoms to AMI. The TTC symptoms include chest pain and electrocardiographic changes, mainly in the absence of obstructive coronary artery disease. In the study from Jaguszewski et al., a unique signature comprising miR-1, miR-16, miR-26a, and miR-133a differentiated TTC from healthy people and ST-segment elevation acute myocardial infarction (STEMI), which had an important meaning for diagnosis [176].

15.4.3.4 Hypertension

Hypertension is an epidemic condition. Approximately 90–95% of hypertension belongs to the essential hypertension subtype. Li et al. [147] demonstrated a novel link between human cytomegalovirus (HCMV) infection and essential hypertension. Twenty-seven differentially expressed circulating miRNAs were identified in 13 essential hypertensive patients and 5 healthy control subjects. The expressions of miR-296-5p, miR-let-7e, and hcmv-miR-UL112 (a human cytomegalovirus-encoded miRNA) were validated independently in plasma samples [147]. Another study detected and analyzed plasma samples from three independent cohorts to identify circulating miRNAs candidates in essential hypertension patients. The results indicated that the plasma hsa-miR-505 was significantly elevated in essential hypertensive patients [88]. Recently, more and more researches focus on the association between circulating miRNAs and pulmonary. A total of 40 human subjects were included in a study, and the degree of pulmonary hypertension (PH) was determined by the mean pulmonary arterial pressure. The study identified several upregulated miRNAs (miR-23b, miR-130a, and miR-191) and downregulated miRNAs (miR-451, miR-1246) in the circulation of PH patients. The results indicated that miRNAs might be considered as potential biomarkers for early diagnosis of PH [177]. One study profiled miRNA expressions in plasma from PH patients and PH rats induced by monocrotaline. The results showed miR-26a decreased in both experimental and clinical PH, and it could be a robust marker and intervention target of PH [178]. Another group reported that reduced miR-150 was closely associated with poor survival in PH patients [179].

15.4.3.5 Coronary Artery Disease

Coronary artery disease (CAD) is a major cause of death and disability worldwide. Atherosclerosis is the main cause of CAD, which is characterized by endothelial activation, plaque formation, and structural remodeling of arterial wall. Currently,

fibrinogen and C-reactive have been used as CAD markers. Still these markers are not ideal for the diagnosis of cardiovascular conditions, because they can be affected by CAD-unrelated environmental factors and disease backgrounds [180]. It has been reported that plasma levels of endothelial cell enriched miRNAs (miR-126, miR-92a, and miR-17), smooth muscle-enriched miR-145, and inflammation associated miR-155 significantly reduced in CAD patients compared with healthy controls [181]. Another research detected 157 different miRNAs in PBMCs of CAD patients by miRNA microarray. The result showed that miR-135a increased and miR-147 decreased significantly in plasma of CAD patients, and miR-135a/miR-147 ratio could be used for CAD diagnosis. It also indicated that patients with unstable angina could be differentiated from patients with stable angina by their increased level of miR-134, miR-198, and miR-370, which suggested circulating miRNAs could be used to identify patients at risk for acute coronary syndromes [182]. Furthermore, miR-149 was closely associated with increased risk for CAD in Chinese Han people [183]. Serum miR-31 was found higher in CAD patients with restenosis compared to CAD patients without restenosis [161]. Circulating miR-133a and miR-208a levels were upregulated, while miR-126, miR-17, miR-92a, and miR-155 levels were significantly downregulated in patients with stable coronary artery disease compared with healthy controls [181]. Lu et al. reported a beneficial effect of miR-214 in CAD patients, and loss of miR-214 mediated protection might lead to increased level of placental growth factor and worsening atherosclerosis [184]. Moreover, a study revealed increased circulating miR-122 and miR-370 might be associated with the presence and the severity of CAD in hyperlipidemia patients. The levels of miR-122 and miR-370 were also positively correlated with total cholesterol, triglyceride, and low-density lipoprotein cholesterol (LDL-C) levels [185]. Taken together, circulating miRNAs have the potential to improve the CAD diagnosis.

15.4.3.6 Cancer

Cancer has been a major focus of miRNA research over the past decade, and accumulating studies have demonstrated the involvement of miRNAs in cancer biology through controlling expression of their target mRNAs to facilitate tumor growth, invasion, angiogenesis, and immune evasion [186, 187]. Circulating lipid vesicles may play an important role in cell-to-cell communication. It has been shown that cancer cells release vesicles containing angiogenic factors to induce angiogenesis [188, 189]. MiRNAs in lipid vesicles may be picked up by cells through endocytic uptake, membrane fusion, or binding to specific cell surface receptors and thereby affect mRNA targets in recipient cells, leading to widespread consequences [190]. Because the cargos carried by these membrane vesicles reflect their cellular origins, there is great interest in identifying biomarkers from cancer cell-derived vesicles. In addition, analyzing the molecular content of these vesicles may shed light on perturbed molecular processes in cancer cells [191, 192]. The identification of miRNAs that target current biomarkers may be applied to miRNA-based tests as an

alternative to mRNA/protein expression for prognosis assessment. MiRNAs have been shown to have a role in cancer progression [193] and might be useful for the prediction of metastatic outcomes for patient management. Specific miRNAs have been shown to support endothelial recruitment to metastases in breast cancer and might serve as efficient biomarkers for predicting this event [194]. MiRNA signatures associated with known inducers of EMT have also been developed and shown to be relevant in both in vitro and in vivo models of EMT in endometrial cancer [195]. The identification and characterization of miRNAs have been well summarized by several groups to facilitate patient diagnosis, prognosis, monitoring, and treatment in the oncology field [196–199].

15.5 Utility of MicroRNAs as Stroke Biomarkers

15.5.1 MicroRNAs as Diagnostic and Therapeutic Biomarkers in Rodent Stroke Models

Considering altered miRNA expression levels in rodent stroke models, blood miRNAs may serve as potential noninvasive biomarkers of ischemic stroke (summarized in Table 15.2). In the first miRNA expression profiling study of cerebral ischemia with rat MCAO models, miR-290 and miR-494 were upregulated, and let-7i was downregulated in both brain and blood at 24 and 48 h following MCAO [17]. In another study, two miRNAs (miR-292-5p and miR-451) were upregulated and five miRNAs (miR-23a, miR-150, let-7a, let-7d, and let-7f) were downregulated only at 24 h after the surgery, whereas five miRNAs (miR-140*, miR-150, miR-214, miR-320, and miR-328) were upregulated and four miRNAs (miR26a, miR26a-26b, miR26a-195, and miR26a-352) were downregulated only at 48 h after the surgery in both brain and blood samples [17, 200].

In addition, by utilizing quantitative PCR analysis to investigate liver-, muscle-, and brain-specific circulating miRNAs as potential biomarkers of tissue injury, miR-124 was found to be increased in plasma specifically after the brain injury, which was observed 8 h after rats subjected to transient MCAO. MiR-124 expression was further increased 24 h after stroke for both transient and permanent MCAO. These results suggested that miR-124 might serve as a sensitive plasma biomarker for ischemic stroke [201]. In a later study by using miRNA array analysis in various tissues, miR-124 was shown almost exclusively expressed in the brain. Brain-specific miR-124 was further found significantly elevated in the plasma at 6 h and remained at a high level at 48 h after MCAO in rat; thus, plasma miR-124 was confirmed to serve as early detection biomarker of cerebral infarction [202].

In another microRNA expression profiling study, rat brain and whole-blood miRNA expression profiles were assessed 24 h after ischemic stroke using TaqMan rodent miRNA arrays. The results showed that, among brain injuries including ischemic stroke, intracerebral hemorrhage, and kainite seizures, 2 miRNAs were upreg-

Table 15.2 MicroRNAs as non-invasive biomarkers of ischemic stroke

Subjects	Sample resources	MicroRNAs alteration
Rats	Male rat brain and blood following MCAO	MiRNAs in both brain and blood at 24 and 48 h following MCAO:
		Up: miR-290, miR-494
		Down: let-7iMiRNAs
		In both brain and blood only at 24 h following MCAO:
		Up: miR-292-5p and miR-451
		Down: miR-23a, -150, let-7a, -7d and 7f
		In both brain and blood only at 48 h following MCAO:
		Up: miR-140*, -150, -214, -320 and -328
		Down: miR26a, -26b, -195 and -352
Rats	Plasma samples from rats undergone MCAO	MiR-124 specificly expressed after the brain injury, and highly increased at 24 h post transient or permanent MCAO
Rats	Various tissues including brains	MiR-124 almost exclusively expressed in the brain, upregulated in the plasma at 6 h, and remained high levels at 48 h after MCAO
Rats	Brain and blood samples from rats undergone ischemic stroke, intracerebral hemorrhage and kainite seizures	MiR-182 was upregulated, miR-223 and miR-210 were downregulated in both brain and blood specifically by ischemia
Mice	Brain and blood samples from rats undergone MCAO	MiR-29b level was significantly downregulated
Stroke patients	Blood of young ischemic stroke patients (LA, SA, Cemb and UND)	Highly expressed in all the subtypes: hsa-let-7e, miR-1184, -1246, -1261, -1275, -1285, -1290, -181a, -25*, -513a-5p, -550, -602, -665, -891a, -933, -939, -923
		Poorly expressed across 3 subtypes of stroke (LA, SA and Cemb): hsa-let-7f, miR-126, -1259, -142-3p, -15b, -186, -519e, -768-5p
Stroke patients	Blood of ischemic stroke patients without any or minimal risk factor	4 miRNAs downregulated (miR-25*, -34b, -483-5p, -498)
Stroke patients	Blood of ischemic stroke patients	MiR-210 significantly decreased at 7 days and 14 days after the onset of stroke
Stroke patients	Blood of ischemic stroke patients	MiR-145 exhibits higher level in ischemic stroke patients
Stroke patients	Blood of ischemic stroke patients	miR-30a and miR-126 levels were significantly downregulated in all ischemic stroke until 24 weeks after the symptoms onset;let-7b was lower in ischemic stroke patients only with large-vessel atherosclerosis, whereas was higher with those of other kinds of ischemic stroke patients until 24 weeks after the symptoms onset.

(continued)

Table 15.2 (continued)

Subjects	Sample resources	MicroRNAs alteration
Stroke patients	Serum of ischemic stroke and atherosclerosis patients	Serum miR-21 exhibits significantly higher level and miR-221 exhibits significantly lower level in ischemic stroke and atherosclerosis patients
Stroke patients	Inhibited angiogenesis by decreasing cell proliferation, migration and tube formation	MiR-125b-2*, -27a*, -422a, -488 and -672 consistently increased during acute stroke
Stroke patients	Blood of ischemic stroke patients	MiR-122, miR-148a, let-7i, miR-19a, miR-320d, and miR-4429 were decreased;miR-363 and miR-487b were increased in peripheral blood cells of stroke patients
Stroke patients	Blood cells of ischemic stroke patients	MiR-15a, miR-16 and miR-17-5p increased 8.3 fold, 42 fold and 9.9 fold in acute ischemic stroke patients, respectively;serum miR-17-5p level was a significant and independent predictor for determining the presence of acute ischemic stroke
Stroke patients	Serum of acute ischemic stroke patients	Serum miR-32-3p, miR-106-5p and miR-532-5p were differentially expressed and were found associated with ischemic stroke
Stroke patients	Serum of ischemic stroke patients	Serum and CSF let-7e were both significantly increased in acute stage of ischemic stroke patients
Stroke patients	Serum and CSF of acute ischemic stroke patients	Blood let-7e-5p was significantly higher in ischemic stroke patients, increased blood let-7e-5p associates with increased occurrence and increased risk of ischemic stroke

ulated and 44 miRNAs were downregulated more than twofold specifically by ischemic stroke in the blood samples. Of note, miR-182 was upregulated and miR-223 and miR-210 were downregulated in both brain and blood specifically by ischemia. These selected blood miRNAs correlate with miRNAs changes in the brain and could be used as biomarkers for ischemic brain injury [76].

Furthermore, Wang et al. demonstrated that the level of miR-29b was evaluated in patients and mice after ischemic stroke. Results reveled that miR-29b level was significantly downregulated in both stroke patients (white blood cells) and ischemic mice (brain and blood), and miR-29b downregulation was negatively associated with the stroke outcomes and infarct volume, suggesting that blood miR-29b is a circulating biomarker as well [203].

15.5.2 MicroRNAs as Diagnostic and Therapeutic Biomarkers in Stroke Patients

Experimental data from peripheral blood isolated from stroke patients also implicate that circulating miRNAs could be used as potential diagnostic and prognostic biomarkers for ischemic stroke. In the first study of expression profile of miRNAs

in blood of young ischemic stroke patients, Tan et al. reported that 157 miRNAs were differentially regulated across stroke samples and subtypes (large artery stroke, LA; small artery stroke, SA; cardioembolic stroke, CEmb; and undetermined cause, UND). Among the 138 upregulated miRNAs, 17 miRNAs (hsa-let-7e, miR-1184, miR-1246, miR-1261, miR-1275, miR-1285, miR-1290, miR-181a, miR-25*, miR-513a-5p, miR-550, miR-602, miR-665, miR-891a, miR-933, miR-939, miR-923) could be identified as highly expressed in all the subtypes. On the other hand, among 19 downregulated miRNAs, 8 miRNAs (hsa-let-7f, miR-126, miR-1259, miR-142-3p, miR-15b, miR-186, miR-519e, miR-768-5p) were observed poorly expressed across the three subtypes of stroke (LA, SA, and CEmb) [18]. Later on, by analyzing ischemic stroke patients without any or minimal risk factor, the same group found that four miRNAs (miR-25*, miR-34b, miR-483-5p, miR-498) were downregulated in these patients in the blood. These four miRNAs were also proved to be specific for stroke pathogenesis in low-risk stroke patients [204]. Therefore, these dysregulated miRNAs could serve as potential biomarkers of different subtype of strokes.

Additionally, miR-210 was suggested to be a novel sensitive biomarker in acute cerebral ischemia as well. MiR-210 was significantly decreased in blood of stroke patients, especially at 7 days and 14 days after the onset of stroke. Also, the expression level of miR-210 in stroke patients with poor outcome was significantly lower than that of patients with good outcome [205]. Circulatory miR-145 expression profile was determined among ischemic stroke patients as well, which exhibited higher level in ischemic stroke patients than health group, implying miR-145 as another potential biomarker [206]. Another study from ischemic stroke patients revealed that circulating miR-30a and miR-126 levels were significantly downregulated in all patients with ischemic stroke until 24 weeks after the onset of symptoms [207]. Circulating let-7b was lower in ischemic stroke patients only with large-vessel atherosclerosis, whereas it was higher with those of other kinds of ischemic stroke patients until 24 weeks after the symptoms onset. All three miRNAs returned to normal expression levels 48 weeks after stroke. This unique expression profile suggested that miR-30a, miR-126, and let-7b might be useful biomarkers for ischemic stroke in human [207].

Moreover, by studying serum miRNAs in 167 patients with ischemic stroke, 66 atherosclerosis patients with any carotid plaque score, and 157 healthy controls. Tsai et al. found that serum miR-21 exhibited significantly a higher level and miR-221 exhibited significantly a lower level in ischemic stroke and atherosclerosis patients than in healthy controls. The results suggested miR-21 and miR-221 could be served as independent predictors for stroke risk and possible biomarkers for both diseases [208]. Furthermore, based on stroke patients blood miRNA profiles, miR-125b-2*, miR-27a*, miR-422a, miR-488, and miR-672 were found to be consistently increased during acute stroke irrespective of age or severity or confounding metabolic complications. The expression profile of these five miRNAs could also be confirmed by results from rat stroke models, implying these five circulating miRNAs as biomarkers of acute stroke [3]. Later, highly elevated hsa-miR-106b-5P and hsa-miR-4306 in patients with acute stroke and quite low abundance of hsa-miR-

320e and hsa-miR-320d were found in plasma of stroke patients compared with health individuals. These altered circulating miRNAs were suggested as early detection biomarkers for acute stroke in humans [209].

Even more, Jickling et al. observed that in patients with acute ischemic stroke, miR-122, miR-148a, let-7i, miR-19a, miR-320d, and miR-4429 expression were decreased and miR-363 and miR-487b were increased in peripheral blood cells compared to vascular risk factor controls. By characterizing the gene targets and pathways associated with ischemic stroke regulated by these miRNAs, it was predicted that these miRNAs might regulate leukocyte gene expression in ischemic stroke including pathways involved in immune activation, leukocyte extravasation, and thrombosis [210]. Wu et al. showed that serum expression of miR-15a, miR-16, and miR-17-5p increased ~8.3-fold, ~42-fold, and ~9.9-fold in acute ischemic stroke patients compared to controls, respectively. Multivariate logistic regression showed that serum miR-17-5p level was a significant and independent predictor for determining the presence of acute ischemic stroke [211]. Li et al. demonstrated that serum miR-32-3p, miR-106-5p, and miR-532-5p were differentially expressed and were associated with ischemic stroke, suggesting their potential use as diagnostic biomarkers [212]. Peng et al. found serum let-7e expression levels were significantly increased in ischemic stroke patients compared to healthy controls, with the highest level in the acute stage and the lowest level in the recover stage. In addition, its expression was also markedly upregulated in cerebral spinal fluid at the acute stage and consistent with the serum samples [213]. Later, Huang et al. suggested the potential use of let-7e-5p as biomarker of ischemic stroke, since its blood level was significantly higher in ischemic stroke patients, which was also associated with increased occurrence and increased risk of ischemic stroke [214].

15.6 Conclusion and Future Directions

With the properties of relative stability, specificity, and reproducibility, miRNAs are considered as more promising markers and better candidates than proteins and genes for early recognition of the onset of disease. The emerging roles of circulating miRNAs in ischemic stroke pathogenesis reviewed in this chapter imply the application of circulating miRNAs as biomarkers for the early noninvasive diagnosis and prediction of stroke.

Imaging technologies such as magnetic resonance imaging (MRI) remain the major diagnosis approach for patients suffering stroke at present. Unlike in myocardial infarction, specific plasma/serum markers that may be used to diagnose and/or assess the severity of ischemic brain injury have yet to be established. Although it has been reported that several protein markers are found elevated in stroke patients, neither of them can be detected in the early phase of stroke nor are specific for ischemic brain injury. Given that computed imaging techniques are either unavailable or yield no obvious acute abnormalities for many patients, a quick and reliable miRNA screening may be useful for the early diagnosis of stroke as well as the prediction of

stroke outcome of the patients. Therefore, an accurate circulating miRNA expression profile is needed to serve as the fingerprint of a pathophysiological condition in ischemic stroke injury. Studies on the regulatory mechanisms of the miRNAs in the disease model may lead to a better understanding of the roles of circulating miRNAs, which may provide a promising screening tool for the faster and more accurate prediction and diagnosis of subtypes of stroke.

However, limitations for using circulating miRNAs as biomarkers for diagnosis and prognosis of stroke still exist. For one thing, current studies on identifying circulating miRNAs as biomarkers of ischemic stroke are relatively small-scale studies. More independent and large cohort studies may help validate the results and drive reliable conclusions. For another, it is quite time-consuming for isolating RNA from blood and subsequent quantifying by real-time PCR. Further, it is rather difficult to set appropriate endogenous controls given circulating miRNAs expression may be different depending on the disease state, patients' age, and gender. Therefore, a better method to detect circulating miRNAs in patients is needed for clinical settings. In recent years, high-throughput platforms including multiplex PCR, microarrays, and next-generation-sequencing (NGS) technology have been quickly applied to the research of circulating miRNAs. In the future, circulating miRNAs may be extensively used in clinical diagnosis of stroke. Even more, circulating miRNAs have the potential to detect and predict therapeutic outcome of stroke.

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Chapter 16
Animal Models for Ischemic Stroke

Liping Wang, Chuan Qin, and Guo-Yuan Yang

Abstract Developing reliable and reproducible animal model is of great importance in the therapeutic research of ischemic stroke. The location and volume of injury are varied in different animal models. Researchers choose different animal models according to the research purposes. In this chapter, we summarized the system of ischemic stroke models.

Keywords Animal model • Application • Distal • Embolic • Evaluation • Focal • Global • Ischemic stroke • Middle cerebral artery occlusion • Method and key point • Neonatal hypoxic-ischemic • Photochemically

Abbreviations

2-VO	Two-vessel occlusion
4-VO	Four-vessel occlusion
CBF	Cerebral blood flow
CCA	Common carotid artery
ECA	External carotid artery
EPCs	Endothelial progenitor cells
ICA	Internal carotid artery
MCAO	Middle cerebral artery occlusion
mNSS	Modified neurological severity score
PPA	Pterygopalatine artery
rt-PA	Recombinant tissue plasminogen activator

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16.1 Introduction

In order to study the pathophysiology and treatment of stroke, developing reliable and reproducible ischemic animal models is of great importance. The ideal animal model can excellently mimic features of human ischemic characteristics. Cerebral ischemic animal models could be divided into focal cerebral ischemic models and global cerebral ischemic models. Focal cerebral ischemic models include suture transient middle cerebral artery occlusion and permanent middle cerebral artery occlusion, embolic middle cerebral artery occlusion model, distal middle cerebral artery occlusion, photochemically induced middle cerebral artery occlusion model, and neonatal hypoxic-ischemic brain damage model. Global cerebral ischemic models include bilateral carotid artery ligation (2-VO), four-vessel occlusion (4-VO), and cardiac arrest-induced forebrain ischemia. This chapter outlines methods and key points, applications, advantages, and limitations of different animal models.

16.2 Focal Ischemic Stroke Model

16.2.1 Suture Transient Middle Cerebral Artery Occlusion (tMCAO)

16.2.1.1 Methods and Key Points

Adult male rats weighing 250–300 g or adult male mice weighing 30–35 g were ideal for this procedure. Isoflurane inhalation anesthesia and intraperitoneal anesthesia with ketamine (80–100 mg/kg) and xylazine (5–10 mg/kg) were the most commonly used methods of anesthesia. To continuously monitor arterial blood pressure and sample for analysis of blood gases and blood pH, a PE-50 catheter for rats or a PE-10 catheter for mice was inserted into the femoral artery. Body temperature is maintained at 37 ± 0.3 °C using a heating pad. Under the operating microscope, a midline incision on the neck is made. Then, the left common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) are exposed and isolated (Fig. 16.1). A 2.5-cm length of 4-0 suture for rats or a 1.5-cm length of 6-0 suture for mice coated with silicone was used. The suture was introduced into the transected lumen of the ECA and gently advanced from the ICA across to the opening of the MCA until a slight resistance was felt. The distance from the tip of the suture to the bifurcation of CCA was about 18 ± 1 mm in rats, while it was about 10 ± 1 mm in mice. A laser Doppler flowmetry was used to test the cerebral blood flow. The reduction of the cerebral blood flow down to 10% of the baseline indicates the success of the occlusion. For reperfusion, the suture was withdrawn to restore blood flow following 60–120 min of MCAO. The dissected skin was sutured, and the animal was returned to the cage when it recovers from the anesthesia [1–3].

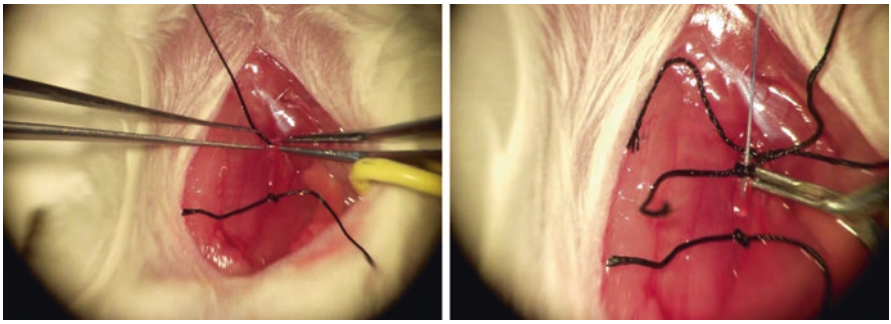

Fig. 16.1 Separation of cervical vessels and insertion of the suture under surgical microscope in the operation of suture MCAO model

16.2.1.2 Application of Transient MCAO

In recent years, many ischemic stroke animal models were established to mimic the clinical patients. In stroke research field, the aim of these stroke models was to understand the pathophysiological changes in the stroke development and the post-stroke recovery. We could use transient MCAO model to mimic the blood restoration following acute ischemia in the clinical situation. This was important to understand cellular and molecular mechanisms of stroke recovery even in the prognosis.

Transient MCAO model was worldwide used in the research trial since this model mimicked the human ischemic stroke and was highly reproducible. In addition, we could control the duration of ischemia and reperfusion, which was necessary for the study of mechanism of ischemia and reperfusion injury, as well as proper treatment during different time points.

The first main advantage of transient MCAO model was to understand the cellular and molecular mechanism in the process of cerebral ischemia. During the experiment, we could obtain the latest knowledge when the neurovascular unit was injured in the brain. We could make our hypothesis and develop a novel approach to attenuate ischemic brain injury and reverse the degeneration. Actually scientists have found many methods to inhibit or attenuate stroke occurrence and development such as signal pathway inhibitors, gene therapy, or stem cell therapy. Based on viral vector gene transfection, gene therapy was beneficial for stroke repair and recovery. Moreover, many kinds of stem/progenitor cells were effective for the stroke therapy. Endothelial progenitor cells (EPCs) were a kind of stem cells, which could differentiate into the endothelial cell for vascular repair after brain injury. EPCs could secret growth factors such as VEGF and FGF, which was necessary for the repair of vessels. The second advantage of tMCAO was this model could be used to study the restoration of blood flow during the stroke recovery stage. Recombinant tissue plasminogen activator (rt-PA) was the only proven drug for acute ischemic stroke; however, the clinical time window was very limited (within 4.5 h). The important advantage of intravenous rt-PA was that it could be used immediately

after clinical assessment. Although intravenous rt-PA has the evidence for the acute ischemic stroke therapy, the limitation including low rates of recanalization was driving people to find new approaches to prolong the therapeutic time window and to increase recanalization rates. Cerebral blood flow (CBF) was the critical issue for the stroke recovery. Effect of endovascular thrombectomy should be assessed by the recovery of blood flow. As we mentioned above, CBF changes following endovascular thrombectomy were similar to the changes observed in the transient MCAO models. Studies showed the similarities between the endovascular thrombectomy and transient MCAO animal procedure in CBF and pathophysiological characteristics, suggesting the rationale for the use of transient MCAO to mimic the potential outcomes of adjunct therapies for endovascular thrombectomy in humans. We could perform preclinical trials on animals and find more efficient drugs. Therefore, future clinical trials for neuroprotection should consider the transient MCAO model to assess preclinical efficacy.

16.2.1.3 Advantages

Intraluminal suture transient MCAO model was one of the widely used focal ischemia models. Suture-induced brain lesion is located both in the cortex and striatum. Postischemic restoration occurs frequently in human ischemic stroke induced by embolism. Transient MCAO model could mimic the clinical situation. It provides a unique tool for the mechanistic studies of cerebral ischemia in vivo. There was no need to perform craniotomy; therefore, the suture MCAO model was relatively simple.

16.2.1.4 Limitations

Approximately 10% of the animals died either during procedure even in experienced hands. The mortality was mainly due to subarachnoid hemorrhage, which was caused by inappropriate insertion of the suture.

Suture transient MCAO model had a high variation of infarct volume. Many factors contributed to the variation including brain and body temperature, method of anesthesia, physical properties of suture, duration, animal strain and weight, brain vascular anatomy, and collateral circulation [4, 5]. Many modifications were made to improve the stability.

Suture-induced thrombosis is a vital factor which causes the failure of reperfusion and reproducibility. Thrombosis could affect the infarct volume and neurobehavioral outcomes. Heparin injection 10 min before suture withdrawal could prevent thrombosis after suture withdrawal and does not reduce brain infarct volume or neurological score [2].

16.2.2 Suture Permanent Middle Cerebral Artery Occlusion

16.2.2.1 Methods and Key Points

The method for permanent MCAO is similar to that of transient MCAO described above. The only difference is that the suture stays inside the ICA without withdrawing it [6, 7].

16.2.2.2 Application of Permanent MCAO

The permanent MCAO was mainly established for the long-term ischemia of the brain [8]. In this model, the suture or other emboli were placed in the bifurcation of the artery to prevent the blood from flowing to the brain tissue. The ischemia period lasted a long time, and it could mimic the clinical pathology of stroke, which was the first leading cause of disabilities in the world. The model had a stable infarction area, but the damage to animal was so horrible at the same time that the mortality of this model was relatively high [9].

The huge territorial infarction and the considerable mortality rate in suture permanent occlusion were important features of the clinical malignant stroke, which was a devastating disease pattern and consists of 10% of all stroke patients. While neuroprotective therapies had frequently produced a significant reduction in filament-induced brain infarction following tMCAO, such protective effects were consistently absent in permanent ischemia [10]. Thus, the experimental model to mimic malignant stroke without high mortality rate was needed. Up to now, many modified permanent MCAO models had been explored to get a better animal performance and lower mortality rate [11]. These results showed that these new methods strongly reduce mortality. Using these methods, researchers could extend the studies over days or even weeks and study the mechanisms of tissue damage, edema resolution, and tissue remodeling.

By using this long-term period model, we could track the animal reactions during stroke. Collateral circulation was an important physiological accommodation; it could connect the vessels between artery and ischemic region where blood flow was needed [12]. In the transient middle cerebral artery occlusion model, it was harder to find the opening collateral circulation because there was no need to use the extra vessels when the artery still had function. So the permanent MCAO model was widely used to study the collateral circulation, the medicines which had the potential to open them, and the mechanisms during process.

As we claimed before, permanent MCAO model could mimic the clinical situation of stroke; many strategies were performed on this model. The pretreatment was the most widely ongoing research because we hoped to prevent the stroke from happening.

16.2.2.3 Advantages

Permanent MCAO suture model could mimic the cerebral injury caused by ischemic stroke without reperfusion. The stability of permanent MCAO suture model was better than that of transient MCAO suture model.

16.2.2.4 Limitation

Because the injured area of permanent MCAO was severer, the mortality of permanent MCAO was higher than that of transient MCAO. Furthermore, this model could not mimic the inflammation caused by reperfusion.

16.2.3 Embolic Middle Cerebral Artery Occlusion

16.2.3.1 Methods and Key Points

Sprague-Dawley rats, Wistar rats, and spontaneous hypertensive rats are the most common used strains for the procedure. First homologous clot is prepared 1 day before the experiment. Anesthetize a blood donor rat and expose the femoral artery. Insert the femoral artery with a 25-cm PE-50 tube. Fresh arterial blood is withdrawn by 2.5-ml syringe into the tube to form homologous clot. The clot is kept in the tube at room temperature for 2 h and subsequently stored at 4 °C for 22 h. The PE-50 tube containing the clot is cut to the length of 4 cm. Use a 2.5-ml syringe filled with saline to connect to the 4-cm tube and transfer the clot into a dish containing saline. Connect a 20-cm PE-04 with a 20-cm PE-50 tube. Inhale the clot into this PE-04-50 tube with a 2.5-ml syringe (Fig. 16.2).

Animals were anesthetized by isoflurane or ketamine and xylazine. Temperature was maintained 37 ± 0.3 °C during surgery by a heating pad. An incision was made in the middle of the neck of the rat, and the CCA, ICA, and ECA were exposed under the microscope, and then the bifurcation of the ICA and pterygopalatine artery (PPA) was exposed. The distal end of the ECA and the origin of the PPA were ligated by 5-0 suture. The proximal side of CCA and ICA was temporally clamped with microvascular clip, and then an incision was made at the ECA lumen close to the ligation. The PE-04 catheter containing a single fibrin-rich clot was introduced into the ECA lumen through the small incision. Then the clip was de-clamped at the ICA, and the catheter was advanced into the ICA. The tip of the catheter will be about 2–3 mm from the origin of MCA. The clot was gently injected with a 2.5-ml syringe filling with saline. After 5 min, the catheter was withdrawn from the ECA. The skin was closed with 3-0 suture. The animal was put back to the cage after it was awake from anesthesia [13–15].

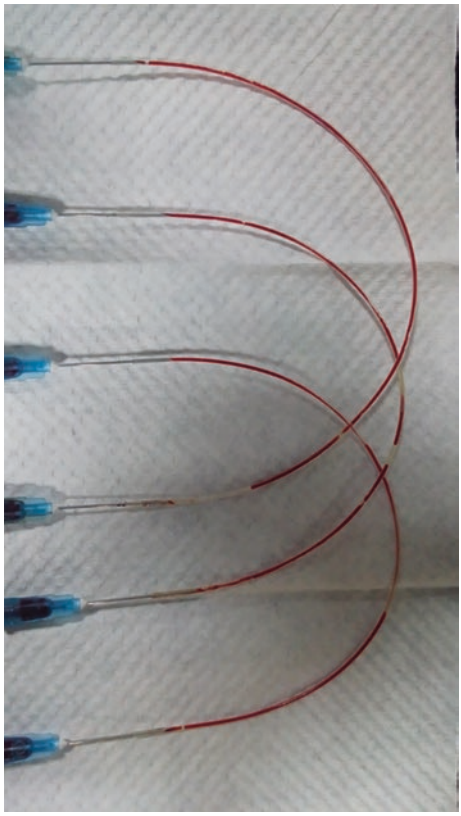

Fig. 16.2 Preparation of thrombus

16.2.3.2 Application of Embolic MCAO

When human stroke was mostly caused by cerebral thromboembolism, a number of animal models had been developed to mimic the embolic occlusion of brain arteria. Embolic stroke models could be performed by injecting some large or small synthetic microspheres into the internal carotid artery to the bifurcation of middle carotid artery. In the first case, large infarcts were similar to those produced by the permanent occlusion of the MCA which could be induced. But in the latter case, some smaller and multifocal infarcts could occur [16, 17]. To study thrombolytic therapies (a clinically very relevant therapeutic issue), several models of vascular occlusion were developed using autologous blood clots that are injected into the internal carotid artery [18]. Embolic stroke models were variable infarcts, which made it more difficult to assess the neuroprotective results; and meanwhile they were well suited to study the reperfusion therapies.

Thromboembolic models of focal cerebral ischemia were mostly used to reestablish human thromboembolic stroke, particularly to investigate the thrombolytic medicine for acute focal cerebral ischemia. In order to find new strategies that may

increase thrombolytic efficiency and reduce the fail rate, the reproducible animal model is required.

Embolic models were more closely representative of the pathophysiology of human ischemic stroke; they offered potential to test more thrombolytic agents [19], assess reperfusion damage after thrombolysis [20], and study potential combination therapies, such as thrombolytic agents combined with neuroprotective drugs [21, 22]. At first, researchers had willings to use the human blood clot suspension of homologous small clot fragments [23]. However, they induced infarcts where spontaneous recanalization took place, and later on autologous clot attracted researcher's attention [24]. Some researchers had described a model of microsphere embolic stroke which had been proved to reduce variability in lesion and more close to the evolution of human disease [25, 26]. However, a definitive microsphere model remains to be defined, because one half of microspheres failed to place in the cerebral arterial circulation [27].

The thromboembolic stroke model showed an excellent reproducibility, and the purified thrombin could be performed to the MCA with good precision [28]. Previously this model was given poor consideration in neuroprotection studies after several failures in applying animal results in human. Moreover, the supraphysiological thrombin content in clots generated by this method might confer resistance to currently available thrombolytic agents [29]. Compared with the mechanical models that had greater accuracy, thrombolytic model did reflect hemodynamic characteristics of blood reperfusion and influencing factors of brain tissue responding to neuroprotective agents.

16.2.3.3 Advantage

Embolic MCAO model in rats could be used to mimic human thromboembolic stroke. So this reproducible animal model could be applied to investigate thrombolytic therapies for cerebral embolism.

16.2.3.4 Limitation

The infarct volume of this model was not stable. The variation was relatively high because of the properties of thrombus. One main reason for failure was spontaneous vascular thrombolytic recanalization.

16.2.4 Distal Middle Cerebral Artery Occlusion

16.2.4.1 Methods and Key Points

Adult male Sprague-Dawley rats weighing 250–300 g were anesthetized by isoflurane or ketamine and xylazine, and an incision was made between the orbit and the tragus under an operation microscope. The temporal muscle was retracted laterally, and a 3-mm diameter craniotomy was made just rostral to the foramen ovale. The dura was incised with a tiny hook, and the MCA was exposed. The arachnoid was then opened, and the MCA was ligated for the permanent MCAO.

For adult male CD-1 mice weighing 30–35 g or adult male C57BL/6 mice weighing 25–30 g, the process was similar to that of rats. Mice are anesthetized by isoflurane or ketamine and xylazine, and a 1-cm incision is made between the orbit and the tragus. The arachnoid is opened, and the MCA is ligated for the permanent MCAO. The reduction of the cerebral blood flow is verified by a laser Doppler flowmetry. The incised skin is sutured. The infarct produced by this model restricts to the cerebral cortex.

16.2.4.2 Application of Distal MCAO

Compared with the other models of MCAO, distal model was the most stable model in all of them, and the infarct area of distal MCAO model was always in the cortex whatever degree of injury was, while the infarct size was affected by whether the MCA and CCAs are permanently or transiently occluded. Cerebral ischemia induced by this method damaged most of the frontal, parietal, temporal, and rostral occipital cortices, the underlying white matter, and a marginal part of the striatum. This technique avoided the thalamic, hypothalamic, hippocampal, and midbrain damage that was seen in the suture transient MCAO model [30]. In the distal MCAO model, it was more common now to ligate the MCA at a more distal location and leave the zygomatic arch intact. This made the surgery less intrusive and speed up recovery time; this procedure induced a smaller infarct compared to the suture MCAO model.

There were several advantages of this model, which has good reproducibility in infarct size and neurologic deficits, low mortality, and visual confirmation of successful MCAO surgery. The main disadvantage was the craniectomy technique, which might lead to injury of the underlying cortex or rupture of a vessel by drilling or electrocoagulation. Additionally, this technique allowed subsequent reperfusion of ischemic tissue. Furthermore, this procedure affected intracranial pressure and blood-brain barrier function and required significant surgical skills to perform it. In order to overcome this limitation, researchers preferred to induce distal MCAO using a photochemical approach through the intact skull of the mouse, which was another kind of MCAO model.

Cortex injury induced by the distal MCAO model was much more stable than other models, so the distal model was becoming more and more widely used not only for its stability but also the convenience in animal experiment. And in some cases where we just want to create a cortex injury excluding the striatum, distal model is the best choice; it is enough to produce the damage that we want.

16.2.4.3 Advantage and Limitation

The distal MCAO model had high stability and strong repeatability. The operation was relatively simple. This model produced ischemic brain injury that was restricted to the cortex of the ipsilateral hemisphere and similar to the pattern of cerebral ischemia in human. Although craniotomy was performed, the overall mortality rate remained low. The limitation was that the infarct volume might be too small to evaluate the therapeutic effect of intervention.

16.2.5 Photochemically Induced Cortical Ischemia

16.2.5.1 Methods and Key Points

Male C57BL/6 mice (20–25 g) were used in the photothrombotic cortical ischemia model. The animal was anesthetized by isoflurane or ketamine and xylazine. The body temperature was monitored continuously and maintained at 37 ± 0.5 °C during surgery using a heating pad. Under an operational microscope, an incision was made between the right orbit and the external auditory canal. A fiber-optic bundle of a KL1500 LCD cold light source with a 4-mm aperture was focused on the selected arteriole. Immediately after the intravenous injection of rose bengal, the brain was illuminated through the exposed intact skull for 2 min. Finally, the surgical wound was sutured [26, 31, 32].

16.2.5.2 Application, Advantage, and Limitation

One of advantages of this model was its high reproducibility and the minimal variation in infarct size combined with a very low mortality. This made it a predestined model to study repair mechanisms and related long-term functional outcome. Another advantage of this model was that the researchers could select a specific cortical brain region of interest to make the ischemia by using stereotactic coordinates [33]. The major issues with this experimental model were fundamental differences compared to the situation in acute human stroke. In this kind of stroke model, cytotoxic and vascular edema and the rapid breakdown of blood-brain barrier were all in an acute phase [34].

But in this model we could not reopen the vessel that was blocked by the thrombus induced by light. It could only be a permanent ischemic model. Compared to the other focal stroke models, this model involved the nonphysiological treatment that made a lesion [35]. Further, because of the damage of the vessels induced by the "photothrombosis," substantial local vasogenic edema formed early after infarction; this was not similar to a clinical patient.

16.2.6 Neonatal Hypoxic-Ischemic Brain Injury

16.2.6.1 Methods and Key Points

Originally 7-day-old Wistar or Sprague-Dawley rats weighing 14–18 g were commonly used in this model. Isoflurane or halothane was delivered from a vaporizer in a mixture of oxygen and nitrous oxide (1:1), 3% for induction and 1.0–1.5% for maintenance. The duration of anesthesia should be less than 5 min. A cervical incision was made, and the common carotid artery was exposed. The common carotid artery using two sutures was ligated; the artery was cut off between the two ligatures. The wound was sutured and infiltrated with a local anesthetic. The animal was kept at around 33 °C temperature before and after procedure. The animal was not anesthetized during hypoxia. The animal was allowed to recover for 1 h. After recovery, the animal was put in the incubator, and the temperature was kept strictly to 36 °C by flowing heated, moisturized air through the incubator for 10 min. The gas flow rate was kept at 3.0 L/min and switched from 7.8% air for rats to 10% oxygen for mice balanced with nitrogen for 50 min. The hypoxic gas mixture was heated and moisturized prior to flow through the incubator and kept at a constant flow of 3.0 L/min. The hypoxia time should be modified according to the lab conditions and different animal strains. Gas flow was switched from 7.8% for rats or 10% for mice oxygen to air for another 10 min. The animals were put back after ischemia [36, 37].

16.2.6.2 Application

In the last decades, researchers had found some similarities in the mechanisms of brain injury and damage evolution between rodents and human infants with cerebral palsy during HI (hypoxia/ischemia) [38]. Modified HI models successfully reproduced hypoxic-ischemic conditions, white and gray matter damage in apoptotic-necrotic pattern, and neuromotor impairments. However, the biggest limitation of the HI rodent models was the difference between rodents and humans in the overall complexity of brain organization and the discrepancies compared with human in the rate of maturation in any period of time [39].

Hypoxia-ischemia rodent models could be used to induce brain injury at the similar stage of cellular development: between 24 and 32 weeks of gestation in human. Most of the oligodendroglial lineage cells were at the stage of pre-OLs at these time points, and they were maximally vulnerable to hypoxic-ischemic injury.

16.2.6.3 Advantage

The neonatal rodent cerebral hypoxia-ischemia model was the most widespread perinatal brain injury model. Ischemic brain damage was produced by unilateral common carotid artery ligation, which induced moderate hypoxia.

The operation was technically simple; this model produced reproducible brain injury with low mortality. This model had proven to be an excellent tool to study physiological and molecular mechanisms of perinatal hypoxia-ischemia brain damage and test potentially protective treatments.

16.2.6.4 Limitation

The variation of the injury degree of brain tissue was considerable. So in order to compensate for the variation, a large number of animals were needed in each group [40].

Another disadvantage was that this model could not produce white matter lesions easily. As white matter lesions were often observed in premature infants, this model had the limitation in precisely mimicking the clinical situation.

16.3 Global Cerebral Ischemia

16.3.1 Bilateral Carotid Artery Ligation (2-VO)

16.3.1.1 Methods and Key Points

Male Wistar rats weighing 250–300 g were commonly used for the procedure. After the rat was anesthetized, the neck skin was shaved and scrubbed with alcohol. A midline cervical incision is made. Both common carotid arteries were gently exposed and isolated from nerve fibers and the surrounding tissues. Then bilateral common carotid arteries were ligated with 5-0 suture. The skin was closed with 3-0 suture. The body temperature was kept at 37 ± 0.5 °C during the procedure until the animal recovery from anesthesia [41].

16.3.1.2 Application of 2-VO

Two-vessel occlusion was the most common model for the chronic cerebral hypoperfusion [42]. In clinic, the chronic ischemic represented many disease situations like Alzheimer. In human, the most common state of aging was the hypoperfusion of the brain tissue, especially the functional cortex that controls the movement and memory [43]. Due to the importance of learning and memory, it was meaningful to perform the chronic hyperfusion research. Though there were several models mimicking the chronic hypoperfusion situation, the two-vessel occlusion was the most widely used model. The aim of two-vessel occlusion was to produce a global ischemic damage in which the onset and the reversal of ischemic process were very rapid. The damage that is produced by the two-vessel occlusion changes the cell morphology in the ischemic brain areas including the hippocampal CA1 subfield, striatum, and cortex. And it had been well documented that the two-vessel occlusion was neither to bring the cerebral blood flow down below the ischemic threshold nor to upset the energy state of the brain tissue to an extent to induce the cell death [44]. With the management of the two-vessel occlusion, most of the brain areas had similar levels of damage, except the Purkinje fibers of the cerebellar brain stem.

With the establishment of the two-vessel occlusion model, pharmacology of many medicines had been studied in the rat or mouse. A number of results were found, and most of these experiments could be divided into these parts. The first purpose was to detect the difference of the region blood flow between treatment and control [42]. The trials were mainly wanted to study the effect of the regulation ability of a medicine or chemical compound. And the other aim of the model was to study the protection mechanism of some medicine or compound. The ischemia often led to the trouble of learning and memory ability, for the decrease of brain blood flow reduced the activity of brain. Based on the theory, the 2-VO rat or mouse was used as the learning- and memory-deficient model; scientists used this model to study methods that could relieve or even reverse the symptom. The second purpose of the model was to mimic the hypoperfusion microenvironment and assess the ability of some treatment that regulates the cerebral blood flow and the function of specific region of the brain like the cortex and striatum. For example, researcher reported effects of chronic guanosine treatment on hippocampal damage and cognitive impairment of rats induced by chronic cerebral hypoperfusion [45]. This study aimed at the reperfusion of the cerebral blood flow in the whole brain and how these treatments could increase the perfusion level, such as the collateral circulation or other way.

16.3.1.3 Advantage and Limitation

The surgical procedure was relatively simple. The success rate of this model is more than 90%. This model could induce high reproducible ischemic damage with great animal survival rate. This model had selective neuronal vulnerability and delayed

neuronal death. For example, typically CA1 pyramidal neurons of the hippocampus were most vulnerable.

The limitation was that brain ischemia could not be induced in awake rats, and it required systemic control of hypotension.

16.3.2 Four-Vessel Occlusion (4-VO) Model

16.3.2.1 Methods and Key Points

Male Wistar rats weighing 250–300 g were used in this model. The animal was anesthetized, and a dorsal neck incision was made from the occipital bone to the second cervical vertebra. The paraspinal muscles were separated to expose the alar foramina of the first cervical vertebrate. The vertebrate arteries were occluded by electrocautery. On the following day, the bilateral common carotid arteries were occluded with aneurysm clips for 10 min to induce ischemia. The animal lost its righting reflex within 30 s, and its pupils were dilated and unresponsive to the light. After 10-min ischemia, the aneurysm chips were removed for reperfusion. The body temperature was maintained at around 37 °C during the procedure [46].

16.3.2.2 Application of 4-VO

The 4-VO was mainly used to produce a global cerebral ischemic pathology. Moreover, this model was always compared with the two-vessel occlusion model; the difference was that the posterior circulation is still working in two-vessel occlusion model; the post-cerebral artery blood could be compensatory supplied to the hindbrain, which only lead to forebrain ischemia [47]. When there is no compensatory blood from the post-cerebral artery in the occlusion of vertebral artery, we could create a permanent global cerebral ischemia and give the reperfusion after a short time in the occlusion of the bilateral common carotid arteries.

There were always some collateral circulations or blood compensation involved in other ischemic model, and that is why we could only induce a regional ischemic model. In order to perform the global ischemic model, the four-vessel occlusion was proposed. In the complete non-blood environment, apoptosis of neuron in the cortex or hippocampus was studied. The neuropathological examination of the model shows a good repeatability of the ischemic damage in the target brain region. The vulnerable brain regions to ischemia were the CA1/2 region of the hippocampus, subiculum, the CA3 region of hippocampus, the CA4 region of hippocampus, superior pyramidal lobe and inferior pyramidal lobe of dentate gyrus, and neocortex in order. The CA1/2 hippocampus and subiculum were most vulnerable to ischemic damage. With the increase of ischemic duration, the damage of the neurons from the outer segment of CA1/2 hippocampus to subiculum was intensified gradually.

Ischemic damage of the neurons in neocortex varied greatly in different individuals; no evident change appeared even after 15 min of ischemia in some animals.

Combining 4-VO with arterial hypotension could considerably enhance the severity of the ischemia by nearly eliminating all of CBF for sustaining tissue. Indeed, total circulatory arrest in the brain was observed only when systemic hypotension was employed and never when the blood pressure was normal. Global cerebral ischemia caused by 4-VO in the rat shows variation in its severity among regions of the brain. Fortunately, the blood supplied to the cardiorespiratory centers in the medulla is relatively spread, which maintained the viability of the animal. Arterial hypotension during 4-VO considerably enhanced the degree of ischemia and permits complete circulatory arrest to be achieved in the cerebrum [48].

16.3.2.3 Advantage

4-VO model in rat was well established and produces reproducible neuropathological results. The cost in purchase and maintenance was relatively low. 4-VO model induced transient forebrain ischemia that mimicked cardiac arrest in clinical situations. This model produced reliable outcome with selective, delayed cell death. For example, CA1 pyramidal neurons in the hippocampus died 2–3 days after ischemia, while CA3 neurons survived after the same insult.

16.3.2.4 Limitations

A common complication of this model was seizure. The incidence of seizures had positive correlation with the severity and duration of ischemia. Ten percent of the animals died during ischemia or after reperfusion. One major complication that contributed to the animal's death during ischemia was the respiratory failure during the first 2–3 min of occlusion. Death after reperfusion might have correlation with severe ischemia. The electrocauterization of the vertebral arteries which are hidden underneath the alar foramina was a difficult task. Unsuccessful electrocauterization was the major reason for incomplete ischemia.

The damage of 4-VO might vary among animals. Many factors, including cerebral collaterals, brain temperature, anesthesia, etc., influenced the ischemic outcome. So in order to obtain stable and consistent results, strictly control variables and optimize experimental conditions in the surgical process.

16.3.3 Cerebral Ischemia Induced by Cardiac Arrest

16.3.3.1 Methods and Key Points

Male adult Sprague-Dawley rats weighing 300–350 g were used in the asphyxia cardiac arrest model. Anesthesia was induced with 4.0% halothane in oxygen, which was then titrated to 1.5% halothane during the surgery. ECG electrodes were put on each forelimb and hind limb. The left femoral area was shaved and prepared with povidone-iodine. The femoral artery and vein were dissected apart carefully. The left femoral vein and artery were inserted with catheters. Arterial blood pressure and blood gases were monitored. The rat was paralyzed by slowly infusing vecuronium (2 mg/kg) intravenously in order to induce asphyxial arrest. During administration of vecuronium, halothane was discontinued. The oxygen source was disconnected, and ventilation with room air was allowed for 2 min. Asphyxia was induced by turning off the ventilator at end expiration. Blood pressure and heart rate would begin to decrease within 30 s. There was no pressure gradient when arterial pressure fell to near 20 mmHg or when pulsatile signals were absent from the arterial tracing. Complete circulatory arrest occurred reliably within 180 s after onset of asphyxia. After 8 min, the ventilator was reconnected, and ventilation resumed with 100% oxygen at a rate of 60 breathes/min. Rapidly administer the resuscitation drugs and deliver chest compressions at a rate of 200 beats/min. Chest compressions continued until spontaneous cardiac activity was detected. The rat would require ventilator support for at least 60 min. When the rat breathes well on its own, the tubes were withdrawn. Rats would not feed properly and might be in a coma for several days after cardiac arrest. Subcutaneous fluids (e.g., 5% dextrose in 0.9% saline) were administered (20–40 ml/kg/day) until rats could feed independently [49, 50].

16.3.3.2 Application

The brain injury associated with cardiac arrest was more global with evidence of neuronal injury in multiple brain regions. Thus, rats surviving cardiac arrest might be expected to have deficits in a variety of behavioral tests in addition to memory [49]. The global assessment of neurological function used here had more relevance to the clinical situation of cardiac arrest, which caused coma followed by global dysfunction.

An important limitation of many porcine cardia arrest models was that juvenile, disease-free pigs were generally used. In clinical settings, patients who experience cardiac arrest typically were elderly and suffered from chronic disorders such as hypertension, atherosclerosis, congestive heart failure, diabetes, emphysema, or end-stage renal disease [51]. The Ossabaw swine, which was predisposed to develop metabolic syndrome when consuming a high-fat diet, provided a unique, clinically relevant experimental model; it was suitable for studying cardiac arrest and

resuscitation superimposed on metabolic syndrome [52]. Indeed, under anesthesia, these swine developed severe arrhythmias, responsive to amiodarone, that might deteriorate into cardiac arrest.

16.3.3.3 Advantage

Sudden cardiac arrest resulted in neurological injury, affecting the majority of person who initially had restoration of pulses. The rat asphyxial cardiac arrest was an established model which mimicked neurological injury after cardiac arrest. This model could reproduce many aspects of neurological injury, for example, transient coma, evolving motor deficits, persistent sensorimotor deficits, and the systemic metabolic disruptions.

16.3.3.4 Limitation

The process of cardiac arrest requires 3 h of special care. The animals need 2–3 days' care after operation. Acute death is not common. Most of the death time is 1–5 days after cardiac arrest which is related to the severity of neurological impairment. The main cause of death is respiratory failure.

16.4 Methods of Evaluation

16.4.1 Cresyl Violet Staining

Cresyl violet staining was used for infarct volume measurement. Following MCAO, the animals were killed, and the brains were removed and frozen immediately in isopentane at −42 °C for 5 min. A series of 20-μm-thick coronal sections from anterior commissure to hippocampus were cut. The distance between adjacent sections on the slide was 200 μm. The sections were dried and stained with Cresyl violet [7]. The infarct area that could not be stained by Cresyl violet (Fig. 16.3) was calculated. Using Image J software, the ischemic area was delineated, and the infarct volume was calculated by multiplying the infarct area by the thickness of the section.

16.4.2 2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

Another method of detecting infarct area was TTC staining which was simple and fast. After MCAO, animal brains were removed immediately and six (for rats) or four (for mice) coronal slices were dissected using a brain slicer. The brain slices

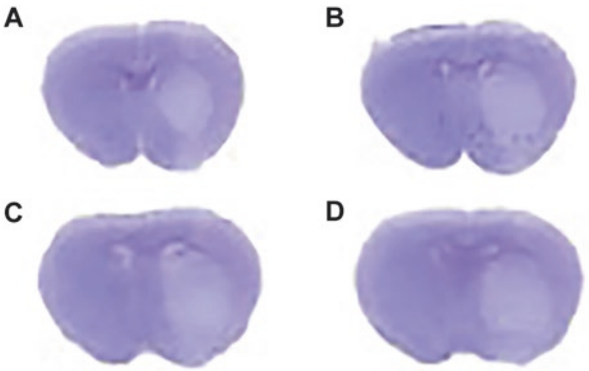

Fig. 16.3 A set of Cresyl violet-stained mouse brain coronal sections. The mouse underwent 90 min of MCAO, followed by 24 h of reperfusion

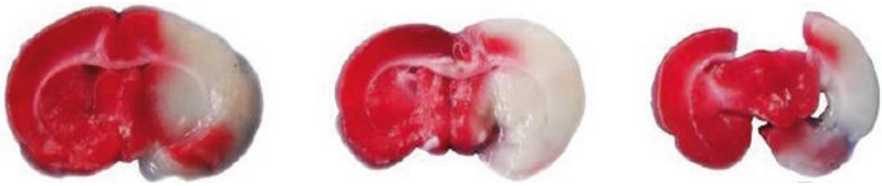

Fig. 16.4 A set of TTC-stained mouse brain coronal sections of a rat 24 h after embolic MCAO

were stained with 2% TTC in Dulbecco's phosphate buffer (pH 7.4) at 37 °C for 20 min [53]. The ischemic lesion area could not be colored red by electron transport in active mitochondria. The volume of infarction is calculated by multiplying the distance between sections (Fig. 16.4).

16.4.3 Brain Water Content Assay

Rats or mice were sacrificed at 1 or 3 days after acute ischemia. Brain samples were weighted before and after dehydration in an oven at 95 °C for 24 h. Brain water content was calculated using the formula: ((wet tissue weight – dry tissue weight)/wet tissue weight) ×100%. The water content of normal brain tissue was about 78% while that of the ischemic brain tissue might elevate to 82–85% [54].

16.4.4 Neurological Severity Scores

modified Neurological severity score (mNSS)	
Raising rat by the tail	
Flexion of the forelimb	1
Flexion of the hind limb	1
Head moved >10° to vertical axis within 30 s	1
Placing rat on the floor (normal=0; maximum=3)	
Normal walk	0
Inability to walk straight	1
Circling toward the paretic side	2
Fall down to the paretic side	3
Beam balance tests (normal=0; maximum=6)	
Balances with steady posture	0
Grasps side of beam	1
Hugs the beam and one limb falls down from the beam	2
Hugs the beam and two limbs fall down from the beam or spins on beam (>60 s)	3
Attempts to balance on the beam but falls off (>40 s)	4
Attempts to balance on the beam but falls off (>20 s)	5
Falls off: No attempt to balance or hang on to the beam (<20 s)	6
Reflexes absent and abnormal movements	
Corneal reflex (eye blink when lightly touching the cornea with cotton)	1
Startle reflex (motor response to a brief noise from snapping a clipboard paper)	1

16.4.5 Rotarod Test

Mice or rats were placed on a rotating rod at a fixed speed for 1 min to make an adaptation. The rod was slowly accelerated from 20 to 40 rpm in 5 min. Duration that mice stayed on the rod (fall latency) was recorded and analyzed as mean duration of three trials on the rotarod. Before performing stroke model, mice or rats were trained for 3 consecutive days to get stable baseline values. The baseline values represented normal neurobehavioral function. Rotarod test could be performed at 7 days, 14 days, 21 days, or 28 days after stroke [1, 55, 56].

16.4.6 Morris Water Maze Test

Water maze test was used to detect the capacities of spatial learning and memory. The water maze was located in a large room with several visual signs around the tank to show the orientation. The visual signs were used by the rats or mice for

spatial orientation. The position of the cues remained unchanged throughout the experiment. The round water tank is divided into four quadrants (north, south, east, and west). Water was filled to a height of 50 cm. The water temperature was kept at 21–24 °C. A circular platform was placed in the center of one of the four quadrants and was 1–2 cm for rats and 0.5–1 cm for mice under the water surface, hidden from the animal's view.

In the test, the animals were first trained to find the hidden platform for 5 days. The animal was placed in the water facing the wall at a start position in any of the four areas. Each animal was allowed to find the platform. If the animal failed to find the hidden platform within 120 s for rats and 60 s for mice, it was placed on the platform for 15 s. The procedure was repeated for all the four start locations. On the sixth day, the platform is removed and the animal is placed in a novel starting position. The number of platform-site crossovers, time, and distance spent in the target quadrant was recorded [57–59].

16.5 Conclusion and Future Directions

Ischemic stroke models need high level of operation to ensure the model's success and stability. Patience, carefulness, and practice are required. Each model remains huge space for development, and stroke models need continuously being improved.

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Part III
Stroke Therapy

Chapter 17
Neuroprotection of Heat Shock Proteins (HSPs) in Brain Ischemia

Jong Youl Kim, Jong Eun Lee, and Midori A. Yenari

Abstract Heat shock proteins (HSPs) were among the first early genes recognized to be upregulated following brain ischemia. This upregulation was thought to be part of an endogenous response to stress, but the significance of these proteins was not fully understood until genetic manipulation to overexpress or delete these genes showed that they indeed participated in the prevention of cell death. The mechanisms by which HSPs prevent cell death are multifold. In addition to their chaperone properties where they can assist in nascent protein folding and the prevention of protein aggregation, HSPs seem to also participate in specific cell death pathways and inflammation. The most studied of these proteins is the 70 kDa inducible HSP also known as HSP70. The availability of a few pharmacological inducers has shown that this same neuroprotective effect can be recapitulated in wildtype animal models. Further, some of these inducers have already been studied in humans for treatment of other conditions. HSPs should be further investigated for their translational relevance in the treatment of stroke and related conditions.

Keywords Heat shock protein • Chaperone • Cerebral ischemia • Apoptosis • Inflammation • Geldanamycin

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Abbreviations

17-AAG	17-(Allylamino)geldanamycin
AIF	Apoptosis-inducing factor
Apaf-1	Apoptosis protease activating factor-1
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
DIABLO/smac	Direct IAP-binding mitochondrial protein/second mitochondria-derived activator of caspases
HO-1	Heme oxygenase-1
HSC	Heat shock cognate
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein
IkB	Inhibitor of kappaB
IKK	IkB kinase
IL-1	Interleukin-1
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
MCA	Middle cerebral artery
MMP	Matrix metalloproteinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor-kappaB
NOS	Nitric oxide synthase
OGD	Oxygen glucose deprivation
PKB	Protein kinase B
STAT-1	Signal transducer and activator of transcription factor-1
TLR	Toll-like receptor
TNF	Tumor necrosis factor

17.1 Introduction

Following a variety of brain injuries including ischemia, the brain undergoes a coordinated stress response which allows it to protect itself from harm. In the injured brain, the most widely studied protein surrounding neuroprotection are the heat shock proteins (HSPs), initially described when cells were exposed to sublethal heat stress. HSPs are a highly conserved family of ATP-dependent, cytosolic chaperone function which is primarily in facilitating protein folding, degradation, complex assembly, and translocation, consequently preventing harmful protein aggregation and assembly of polypeptide of newly synthesized proteins [1]. Constitutively expressed members exist within all subcellular compartments and appear essential for normal development and cellular function. Inducible forms increase following a variety of

external stress, including brain injury, but were originally described following heat stress [2]. Work over the past two decades has also established that some HSPs function as cytoprotectants. HSPs have long been known to serve as protein chaperones in the sense that they assist in protein folding and the correct attainment of a functional three-dimensional configuration while preventing incorrect folding and protein aggregation [3]. They have also been shown to affect cellular signaling and have been extensively studied to provide protection against different types of experimental brain injury models.

17.2 Heat Shock Proteins

At the initiation of brain injury, synthesis of most cellular proteins is suppressed with the exception of a small class of proteins. These proteins include HSPs. HSPs are classified according to their molecular mass and include HSP100, HSP90, HSP70, HSP60, HSP40, and the small HSP families. Constitutive HSPs, such as HSP90, HSP40, and HSC70, perform housekeeping functions within cells [4]. Induced HSPs are involved in folding proteins correctly and preventing the aggregation of unfolded proteins [4]. In brain cells, hyperthermia triggers a robust expression of induced HSPs, such as HSP70, HSP32, and HSP27 [5]. The best-studied class of neuroprotective HSPs is HSP70 or the 70-kDa class which includes an inducible form also known as HSP72, HSP70i, or simply HSP70. HSP70 interacts with hydrophobic peptide segments of unstructured proteins in an ATP-dependent manner. HSP70 also contains a C-terminal substrate-binding domain which identifies unstructured polypeptide segments and an N-terminal ATPase domain which assists in protein folding, alternating between an ATP-bound, open state with low substrate affinity and an ADP-bound closed state with high substrate affinity [1]. In studies of cerebral ischemia, HSP70 was observed to be induced in brain regions that were relatively resistant to ischemic insults. Hence, the notion of a "molecular penumbra" was introduced and raised questions as to whether this expression was epiphenomena of the injury or an active participant in cell survival [3]. Subsequent studies using strategies to increase or inhibit HSP70 expression have consistently shown that HSP70 protects the brain and brain cells against experimental cerebral ischemia, neurodegenerative disease models, epilepsy, and trauma. Through its chaperone properties, it has been shown to reduce protein aggregates and intracellular inclusions [2]. Two other stress proteins studied in brain ischemia include HSP27 and HSP32 (also known as heme oxygenase or HO-1) [6]. In addition to their function in protein processing, HSPs appear to protect the brain by affecting several cell death and immune response pathways [1, 7].

17.2.1 The Heat Shock Response

HSPs are thought to play roles in signaling cascades involved cell growth and differentiation under non-injury conditions. They are rapidly upregulated in response to cell stress including brain injury. The molecular mechanism for regulation of HSP overexpression depends on the activity of a unique transcription factor-heat shock factor 1 (HSF1) that can bind to the 5′ promoter regions of all HSP genes and trigger transcription [8]. Under homeostatic conditions, HSPs are located intracellularly and are bound to HSF1 [7]. Following an appropriate stress such as heat, ischemia and other causes of accumulation of unfolded proteins, HSPs dissociate from HSF, leaving HSPs free to bind target proteins. In the stressed cell, dissociated HSF is transported to the nucleus where it is phosphorylated, possibly by protein kinase C, to form activated trimers. These trimers bind to highly conserved regulatory sequences on the heat shock gene known as heat shock elements (HSEs). Once bound to HSEs, HSFs bind to the promoter region of HSP genes, leading to more HSP generation [8]. Newly generated HSPs can then bind denatured proteins and act as a molecular chaperone by contributing to repair, refolding, and trafficking of damaged proteins within the cell. HSP90 can also influence HSP70. HSP90 is bound to HSF1 where it acts to prevent HSF1 from entering the nucleus. When HSP90 dissociates from HSF1, HSF1 is liberated and binds to HSEs, leading to more HSP70 induction.

17.2.2 HSP in Cell Death Signaling Pathways

HSPs influence several different steps in the cell death pathway, such as apoptosis (Fig. 17.1). HSP70 interacts with components of the programmed cell death machinery upstream [9, 10] and potentially downstream of mitochondrial events [11]. HSP70 is able to interrupt cytochrome c release in experimental stroke models [12, 13] and blocks apoptosis-inducing factor (AIF) translocation to the nucleus [14] while reducing ischemic brain injury in experimental stroke models. Previous study observed that HSP70 overexpression by transgenic mice has been shown to interfere with recruitment of procaspase-9 into the apoptosome and to sequester AIF [15]. HSP70 also inhibited release of the proapoptotic protein Smac/DIABLO from myocyte mitochondria [16]. Mitochondrial HSP70/HSP75/mortalin, assistants to maintain mitochondrial membrane potential may lead to the preservation of mitochondrial function76 and mitochondrial protein import [17]. HSP27 can also reduce procaspase-9 activation by interrupting formation of the apoptosome and interaction with cytochrome c and can inhibit release of cytochrome c from mitochondria [18]. HSP27 can directly interact with procaspase-3 and inhibit caspase-3 activation [18]. Overexpression of HSP70 in astrocytes reduced their vulnerability to in vitro ischemia-like injury (oxygen glucose deprivation, OGD) and preserved higher ATP levels in stressed cells [19]. These results were associated with reduced

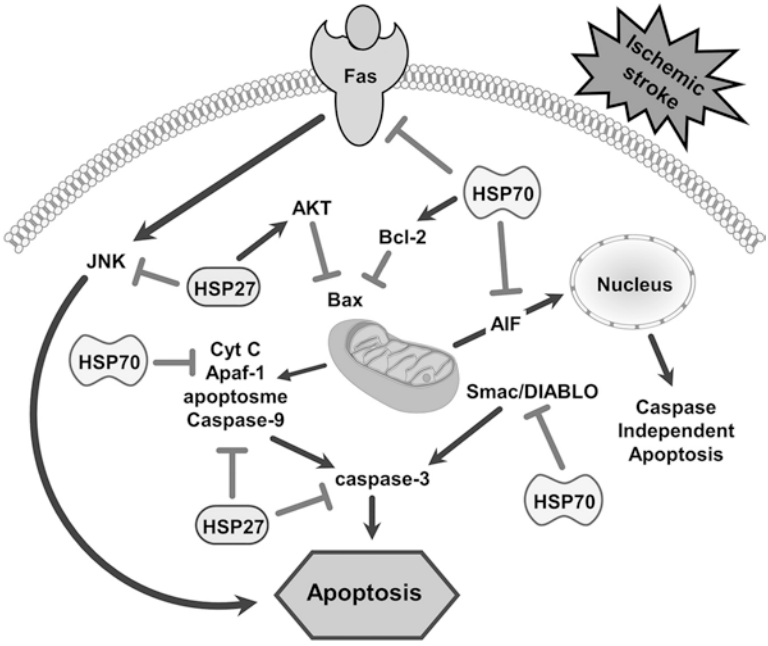

Fig. 17.1 Influence of HSPs in apoptosis. Ischemic stroke induces apoptotic cell death by several distinct pathways, and heat shock proteins (HSP70 and HSP27) influence these pathways at several levels. Ischemic stroke can trigger apoptosis through both intrinsic (mitochondrial) and extrinsic (receptor-mediated) pathways. Ischemic leads to mitochondrial stress, which causes the release of proapoptotic factors such as cytochrome c, Smac/DIABLO, and AIF. Cytochrome c induces oligomerization of Apaf-1, which recruits and activates procaspase-9 to caspase-9. Caspase-9 then activates effector caspase-3. Ischemia also leads to the release of factors that can bind death receptors such as Fas which leads to apoptosis through activation of the JNK stress kinase. HSP70 and HSP27 have been shown to inhibit apoptosis at the sites shown. This includes prevention of mitochondrial release of proapoptotic factors, inhibition of caspase-3 activation, and downregulation of Bax while upregulating the anti-apoptotic molecule Bcl-2. HSPs also interfere with receptor-mediated apoptosis by downregulating Fas and inhibiting JNK (*AIF* apoptosis-inducing factor, *Akt* protein kinase B, *Apaf-1* apoptosis protease activating factor-1, *Cyt C* cytochrome c, *JNK* c-Jun N-terminal kinase)

reactive oxygen species (ROS) formation and better maintained mitochondrial membrane potential in an in vitro model of ischemic stroke [20] and with better preservation of glutathione levels [21]. In myocardial cells, overexpression of HSP70 was shown to increase the activity of the mitochondrial antioxidant enzyme manganese superoxide dismutase [22].

Bcl-2 is known as an anti-apoptotic protein, whose function in apoptosis is to block release of cytochrome c and AIF, thus preventing effector caspase activation. HSP70 overexpression using a viral vector led to increased levels of Bcl-2 protein in hippocampal neurons [7]. The balance between pro- and anti-apoptotic members of the Bcl-2 family determines whether cells undergo apoptosis by regulating the mitochondrial membrane permeability transition [23]. Thus, HSP70 overexpression

can decrease induction of apoptosis upstream of mitochondria in ischemic stroke both directly and via increased Bcl-2 levels. HSP70 reduces heat-induced apoptosis primarily by blocking translocation of the proapoptotic Bcl-2 family member Bax, thereby preventing the release of proapoptotic factors from mitochondria [10]. Recent studies showed that HSP27 can indirectly suppress stress-induced Bax oligomerization and translocation to the mitochondria [24]. HSP27 anti-apoptotic activity has been demonstrated to activate the protective kinase Akt/PKB [25] or to inactivate the pro-death c-Jun N-terminal kinase (JNK) pathway [26]. HSP70 also interferes with the activity of apoptosis protease activating factor-1 (Apaf-1), which is required for formation of the apoptosome and activation of caspase-9 [15], but also Steel et al. demonstrated a lack of direct interaction with Apaf-1 [9]. Recently, our laboratory showed that dynamin trafficks Fas to the neuronal cell's surface and HSP70 overexpression prevents this trafficking after ischemic stroke [27]. This study indicated that HSP70 also prevented the extrinsic or receptor-mediated apoptotic pathway through specific chaperone interactions.

17.2.3 HSP in Inflammation

HSPs are known to have significant modulating roles both acting as a pro-inflammatory stimulator and inhibitor. Extracellular HSPs are also capable of immunomodulatory functions that trigger immunological responses [2]. HSP70, perhaps the most studied of the HSPs with respect to its role in inflammation, appears to play dual roles depending on the nature of the stimulus and the ensuing immune response. In the extracellular environment, HSPs have been studied in terms of their role in adaptive immunity where they appear to assist in and potentiate adaptive immune responses. HSPs complexed with peptides elicit CD8+ T-cell responses after exogenous administration [28]. Immunization of mice with these same complexes can elicit CD4 responses, indicating that HSPs can act as adjuvants [29]. Extracellular HSP70 can also interact with the macrophage/dendritic cell CD40, CD91, or LOX-1 receptor and aid in antigen presentation [30]. Extracellular HSPs also appear to participate in innate immune responses (Fig. 17.2). In innate immune responses, HSP70 can interact with macrophages, microglia, and dendritic cells through Toll-like receptors (TLRs) and lead to nuclear factor-kappaB (NF-kB) activation with subsequent upregulation of pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS) [31]. HSP60 and HSP70 are both thought to interact with TLR 2 and TLR4 [32].

As an anti-inflammatory molecule, HSPs have also been shown to interact with pro-inflammatory factors such as NF-kB, matrix metalloproteinases (MMPs), and ROS, leading to an anti-inflammatory state. Intracellular overexpression of HSP70 or its intracellular induction by heat stress has been shown to reduce inflammatory cell production of nitric oxide and iNOS expression while decreasing NF-kB activation in astrocytes [33]. Heat shock has also been correlated to decreased secretion of tumor necrosis factor-alpha (TNF-α) and reduced generation of ROS. HSP70 can

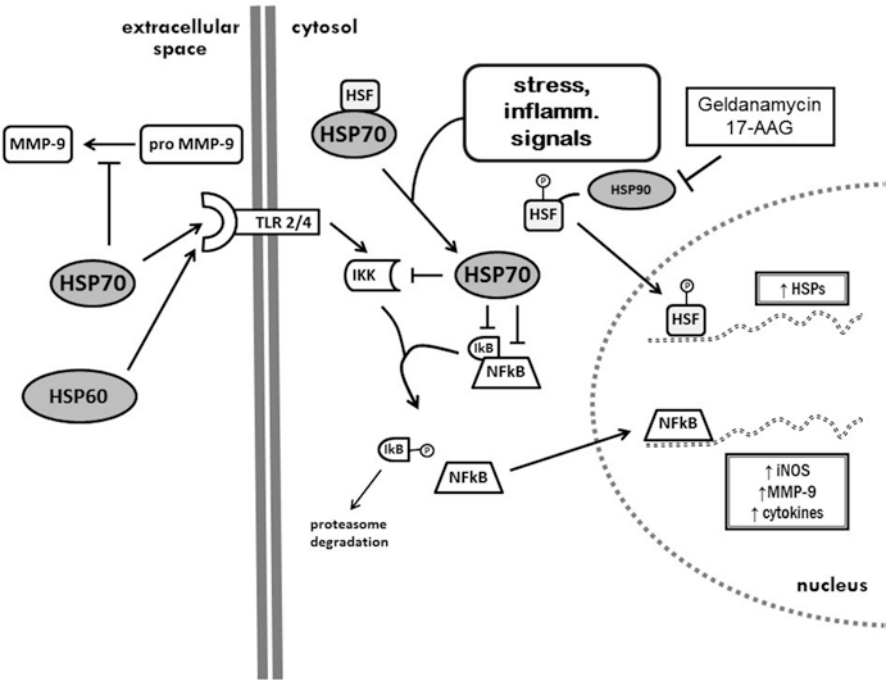

Fig. 17.2 Influence of HSPs in innate immunity. For details, see Sect. 17.2.3. HSPs have been shown to affect innate immune responses both intracellularly and extracellularly and lead to both pro- and anti-inflammatory responses depending on the compartment in which they reside. Following cerebral insults, HSPs are thought to be upregulated in brain cells and, when these cells lyse, are then liberated into the extracellular space. In the extracellular space, HSP70 appears to prevent activation of MMPs by preventing their conversion from their pro-form. HSPs, including HSP70 and HSP60, have also been shown to act as ligand for Toll-like receptors 2 and 4 (TLR 2/4) typically found on immune cell such as microglia. When activated, TLRs lead to signaling which activate the kinase of NF-kB's inhibitor protein (IKK). When NF-kB's inhibitor protein (IKB) is phosphorylated, it liberates NF-kB to enter the nucleus where it can bind its consensus sequences and upregulated a variety of pro-inflammatory molecules. Phosphorylated IkB is then degraded in the proteasome. Intracellularly, HSPs have been shown to inhibit the activation of IKK, as well as bind both IkB and NF-kB and prevent NF-kB's activation and nuclear translocation, respectively, thus preventing the upregulation of various pro-inflammatory molecules. Pharmacological induction of HSP70 is possible through inhibitors of HSP90 (geldanamycin, 17-AAG). HSP90 normally inhibits HSF1 from translocating to the nucleus and upregulating HSP70

also prevent responses to inflammatory cytokines such as TNF-α and interleukin-1 (IL-1) [34], while overexpression of HSP70 in macrophages blocked LPS-induced increases in TNF, IL-1, IL-10, and IL-12 [35]. In a model of intracerebral hemorrhage, upregulation of HSP70 decreased TNF-α expression and attenuated blood-brain barrier (BBB) disruption, edema formation, and neurological dysfunction [36].

Induction of HSP70 by heat shock inhibits NADPH oxidase activity in neutrophils and enhances superoxide dismutase, which scavenges superoxide, in phago-

cytes [37]. We previously showed that overexpression HSP70 also interrupts the phosphorylation of IkB, JNK, and p38 and blunts DNA binding of their transcription factors, such as NF-kB, activator portien-1, and signal transducer and activator of transcription factor 1 (STAT-1), effectively downregulating the expression of pro-inflammatory genes in heat-pretreated astrocytes [38]. Other studies have also shown that prior heat stress leads to inhibition of the inflammatory response, and was associated with overexpression of HSP70 which blocked nuclear NF-kB translocation [39, 40]. It has been speculated that HSP70 could interact with inhibitor of kB (IkB) and interrupt NF-kB dissociation by inhibition of IkB phosphorylation [33]. A few studies have shown that HSP70 binds to and inhibits NF-kB and/or its regulatory proteins [41, 42], although how it does this may depend on the nature of the stimulus. In a model of TNF-α induced cell death pathway, HSP70 directly inhibited IkB kinase (IKK) activity, whereas in a model of ischemic stroke, HSP70 appeared to associate with NF-kB and IkB, thus preventing IkB phosphorylation by IKK. The inhibition of NF-kB by HSP70 led to blocked transcription of several immune genes and improved neuroprotection.

Our study showed that MMP-9, one of several immune genes regulated by NF-kB, was reduced in cultured HSP70-overexpressing astrocytes exposed to ischemia-like insults. Consistent with the observation that HSP70 overexpression by viral vectors may regulate inflammatory protein expression at the transcriptional level, MMP-9 mRNA was also lower in HSP70-transfected astrocytes [43]. Furthermore, HSP70 expressed in astrocytes seems not only to decrease expression of MMP-9 at both the transcriptional and translational levels but also to decrease MMP-2 [43]. Interestingly, MMP-9 expression is regulated by NF-kB, whereas MMP-2 is not, suggesting that HSP70 may interfere with transcriptional responses in systems other than NF-kB. In fact, studies in alveolar macrophages suggest that heat stress-induced HSP70 can inhibit the STAT-1 signaling pathway [44], and this pathway has been linked to MMP-2 expression [45]. HSP70 also appears to prevent MMP processing from its pro- or inactive form to its cleaved or active form. Thus, it is clear that HSPs have a myriad of roles, some of which modulate immune responses, both adaptive and innate, toward both pro- and anti-inflammatory phenotypes.

17.2.4 HSP in Ischemic Stroke

Overexpression of HSPs in experimental models leads to protection against a variety of acute insults in ischemic stroke [6]. During homeostatic conditions, inducible HSP levels are low; however, its expression is significantly increased following injury. In experimental stroke models, HSPs have been shown to lead to neuroprotection [7].

HSP70 is the most abundant HSP found in cells. Many studies have shown the extensive link between HSP70 overexpression and tolerance in ischemic stroke. Following 10 min of focal cerebral ischemia, HSP70 protein is induced in the middle cerebral artery (MCA) territory 24 h later. After 1.5 h MCA occlusion, there is HSP70 expression at the watershed zone between the middle and anterior cerebral arteries. In the penumbra, HSP70 induction occurs primarily in neurons [3]. In areas of infarction, or areas adjacent to the infarction, HSP70 can be induced in endothelial and glial cells, such as astrocytes and microglia [3]. Viral vector-mediated HSP70 overexpression has been shown to improve survival of neurons and astrocytes from ischemic and ischemia-like insults, including oxygen glucose deprivation and focal and global cerebral ischemia [1]. Similarly, transgenic mice that overexpress HSP70 are protected from these ischemic insults, whereas their deficiency exacerbates transport across the [27, 46]. Intravenous TAT-Hsp70, a Hsp70 attached to a TAT motif to improve transport across the BBB, led to decreased infarct volumes, improved neurological outcomes, and improved survival of neural progenitors in an experimental stroke model [47]. This neuroprotection is not only associated with less apoptotic cell death and increased expression of anti-apoptotic proteins, but has been shown to affect various intracellular immune pathways and signaling.

HSP27 possesses many similarities to HSP70, with the exception that it does not require ATP for its actions [18]. Transgenic mice overexpressing HSP27 subjected to cerebral ischemia demonstrated neuroprotective benefits due to this overexpression [48]. Viral vector-mediated HSP27 expression in vitro and in vivo has been shown to protect against ischemic brain and kainate-induced neuronal cell death [6]. Mechanisms of this protective effect have largely been attributed to HSP27's ability to interfere with apoptosis. Like HSP70, HSP27 has been shown to prevent mitochondrial release of cytochrome c and prevent formation of the apoptosome. HSP27 may also directly interact with procaspase-3 and prevent Bax translocation to the mitochondria. In ischemic stroke, neuroprotection of Hsp27 has also been performed where the PEP-1-Hsp27 fusion protein, designed to cross the BBB, was given intraperitoneally and protected hippocampal pyramidal neurons against death in the global ischemia model [49].

HSP32 is also known as heme oxygenase 1 (HO-1). This is an enzyme involved in heme catabolism. HO-1 is inducible and is considered a member of the stress protein family, because it contains a HSF in its promoter. It converts heme into biliverdin, carbon dioxide, and ferrous iron. It is induced by similar factors as other HSPs [7]; however, the literature surrounding HO-1 in ischemic brain and related conditions is conflicting. Studies of HO-1 deficiency have shown that HO-1 knockout mice have worsened outcomes in ischemic stroke models but improved outcome in models of brain hemorrhage [50]. Reasons for these discrepancies may be due to differential effects of its metabolites and the setting and location where HO-1 is active.

17.3 HSP Therapy for Neuroprotection

Pharmacological induction of HSP70 is also possible and has been shown to protect the brain in experimental models. The best-studied Hsp70 inducers are the ansamycins, geldanamycin, and 17-(allylamino)geldanamycin (17-AAG). These compounds upregulate inducible HSP70 through their ability to inhibit HSP90 and have been shown to protect the brain from brain injury [8]. Additionally, geldanamycin and 17-AAG have been studied in clinical trials, albeit for other indications. Also, other HSP70-inducer groups are the purine-based compounds, the resorcinols, and other novel chemotypes. The purine series is a synthetic class that was modeled after the way ansamycins co-opt ADP to bind the HSP90 ATP binding site [51]. The best-known purine-based HSP70-inducer to date is BIIB021 (also named CNF-2024), which has been demonstrated to be a potent Hsp90-inhibitor [52]. Some of the variants designed to counter this pharmaceutical disadvantage are NVP-AUY922 and AT-13387. To date, there have been no studies evaluating resorcinol's effect on brain injury [52], though ostensibly its function as an HSP70 inducer would be expected to provide protection. There are also novel chemotypes such as EC144, a synthetic HSP90 inhibitor, which has been shown to effectively block both innate and adaptive immune response pathways [53]. As with many other HSP70 inducers, none of the novel chemotypes have yet been tested for BBB permeability or applied to the brain injury models.

17.4 Conclusions

Numerous studies suggest the neuroprotective effect of HSPs in brain injury. HSPs have shown consistent neuroprotective effects in different injury models. It appears to have multiple protective mechanisms and can be pharmacologically induced with agents that have been tested in humans for other indications. Other stress genes and their respective proteins also hold promise as beneficial endogenous protectants but may be less developed in terms of how this property may be applied clinically.

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Chapter 18
Photobiomodulation for Stroke

Michael R. Hamblin

Abstract Photobiomodulation (PBM) or low-level laser (light) therapy (LLLT) describes the use of red or near-infrared light from lasers or LEDs to heal, stimulate, and protect damaged tissues. It was first used for wound healing and pain relief but in recent years has been extensively investigated for brain disorders. One of the first applications of transcranial PBM in the brain was for acute stroke. The mechanisms of action of PBM are multifunctional. PBM applied to the head can increase cerebral blood flow by releasing nitric oxide and improve tissue oxygenation in the brain. It can stimulate mitochondrial metabolism and increase ATP production. Protective mechanisms are activated that can reduce neuronal cell death and oxidative stress occurring as a result of hypoxia, while neuroinflammation is also reduced. PBM can stimulate the formation of new neurons from neuroprogenitor cells in the hippocampus and subventricular zone. Finally, PBM can stimulate synaptogenesis and neuroplasticity (formation of new connections between existing neurons). This chapter will cover animal studies of PBM for acute stroke (caused by a variety of techniques) carried out in rats, rabbits, and dogs. The three major clinical trials for acute stroke in human patients (NEST-1, NEST-2, NEST-3) and the reasons for the eventual failure of NEST-3 will be discussed. Finally, the possibility of using PBM for rehabilitation of chronic stroke patients will be addressed.

Keywords Photobiomodulation • Low-level laser therapy • Mechanism of action • Acute stroke • Animal studies • Clinical trials

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18.1 Introduction

Photobiomodulation (PBM) (previously known as low-level laser (light) therapy, LLLT, or near-infrared laser therapy, NILT) is approaching its 50th anniversary, after being discovered by Endre Mester working in Hungary in 1967 [1]. Mester was studying the effects of the newly discovered ruby laser (694 nm) on cancer in small animal models. He found that his laser beam was neither able to cure cancer nor to cause cancer in the rats. However to his surprise, the laser was able to stimulate wound healing in the incision used to implant the tumor and to stimulate hair growth in the shaved skin sites that had been irradiated by the laser beam. These effects were originally thought to be a property of red lasers (600–700 nm), but now PBM has broadened to include near-infrared (NIR) wavelengths 760–1200 nm and even blue and green wavelengths. Moreover the advent of inexpensive and safe light-emitting diodes (LEDs) has supplanted the use of expensive lasers in many indications. The better tissue penetration properties of NIR light, together with its good efficacy, has made it the most popular wavelength range overall.

The best-known medical applications of PBM have been for indications such as stimulation of wound healing [2, 3], reduction of pain and inflammation in orthopedic and musculoskeletal conditions [4, 5], and mitigation of cancer therapy side effects [6, 7]. However in recent years, there has been growing interest in the use of PBM in various brain disorders [8–11]. The almost complete lack of any adverse side effects of PBM, coupled with growing disillusion with pharmaceutical drugs that affect brain function, has combined together to suggest an alternative physical therapy approach to improving brain function.

18.2 Mechanisms of PBM

18.2.1 Chromophores

It is a fundamental law of photobiology that a photon of light must be absorbed by a molecule located within the cells or tissues to have any biological effect (see Fig. 18.1). These photoacceptor molecules are called chromophores. While well-known biological chromophores in the red region of the spectrum are hemoglobin, myoglobin, and melanin, it is thought that the enzyme cytochrome c oxidase (CCO) is the main player in PBM effects. CCO is unit IV in the mitochondrial respiratory chain and is responsible for using electrons produced by units 1–III to reduce oxygen to water while at the same time producing the proton gradient needed to drive the production of adenosine triphosphate (ATP) by unit V (ATP synthase). CCO has two copper atoms and two heme groups which can be either reduced or oxidized, and each state has a different absorption peak in the red/NIR spectrum. CCO is one of the few biological molecules that has significant absorption in the NIR region 750–950 nm. The current theory is that nitric oxide (NO) can bind to copper and

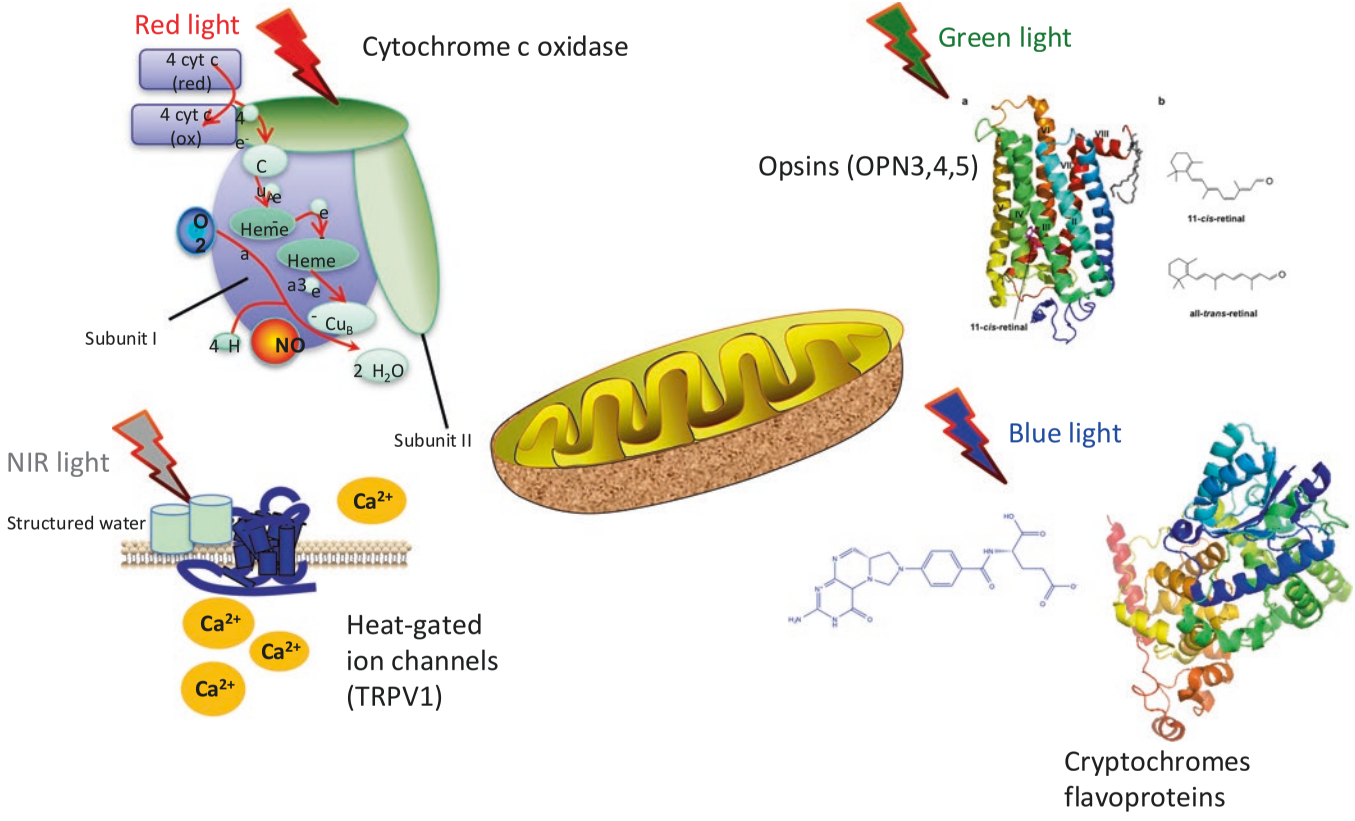

Fig. 18.1 Chromophores implicated in the absorption of different wavelengths of light. CCO absorbs *red light* (and NIR light around 800 nm) possibly dissociating inhibitory NO. Light-sensitive proteins (opsins) can absorb *blue/green light* via cis-retinal. Cryptochromes can absorb *blue light* via flavins. TRP (heat-gated ion channels) can absorb long-wavelength NIR light via structured water

heme centers, thus competitively inhibiting the binding of oxygen and reducing cellular energy metabolism. Absorption of light is proposed to photodissociate the relatively weak binding of NO from CCO, thus restoring normal levels of ATP production. The released NO is thought to be responsible for the vasodilation and increased blood flow seen after PBM. Moreover the extra ATP synthesized can go on to form cyclic AMP.

There is accumulating evidence that there are other biological chromophores, especially those which absorb shorter wavelengths (blue and green light) and longer wavelengths (long-wavelength NIR). These chromophores appear to be connected with membrane ion channels. These chromophore(s) may take the form of light-sensitive proteins (cryptochromes or opsins at blue/green wavelengths) or even nanostructured water (particularly at infrared wavelengths). The net result is opening of ion channels such as transient receptor potential (TRP) calcium channels, rise in the intracellular calcium concentration, and activation of calcium-sensitive second messenger signaling pathways. TRP channels are known to function as light-gated [12] or heat-gated [13] calcium ion channels.

18.2.2 Signaling Pathways and Gene Transcription

The production of signaling intermediates immediately after light absorption goes on to activate a wide range of cellular pathways (see Fig. 18.2). Examples of these signaling intermediates are calcium, NO, and reactive oxygen species (ROS). Another signaling pathway that is activated is based upon cyclic AMP which is formed from the extra ATP produced by the light [14]. A brief controlled burst of ROS is produced by the changes caused in the mitochondria (increase in mitochondrial membrane potential). There are many ROS molecular sensors inside cells, whose function is to detect oxidative stress and to activate inducible antioxidant defenses against it [15]. Antioxidant enzymes such as superoxide dismutase and glutathione peroxidase are activated by PBM [16], so the long-term consequence is a reduction (not an increase) in the level of oxidative stress in the tissues [17]. Another family of protective factors that is activated by PBM consists of antiapoptotic proteins such as survivin [18] and Bcl2 [19].

It is clear that changes in gene transcription must follow PBM as a single exposure to light can have very long-lasting effects (days or weeks) in some organisms [20]. A wide range of transcription factors have been shown to be activated in PBM including NFkB [21] and stem cell-, bone-, and muscle-specific transcription factors. Although NFkB is accepted as a pro-inflammatory transcription factor, the result of PBM in tissues where inflammation is present is a reduction, rather than an increase in inflammation [22].

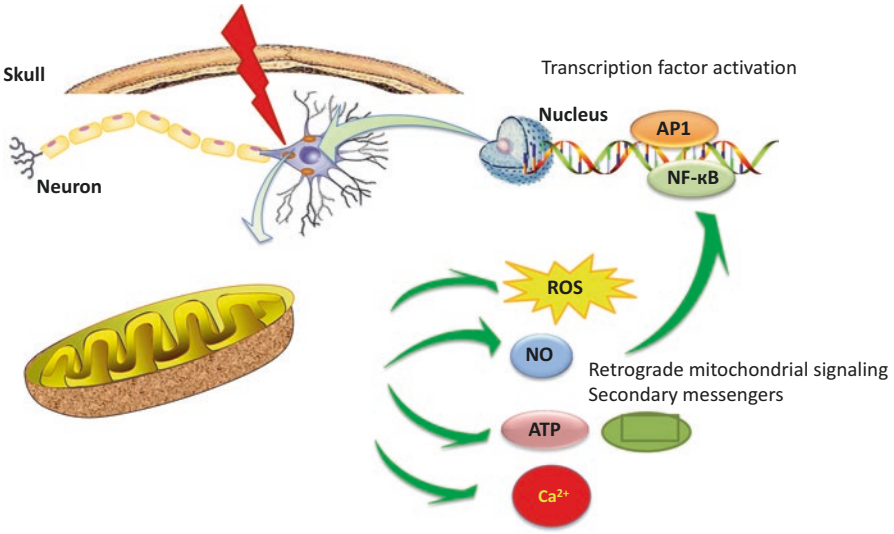

Fig. 18.2 Mechanisms of PBM occurring in neurons. Second messengers and signaling molecules are produced after the primary photon absorption event. These include reactive oxygen species (*ROS*), nitric oxide (*NO*), cyclic adenosine monophosphate (*cAMP*), and calcium (*Ca2+*). These messengers go on to lead to activation of transcription factors such as NFkB and AP1

18.3 Mechanisms of PBM in the Brain

In addition to the foregoing, there are some PBM tissue mechanisms that are specific to the brain (see Fig. 18.3). One of the most important is an increase in cerebral blood flow often reported after transcranial PBM (tPBM) [23], leading to increased tissue oxygenation in the cortex and more oxidized CCO as measured by NIR spectroscopy [24]. PBM is thought to increase mitochondrial function within the brain, and mitochondrial dysfunction is accepted as a pathognomonic factor common to many (if not most) brain disorders [25]. Moreover PBM is also able to stimulate mitochondrial biogenesis [26] (the process by which mitochondrial can proliferate) to meet increased demands for energy [27]. The mechanistic target of rapamycin (mTOR) pathway regulates mitochondrial biogenesis to coordinate energy homeostasis with cell growth [28]. It is well known that exercise induces the proliferation of mitochondria within the cells, particularly mitochondrial biogenesis within muscle cells [29]. tPBM has been shown to reduce activated microglia in the brains of TBI mice as measured by IBA1 (ionized calcium-binding adapter molecule-1) expression thus demonstrating reduced neuroinflammation [30]. tPBM has been shown to increase neurogenesis (formation of new brain cells derived from neuroprogenitor cells) [31] and synaptogenesis (formation of new connections between existing brain cells) [32] both in mice that had received a traumatic brain injury (TBI). Moreover tPBM in TBI mice was also shown to increase the expression of the neutrophin known as brain-derived neurotrophic factor (BDNF) [32]. BDNF

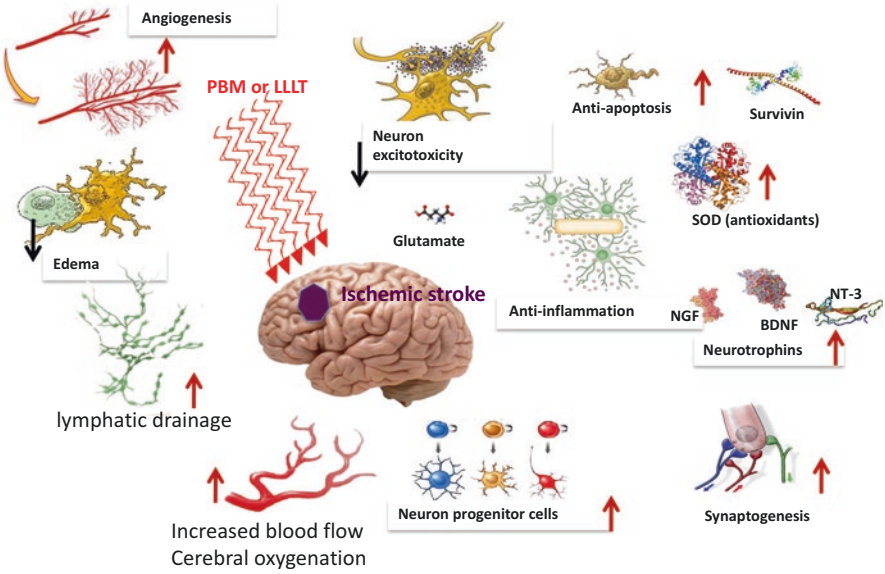

Fig. 18.3 Mechanisms of PBM occurring in the brain. A large range of mechanisms can operate after PBM that will beneficially affect the brain after an ischemic stroke. These include reductions in edema, increases in blood flow, cerebral oxygenation, angiogenesis, and lymphatic drainage. Neuroprotection is afforded by increases in antioxidants (SOD), survivin, and BCL2. Excitoxicity caused by glutamate is reduced together with neuroinflammation. Brain repair pathways are activated by neurotrophins (BDNF, NGF, NT-3), neuroprogenitor cells, and synaptogenesis

acts as a pleotropic factor that benefits most brain functions [33]. Moreover it is also possible that PBM can modulate NO metabolism in the brain which could be beneficial. NO can act as a Janus molecule within the brain with overproduction of NO by activated microglial cells being detrimental [34], while controlled release of NO can be beneficial [35].

18.4 Light Sources and Light Delivery

18.4.1 Light Sources

PBM was originally developed using lasers and is still often called "laser therapy." For many years the stimulating effects of PBM were thought to be a particular function of laser light (in particular its coherence and monochromaticity). Now it is becoming accepted that noncoherent light-emitting diodes (LEDs) typically having a 30 nm full width half maximum bandspread are equally as effective as lasers that have comparable parameters (power density and spot size). For transcranial applications, wavelengths in the NIR region (800–1100 nm) have been the most often used,

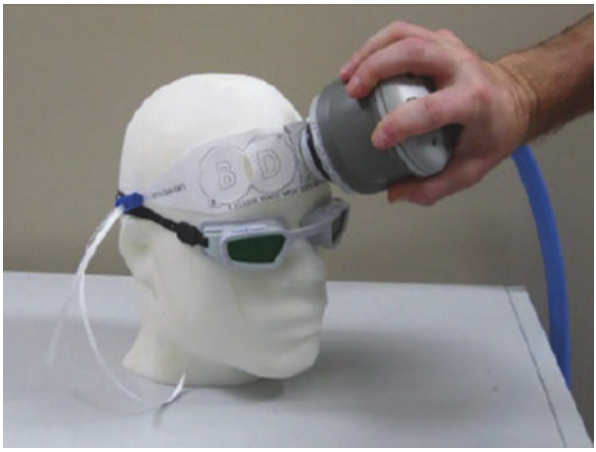

Fig. 18.4 NeuroThera Laser. This was the same device as used in the NEST trials for stroke and was also used for depression [44]

while red wavelengths have sometimes been used either alone or in combination with NIR. Power levels have also varied markedly from class IV lasers with total power outputs in the region of 10W [36] to lasers with more modest power levels (circa 1W). LEDs can also have widely varying total power levels depending on the size of the array and the number and power of the individual diodes. Power densities can also vary quite substantially from the PhotoThera laser [37] (see Fig. 18.4) and other class IV lasers, which required active cooling (~700 mW/cm^2) to LEDs in the region of 10–30 mW/cm^2.

18.4.2 Light Penetration into the Brain

Due to the growing interest in PBM of the brain, several tissue optics laboratories have studied the penetration of light of different wavelengths through the scalp and the skull and into the brain. This is an intriguing question to consider, because at present it is unclear exactly what threshold of power density in mW/cm^2 is required at what depth in the brain in order to have a biological effect. There clearly must be a minimum threshold value below which the light can be delivered for an infinite time without doing anything, but whether this is in the region of μW/cm^2 or mW/cm^2 is not known at present.

Functional near-infrared spectroscopy (fNIRS) using 700–900 nm light has been established as a brain imaging technique that can be compared to functional magnetic resonance imaging (fMRI) [38]. Haeussinger et al. estimated that the mean penetration depth (5% remaining intensity) of NIR light through the scalp and skull was 23:6 + 0:7 mm [39]. Other studies have found comparable results with variations depending on the precise location on the head and wavelength [40, 41].

Jagdeo et al. [42] used human cadaver heads (skull with intact soft tissue) to measure penetration of 830 nm light and found penetration depended on the

anatomical region of the skull (0.9% at the temporal region, 2.1% at the frontal region, and 11.7% at the occipital region). Red light (633 nm) hardly penetrated at all. Tedord et al. [43] also used human cadaver heads to compare penetration of 660 nm, 808 nm, and 940 nm light. They found 808 nm light was best and could reach a depth in the brain of 40–50 mm. Lapchak et al. compared the transmission of 810 nm light through the skulls of four different species and found that mouse skull transmitted 40%, while for rat, it was 21%, for rabbit it was 11.3, and for human skulls it was only 4.2%.

18.4.3 Anatomical Application Sites

Mice and rats have very small heads and a 1-cm diameter spot of light delivered to the top of the head fills the whole skull up with light. Humans by contrast have rather large heads, and the site of application becomes important. The NEST clinical trials (see later) used a high-power laser (808 nm) with a 3 cm diameter spot designed to treat 20 separate locations on the shaved head for 2 min each. Figure 18.4 shows how the laser spot was delivered to the forehead in a clinical study carried out for depression [44]. Schiffer et al. also carried out a clinical study for depression using an 810 LED spot applied to the forehead [45] (see Fig. 18.5). Naeser et al. believe that the particular sites on the head that should be targeted with light are of critical importance [46]. For chronic TBI, they targeted 11 locations on the scalp: midline from front-to-back hairline and bilaterally on frontal, parietal, and temporal areas [46]. Saltmarche et al. used different areas of the head to deliver PBM to treat Alzheimer's disease [47]. The VieLight Neuro LED device (see Fig. 18.6) targets the default mode network nodes: (a) mesial prefrontal cortex, (b) precuneus, (c) posterior cingulate cortex, and (d) inferior parietal lobe. In addition there was an intranasal LED that is hypothesized to target the hippocampus.

18.4.4 Systemic Effects

In actual fact it is very likely that the beneficial effects of PBM on the brain cannot be entirely explained by penetration of photons through the scalp and skull into the brain itself. There have been some studies that have explicitly addressed this exact issue. In a study of PBM for Parkinson's disease in a mouse model [48], Mitrofanis and colleagues compared delivering light to the mouse head and also covered up the head with aluminum foil so that they delivered light to the remainder of the mouse body. They found that there was a highly beneficial effect on neurocognitive behavior with irradiation to the head, but nevertheless there was also a statistically significant effect (although less pronounced benefit, referred to by these authors as an "abscopal effect") when the head was shielded from light [49]. Moreover Oron and coworkers [50] have shown that delivering NIR light to the mouse tibia (using either

Fig. 18.5 Transcranial LED device. This device was used in the Schiffer et al. study for depression [45]

Fig. 18.6 VieLight Neuro and intranasal device. This transcranial device together with intranasal LED was used in trial for Alzheimer's disease [47]

surface illumination or a fiber optic) resulted in improvement in a transgenic mouse model of Alzheimer's disease (AD). Light was delivered weekly for 2 months, starting at 4 months of age (progressive stage of AD). They showed improved cognitive capacity and spatial learning, as compared to sham-treated AD mice. They proposed that the mechanism of this effect was to stimulate c-kit-positive mesenchymal stem cells (MSCs) in autologous bone marrow (BM) to enhance the capacity of MSCs to infiltrate the brain and clear β-amyloid plaques [51]. It should be noted that the calvarial bone marrow of the skull contains substantial numbers of stem cells [52].

18.5 PBM for Animal Models of Acute Stroke

The story about the development of PBM for stroke starts with work from Uri Oron's laboratory in Israel. Oron had shown that PBM could have remarkable effects on heart attacks or myocardial infarction in various animal models. Oron et al. used two different animal models of heart attack (occlusion of coronary artery in both rats and dogs) [53]. They used three different power densities of an 810 nm laser applied to the infarcted area of the heart (open chest procedure) immediately after the heart attack. They found an interesting biphasic dose response in rats with the best reduction in infarct area (60%) seen with 6 mW/cm^2 with lesser improvements seen with 2.5 mW/cm^2 or 20 mW/cm^2. Dogs also showed a good reduction in infarct area (4%). Oron's laboratory went on to publish three further papers on this remarkable discovery [54–56].

The company PhotoThera Inc. was formed by Jackson Streeter to commercialize this discovery, and it was decided to concentrate on stroke because they realized that light could be delivered to the brain in a noninvasive transcranial manner, while light delivery to the heart would almost certainly require a surgical approach. The pathological similarities between the processes occurring after ischemic stroke and myocardial infarction leading to growth of a necrotic lesion in the brain or the heart were remarked upon [57].

The first experimental study was carried out in rats who received an acute stroke by permanent occlusion of the middle cerebral artery using a filament introduced via the carotid artery [58]. Rats were tested for neurological damage 24 h poststroke and then received laser treatment by application of the tip of the fiber from an 808 nm laser to three different sites on the shaved head (ipsilateral, contralateral, or both sides). Although the precise power density on the head is not given, it was stated that it delivered 7.5 mW/cm^2 to the surface of the brain and 0.9 J/cm^2 over 2 min. All three sites showed significant improvements in neurological severity score steadily increasing over 28 days, with the contralateral side being the best. The next study investigated the safety of the treatment by testing very high power densities of laser energy to the head [59]. Only the very high power density (750 mW/cm^2 CW to the brain, 100× the optimal dose) showed any neurological and histological damage. The next study explored the timing of the PBM (either 4 h or 24 poststroke) [60]. Strokes were induced by two different methods, filament insertion or middle cerebral artery occlusion via a craniotomy. PBM was 7.5 mW/cm^2 (2 min) to the contralateral side either CW or pulsed. Treatment at 4 h did not show any significant improvement, but the improvements with laser at 24 h were significant with CW laser being somewhat better than pulsed. The number of neuroprogenitor cells in the ipsilateral subventricular zone (determined by BrdU injection at day) was increased by CW PBM. Markers of migrating neuroprogenitor cells TUJ1 (neuron-specific class III beta-tubulin) and DCX (double cortin) were increased in the CW group.

Lapchak's laboratory next went on to study tPBM in a rabbit small clot embolic stroke model (RSCEM) [61]. In this model blood is taken from a rabbit, allowed to

clot, and then a fine suspension of clots is labeled with a radiotracer (to allow the amount of clot that lodges in the brain to be calculated), before being injected into the carotid artery. In this model the effectiveness of a therapy is shown by an increase in the dose of clots (mg) needed to produce a 50% neurological deficit. They compared PBM with 808 nm laser at 7.5 mW/cm^2 or to the cortex delivered either 1, 3, 6, or 24 h poststroke. The best results were found with 6 h while 1 and 3 h (but not 24 h) were also effective. They next used the RSCEM model to compare CW with two pulsed regimens (300 us at 1 kHz and 2 ms at 100 Hz) and delivered either at 6 or 12 h post-embolization [62]. The best results were seen with pulsed mode PBM delivered at 6 h poststroke. Another study used a similar large clot RSCEM model to test whether tPBM was compatible with administration of the clot-busting drug, tissue plasminogen activator (tPA, alteplase) [63].

18.6 Clinical Trials of PBM for Acute Ischemic Stroke

There were a series of three NeuroThera Effectiveness and Safety Trials (NEST-1, NEST-2, and NEST-3) to evaluate the safety and effectiveness of the NeuroThera Laser System to improve 90-day outcomes in ischemic stroke patients treated within a 24 h period from the stroke onset [64]. The NEST-1 clinical trial was a prospective, intention-to-treat, multicenter, international, double-blinded trial involving 120 ischemic stroke patients, randomized in a 2:1 ratio, with 79 patients in the active treatment group and 41 in the sham (placebo) group. Only patients with baseline stroke severity measured by National Institutes of Health Stroke Scale (NIHSS) scores of 7–22 were included. Treatment consisted of the application of a handheld 808 nm laser probe to 20 predetermined locations on the shaved scalp for 2 min in each location. The mean time to treatment was 16 h (range 2–24 h). Patients receiving active treatment had more successful outcomes (70%) than did controls (51%) ($P = 0.035$ stratified by stroke severity and time to treatment; $P = 0.048$ stratified only by severity). Similar results were found for the Barthel Index and Glasgow Stroke Outcome Scale. Mortality rates did not differ significantly (8.9% active vs 9.8% placebo), nor serious adverse events (SAEs) (25.3% active vs 36.6% placebo).

These highly encouraging results led to a second clinical trial NEST-2 being conducted [65]. This study was a prospective, double-blind, randomized, sham-controlled, parallel group, multicenter study that included sites in Sweden, Germany, Peru, and the USA and enrolled 660 subjects. Subjects were followed for 90 days poststroke onset. The primary endpoint for this study was a simple binary division that defined success as a modified Rankin Scale (mRS) score of 0–2 and failure as a mRS score of 3–6 at 90 days post-therapy or at the last rating. PBM was applied within 24 h from the stroke onset. The study demonstrated safety but did not meet the formal statistical significance criteria for efficacy. Out of a total recruitment of 658 randomized patients, 331 received PBM and 327 received sham. One hundred twenty patients (36.3%) in the TLT group achieved a favorable outcome vs 101

(30.9%), in the sham group ($P = 0.094$). Comparable results were seen for the other outcome measures. A post hoc analysis of patients with a baseline NIH Stroke Scale (NIHSS) score of <16 did show a statistically favorable outcome at 90 days on the primary endpoint ($P < 0.044$). Mortality rates and serious adverse events did not differ between groups with 17.5% and 17.4% mortality and 37.8% and 41.8% serious adverse events for TLT and sham, respectively.

A subsequent study [66] used brain scans (CT or MRI) to analyze additional measures in the patients in NEST-2. These included the infarct volume in the cortex among patients with clinical presentations suggesting cortical involvement and assessing the cortical Alberta Stroke Program Early CT Score (cASPECTS) components (M1–M6, anterior, posterior) on a 0- to 8-point modified scale.

A total of 640 subjects had scans on day 5. The total ASPECTS was correlated with total infarct volume ($r = 0.71$). In the overall study population, there was no impact of PBM on total infarct volume ($P = 0.30$), total ASPECTS ($P = 0.85$), or cASPECTS ($P = 0.89$).

The next study was a pooled analysis of NEST-1 and NEST-2 [67, 68] based on a total of 778 patients. Baseline characteristics and prognostic factors were balanced between the two groups. The success rate in the active PBM group ($n = 410$) was significantly higher compared with the sham group ($n = 368$) ($P = 0.003$). The distribution of scores on the 90-day mRS was significantly different in PBM compared with sham ($P = 0.0005$). Subgroup analysis identified moderate (rather than severe) strokes as a predictor of better treatment response.

Despite NEST-2 missing its formal endpoint, a decision was made to carry out the NEST-3 trial. NEST-3 was planned to be a double-blind, randomized (1:1), sham-controlled, parallel group, multicenter, pivotal study that would enroll 1000 subjects at up to 50 sites. All subjects were to receive standard medical management based on the American Stroke Association and European Stroke Organization Guidelines. Both groups (real and sham) were to be treated with PBM between 4.5 and 24 h of stroke onset [69]. The large trial was prematurely terminated by the data safety monitoring board for "futility" (an expected lack of statistical significance) [37]. An interim analysis of 566 completed patients found no difference in the primary endpoint (real PBM had 140/282 [49.6%] versus sham with 140/284 [49.3%]) for good functional outcome using the modified Rankin Scale, 0–2. The results remained stable after inclusion of all 630 randomized patients. Many commentators have asked how tPBM could work so well in NEST-1 and NEST-2, yet fail in the third NEST-3? Insufficient light penetration, too long an interval between stroke onset and PBM, inappropriate stroke severity measurement scale, use of only one single tPBM treatment, and failure to illuminate different specific areas of the brain for individual patients have all been suggested as contributory reasons [9]. In fact, Lapchak and Boltano [70] published a study subtitled "How to Recover from Futility in the NEST-3 Clinical Trial" suggesting that the main reasons for the failure were firstly that the trial was conducted at a period when the Stroke Treatment Academic Industry Roundtable (STAIR) criteria and RIGOR guidelines were not adhered to. Secondly the PBM procedure was not optimized in multiple species, because light penetration profiles differ across the skulls of four different species

(mouse, rat, rabbit, and human). They found extensive attenuation of light penetration across the human skull, compared with animal skulls.

18.7 PBM for Rehabilitation in Chronic Stroke

Considering the growing body of evidence concerning the benefits obtained by applying PBM to the head, in a diverse range of brain disorders [9–11, 71–73], it is somewhat surprising that more attention has not been paid to the problem of chronic stroke. There appears to have only been the occasional report published to date. Naeser reported in an abstract the use of tPBM to treat chronic aphasia in poststroke patients [74]. Boonswang et al. [75] reported a single patient case in which PBM was used in conjunction with physical therapy to rehabilitate chronic stroke damage. However the findings that PBM can stimulate synaptogenesis in mice with TBI do suggest that tPBM may have particular benefits in rehabilitation of stroke patients. Norman Doidge, in Toronto, Canada, has described the use of PBM as a component of a neuroplasticity approach to rehabilitate chronic stroke patients [76].

18.8 Future Outlook

The resounding failure of the NEST-3 trial has had a long-lasting effect on the medical profession's opinion of the validity of tPBM for stroke. Some voiced the opinion that "PBM has been shown not to work." This sense of failure surrounding application of tPBM in acute ischemic stroke contrasts sharply with the sense of optimism in other applications of tPBM for brain disorders, especially for chronic TBI [77, 78], major depression [44, 45], Alzheimer's disease [47, 79], and Parkinson's disease [80, 81]. Whether the solution to overcoming futility lies in optimizing tissue penetration of light through the human skull, in tailoring the actual sites of application of the light to the head to correlate with the actual site of the stroke lesion, or in applying tPBM several times in the days following the stroke, I believe that tPBM for acute ischemic stroke will be tested again in the near future, because the data from NEST-1 and NEST-2 were so tantalizing and promising. Besides the use of clot-busting drugs, effective therapies for stroke are thin on the ground [82]. While many experimental approaches to ischemic stroke therapy have shown some success in animal models, none are as yet approved for use in humans [83].

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Chapter 19
In Vitro Enzymatic Clot Lysis Using Focused Ultrasound Waves as an Adjunct to Thrombolytic Drug Tenecteplase and in Combination with Microbubbles

Nicolaos Papadopoulos and Christakis Damianou

Abstract The low and incomplete recanalization performance of thrombolytic therapy in stroke patients has created the need to use focused ultrasound (FUS) energy as a way to enhance thrombolysis efficacy. The aim of the study was to establish an optimized treatment protocol that through nonthermal mechanisms maximizes the thrombolytic activity of thrombolytic drug tenecteplase (TNK-tPA), leading to thrombolysis enhancement.

Using an in vitro circulating flow clot model, designed to reproduce the physiologic situations of a middle cerebral artery occlusion occurred either 4 cm deep into a brain tissue or superficially, the role of various experimental parameters on thrombolysis efficacy were evaluated. For this purpose, fully retracted porcine blood clots were treated with FUS waves as an adjunct to thrombolytic TNK-tPA, in the presence or absence of microbubbles (MBs). A spherically FUS transducer (4 cm diameter), focusing at 10 cm and operating at 1.18 MHz, was used. In all the proposed parametric studies, temperature elevation at beam focus never exceeded 1 °C, providing that the contribution of thermal mechanisms to clot lysis was negligible.

The effect of experimental parameters such as temperature, FUS alone, TNK-tPA alone and in synergy, sonication time, standing waves, flow rate, acoustic power and MB administration in thrombolysis efficacy was investigated. The degree of thrombolysis achieved by each parameter was measured either as the relative reduction in the mass of the clot or in mg of mass clot removed.

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Study findings clearly demonstrated that the combination of 1.18 MHz FUS pulses with MBs strongly accelerated the thrombolytic action of TNK-tPA. Experimental results have been very promising, specifically in the case of a superficial occlusion, where 1,050 mg of mass clot was removed after 30 min of treatment. Since stroke is time dependent, this thrombolytic rate should be sufficient for timely recanalization of an occluded cerebral artery.

Keywords FUS • Stroke • Blood clot • TNK-tPA • MBs • Agar

19.1 Introduction

Stroke is the leading cause of morbidity and mortality worldwide [1]. According to the World Health Organization (WHO), each year 15 million people worldwide suffer a stroke, and of these, nearly 6 million die and another 5 million are left permanently disabled [2] causing a tremendous burden on the healthcare system of each country, since it is associated with expensive long-term rehabilitation care [3].

The pathological background for stroke may be either due to ischemic or haemorrhagic disturbances of the cerebral blood circulation. About 87% of the annual incidences of stroke are caused by ischemia and the remaining roughly 13% by haemorrhage [4]. Acute ischemic stroke (AIS) is the sudden obstruction of the blood supply to a part of the brain due to an occluded blood vessel, which rapidly leads to severe brain damage and cerebral infarction. Consequently, the most vital factor for the recovery of a stroke patient is to resupply blood flow to the affected area before brain cells begin to die. Haemorrhagic stroke occurs when a weakened blood vessel in the brain ruptures, causing blood leakage into the brain.

At present, intravenous (iv) recombinant tissue plasminogen activator (rt-PA), reflecting its method of manufacture, is the only thrombolytic treatment for AIS, approved by the United States Food and Drug Administration (FDA) [5]. This artificially produced lytic agent, also known by specific chemical names such as alteplase, reteplase and tenecteplase, is identical to the naturally occurring endogenous plasminogen activator (PA), a naturally occurring fibrinolytic enzyme found in vascular endothelial cells in humans.

If rt-PA is given within 4.5 h from symptom onset, then it can increase patient outcome [6]. However, less than 5% of patients with AIS receive iv rt-PA [7]. Of these, arterial recanalization is achieved in only 30–40%, and the recanalization is complete and sustained in only 18% [8–10]. As a result, many patients are left with a substantial brain damage, with high rates of disability and mortality [11]. Furthermore, rt-PA treatment involves a major risk of symptomatic brain haemorrhage [5].

Since thrombolytic treatment is often not sufficient for timely vascular recanalization [12], new therapeutic strategies that aim to improve recanalization rates and clinical outcomes after AIS are needed. A promising strategy, which represents an

important breakthrough, is the application of US energy to improve the action of thrombolytic drugs. US-enhanced thrombolysis, or sonothrombolysis, is a new and promising therapy for the treatment of AIS, in which the effectiveness of thrombolytic drugs can be increased when combined with transcranial US [13–15]. Many, in vitro [16–17] and in vivo [18–19], studies have shown that US energy accelerates thrombolysis due to a possible increase of the enzymatic effect of thrombolytic drugs.

Although the mechanisms involved in sonothrombolysis are not fully understood, it is hypothesized that US energy:

(a) Promotes motion of fluid around the clot surface (microstreaming), leading to increase delivery of the rt-PA near occlusion [20, 21]
(b) Weakens fibrin cross-links, leading to increases in uptake, penetration and concentration of rt-PA to the binding sites of the clot [22–23]

In addition to thrombolytic drugs, the administration of gaseous microbubbles (MBs), i.e. ultrasound contrast agents (UCA), may further increase US-enhanced rt-PA-induced thrombolysis, according to many in vitro [24–25] and in vivo [26–27] studies. When MBs exposed to US field, two possible events may occur. The first possibility is that the bubbles oscillate in phase with the applied sound wave near their linear resonance size, contracting during compression and expanding during rarefactions, producing stable cavitation (microstreaming). The other possibility is that the bubbles may become unstable and violently collapse within a single acoustic cycle or over a small number of cycles, producing inertial cavitation (microjetting). Stable cavitation, which is associated with sustained bubble activity, agitates the fluid where the spheres are dissolved, improving rt-PA delivery and penetration inside the clot [28, 29]. Inertial cavitation, which is associated with a rapid increase in size of bubbles, followed by a violent collapse, causes mechanical fragmentation of the thrombus [30–31].

In the last few years, focused ultrasound (FUS) has emerged as a relatively new approach to sonothrombolysis. This novel emerging therapeutic modality has received attention lately, as it can transmit high pressures noninvasively into millimetre-sized focal volumes within the body, without harming adjacent normal tissue, due to the little power deposition outside beam focus. Numerous studies, both in vitro [32–33] and in vivo [34–35], have shown that FUS waves in combination with rt-PA significantly improved clot lysis efficacy over both rt-PA alone and FUS alone.

Despite the encouraging results of the preclinical studies, the results from clinical trials are not very optimistic. In the past decade, there have been some significant clinical trials concerning the application of therapeutic US in the treatment of stroke.

One of the first clinical trials on sonothrombolysis was the transcranial low-frequency US-mediated thrombolysis in brain ischemia (TRUMBI), which was designed to treat stroke patients with either rt-PA alone or 300 kHz US plus rt-PA [36]. The study was stopped prematurely due to very high rate of symptomatic

intracranial haemorrhage (sICH) in patients treated with US and rt-PA. Since then, low-frequency US has not been available for therapeutic purposes in clinical trials.

The safety of higher frequencies (2 MHz) was studied using low-intensity US (similar to that used for diagnostic purposes), in the Combined Lysis of Thrombus in Brain Ischemia Using Transcranial Ultrasound and Systemic rt-PA (CLOTBUST) trial [37]. Study results have shown that the combination of rt-PA with 2 h of continuous transcranial Doppler (TCD) increased recanalization rates compared with rt-PA alone.

In another clinical trial, patients with proximal MCA occlusion were treated with 1.8 MHz transcranial colour-coded duplex (TCCD) technology in association with rt-PA [38]. Study results, also demonstrated improved recanalization rates when TCCD was combined with rt-PA in patients with AIS.

More recently, clinical research has been focused on the boosting effect of MBs to enhance sonothrombolysis. In 2009, Molina et al. performed the transcranial ultrasound in clinical sonothrombolysis (TUCSON) clinical trial [39]. In this study, stroke patients were treated with 2 MHz TCD in combination with rt-PA and continuous infusion of MBs (MRX-801). Although at low doses of MBs 50% reperfusion enhancement was detected, when the dose was double, reperfusion enhancement was ceased and haemorrhaging occurred. As a result the clinical trial was terminated, showing that further research in the field of MB-enhanced sonothrombolysis has to be done.

This research focuses on the beneficial effect of FUS to accelerate the thrombolytic efficacy of the third-generation thrombolytic agent tenecteplase (TNK-tPA). The study's outcome can be applied in the future for clinical use, providing alternative techniques for noninvasive treatment of stroke. The main objective of this research was to propose a pulsed US treatment protocol that through nonthermal mechanisms maximizes the enzymatic fibrinolysis induced by TNK-tPA, leading to enhanced thrombolysis. In order to succeed this, various parametric studies were conducted in vitro to obtain the optimum value for each experimental parameter that influence thrombolysis efficacy.

To study the effect of FUS on thrombolysis efficacy, two different in vitro circulating flow clot models were developed. Fully retracted porcine blood clots were treated with FUS waves as an adjunct to TNK-tPA, in the presence or absence of MBs. Each one of the flow clot models used was designed to reproduce a different physiologic situation of a middle cerebral artery (MCA) occlusion, which is the most common cause of stroke. In all the proposed parametric studies, the temperature elevation at beam focus never exceeded 1 °C, providing that the contribution of thermal mechanisms to enzymatic clot lysis was negligible.

The first flow clot model was designed to reproduce the physiological situation of a MCA occlusion occurred 4 cm deep into a brain tissue. In order to provide a more realistic clinical environment, the study was conducted into a brain tissue-mimicking phantom [40]. Agar gel was selected as the base of the phantom, as it ensures mechanical characteristic similar to that of soft tissue. The agar powder used (Himedia Laboratories, Mumbai, India) produced a gel flexible enough to withstand high-intensity US compression forces without cracking. To control the

gel's scattering coefficient, crystalline silica dioxide powder (Merck Millipore, Darmstadt, Germany) was added as scattering material to reproduce the acoustic properties of brain tissue. Furthermore, evaporated milk was also added to the mixture in order to raise its attenuation coefficient to the average value of the brain tissue, which is approximately 0.6 dB/cm.MHz [41]. The flow rate was selected to be at 20% of the maximum value occurring in an open MCA, and the TNK-tPA concentration was selected to be at 30% of the average maximum concentration in the blood. Using 1.18 MHz FUS pulses, the role of various experimental parameters, such as sonication time, FUS alone, TNK-tPA alone and in synergy, standing waves and flow rate in thrombolysis efficacy, was evaluated, with the purpose of establishing the optimum treatment protocol that maximizes the thrombolytic activity of TNK-tPA.

The second in vitro flow clot model was designed to reproduce a physiological situation of MCA occlusion, occurred superficially. This time, the flow rate was selected to be at 80% of the maximum value occurring in an open MCA, and the TNK-tPA concentration was selected to be at 60% of the average maximum concentration in the blood. The optimum experimental parameters obtained before were employed in this study in order to investigate the influence of acoustic intensity and MB administration in thrombolysis efficacy. In addition, using a simple and effective in vitro clot lysis model, the effect of temperature on the thrombolytic activity of TNK-tPA was also investigated.

19.2 Materials and Methods

19.2.1 In Vitro Clot Preparation

Blood clots were obtained by natural coagulation of fresh porcine blood, collected from healthy pigs. Blood clots were prepared by aliquoting blood samples of the same volume into pre-weighed tubes, tubing or custom-made containers (depending on the study). The blood aliquots were incubated in a 37 °C water bath for 3 h before refrigerated at 5 °C for 72 h to ensure maximal clot retraction [42].

After clot formation, the produced serum was completely removed from each sample with great caution in order not to disturbed the formed clot. Then, each tube, tubing or container comprising clot was weighed again to determine the net mass clot weight (C_{NW}):

$$C_{NW} = W_C - W_T \tag{19.1}$$

where W_C is the weight of the container (tube or tubing) plus the clot and W_T are the weight of the container (tube or tubing) alone.

At the termination of treatment, clot's residual was carefully removed from the container and was left to dry for 60 min before weighted again to obtain the mass clot removed due to thrombolysis.

The effects of temperature, modality used, sonication time, type of wave and flow rate on the efficacy of thrombolytic treatment (T) were measured, where T is the relative reduction in the mass of the clot before and after treatment:

$$T = \frac{W_{\text{In}} - W_{\text{Fi}}}{W_{\text{In}}} x100 \tag{19.2}$$

W_{in} is the initial clot weight and W_{fi} is the final clot weight.

In the remaining experimental studies (acoustic power and bubbles administration), the efficacy of thrombolytic treatment was evaluated in mg of mass clot removed:

$$T = W_{\text{In}} - W_{\text{Fi}} \tag{19.3}$$

In all experimental studies, the mass of the clot before and after treatment was measured precisely on a digital balance (Scaletec, SM001, Heiligenstadt, Germany).

19.2.2 Preparation of TNK-tPA

TNK-tPA (Boehringer, Ingelheim, Germany) is a third-generation modified tissue PA that is more fibrin-specific and more resistant to PA inhibitor. Due to higher thrombolytic potency and longer half-life than rt-PA, TNK-tPA can be administered as an iv bolus [43–44]. Although this thrombolytic agent is an approved safe drug for acute myocardial infarction, due to its safe administration and ease of use, it can be a new choice for AIS treatment [45]. TNK-tPA was obtained as powder mixed with sterile water as per manufacturer's instructions.

19.2.3 Preparation of SonoVue MBs

To study the effect of cavitation nuclei on sonothrombolysis efficacy, UCA were used. UCA are micron-sized MBs, with inert, high-molecular-weight gas core, surrounded by a lipid, surfactant, or biocompatible polymer shell. SonoVue (Bracco, Milan, Italy) MBs contain a sulphur hexafluoride (SF6) gas core, which is a very stable molecule. The shell consists of a highly flexible phospholipid membrane, allowing the MBs to change the size and shape. The composition of the shell determines the stiffness of the bubbles and their resistance to rupture in the US field. As a result, MBs decreased solubility, and low diffusion coefficient prolongs their

lifespan within the circulation. The mean size of these MBs is 2.5 μm, and the resonance frequency ranges between 1 and 4 MHz [46]. SonoVue was obtained as a kit with a vial containing a lyophilized powder (25 mg) and a syringe prefilled with sterile saline solution (5 mL). The powder and the saline solution were mixed as per manufacturer's instructions, a manual process that takes less than 1 min. The resultant suspension with a concentration of $1–5 \times 10^8$ MBs/mL was injected in the reservoir of the circulating flow system on a constant rate to replace the bubbles that were destroyed in process and sustain cavitation.

19.2.4 Experimental Apparatus

The experiments were carried out in an acrylic tank ($42 \times 24 \times 23$ cm^3) filled with degassed water to prevent reflections that may affect the propagation of the beam. The US system used included a single element spherically shaped focused transducers (100 mm focal length and 40 mm in diameter), made from piezoelectric ceramic (Piezo Technologies, Etalon, Lebanon, IN, USA). The transducer operates with 1.18 MHz centre frequency and was driven by a radio frequency (RF) generator/amplifier (750W, JJ&A Instruments, Duvall, WA, USA). Its acoustic output was measured with an US power metre (UPM-DT100N, Ohmic Instruments Co. Easton MD 21601).

The container comprising the clot was fixed on a custom-made plastic holder and immersed into the water tank. The rear surface of the container was lined with an absorbing rubber to minimize reflections and to prevent the generation of standing waves, since the enhancement of clot dissolution is much more pronounced in travelling than in standing acoustic waves [47].

The physiological situation of flow in a MCA occlusion was reproduced by exposing the clots to a circulating pulsatile flow using a Masterflex peristaltic pump (Cole-Parmer, 7518-40, Vernon Hills, IL, USA). The flow system included a reservoir connected to a peristaltic pump and the container with the clot. The outlet tubing from the reservoir after passing the peristaltic pump, where the pulsatile flow was generated, was connected to the upper left side of the container. The inlet tubing was connected to the opposite right side, in order to sustain circulation. The reservoir was filled with degassed water (fluid for the flow system) along with TNK-tPA.

The transducer was mounted on the arm of an automated robotic system (VXM, Velmex Inc., Bloomfield, NY, USA), immersed in the water tank and arranged opposite the blood clot. The robotic system has three user-controlled degrees of freedom, capable to guide the transducer in X, Y and Z direction with submillimetre accuracy.

All custom-made 3D plastic models used in this study such as containers, holders, needles, arm of the robotic system, etc. were manufactured using a Stratasys 3D printer (FDM400, Eden Prairie, Minnesota, USA). The machine uses a common thermoplastic non-magnetic material known as acrylonitrile butadiene styrene (ABS).

19.2.5 Beam Positioning

To focus the US beam precisely on target, a custom-made plastic needle with length equal to the focal length of the transducer was attached at the transducer's face. Then, the needle was moved by the robotic system until its tip touched the target (Figs. 19.1 and 19.2). Beam focus was always positioned at clot's edge in order to increase the uptake, penetration and binding of the thrombolytic drug into the clot. When it was confirmed that the focus of the US beam was positioned precisely on target, the plastic needle was carefully removed.

Two different flow clot models were designed to study the effect of FUS on thrombolytic efficacy. In the case where the blood clot was formed into a plastic tubing mimicking MCA occlusion occurred 4 cm deep into a brain tissue, precise beam positioning was achieved when the tip of the needle touched anteriorly the mid-height of the tubing at clot's edge (Fig. 19.1). After precise beam positioning, the brain tissue-mimicking phantom (specially designed to allow the clot tubing to be inserted through it, leaving no air gap) was carefully placed over the tubing, simulating an occluded MCA embedded in the brain tissue.

In the case where the blood clot was formed into a container mimicking MCA occlusion, occurred superficially, precise beam positioning was achieved when the tip of the needle touched anteriorly the clot's edge (Fig. 19.2).

19.2.6 Experimental Protocol

The main aspect for establishing a treatment protocol was to ensure the negligible contribution of thermal effects to clot lysis, i.e. providing that thrombolysis occurred mainly through nonthermal mechanisms.

Regarding thermal mechanisms for biological effects of US, Miller and Ziskin 1989 [48] suggested that a temperature rise of 1–2 °C in an afebrile patient would not be likely to have a damaging effect. However, for exposures resulting in temperature rises of greater than 2 °C, the duration of exposure becomes an important consideration in risk/benefit assessment. Moreover, a comprehensive review on thermal bioeffects by the World Federation of Ultrasound in Medicine (WFUMD) concluded that "Based solely on a thermal criterion, an US exposure that produces a maximum temperature rise of 1.5 °C above normal physiological levels (37 °C), may be used without reservation in clinical examinations" [49].

Based on the above-mentioned thermal criterion, in all experimental studies, the temperature increase within the clot did not exceed 1 °C (called safe temperature). This means that during sonication, the temperature of the medium surrounding the clot was held constant with a localized temperature increase of 1 °C at the target. For the assessment of heat produced during sonication, a thermocouple (OMEGA Engineering, INC. Stamford, Connecticut, USA) was fixed at the position of target. The thermocouple was connected to a data acquisition-DAQ interface (National

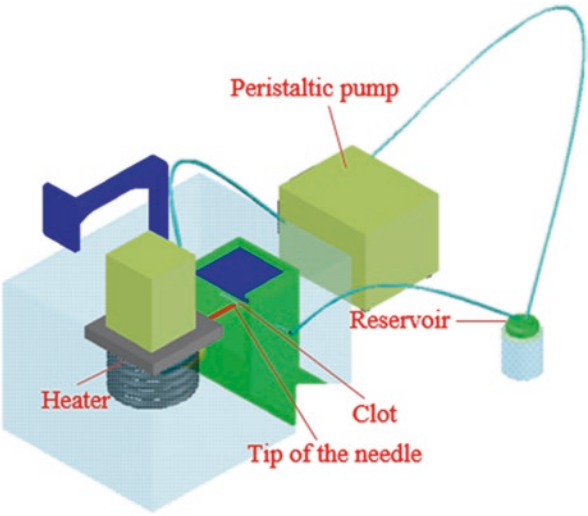

Fig. 19.1 Beam positioning for clot within an agar-based phantom

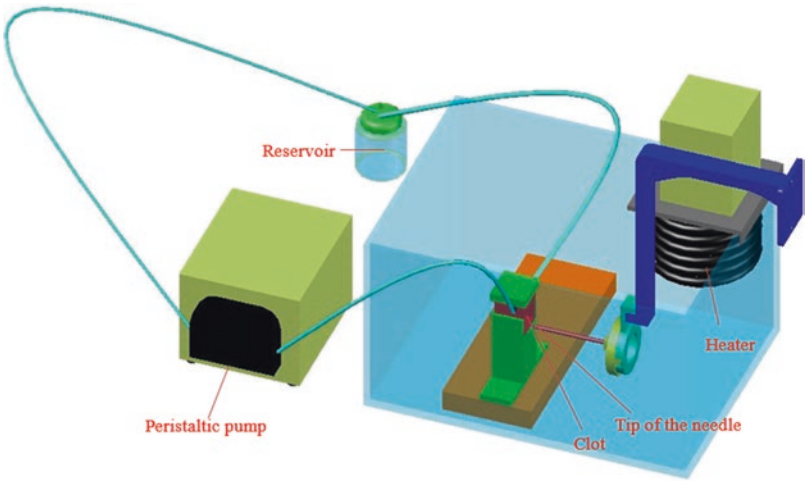

Fig. 19.2 Beam positioning for directly sonicating clot

Instruments Corporation, Austin, Texas, USA) that fed real-time temperature measurements to a computer.

Pulsed US protocols that maintained temperature elevation within the clot of 1 °C were applied. Each sonication set included a pulse *ON* period followed by a pulse *OFF* period, and both the *ON* and *OFF* pulses were repeated in succession for the duration of the treatment. In each experiment, only one parameter was varied, while all others parameters were kept constant provided that no excessive heating (>1 °C) was produced.

The optimal frequency for US applications varies by treatment and is always a trade-off between the depth of penetration and the sharpness of the focus. For transcutaneous sonothrombolysis, frequencies up to 2 MHz are employed as a best trade-off between focus and penetration. Although US frequencies in the kilohertz (kHz) range penetrate better with minimal heating, an experimental kHz delivery system in combination with rt-PA resulted in a very high rate of sICH in patients with AIS, due to the formation of standing waves [36] [50]. Taking into consideration that US frequencies in the kHz range are associated with an increased risk of causing sICH, the use of 1 MHz US frequency seems to be the best choice for transcutaneous sonothrombolysis, since it penetrates the temporal bone with less attenuation than higher frequencies (>1 MHz) and hence reduced heating [51–52].

The acoustic parameters that mainly control heating are the AP, which is the amount of power sent to the transducer's surface and the DF, which is the fraction of time that the US system is transmitting. Although longer DFs achieve better thrombolysis efficiency, the use of shorter DFs (≤10%) is very attractive, since thrombolysis efficiency can be enhanced by increasing AP levels and the desired reduction in heating can be achieved. Since the temperature at the target was continuously monitored, the value of AP was set to the appropriate level, where no excess heating was produced ($\Delta T \leq 1$ °C).

All experiments were conducted in a circulating unidirectional flow system, simulating blood flow in MCA occlusion. The peristaltic pump was set to maintain the appropriate flow rate for each study, and degassed water was used as a fluid for the closed-loop flow system. The values for flow rate and TNK-tPA concentration were selected to be well below the maximum levels occurred in a physiological situation of MCA occlusion, following iv thrombolysis with thrombolytic drug.

During treatments, the temperature in the water tank was kept constant at 37 °C, using a heating element (Thermo Scientific, SC001, Haake, Germany). For each experimental study, a group of five clots was used. The group of clots treated with neither US exposure nor TNK-tPA was served as the control or untreated group.

19.3 Determination of the Experimental Parameters That Influence Thrombolysis Efficacy

To optimize US-enhanced TNK-tPA-mediated thrombolysis for therapeutic purposes, it is critically important to determine and evaluate all experimental parameters (operating and physical) involved in this technique. Therefore, for the establishment of an optimized treatment protocol, it was necessary to elucidate the role that each one of the following experimental parameters plays on thrombolysis efficacy.

19.3.1 Effect of Temperature

To study the effect of temperature on the lytic efficacy of TNK-tPA, three groups of clots were treated with TNK-tPA alone. Percentage mass loss was measured for clots treated at 37, 39 and 41 °C, respectively.

19.3.2 Effect of Each Modality Alone and with Synergy

To determine the enhancing effect of FUS waves on clot lysis, the decrease in clot weight of the group treated with FUS + TNK-tPA was compared with that of the group receiving the same TNK-tPA concentration but no FUS or with that of the group treating with FUS alone.

19.3.3 Effect of Sonication Time

To evaluate the effect of sonication time on thrombolysis efficacy, three groups of clots were treated with FUS + TNK-tPA. Clot mass loss was assessed for exposure times of 15, 30 and 60 min, respectively.

19.3.4 Effect of Standing Waves

To estimate the effect of standing acoustic waves on thrombolysis efficacy, two groups of clots were treated with FUS + TNK-tPA. Data on clot mass loss were taken for clots treated with and without the use of absorber, which prevents the formation of standing waves.

19.3.5 Effect of Flow Rate

To investigate the effect of flow rate on thrombolysis efficacy, two groups of clots were treated with FUS + TNK-tPA. Percentage mass loss was measured for clots exposed to a flow rate of 10 mL/min compared with clots exposed to a flow rate of 30 mL/min.

19.3.6 Effect of Acoustic Power

To examine the effect of AP on clot lysis, three groups of clots were treated with FUS + TNK-tPA. The degree of thrombolysis was determined for clots treated at AP of 20, 30 and 60 W, respectively.

19.3.7 Effect of Bubbles

To study the effect of MBs as a way to further enhance FUS-mediated TNK-tPA thrombolysis, two groups of clots were used. The one group was treated with FUS + TNK-tPA, while the other one was treated with FUS + TNK-tPA in association with MBs (SonoVue; Bracco SpA, Milan, Italy). A 0.5 mL bolus of MBs was injected in the reservoir of the flow system at the beginning of insonation, and the procedure was repeated every 5 min, for the duration of a 30 min treatment (i.e. a total amount of 3 mL was used).

19.4 Results

19.4.1 Effect of Temperature

Firstly, data on the temperature dependence of TNK-tPA lytic efficacy would be useful in establishing an optimal treatment protocol. To study the effect of temperature on the lytic efficacy of TNK-tPA, three groups of clots were treated with TNK-tPA alone. Percentage mass loss was measured for clots treated at 37, 39 and 41 °C, respectively. Figure 19.3 shows the thrombolysis vs. temperature for treatment time = 120 min, TNK-tPA concentration = 10 μg/mL and no flow rate. Study results demonstrated that the peak fibrinolytic activity of TNK-tPA was achieved at 37 °C.

19.4.2 Effect of Each Modality Alone and in Synergy

Next, the effect of each modality alone (FUS, TNK-tPA) and in in synergy was evaluated. Figure 19.4a refers to the situation of an occluded artery occurred 4 cm deep into brain tissue and shows the effect of no FUS, no TNK-tPA (control), TNK-tPA alone, FUS alone and FUS + TNK-tPA for f = 1.18 MHz, DF = 10%, *AP* = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min,

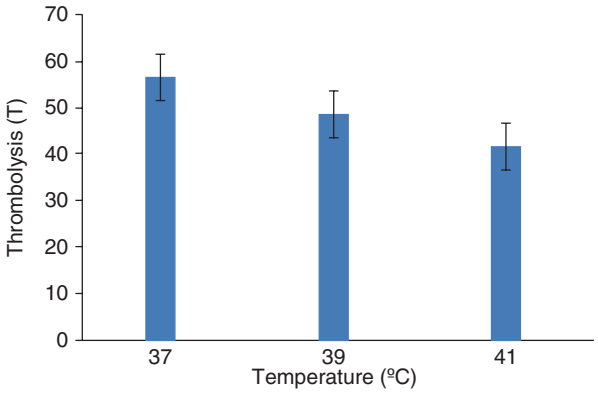

Fig. 19.3 The effect of temperature on the lytic efficacy of TNK-tPA. Treatment parameters: no FUS, no MBs, no flow rate, treatment time = 120 min and TNK-tPA concentration = 10 μg/mL

TNK-tPA concentration = 3.5 μg/mL and no MBs. This result shows that even with no treatment, there is a 30% reduction in mass due to the placement of the clot in the experimental setup. Figure 19.4b refers to the situation of an occluded artery occurred superficially and shows the effect of no FUS, no TNK-tPA (control), TNK-tPA alone, FUS alone and FUS + TNK-tPA for f = 1.18 MHz, DF = 10%, AP = 60 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7 μg/mL and no MBs. This result shows that even with no treatment, the placement of the clots in the circulating flow system resulted in 1,200 mg of mass clot removed. In both cases, treatment with FUS alone has no effect, while treatment with TNK-tPA alone has some effect, but clearly the combination of the two has enhanced effect.

Similarly, by subtracting thrombolysis achieved in the control group, the enhancing effect of FUS on TNK-tPA mediated thrombolysis was assessed. Therefore, in all subsequent experiments the synergy of FUS with TNK-tPA was implemented.

19.4.3 Effect of Sonication Time

The effect of sonication time was evaluated at 15, 30 and 60 min, respectively. Figure 19.5 shows the thrombolysis vs. time for f = 1.18 MHz, DF = 10%, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 μg/mL and no MBs. This result shows that saturation was achieved in 30 min; therefore in all subsequent experiments, the 30 min duration was used.

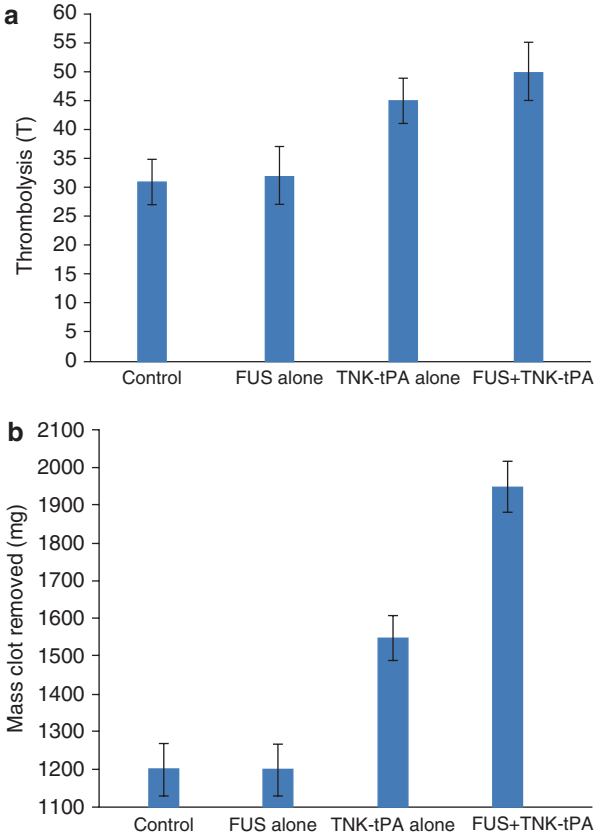

Fig. 19.4 The degree of thrombolysis in untreated clots, in clots treated with FUS alone, in clots treated with TNK-tPA alone and in clots treated with FUS+TNK-tPA, for (**a**) sonothrombolysis occurred 4 cm deep into agar gel and (**b**) for sonothrombolysis occurred superficially. (**a**) FUS parameters*:* f = 1.18 MHz, DF = 10%, AP = 20 W, pulse length = 1 ms, PRP = 10 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, water bath temperature = 37 °C and no MBs. (**b**) FUS parameters: f = 1.18 MHz, DF = 10%, AP = 60 W, pulse duration = 1 ms, PRP = 10 ms and PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, water bath temperature = 37 °C and no MBs

19.4.4 Effect of Standing Waves

The effect of standing as well as travelling acoustic waves was estimated by using an absorbing medium. Figure 19.6 shows the thrombolysis vs. presence or absence of absorbing material for f = 1.18 MHz, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 μg/mL and no MBs.

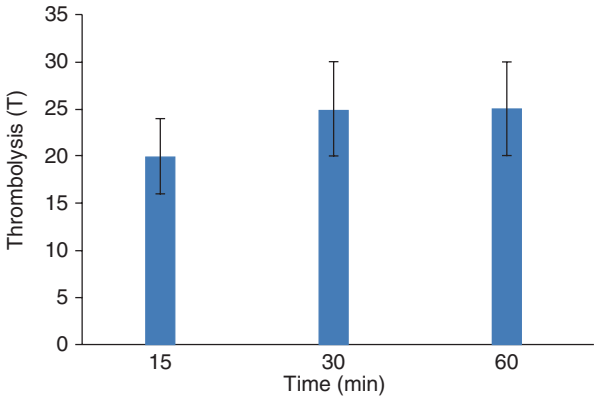

Fig.19.5 The effect of time on thrombolysis efficacy. FUS parameters: f = 1.18 MHz, DF = 10%, AP = 20 W, pulse length = 1 ms, PRP = 10 ms, PRF = 100 Hz, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 μg/mL, water bath temperature = 37 °C and no MBs

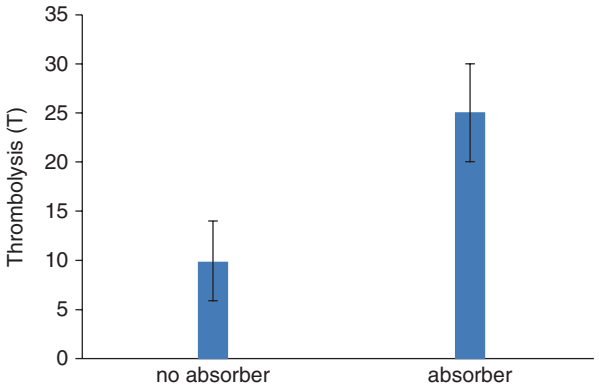

Fig. 19.6 The impact of standing acoustic waves on thrombolysis efficacy. FUS parameters: f = 1.18 MHz, AP = 20 W, pulse length = 1 ms, PRP = 10 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 10 mL/min, TNK-tPA concentration = 3.5 μg/mL, water bath temperature = 37 °C and no MBs

19.4.5 Effect of Flow Rate

To investigate the effect of flow, two different rates were used (10 mL/min and 30 mL/min). Figure 19.7 shows the thrombolysis vs. flow rate for f = 1.18 MHz, DF = 10%, AP = 20 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, TNK-tPA concentration = 3.5 μg/mL and no MBs.

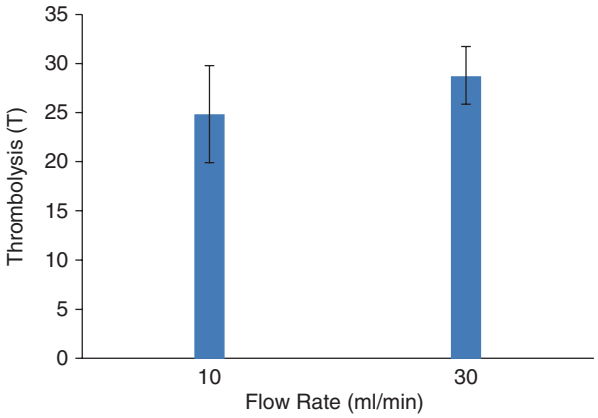

Fig. 19.7 The beneficial role of flow rate on thrombolysis efficacy. FUS parameters: f = 1.18 MHz, DF = 10%, AP = 20 W, pulse length = 1 ms, PRP = 10 ms, PRF = 100 Hz, treatment time = 30 min, TNK-tPA concentration = 3.5 μg/mL water bath temperature = 37 °C, and no MBs

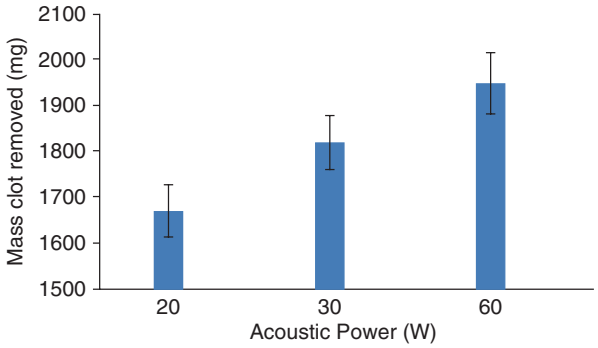

Fig. 19.8 The effect of AP on clot lysis. FUS parameters: f = 1.18 MHz, DF = 10%, pulse duration = 1 ms, PRP = 10 ms, PRF = 100 Hz. treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 μg/mL, water bath temperature = 37 °C and no MBs

19.4.6 Effect of Acoustic Power

The degree of thrombolysis was examined over a range of AP up to the level where the temperature at beam focus elevated 1 °C. To examine the effect of AP on clot lysis, three groups of clots were treated with FUS + TNK-tPA. The degree of thrombolysis was determined for clots treated at AP of 20, 30 and 60 W, respectively. Figure 19.8 shows the thrombolysis vs. AP for f = 1.18 MHz, pulse duration = 1.0 ms, PRP = 10 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 μg/mL, temperature of the water bath = 37 °C and no MBs.

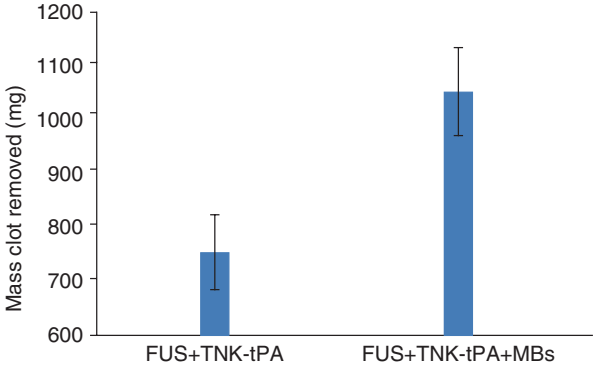

Fig. 19.9 The effect of MBs administration on thrombolysis efficacy. FUS parameters: f = 1.18 MHz, DF = 10%, AP = 60 W, pulse duration = 1 ms, PRP = 10 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7.0 μg/mL, Water bath temperature = 37 °C and MBs rate = 0.5 mL/5 min

19.4.7 Effect of Bubbles

The effect of cavitation nuclei was investigated by injecting 3 mL of MBs (0.5 mL/5 min). Figure 19.9 shows the thrombolysis vs. MBs for f = 1.18 MHz, AP = 60 W, pulse length = 1 ms, PRF = 100 Hz, treatment time = 30 min, flow rate = 50 mL/min, TNK-tPA concentration = 7 μg/mL and MBs rate = 0.5 mL every 5 min.

19.5 Discussion

In this study, the influence of various experimental parameters on thrombolysis efficiency was evaluated, in order to optimize the treatment protocol for 1.18 MHz FUS pulses that maximizes the thrombolytic efficiency of TNK-tPA.

To investigate the effect of temperature on the thrombolytic efficacy of TNK-tPA, an in vitro clot model was used. In this model, blood clots were exposed to a certain concentration of TNK-tPA, at fixed temperatures (37, 39 and 41 °C). Temperatures below the body baseline temperature of 37 °C were not investigated because:

1. Some other in vitro studies showed that the thrombolytic efficacy of rt-PA is reduced at lower temperatures (T ≤35 °C), which anticipated for clinical hypothermic therapy [53–54].
2. Hypothermic studies showed an increased risk of haemorrhage [53].

Study results showed that temperature has a significant impact on thrombolysis efficacy, in clots exposed to TNK-tPA. The study also demonstrated that the peak

fibrinolytic activity of TNK-tPA occurred at 37 °C and decreases approximately 4%/°C increase in temperature (Fig. 19.3). These findings are in good agreement with those from another in vitro study [54], which has shown that the fibrinolytic activity of rt-PA decreases 5%/°C increase in temperature.

Treatment with TNK-tPA alone produced more thrombolysis than that achieved either in the control or in the FUS alone (Fig. 19.4). The effect of FUS alone on thrombolysis efficacy is practically negligible (Fig. 19.4). In the case of a target embedded 4 cm deep into a brain tissue-mimicking phantom, clots treated with FUS plus TNK-tPA exhibited almost 25% (56.2/45) greater degree of thrombolysis compared with clots treated with TNK-tPA alone (Fig. 19.4a). In the case of a superficial target, clots treated with FUS plus TNK-tPA exhibited almost 26% (1950/1550) greater degree of thrombolysis compared with clots treated with TNK-tPA alone (Fig. 19.4b). Study results are supported by the results of another in vitro study conducted by Frenkel and colleagues [32]. They demonstrated that clots treated for 30 min with 1 MHz FUS + rt-PA (10 μg/mL), utilizing AP of 60 W and 100 ms pulses, exhibited 19% greater degree of thrombolysis compared to clots treated with TNK-tPA alone. Thrombolysis enhancement was increased to 50% when the degree of thrombolysis achieved in the control group was subtracted. In the case of our superficial target, thrombolysis enhancement was increased to 114% (750/350), when the degree of thrombolysis achieved in the control group was subtracted. Although the levels of acoustic parameters applied in both studies were the same (DF = 10%, AP = 60 W), a significant greater degree of thrombolysis exhibited in our study, which might be probably due to the following effects:

1. The presence of perfusion gradient through occlusive clots due to flow. The penetration of the thrombolytic agent into clots by perfusion is much more effective than by diffusion [55].
2. The type of the thrombolytic agent. TNK-tPA has a longer half-life and greater binding affinity for fibrin than rt-PA [43].
3. The 18% difference between the two operating frequencies used (1.18 vs. 1.0 MHz), since it is reported that the higher the operating frequency, the higher the associate acoustic radiation force (ARF) [32–33].

Regarding the influence of time on the thrombolytic activity of TNK-tPA, study findings showed a steep increase in thrombolysis efficacy during the first 15 min of FUS exposure, where 90% of clot mass loss was observed. After 30 min of treatment, the peak thrombolytic activity of TNK-tPA was measured, followed by a plateau for longer exposure times, where no further thrombolysis occurred (Fig. 19.5). The phenomenon that no thrombolytic activity took place between 30–60 min of treatment can be explained from the time dependence curve of TNK-tPA concentration in blood, as presented in TIMI 10B trial [56], which was designed to compare prospectively the efficacy and safety of rt-PA and TNK-tPA. The blood concentration of TNK-tPA over time in the TIMI 10B trial shows that after 30 min of treatment, the concentration of the agent reduces below 50% of its initial value (17–24 min half-life) and drops down to 25% at 60 min. Taking into consideration that FUS alone did not enhance thrombolysis compared to control clots, it is reasonable to

assume that that the thrombolytic activity of TNK-tPA between 30 and 60 min of treatment was probably very low to cause further fibrinolysis.

Concerning the effects of standing and travelling US waves on thrombolysis enhancement, experimental results have shown a significant difference in clot mass loss in relation with the wave type applied. Results demonstrated that the impact of travelling acoustic waves on enzymatic fibrinolysis is much more pronounced than in standing waves, resulting in a 163% (25.2/9.6) enhancement in thrombolysis efficiency (Fig. 19.6).

The effect of flow on the speed of fibrinolysis is always considered beneficial, since it is directly responsible for increased transport and penetration of PAs in blood clots, leading to enhanced thrombolysis. In the case of full occlusion, a situation occurred in our study: lysis proceeded from the inside, due to the non-uniform permeation of PAs into the blood clot, through the least permeation resistant. In a highly ischemic MCA, flow rates can vary between 0 and 15 cm/s [57]. In contrary, flow rates in an open MCA (without occlusion) can be as high as 50 cm/s [58]. In our experimental study, flow rate was set six times lower (8.3 cm/s). A threefold increase on the flow rate (25 cm/s) resulted in 17% (29/25.2) enhancement on thrombolysis efficacy, indicating that flow rate is directly proportional to pressure gradient (Fig. 19.7). In addition, it should be taken into account that in our in vitro case, the clot is located at a dead end, and thus flow was minimal. Normally, in an in vivo case, there is also flow from the veins resulting in an increased concentration of the thrombolytic agent that is in direct contact with the clot. So, using this in vitro model, our results are underestimated.

Investigating the effect of acoustic power on thrombolysis efficacy, a linear relationship was observed, in the range between 20 and 60 W (Fig. 19.8). Thrombolysis efficacy at AP levels above 60 W was not attempted since the temperature at beam focus would exceeded our prerequisite of 1 °C. The application of 20 W AP resulted in 1,670 mg of mass clot removed. When AP was increased from 20 to 40 W, a significant increased rate in thrombolysis efficacy was achieved, leading to 1,820 mg of mass clot removed. A second increase in AP from 40 to 60 W caused a further significant increase in thrombolysis efficacy with 1,950 mg of mass clot removed, showing that a linear increase of AP is directly related with enhanced thrombolysis, probably due to a proportional increase in the ARF. It is well established that in the case of fluids, ARF creates acoustic streaming [59], while in tissues it causes motion [60]. The ARF generated in tissue as a result of FUS exposures has been reported to cause repetitive micron-sized tissue displacements [61–62]. Hancock et al. [61] and Wright et al. [62], using FUS pulses at 1.0 and 1.5 MHz, respectively, observed a linear correlation between transmit AP and the magnitude of clot displacement, as predicted for the generation of radiation forces [63]. Similarly, Frenkel et al. [32], using 1 MHz FUS pulses, demonstrated a linear increasing dependence between transmitted AP and thrombolysis efficacy, in the range of 20–80 W, suggesting that FUS exposures created structural changes that enabled greater amounts of thrombolytic agent to penetrate the clots, to expose additional binding sites and hence to increase thrombolysis efficacy.

In view of the findings in the above-mentioned studies, in association with the results of the present study, it is speculated that ARF is the mechanism of action that dominantly appears in the propagation of pulsed FUS waves through a blood clot and is accompanying by enhanced thrombolysis.

The largest thrombolysis enhancement was assessed when clots were treated with FUS plus TNK-tPA in the presence of MBs. Study outcome demonstrated that the interaction of FUS waves with MBs provided more efficient use of thrombolytic enzymes. The synergistic effect of FUS in combination with MBs on the enzymatic fibrinolysis induced by TNK-tPA enhanced thrombolysis efficacy by 40% (1,050/750), compared to thrombolysis induced using only FUS + TNK-tPA (Fig. 19.9). This phenomenon can be explained by the ability of MBs to act as cavitation nuclei, reduce the acoustic cavitation threshold, and give rise to cavitation activity. It is well established that cavitation activity induces changes in the fibrin matrix and accelerates the transport and penetration of fibrinolytic agents into the clot, leading to faster clot dissolution [64]. Also, it is important to bear in mind that the cavitation properties of MBs depend on their size which influences their resonant frequency. A simple relationship that can relate the resonance size of the bubble with the frequency is given by equation:

$$F\,x\,R \oplus 3 \tag{19.4}$$

where F is the frequency in Hz and R is the bubble radius in m. Note that this equation gives only a very approximate theoretical resonance size [65].

Using Eq. 19.4, the theoretical resonance radius of MBs in 1.18 MHz US field is approximately 2.5 μm. Based on this approximation, it is not unreasonable to hypothesize that the bubbles in our study were driven near their resonance frequency, which is correlated with enhanced thrombolysis [66]. Although cavitation activity was not detected in our experiments, we can postulate that MB-enhanced sonothrombolysis is related with cavitation-based phenomena.

19.6 Conclusions

In conclusion, this experimental study clearly demonstrated that thrombolysis efficacy can be significantly enhanced in vitro when 1.18 MHz pulsed FUS exposures are combined with the thrombolytic agent TNK-TPA.

Furthermore, the administration of MBs on a constant rate to sustain cavitation activity was established as a way to further accelerate FUS-enhanced TNK-tPA-mediated thrombolysis. Consequently, MB-mediated sonothrombolysis may offer a new approach to improve outcomes of AIS.

In addition, study results showed that the fibrinolytic activity of TNK-tPA varies with temperature, and it is reasonable to say that temperature significantly affects thrombolysis in terms of both thrombolysis efficacy and haemorrhage risk.

Taking into consideration that for intracranial occlusion, low-frequency US has the potential to cause sICH in association with the results of the present study, it is confirmed that for transcranial sonication, a frequency of 1.18 MHz, is employed as a best trade-off between focus and penetration. Study findings, which are all associated with enhanced thrombolysis, helped us to optimize the treatment protocol for 1.18 MHz FUS that maximize the thrombolytic efficacy of TNK-tPA.

Moreover, emphasis was given to the biological effects produced when FUS interacted with blood clots. In all parametric studies, due to low-energy deposition rates, thermal rise at beam focus was minimal ($\leq$1 °C), making sure that no bioeffects were generated from thermal mechanisms. As a result, the enhancement of enzymatic fibrinolysis occurred mainly through nonthermal mechanisms.

Although the lytic effect of FUS exposures was clearly demonstrated, the mechanisms behind clots dissolution were not elucidated. It is speculated that ARF and cavitation activity are probably the most important mechanisms of FUS that dominantly appeared in our research, contributing to thrombolysis enhancement.

As a final point, this study has shown promising results concerning the enhancing effect of FUS pulses in synergy with MBs, on TNK-tPA-induced thrombolysis with no excess heating. However, taking into consideration that stroke is time dependent, it is possible that even with this optimization, the proposed treatment protocol might not unblock on time large occluded volumes. Therefore, the following needs should be done in order to improve thrombolysis efficacy:

1. Study results even with the use of low flow rates and TNK-tPA concentrations have shown a significant increase in thrombolysis efficacy. Thus, it is likely that our findings would have been even more significant, under more realistic (higher) levels of flow rates and TNK-tPA concentrations.
2. Beam focus was always stationary during the treatment. We hypothesize that if the beam focus was moving with the robotic system across the long axis of the clot following clot's edge (due to clot lysis, the position of clot's edge will change with time), presumably the degree of thrombolysis rate would have been even higher.
3. The use of a transducer that produces a larger beam area on clot's surface, in order to increase the uptake, penetration and binding of the thrombolytic agent into the clot.

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Chapter 20
Gene Therapy for Stroke

Fanxia Shen and Hua Su

Abstract Ischemic stroke is one of the leading causes of death in the world. Current treatment option is limited by therapeutic time window. Gene transfer might be a potential strategy to decrease neurologic dysfunction after stroke. In this chapter, we discussed status of gene therapy for ischemic stroke, available vectors, potential targets, therapeutic genes, and delivery methods.

Keywords Angiogenesis • Gene therapy • Ischemic stroke • Neuroprotection • Vector

Abbreviations

AAV	Adeno-associated virus
Ad	Adenoviral virus
ANG	Angiopoietins
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CNS	Central nerve system
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
FGF	Fibroblast growth factors
G-CSF	Granulocyte colony-stimulating factor
GPE	Glycine-proline-glutamate
GSH peroxidase	Glutathione peroxidase

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HO-1	Heme oxygenase-1
HREs	Hypoxia response elements
Hsp70	Heat-shock protein 70
HSV	Herpes simplex virus
IA	Intra-arterial
IGF-1	Insulin-like growth factor-1
iPSCs	Induced pluripotent stem cells
IV	Intravenous
MCAO	Middle cerebral artery occlusion
MHPs	Microglial healing peptides
miRNAs	MicroRNAs
MMLV	Melony murine leukemia virus
MMP	Matrix metalloproteinase
MSCs	Bone marrow mesenchymal stromal cells
NMDA	N-methyl-D-aspartate
Nox1	Nitrogen oxides
NSC	Neural stem cell
NT3	Neurotrophin-3
ODNs	Antisense oligodeoxynucleotides
PEG	Polyethylene glycol
PPE-EA	Polyaminoethyl propylene phosphate
RA-NP	Retinoic acid-loaded nanoparticles
ROS	Reactive oxygen species
RTL1000	T-cell receptor ligand 1000
rtPA	Recombinant tissue plasminogen activator
SGZ	Subgranular zone
SHR-SP	Stroke-prone spontaneously hypertensive rat
siRNAs	Small interference RNAs
SOD	Superoxide dismutase
SVZ	Subventricular zone
TAT	Transactivating transcriptional activator
TGF	Transforming growth factors
Th	Type-2 helper T cells
TJs	Tight junctions
VEGF	Vascular endothelial growth factor

20.1 Introduction

Stroke patients have a high rate of morbidity, mortality, and disability. Despite considerable research efforts, few treatment options are presently available; the only effective treatment identified to date is early (<4.5 h) thrombolysis and intravenous (IV) administration of recombinant tissue plasminogen activator (rtPA).

Endovascular treatment has been shown to improve functional outcome in five randomized clinical trials with selected patients with acute ischemic stroke. Thrombectomy with stent retrievers is now recommended as a standard care for acute ischemic stroke patients with a proximal large vessel occlusion in the anterior circulation [1]. The short therapeutic time window limited clinical use of both rtPA and thrombectomy [2–4].

Gene therapy is an alternative therapeutic option for improving ischemic stroke outcomes. Gene therapy is broadly defined as the delivery of genetic-based material (e.g., DNA or RNA) to specific cells to modify their gene expression patterns or treating diseases at the genetic level. As early as 1993, antisense oligodeoxynucleotides (ODNs) has already been used in the treatment of experimental ischemic stroke. ODNs are short segments of DNA, which bind to complementary mRNA sequences to stop translation of encoded proteins. Selectively reducing the expression of NMDA (N-methyl-D-aspartate) receptors by antisense oligodeoxynucleotides could prevent neurotoxicity elicited by NMDA in vitro and reduce the cerebral infarct size following experimental occlusion of cerebral artery in rats [5].

An idea gene therapy vector should be able to robustly and specifically delivery and persistently express therapeutic genetic materials (DNA, RNA, oligonucleotides, etc.) to the target tissue or/cell without generating local and/or systemic toxicity. Transgenes can be delivered by either viral or nonviral vectors. Biology, safety, easiness of manufacture, and cost-effectiveness should be considered in designing gene therapy strategies. From a clinical standpoint, it is also important to determine the efficacy on long-term outcomes.

20.2 Gene Therapy Targets

A stroke occurs because of a disturbance or interruption of cerebral blood flow that causes significant reduction of the oxygen and glucose supplies to the neuronal tissue. Following the primary ischemia insult, a subsequent cascade of events amplifies the initial damage and enhances neuronal cell death. The time course over which these effects occur may be longer than that assumed previously and thus potentially provide a wider time window for interventions. Pathophysiological events occur after ischemic stroke including ionic imbalance [6], neuroinflammation [7, 8], excitotoxicity [9], and activity of microglia. All of these contribute to neuronal death and can be the target of gene therapy. Of particular note is that a variety of endogenous proteins are upregulated by ischemic stroke. Modulation of these gene expressions could provide neuroprotection, neurogenesis, and angiogenesis.

Cerebral ischemia induces a cascade of inflammatory reactions that encompass genomic as well as molecular and cellular events. Immune response has been shown to play a major role in ischemic stroke progression [10]. The extent of neuronal damage seems to correlate with the degree of innate immune activity. Numerous studies have demonstrated the critical role of the cellular and humoral immune sys-

tem in postischemic brain injury [11]. The production of inflammatory cytokines could amplify the ischemic lesion, while anti-inflammatory cytokine could attenuate brain damage [12].

The therapeutic targets for gene therapy are designed to mitigate above mention postischemic stroke events.

20.2.1 Neuroprotection

During the past two decades, the effectiveness of neuroprotectants has been demonstrated in rodent experimental stroke models. Although necrosis, excitotoxicity, free radical production, and inflammation are proved to be involved in the mechanisms of cell death in brain ischemia [13], design strategies for neuroprotection following stroke is still quite challenge.

Neuroprotective strategies could be achieved by modulation of immune response, antioxidant defense, and activation or inactivation of pre-existing proteins, inhibiting mitochondrial-dependent apoptotic pathways, and activation or inactivation of pre-existing proteins [14–16]. The neuroprotective genes that have been tested in gene therapies include heat-shock protein 70(Hsp70), brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), glycine-proline-glutamate (GPE), granulocyte colony-stimulating factor (G-CSF), and superoxide dismutase (SOD) [17].

20.2.1.1 Anti-inflammation

The extent of neuronal damage seems to correlate with the degree of innate immune activity. Previous studies have demonstrated the critical role of the cellular and humoral immune system in postischemic brain injury [11]. The production of inflammatory cytokines could amplify the ischemic lesion, while anti-inflammatory cytokine could attenuate brain damage [12]. It has been shown that TAT-Hsp70 ameliorates postischemic inflammation via proteasome inhibition, thus providing an appropriate extracellular milieu for delayed NPC transplantation and culminating in long-term neuroprotection [18].

IL-10, a pleiotropic cytokine produced by type-2 helper T (Th) cells, regulates inflammatory reactions and decrease stroke-associated symptoms. A single intramuscular adeno-associated virus (AAV) vector resulted in sustained IL-10 expression for over 6 months. AAV-mediated IL-10 expression prevents vascular remodeling and target-organ damage in the stroke-prone spontaneously hypertensive rat (SHR-SP) – an animal model of malignant hypertension; no stroke-associated symptoms were observed in the rats transduced with AAV1-RIL10 for more than 7 months after the treatment [19].

20.2.1.2 Antioxidative Stress

Antioxidant genes include catalase and glutathione (GSH) peroxidase, Nox1 short hairpin RNA, heme oxygenase-1 (HO-1) [20], and superoxide dismutase [21].

Overt oxidative stress enhances tissue damage after ischemic stroke [22, 23]. NADPH oxidases, Nox, are a family of isoenzymes, whose primary catalytic function is to produce reactive oxygen species (ROS). Upregulation of NADPH-oxidase is the main endogenous source of ROS during brain ischemia [24]. Nox has been shown to play a significant role in I/R-induced brain injury through superoxide production. AAV-mediated transduction of Nox1 short hairpin RNA (shRNA) into the cortex and striatum of rat significantly reduced infarction size and neuronal death in peri-infarct regions [25]. Antioxidants mitigated the inflammatory response, protected neuronal cells from apoptosis, and reduced edema through protecting the blood-brain barrier damage caused by ROS-mediated reperfusion injury [26].

20.2.1.3 Immune Modulate

Immune modulate has been studied for the treatment of ischemic stroke. Many cytokines have anti-inflammatory property. Studies indicated that the immune response is induced by ischemic stroke activating peripheral leukocytes, which contribute to ischemic brain damage [27]. Mice lacking T and B cells tend to have smaller infarcts than normal mice [28]. Delivery of recombinant T-cell receptor ligand 1000 (RTL1000) through subcutaneous (S.C.) injection protects against ischemic stroke in middle-aged male mice by limiting postischemic inflammation [29, 30].

MHP1, a type of microglial healing peptides (MHPs), designed from RANKL, significantly prevented exacerbation of ischemic brain injury in vivo after intracerebroventricular injection 4 h after the MCAO. This effect was associated with inhibition of TLR2- and TLR4-induced inflammation, which is demonstrated in cultured microglia and macrophages [31].

Administration of exogenous growth factor/hormones has been shown to have neuroprotective effects in ischemic stroke, such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor-1 (IGF-1), glycine-proline-glutamate (GPE), and granulocyte colony-stimulating factor (G-CSF) [32–34].

IGF-1 is a circulating hormone generated in the liver and many cell types in the brain including neurons, glia, and cerebral microvascular endothelial cells. IGF-1 possesses both neurotrophic and angiogenic effects and has been demonstrated to protect the central nervous system from experimental ischemic stroke injury [35, 36]. AAV-mediated IGF-1 overexpression before ischemic stroke enhances neurovascular remodeling and improves functional outcome in a mouse model of permanent middle focal cerebral artery occlusion [37].

The neurotrophic factor is widely expressed in the brain and plays a key role in neuronal survival, differentiation, and plasticity [38]. As a type of secreted proteins, it binds the cognate receptors on the surface of the cells, thereby triggering prosurvival signaling cascades [39]. BDNF rapidly potentiates the spontaneous and

impulse-evoked synaptic activity of developing neuromuscular synapses in culture [40]. BDNF plays a central role in neuronal maturation, circuit formation, and activity-dependent forms of plasticity, such as long-term potentiation of synaptic transmission [41]. Neurotrophin-3 (NT3) plays a role in the development, function, and repair of locomotor circuits [42]. Intramuscular injection of an AAV1 vector expressing NT3 restored sensorimotor function following spinal cord injury/stroke in rats by promoting axon growth and synaptic plasticity in multiple locomotor pathways including the corticospinal tract and proprioceptive pathways [42, 43].

VEGF enhances cortical newborn neurons and their neurite development in adult rat brain after cerebral ischemia. Human VEGF (165)-expressive plasmid (phVEGF) has been injected into the brain lateral ventricles of rats that have subjected to a transient middle cerebral artery occlusion (MCAO). Injection of phVEGF significantly enhances maturation of stroke-induced cortical newly formed neurons and dendritic formation of them [44].

20.2.2 Angiogenesis

Angiogenesis refers to the formation of new blood vessels from pre-existing small vessels. Postischemic angiogenesis plays a crucial role in restoring blood flow in affected brain regions. Restoring neurovascular function through reestablishing cerebral blood flow in the ischemic cerebral blood supply has drawn attention as a potential treatment strategy for ischemic stroke [45]. Newly formed vessels in the ischemic boundary zone may contribute to restore cellular metabolism in surviving neurons and provide the essential neurotrophic support to newly generated neurons. Angiogenic environment attenuated ischemic stroke injury through providing growth factor to damaged tissue. Numerous studies showed a positive correlation between angiogenesis and functional recovery after ischemic stroke in both animal models and stroke patients [46–48]. Pro-angiogenic molecules include matrix metalloproteinases, cytokines, integrins, and growth factors, such as VEGF, fibroblast growth factors (FGF), transforming growth factors (TGF) [49], and epidermal growth factor (EGF).

Most pro-angiogenic factors have both neuroprotection and neurogenesis effects [50]. Among the angiogenic genes, VEGF (121 and 165 amino acid isoforms of VEGF1 and VEGF2) and IGF-1 are relatively well investigated in ischemic stroke animal model.

1. *VEGF*: Exogenous delivery of VEGF reduces ischemic brain injury by inducing neuroprotection, neurogenesis, and angiogenesis. VEGF induces angiogenesis in ischemic area and improved functional recovery following ischemic stroke. VEGF increases in the BBB leakage during acute ischemic stroke. However, there is increasing evidence shown that VEGF is also involved in subsequent repair and regenerative mechanisms during recovery stage [51]. VEGF stands out among many growth factors for its wide-spectrum trophic activities and pro-

angiogenesis effect. VEGF is therefore viewed as a promising therapeutic agent for ischemic stroke. VEGF has a balancing role in injury vs. protection in many pathological conditions and differences in acute and subacute outcomes after stroke. These could be due to balancing roles for VEGF in early inflammation and injury vs. subsequent angiogenesis, neuroprotection, and neurogenesis [52].

2. *Angiopoietin*: The angiopoietins (ANG1, ANG2, ANG3, and ANG4) constitute a family of endothelial growth factors. Angiopoietin-1 functions in vascular remodeling [53]. Overexpression of angiopoietin-1 improves BBB integrity in stroke brain. AAV-mediated co-expression of ANG1 with VEGF in the brain of a mouse MCAO brain improved BBB integrity and resulted in a better reduction of atrophy volume than expression of VEGF alone [54].
3. *IGF-1*: Insulin-like growth factor-1 (IGF-1) is a multifunctional peptide and plays an important role in brain development [55]. IGF-1 is an angiogenic factor and reported to mediate vascular growth in developing an adult brain. Injection of AAV-IGF-1 into the peri-infarct area 3 weeks before distal MCAO increased endothelial cell proliferation as well as microvessels in the peri-infarct region along with increasing local blood flow [56].

In summary, physiology angiogenesis involves endothelial cell proliferation, differentiation, migration, and organization into a branched tubular network. These processes are controlled by specific interaction of various endogenous pro- and anti-angiogenic factors with endothelial cells [57].

Clinical trials have failed to show any beneficial effect of angiogenic therapy for stroke, implicating that stimulation of angiogenesis alone is not sufficient. The strategy for angiogenic therapy has been extended to using combination of drugs or cells that have both angiogenic and other functions, e.g., neuroprotective or neuroregenerative [58].

20.2.3 Neurogenesis

Neurogenesis continues throughout life in the two restricted zones, the hippocampal subgranular zone (SGZ) and the rostral migratory stream, where newly generated immature neurons migrate from the anterior subventricular zone (SVZ) into the olfactory bulb [59]. Brain injury and ischemic insult stimulates neurogenesis in the SGZ and SVZ [60]. Therefore, therapeutic strategy for enhancing neurogenesis after ischemic insult may promote functional recovery in stroke patients.

Neurogenesis could be involved by several growth factors including FGF-2, IGF-1, BDNF, and VEGF, as well as some chemokines such as SDF-1 and MCP-1 [39, 41, 44].

The IGF-I/IGF-IR system regulates the differentiation and maturation of neurons generated from NSCs and progenitors both during embryonic development and in the adult brain largely via the PI3K/Akt pathway [61].

Stereotactic injection of AAV-IGF-1 increases the number of neuronal progenitor cells in both SVZ and subcortical white matter region after ischemic stroke in mice [62].

Polyethylene glycol (PEG)-mediated IGF-I expression has been shown to improve outcomes of ischemic stroke in mice through neuroprotection and promote neurogenesis and axonal sprouting [36].

20.3 Gene Transfer Vectors

Vectors are used to transfer genes of interest into host. Gene delivery vectors are generally divided into two classes: viral and nonviral vectors. The most interesting advance in recent years is the use of site-specific genome editing to provide long-term, stable expression of therapeutic genes.

20.3.1 Nonviral Vectors

Nonviral vectors include plasmid/naked DNA, particle (nanoparticles), and chemical-based (cationic polymer). The disadvantages of nonviral vectors are lower gene transfer efficiency and transient transgene expression. Nonviral vectors do not integrate into the host genome. Therefore, they will not produce insertional genome mutations and thus have less possibility to produce tumor. However, this characteristic also limits their use in conditions requiring long-term gene expression [63].

20.3.1.1 Plasmid Vector

Plasmid DNA is a stable molecule that is relatively inexpensive to produce in large quantities and does not induce an immune response to the vector [64]. The direct gene transfer with naked pDNA is the simplest and safest gene delivery method currently used in ischemic stroke study [44, 65, 66]. However, as cells go through cell division, the non-replicable episomal plasmid copies undergo rapid degradation by nucleases and are cleared by the mononuclear phagocyte system. Therefore, plasmid-mediated therapeutic gene expression is transient.

20.3.1.2 Nanoparticles

The use of nanoparticles as carriers for the delivery of therapeutic materials to target tissues has become popular in recent years and has demonstrated great potentials for the treatments of ischemic stroke. Nanoparticles smaller than 10 nm in diameter penetrate BBB effectively [67]. In the late 1980s, it was found that using liposomes

containing the cationic lipid DOTMA could facilitate the delivery of DNA into cells. The nanoparticles applied in gene delivery can be classified into two major types: solid (including lipids, biodegradable polymers) and liquid (mainly liposomes) [68, 69]. Mixing plasmids with cationic lipid lead to highly efficient and reproducible gene transfer in cultured cells [70]. Most nanoparticles can effectively deliver genes into the brain without disrupting BBB via adsorptive-mediated transcytosis and receptor-mediated transcytosis.

Nanoparticles carry DNA molecules through three different ways: encapsulating DNA inside nanoparticles, forming complexes with DNA through ionic interactions, and loading DNA on the surface via conjugation or trapping with a modified polymer [71].

Administered recombinant human tissue-type plasminogen activator (tPA) via carotid artery at 3 h post stroke in a thromboembolic rat model, followed by sequential administration of antioxidants catalase (CAT) and superoxide dismutase (SOD) genes encapsulated in biodegradable nanoparticles (nano-CAT/SOD), mitigated the inflammatory response, protected neuronal cell apoptosis, and reduced brain edema through protecting the BBB breakdown caused by ROS-mediated reperfusion injury [26].

MMP-12 shRNA-expressing plasmids were formulated as nanoparticles. Intravenous delivery of nanoparticle containing MMP-12 shRNA-expressing plasmids reduced MMP-12 expression in the ischemic brain regions, inhibited myelin degradation in the ischemic tissue, increased the density myelin fibers, and attenuated brain damage [72].

The therapeutic use of retinoic acid-loaded nanoparticles (RA-NP) repairs the ischemic brain safely and efficiently through creating a favorable pro-angiogenic environment enhancing neurogenesis and neuronal restitution [73]. RA-NP reduced ischemia-induced endothelial cell death and stimulated the release of pro-survival, proliferation factors and differentiation cues for neural stem cell (NSC).

Lipid-mediated delivery of DNA or mRNA is usually more rapid than viral vector-mediated delivery, offers a larger payload, and has nearly zero risk of integration. Therefore, lipid-mediated delivery of DNA or RNA is preferable to viral DNA delivery in those clinical applications that do not require long-term expression [74].

20.3.1.3 Other Nonviral DNA Vectors

Liposome-DNA complexes have non-immunogenicity and low toxicity, are relatively easy to produce, and can deliver DNAs of unlimited size [75, 76].

Polyethylenimine/DNA complexes are a controlled release system in which DNA is complexed with PEGylation or coated lipid. This system can delivery therapeutic gene efficiently into the ischemic brain [77].

Polycation/plasmid DNA complexes are also a controlled release system in which DNA is complexed with polyaminoethyl propylene phosphate (PPE-EA).

PPE-EA/DNA complexes have DNA complexed with polyethylenimine (PEI), a nondegradable cationic polymer. Higher levels of gene expression could be detected in the cerebral cortex, basal ganglia, and diencephalons more than 4 weeks after intracistern injection into the mouse cerebrospinal fluid [78]. The purity of pDNA preparations is important for consistency of expression.

20.3.1.4 RNA

A subset of long noncoding RNAs (>200 nt in length), microRNAs (miRNAs), and small interference RNAs (siRNAs) compose an important class of widely used effectors for gene therapy in ischemic stroke [79, 80]. Functioning through the RNA interference (RNAi) mechanism, miRNA and siRNA show potent ability in decreasing postischemic infarction and motor dysfunction [72, 81]. Modified naked siRNAs have increased nuclease stability and gene-silencing efficiency as well as reduced immune responses and off-target effects, when compared to unmodified RNAs.

20.3.1.5 Peptide (p53DM Peptide)

Peptides including growth factors/neurotrophic factor have been used for cerebral ischemia. Transactivating transcriptional activator (TAT) peptides are a kind of cell-penetrating peptides which can effectively cross the BBB [82]. TAT-mediated transduction is able to cargo large molecules, such as proteins across cell membranes, and is becoming a promising tool for the therapy of many diseases. The PTD sequence derived from HIV TAT protein is capable of delivering a large variety of proteins or peptides into the brain and improve neurological functions [83–85]. TAT-14-3-3ε fusion protein was constructed in frame with TAT-PTD domain and 6×His-tag. TAT-14-3-3ε pretreatment had neuroprotective effects and attenuated ischemic stroke in animal model.

More recently, heme oxygenase-1 (HO-1) gene which had anti-apoptotic and anti-inflammatory effects was delivered into the brain using dexamethasone-conjugated polyamidoamine generation 2 (PAMAM G2-Dexa) for the treatment of ischemic stroke [77, 86].

20.3.2 Viral Vectors

Viruses have been under selective pressure for millions of years and have optimized their genome to promote rapid and efficient expression of viral genes within the infected host cell [83]. Despite the various limitations in current gene delivery systems, a spectrum of viral vectors has been successfully used to deliver genes to the CNS.

The most important viruses that have been used as vectors in stroke gene therapy include adeno-associated virus (AAV), adenovirus, herpesvirus, retrovirus, and lentiviruses. Each of these vectors has advantages and disadvantages.

20.3.2.1 AAV Vectors

AAV, belonging to *Parvoviridae* family, is a small particle with protein capsid and a single-stranded DNA genome [87–89]. rAAV vector gene transfer system is currently the best used viral vector for therapeutic gene delivery. The advantages of AAV include (1) not being associated with identified disease, (2) having the ability to transduce both dividing and nondividing cells, (3) having low immunogenicity, (4) mediating long-lasting gene expression, and (5), importantly, being relatively safe [90–92].

AAV-mediated overexpression of VEGF induces angiogenesis in adult mice brain which lasts more than 12 weeks [93]. However, long-term uncontrolled VEGF expression may cause side effects such as hemangioma formation [94]. HIF-1α is an essential regulator of gene expression in response to hypoxia. Hypoxia-responsive element (HRE) is located in the enhancer region of genes where expression is regulated by hypoxia. Our previous study indicated that HRE effectively restricts gene expression only in the ischemic brain and, therefore, reduces the unwanted side effects in normal brain and other organs [95].

In most studies including ours, the AAV vectors were delivered to the brain via stereotactic injection. Direct injection, however, is an invasive procedure that can cause additional brain damage to a critically ill patient. Recombinant AAV packaged in a serotype 9 capsid effectively passes through the blood-brain barrier (BBB). We demonstrated that intravenous (IV)-injected single-stranded AAV9 (AAV-CMVLacZ) mediates LacZ expression in the peri-infarct region of adult brain and other organs. We further showed that IV injection of an AAV9 vector with hypoxia response elements (HREs) that regulated LacZ expression (AAV-H9LacZ) resulted in gene expression specifically in the peri-infarct region of the brain only [96].

20.3.2.2 Adenoviral Vectors

Adenovirus is a non-enveloped virus without double-stranded DNA genome. The transgene capacity is 7–35 kb. Adenoviral vectors infect a broad spectrum of cell types with high transduction efficiency, which leads to a high level of gene expression. However, the gene expression mediated by adenoviral vector is short term and lasts less than 4 weeks. The main disadvantage of adenovirus-derived vectors is being highly immunogenic, which can elicit cellular- and cytokine-mediated inflammatory response [97]. The cytotoxic effects in the brain are vector dose dependent, which limit their usefulness in clinical [98].

Adenoviral vector-mediated eNOS overexpression in ischemic stroke model showed neuroprotection effect through modulating BDNF secretion [99].

Introducing an adenoviral vector expressing SDF1α gene (Ad-SDF1α) into the boundary of the infarcted area 3 days before ischemia resulted an increase in neurogenesis, neuroblast migration, and neural differentiation in the Ad-SDF1α-injected brain area [100]. Injection of an adenoviral vector expressing BDNF into the right caudate putamen 7 days prior to transient MCAO resulted significantly in the improvement of sensorimotor scores and amelioration of ischemic brain injury [101].

20.3.2.3 Gammaretroviral Vectors

Gammaretroviral vectors include lentiviral vectors and MMLV vectors. Due to its improved safety profile and ability to transduce both dividing and nondividing cells, lentiviral vector is generally preferred to gammaretroviral vector.

Gammaretroviruses are enveloped with single-stranded RNA genomes. A subclass of gammaretroviruses has emerged as a vehicle for gene delivery including HIV-based and lentiviral vectors [102]. Gammaretrovirus vectors are characterized by integration into the host genome and mediate long-term transgene expression. However, there are also some safety concerns with insertional mutagenesis and activation of oncogenes in host tissues [103].

1. *Lentiviral vectors:* A number of HIV-1 vector systems have been described in the past. HIV type 1 differs fundamentally from onco-retroviruses in that they are relatively independent of cell division for completion of their replicative cycle.

Lentiviral vectors derived from HIV are able to infect both dividing and nondividing cells and are more efficient in transducing quiescent cells. Lentivirus vector-mediated mmp-9 shRNA ameliorates BBB disruption, which in turn reduces vascular permeability, neuronal cell death, and neurobehavioral deficits in a rat ischemic model [102]. The limitations of lentiviral vectors include limited spread within the brain parenchyma and safety concerns.

2. *Melony murine leukemia virus (MMLV) vectors:* Unlike lentivirus, MMLV depends on cell proliferation for completion of their life cycle. One of the main disadvantages of MMLV vectors is that they are unable to transduce nondividing or slowly dividing cells.

20.3.2.4 Herpes Simplex Virus

Herpes simplex virus (HSV) is a member of *Herpesviridae* and belongs to the subfamily *Alphaherpesvirinae.* HSV is among the largest DNA viruses developed for gene transfer. HSV offers the advantage of large packaging capacity as well as its natural tropism of sensory neurons [104]. Gene expression mediated by HSV system starts as early as 4–6 h, peaks at 12 h, and decreases thereafter [105]. Herpes simplex viral vector-mediated SOD1 overexpression showed neuroprotection in an experimental stroke animal model [106].

20.3.2.5 Sendai Virus

The *Sendai virus* has a genome of negative-strand RNA and belongs to the *Paramyxoviridae* family. It infects the host cell by delivering the nucleocapsid via fusion of its envelope with the host cell membrane and has no interaction with the host chromosomes [107]. *Sendai virus*-mediated GDNF gene transfer showed a significant neuroprotective effect in the ischemic brain of an animal model [108]. Sendai viral vector-mediated overexpression of neurotrophic factors has high therapeutic potency for preventing the delayed neuronal death induced by transient global ischemia [109]. Despite promising data presented to date, viral vectors possess several limitations, which include complex vector construction and production, difficulties in storage, and off-target effects (e.g., undesired toxicity, immunogenicity, and tumorigenicity).

20.3.3 Cell-Based Vector

Ischemic stroke induces endogenous repair processes that include proliferation and differentiation of neural stem cells and increase mobilization of $CD34^+$ cells from the bone marrow into the periphery [110]. Stem cell-based therapies are effective in restoring function in experimental ischemic stroke models. Stem cell therapy improves neuronal function through enhanced neurogenesis, angiogenesis, and synaptogenesis [111–113]. However, the underlying repair processes are unknown, although some studies indicated that it might be correlated with the cell replacement and/or the change of local host environment through providing varieties of bioactive substances, including trophic factors and extracellular vesicles released by transplanted cells [114]. Many cells present transiently a few days after transplantation that provide trophic influences on immune or inflammatory responses. The safety of stem cell therapies is still a big challenge, such as ESC/iPSC therapy that was associated with tumor formation or stroke. The optimal cell dosage and route of administration of stem cells in stroke are another problem that needs to be settled. Investigators often emphasize that allogenic stem cells are immune privileged and safe for recipients but this is not universally accepted.

Cell-based gene therapy often utilizes stem or progenitor cells that overexpress therapeutic genes. Due to their plasticity and differential capacity, cellular vehicles could be genetically modified ex vivo to express therapeutic molecules. In cell-based gene therapy, stem cells are isolated, cultured, and genetically modified ex vivo through nonviral or viral vector-mediated gene transfer. The cells that are successfully modified can be selected and expanded before being transplanted.

EPO is considered as a kind of hematopoietic growth factors with also potent neuroprotectants and potential candidates for the treatment of various neurological diseases in humans. Bone marrow mesenchymal stromal cells (MSCs) overexpressing the EPO gene (EPO-MSCs) promoted neural cell survival and improved neurological function in rat ischemic stroke model [115]. Lentiviral and adenovirus vector are the most used viral vectors for modifying MSCs [116–118].

Transplantation of mobilized peripheral blood cells is currently the method of choice in autologous transplantation. Peripheral blood cells contained a population of progenitor cells. Compared to BM cells, peripheral blood cells are easier to collect and have fewer technical difficulties, lower risk, and considerably less pain. Although NSCs and MSCs have promising potentials in treating stroke, the difficulty in obtaining large numbers of cells and poor proliferating ability limit their application for autologous cell transplantation. Clinical use of fetal NSPCs is not permitted due to ethical limitations.

Induced pluripotent stem cells (iPSCs) are stem cell populations generated from adult somatic cells through reprogramming by transcription factors. iPSCs can be generated by introducing a set of specific transcription factors into somatic cells such as skin fibroblasts ex vivo. iPSCs can be differentiated into all cell types derived from the three embryonic germ layers including neurons and glia [119, 120]. iPSCs also have a great potential for clinical cell therapies with lesser ethical concern and immunogenicity than ESCs. Injected iPS-derived NPCs into the infarct cavity of stroked NSG mice with a hydrogel that contains fibronectin and laminin peptide motifs promoted neuronal differentiation of transplanted NPCs [121, 122].

Advances in iPSC technologies, including improvements in the ease and efficiency of generating iPSCs and iPSC-derived neural cells, have resulted in increased tests of them as an option for the treatment of stroke.

20.4 Delivery Methods

The delivery systems for gene therapy vary among delivery cargos. BBB is a major obstacle of therapeutic gene delivery. The BBB is a complex physical barrier composed of four main cellular elements, namely, brain microvascular endothelial cells, astrocyte end feet, microglial cells, and pericytes [123]. Tight junctions (TJs), present between the cerebral endothelial cells, form a diffusion barrier, which selectively excludes most blood-borne substances from entering the brain [124]. Due to the present of BBB, gene delivery to CNS remains a major challenge. There are two commonly used was for delivery genes into CNS: direct CNS administration and systemic administration. Optimizing the administration techniques to maximize gene distribution and gene expression and to minimize unwanted side effects is an important step in developing gene therapy strategies.

20.4.1 Direct CNS Administration

Directed gene delivery is achievable via stereotactic injection into the lateral ventricle or brain parenchyma. Most of the viral vectors and nonviral vectors are delivered through direct injection because they are unable to cross the BBB. Some of the nonviral vectors are unstable in the blood circulation and easily degraded by the

nuclease. Direction injection has a limited clinical application scope because it is invasively requiring opening the skull and meninges.

20.4.2 Systemic Administration

Systemic administrations can be achieved through intravenous (IV) or intra-arterial (IA) injection.

IV or IA administration of viral vector is less invasive and safer, with fewer risks than intra-brain delivery [125].

Most of the currently used vectors cannot cross the BBB effectively and therefore need to be delivered through direct injection. A notable exception is AAV serotype 9 (AAV9) [126]. AAV9 has been shown to cross the BBB upon intravenous injection [127, 128]. Intravenous delivery of vector can provide widespread distribution of gene expression throughout the entire CNS, which has advanced potential of global CNS gene therapy. However, for focal ischemic stroke, to restrict therapeutic gene expression specifically within the ischemic area is important to minimize adverse effects. The majority of transplanted cells by intravenous delivery might be absorbed by nontarget organs—especially the lungs, spleen, and liver—before they could reach the target organ, the brain. It is crucial to control gene expression in targeted region in the brain. It has been shown that excessively high levels of VEGF expression in the rat myocardium resulted in hemangioma formation. Regulated therapeutic gene expression can be achieved through using tissue-specific promoters, enhancers, and regulatory systems to control gene expression.

Transient disrupt BBB can improve the transduction efficiency of systemically delivered genes. Osmotic can reverse and transiently disrupt the normal BBB and has been investigated as a means to facilitate delivery viral particles to the brain [129]. The most commonly used method of increasing permeability is mannitol-mediated osmotic disruption which has been widely used in preclinical and clinical studies [130].

In addition, ultrasound exposure improves drug or gene delivery efficiency into tissues and cells. The permeability of the BBB can be enhanced by the combination of bubble liposomes and high-intensity focused ultrasound, thereby enabling the delivery of nucleic acids (e.g., antisense oligonucleotide) or plasmid DNA into the brain [131].

20.5 Summary

Various experimental data indicated that gene therapy could provide neuroprotection and promote neurogenesis and angiogenesis. Therefore, it attenuated ischemic brain injury. Many vector systems have been developed with a broad potential. Hybrid delivery vectors, such as incorporation of adeno-associated virus into porous

silicon nanoparticles, which overcome the vector-specific limitation and retain their advantages, might be future directions. The pathophysiologic processes occurring after the onset of ischemic stroke are complex and involve multiple events that have not been fully understood. Many factors have to be considered in designing gene therapy strategy for treating ischemic stroke, which include selecting the appropriate vectors and therapeutic genes, maximizing the efficacy, and minimizing the side effect. The optimal delivery route, doses, or time window of administration after stroke are still under debate. Therefore, there is still a long way for the innovating gene therapy to be successfully moved from bench to bedside.

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Chapter 21
Stem Cell Therapy in Stroke

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Abstract Stem cell-based treatment for ischemic stroke has shown its effectiveness in animal models and clinical trials. In this chapter, we describe different types and delivery routes of stem cells for therapy, the tracing of stem cells after delivery, and the clinical challenges and strategies in the future. Stem cells derived from various tissues have shown their beneficial role for functional recovery after stroke. Although the mechanism of stem cell-based therapy is not fully understood, it may include the releasing of growth factors, microenvironment regulation, and the preparation of repairing the blood-brain barrier integrity. Clinical applications of stem cell-based therapy are still in infancy. The future of clinical study in the stem cell-based therapy in the stroke field needs to focus on the modification of stem cells or combining different types of stem cells to enhance the therapeutic efficacy, mechanisms of stem cells action, and translation to clinical applications. Stem cell treatment is a promising regenerative therapeutic strategy because it can prevent neuronal cell apoptosis, inhibit pro-inflammatory cell recruitment, secrete multiple neurotropic factors, and promote neural differentiation.

Keywords Cell therapy • Clinical trials • Cytokines • Extracellular vesicles • MicroRNA • Stroke • Stem cells • Strategies • Translational

Abbreviations

BBB	Blood-brain barrier
EPCs	Endothelial progenitor cells
ESCs	Embryonic stem cells
iPS	Inducible pluripotent stem
MCAO	Middle cerebral artery occlusion
MSCs	Mesenchymal stem cells

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NSCs	Neural stem cells
SDF-1α	Stromal cell-derived factor 1-α
SVZ	Subventricular zone
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
VPCs	Vascular progenitor cells
VSELs	Very small embryonic-like stem cells

21.1 Stem Cells in Experimental Ischemic Stroke Therapy

Heterogeneous population of stem and progenitor cells is found in the bone marrow, circulating umbilical cord blood, and peripheral blood. Many types of stem cells have been highlighted the therapeutic potentials in the treatment of ischemic stroke due to their multipotency and self-renewal features [1]. Studies for the efficacy of stem cell-based therapy usually focused on mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), neural stem cells (NSCs), embryonic stem cells (ESCs), vascular progenitor cells (VPCs), and induced pluripotent stem (iPS) cell.

21.1.1 Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs, mesenchymal stem cells, or multipotent stromal cells) have attracted attention in reducing the long-term impact of stroke. MSCs, a population of plastic adherent fibroblastic cells, express CD105, CD90, and CD73 ($\geqslant$95% positive), but not CD34, CD45, HLA-DR, and other hematopoietic markers ($\leqslant$2% positive) [2]. MSCs have the ability to differentiate into mesodermal cell lineages including adipocytes, chondroblasts, fibroblasts, osteoblasts, skeletal myoblasts, and neurons both in vitro and in vivo [3, 4]. MSCs could be isolated from the bone marrow, cord blood, and placenta then expanded in vitro [5–7]. Transplanted MSCs could migrate to damaged brain tissues, inhibit apoptosis, and exert neuroprotection by secreting cytokines [8]. MSCs exerted the beneficial role by immunomodulatory, angiogenesis, anti-inflammation, and anti-apoptotic effect in acute and chronic pathological condition [9]. MSCs could differentiate into neurons, astrocytes, and oligodendrocytes to promote brain plasticity and support neural cell growth after injury in vitro [10–12]. MSC-conditioned media decreased the migration and production of monocyte chemotactic protein (MCP-1), which attenuates the immune reactions in ischemic brain [13]. In vivo studies also showed a beneficial role of MSC transplantation in the ischemic stroke therapy. Numerous evidences showed that grafted MSCs could migrate to injured tissues and differentiate into several cell types, then promoted angiogenesis and oligodendrogenesis, and finally improved neurogenesis. Intravenous administration of MSCs in stroke

models reduced cell death and apoptosis in the subventricular zone (SVZ) [14, 15]. MSCs repressed inflammatory cytokines and protected blood-brain barrier (BBB) permeability by reducing the apoptosis of astrocytes after brain ischemia [16]. Grafted MSCs could promote angiogenesis by secreting endogenous vascular endothelial growth factor (VEGF) and ameliorate BBB destruction due to its characteristic of hepatocyte growth factor (HGF) [17]. MSCs regulate BBB permeability and cerebral blood flow in a dose-dependent manner after middle cerebral artery occlusion (MCAO). MSCs enhanced endogenous glial cell-derived neurotrophic factor (GDNF) production in the ischemic boundary zone (IBZ) of adult rat MCAO model [18], where oxygen and glucose delivery was partly maintained. MSCs differentiated into neuronal phenotypes to improve functional recovery after injury [19]. Grafted MSCs could also enhance gliagenesis including oligogenesis and astrocytogenesis, consequently to support neuronal synaptogenesis [20, 21]. Furthermore, intravenous treatment with MSCs enhances angiogenesis in the host brain due to the increase of VEGF and its receptor (VEGFR2), which suggests that administration of MSCs provides a microenvironment to activate endogenous restorative mechanisms of ischemic brain. Recently, studies showed that the extracellular vesicle, such as exosomes shed from MSCs, also enhanced the benefit for neurite and promote functional recovery after stroke [22, 23].

21.1.2 Endothelial Progenitor Cells (EPCs)

Endothelial progenitor cells (EPCs) are hematopoietic in origin and can be found in the peripheral blood, bone marrow, and human umbilical cord blood (*h*UCB) [24–26]. EPC maturation to endothelial cells (ECs) is an important step of the vascular system. Identification of EPCs remains controversial due to lack of specific markers. EPCs were first described as $CD34^+/VEGFR^+$ cells by Asahara, and that double labeling was considered as the prevailing marker for homogenous EPCs [27–29]. Emerging evidence indicated that EPCs played a role in reestablishing the endothelial integrity following its disruption and promoted neurogenesis after cerebral ischemia. In vitro studies showed that EPCs cross-talk to ECs and astrocytes to increase their proliferation and vascular network formation. Hypoxic condition could stimulate EPCs to enhance tube formation. On the other hand, in vivo studies indicated that EPC transplantation promoted angiogenesis and improved long-term stroke outcomes [30]. Administration of $CD34^+$ cells after stroke showed a better functional recovery, the angiogenic environment around the injury induced by EPC suggested to promote neurogenesis [31–35]. EPC transplantation could reduce infarct volume and neuronal cell death; induce neovascularization, angiogenesis, and neurogenesis; and improve neurobehavioral recovery after ischemia [30, 36]. Not only cells but also EPC-conditioned media lead to significant tissue revascularization and functional recovery in a rat model of chronic hind limb ischemia and a mouse stroke model [37–39], which suggested that the relevant trophic factors such as VEGF-A, hepatocyte growth factor, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor

secreted by EPC provide a suitable microenvironment for neuronal regeneration and survival [40]. And endothelial cells also could release factors and chemokines, such as brain-derived neurotrophic factor (BDNF) to promote the function of neurons [41, 42]. Furthermore, endothelial outgrowth cells (EOCs) or endothelial colony-forming cell populations of human EPCs are particularly promising for vascular tissue engineering applications [43].

21.1.3 Neural Stem Cells (NSCs)

Neural stem cells (NSCs) mainly exist in mammalian brain SVZ and hippocampus, and they have the ability to self-renew, proliferate, and differentiate into multiple lineages [44–46]. Exogenous NSCs could be isolated from many regions of the central nervous system and cultured in vitro as neurospheres [47]. After ischemic stroke, neurospheres (NSs) formed from the SVZ and stroke-injured cortex for self-renewal [48]. Transplanted NSCs differentiated into neurons and astrocytes, which demonstrated reduction of brain damage after stroke [49]. The therapeutic potentials of NSCs are enhancing endogenous secretion for angiogenesis, protecting endogenous neurons from apoptosis, then finally reduced infarct volume and improved neurobehavioral recovery [50]. Intravenously delivered mouse NSCs has been reported to suppress inflammation and glia scar formation and promote neuroprotection [51]. Furthermore, experimental evidence indicated that the proliferation of neural progenitor cells (NPCs) in SVZ and their migration could be induced by ischemic brain injury [52, 53]. Endogenous NPCs continuously supply the injured adult brain with new neurons and then improved recovery after stroke, but it may be not enough for the injury recovery [54]. NSCs and NPCs could undergo asymmetric cell division and maintain the stem and progenitor pools for brain repair [55]. Study demonstrated that NPC-derived conditioned media improved neuroprotection and neurological recovery, suggesting that cell transplantation was dispensable and secreted factors played a vital role for the recovery after stroke [56]. Ischemic stroke animal models treated with NSCs showed that the hypoxic injury led to the increase of neurogenesis in the SVZ [52, 53]. Genetically modified NSCs also provided the functional improvement [57–60].

21.1.4 Embryonic Stem Cells (ESCs)

Embryonic stem cells (ESCs) are self-renewing, pluripotent cells derived from blastocysts during the 16-cell stage with the pluripotent characteristic to give rise to all cell types [47, 61, 62]. However, the malignant transformation risk and teratoma formation in receiving animals are the mainly limitations of ESC application in the treatment of stroke [63, 64]. Transplantation of endothelial cells and mural cells

derived from undifferentiated human ESCs markedly enhanced neovascularization in the ischemic brain, which could reduce the infarct volume and apoptosis and promote neuroprotection in a transient MCAO model [65]. ESCs had been found to differentiate into many cell types in vitro [66, 67], and transplantation of ESC-derived tissue-specific differentiated cells rather than undifferentiated ESCs provides a promising way to decrease tumorigenic risk. ESCs could generate specific neuronal differentiation via extrinsic and/or intrinsic signal, which were considered as a suitable candidate for the neuronal replacement. ESC-derived neural precursors could survive and differentiate into mature glial cells and functional neurons after transplanting into the rat stroke model [68]. Transplanting ESC-derived neural precursors into infarct core and periphery of adult rat improved functional recovery and showed a time-dependent increase in mature neuronal and glial markers after transplantation. Recent study demonstrated that uncommitted cells derived from ESCs have the ability to support neuronal differentiation within the adult brain and promote the specific differentiation and survival [69].

21.1.5 Vascular Progenitor Cells (VPCs)

Vascular progenitor cells (VPCs) were first described as Flk-positive cells and derived from ESCs. VPCs can differentiate into endothelial and mural cells and produce the vascular organization process. VPCs could differentiate into both ECs and vascular mural cells with the induction of VEGF and PDGF-BB, respectively [70]. In a hind limb ischemia experiment, transplantation of VPCs formed well-defined vascular structures, improved blood perfusion, and significantly attenuated the ischemic injury [71]. Further study in cerebral ischemia indicated that transplantation of endothelial cells and mural cells derived from human ESCs could significantly promote neurological recovery due to the high ability of proliferation and expanding in large scale, consequently promote vascular regeneration in the infarct area [65]. The co-transplantation of NPCs and VPCs could decrease infarct volume and attenuated functional neurological deficits after transient MCAO, which had a better improvement than transplantation of NPCs alone [72].

21.1.6 Inducible Pluripotent Stem (iPS) Cells

Inducible pluripotent stem (iPS) cells express pluripotency-associated gene OCT4, Sox2, and Nanog before differentiation. iPS cells can be generated using somatic cells such as skin fibroblasts and show the potential for multilineage differentiation. iPS cells shared the same features as ESCs and provided a source for the stem cell-based therapies [73, 74]. The NSCs derived from iPS cells could spontaneously differentiate into neurons and astrocytes [75]. Studies demonstrated that iPS cells could differentiate into specific neuronal subtypes including dopaminergic neurons

and motor neurons [76, 77]. Subdural-transplanted iPS cells showed a better outcome such as lesion area minimization, motor function improvement, and inflammatory reduction after MCAO [78]. Transplantation of iPSC-derived NSCs was demonstrated to survive, migrate into ischemic brain region, and differentiate into mature neural cells, which showed the potential to restore lost neurologic function in MCAO [75]. Furthermore, iPS-NPC transplantation showed greater expression of stromal cell-derived factor 1-α (SDF-1α) and VEGF, which played an important role in directing endogenous neural progenitors to the stroke lesion and promoting endogenous regeneration in the peri-infarct area and function recovery [79]. The formation of teratomas has been used as a landmark for evaluating the pluripotent stem cells. Injection of iPS cells into the brain showed the high risk of teratoma formation in MCAO rats; strategies like using iPS-derived NSCs, capsulated in a hyaluronic acid hydrogel matrix, and pretreatment with plasmids combined with hypoxia were used to decrease cancer formation [80–82]. These data suggested that iPS cells provide a novel approach to produce specific cells for autologous transplantation.

21.1.7 Very Small Embryonic-Like Stem Cells (VSELs)

The very small embryonic-like stem cells (VSELs) are considered as a unique population of stem cells that was discovered by Ratajczak's research team and suggested to be a source of pluripotent stem cells for tissue/organ regeneration [83]. Following experimental stroke, VSELs expressing markers of neural tissue-committed stem cells (TCSCs) increased; these cells were mobilized into the peripheral blood and migrated toward SDF-1, HGF, and LIF gradients [84]. In damaged brain, microglial VSELs showed a potential to differentiate into neurons, oligodendrocytes, and microglias, and these cells could act as donor grafts for neural regeneration. The population of VSELs was epiblast-derived progenitor stem cells and considered as a potential reserve pool of tissue-committed stem cells for various organs and tissues [17, 85, 86]. VSELs were mobile resources of primitive stem cells and subsequently translocated along with HSCs to develop the bone marrow (BM) [87]. A variety of chemical signals of VSELs orchestrate the homing and engraftment into ischemic tissue. The potential prognostic value of VSELs in stroke patients was also demonstrated [88]. Study on stroke patients found that stroke triggered mobilization of $CXCR4^{+}$ VSELs, which may have the prognostic value [89]. It was noted that VSELs not only expressed the markers of pluripotent stem cells but also changed the epigenetic signature of imprinted genes, so these cells should be quiescent to prevent teratoma formation [90]. In conclusion, VSELs were supposed to be purified from BM or mobilized peripheral blood (mPB), and the use for therapeutic neural regeneration after stroke still requires further studies.

21.2 Mechanism of Stem Cell Therapy

21.2.1 Cell Replacement

The nervous system has a limit for self-repair, and the transplantation of various types of stem cells to replace the injury is thought to be an exciting option. Stem cell-based therapy persistently focused on inducing the generation of parenchymal cells. Thus, stroke-induced new neurons mainly differentiated the phenotype of destroyed neurons [52]. Evidence from experimental ischemia models showed that it was possible for neuronal replacement and partial reconstruction of neuronal circuitry [91]. Endogenous NSCs in the adult brain responded to environment demand and continually migrated from the pool of progenitor/stem cells [44, 92]. Replacement of lost or dysfunctional neurons and glial cells even happens in the aging brain [93]. Neuroblast in the adult brain has the ability to migrate toward the injured striatum and cortex where the neuron lost after MCAO [94]. The replacement of lost function could be achieved when synaptogenesis occurred [93]. Various neurotrophic factors and cytokines were involved in the modulation of neurite outgrowth and synaptic recovery after brain injury. Exogenous transplanted cells like iPS cells could also be used for neuronal replacement [95]. Grafted ESC-derived neuron replacement in the ischemic brain also showed functional recovery and synaptic integration [68, 96, 97]. If the new neurons perform function and their formation can be induced, this strategy could apply to the therapy of human stroke. Replacing glial cells could be a better strategy than building new neurons [98]. Upregulating the expression of many developmental signaling molecules could guide neocortical neuron differentiation.

21.2.2 Cytokines

Neuronal replacement and reconstruction are the major mechanisms for the improvement of long-term recovery, but bioactive molecules secreted from stem cells also play a partial role of regeneration. Study demonstrated that neuroprotection induced by stem cells is mainly due to the increase of trophic factor [99]. Conditioned media from stem cells showed the effective therapy for acute phase stroke [100]. Continuous conditioned media treatment reduced the infarct volume and maintained motor function after the experimental stroke [101]. Bioactive factors including IGF-1 (insulin-like factor-1) and BDNF secreted from adipose-derived stem cells protect neuron from injury as well as endogenous repair [102]. Astrocytes could secrete beneficial growth factors such as granulocyte-macrophage colony-stimulating factor, VEGF, transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), and IGF-1 [103–105]. CXCR4 and CXCR7 are the

receptors of CXCL12 (SDF-1) and could be widely detected in the nervous system. In differentiated neurons, CXCR7 is involved in the CXCL12/CXCR4 signaling pathway and could be found in cultured neurons [106]. CXCL12 played a role in neuroprotection, neurogenesis, and regeneration [107–109]. Exogenous CXCL12 increases the synthesis or release of neurotrophic factors. In cerebral ischemic mice, injection of CXCL12 could reduce infarct volume, improve neural plasticity, and enhance local functional cerebral blood flow [110]. CXCL12 gene therapy promoted neurogenesis and angiogenesis and ameliorates white matter injury [111, 112]. In vitro and in vivo studies showed that MSCs increased the release of glial cell-derived neurotrophic factors such as VEGF, basic fibroblast factor (bFGF), and BDNF [18, 113]. EPCs provided a favorable angiogenic environment to support neurogenesis in the SVZ [34, 35]. Transplanted NPCs or intravenously injected NPC-conditioned medium might respond to specific environmental signals to release growth factors, such as BDNF, GDNF, and NGF, which improved functional recovery after injury [56, 114–116].

21.2.3 MicroRNAs (miRNAs)

MicroRNAs are small, noncoding, composed of 20–24 nucleotides. miRNAs interact with target mRNA and play a role in regulating gene expression at the posttranscriptional level [117]. miRNAs are essential in stroke pathogenesis for the contribution of angiogenesis and neurogenesis. miRNAs have their particular function in normal biological process such as cell-cycle regulation, cell differentiation, apoptosis, and metabolism [118–121]. In the past few years, miRNAs have been found to implicate the etiology of various diseases including cancer, cardiovascular disease, and neurodegenerative disease. miRNA profiles are alerted during the ischemic injury and the development of pathologic condition. Study in profiling miRNAs in rat MCAO reported that miRNAs were actively regulated and their expression pattern changed with the reperfusion time [122]. miRNA-15a was involved in the pathogenesis of ischemic injury, which was due to the induction of cerebral vascular endothelial cell death after oxygen-glucose deprivation (OGD) [123]. Furthermore, specific miRNAs are involved in neural differentiation, neurogenesis, and neuronal development function [124–126]. Many studies aimed at elucidating the role of miRNAs in the regulation of angiogenesis after ischemia. miRNA profiles suggested that many miRNAs are related to angiogenesis including miRNA-15b, miRNA-16, miRNA-20, miRNA-21, miRNA-23a and b, miRNA-24, miRNA-29a, miRNA-29b, miRNA-31, miRNA-99a, miRNA-100, miRNA-103, miRNA-106, miRNA-125a, miRNA-125b, miRNA-126, miRNA-130a, miRNA-181a, miRNA-191, miRNA-221, miRNA-222, miRNA-320, miRNA-let-7, miRNA-let-7b, miRNA-let-7c, and miRNA-let-7d [127–129]. miRNAs were highlighted during the regulation of NSCs' fate including neurogenesis, NSCs' self-renew, synaptic formation, and plasticity. miRNA-200 and miRNA-182 could upregulate early and showed a neuroprotective role by downregulating prolyl-hydroxylase-2 level after

stroke [130–133]. miRNA-124 protected neurons against apoptosis in ischemic stroke model both in vitro and in vivo [134]. Endogenous miRNA-124 and miRNA-9 in NPCs promoted neuronal differentiation in mouse brain following cerebral ischemia [135, 136]. miRNA-132/miRNA-212 cluster is highly regulated by synaptic activity in hippocampal neurons [137]. miRNA-126 is enriched in endothelial cells and EPCs and considered a master regulator of angiogenesis. miRNA-126 played an important role in neurorestoration induced by human umbilical cord blood cells (HUVECs) in type-2 diabetic stroke model [138]. MSCs transfected with miRNA-126 also showed a better outcome in angiogenesis and functional recovery in myocardial infarction [139]. miRNA-29b was considered as a circulating biomarker of stroke, and its overexpression reduced brain damage possibly by targeting AQP4 and consequently protected BBB [140]. miRNA-210 was involved in regulating vascular endothelial cell migration and tube formation under hypoxia condition, and a lentiviral vector carrying miRNA-210 could promote both angiogenesis and neurogenesis [141]. miRNA-210, induced by hypoxia-inducible factor (HIF), was involved in cell cycle, DNA repair, and apoptosis [142]. miRNA-200b, 200c, and miRNA-429 possessed their inherently cytoprotective effect following ischemic preconditioning in neural cell culture [130]. In human ischemic stroke, miRNA-223 showed its role in upregulating growth factor such as IGF-1 and could be used as a biomarker for diagnosis of stroke [143]. miRNAs emerged as important regulators of gene expression, and multiple combinations of miRNAs could be an essential part of the therapeutic strategy.

21.2.4 Extracellular Vesicles/Exosomes

Extracellular vesicles (EVs) are nanometer-sized vesicles classified into exosomes, microvesicles/microparticles, and apoptotic bodies and can be released into extracellular fluids including cerebrospinal fluid (CSF) in all living system [144–146]. EVs could transfer DNA, mRNA, noncoding RNA, proteins, and lipids mediating the intercellular connection [147–149]. The function of EVs largely depended on its enriched miRNAs and capability to transverse BBB, which made EVs become a perfect candidate for central nervous system diagnosis or therapy [150]. Exosomes derived from preconditioned cells might encapsulate specific factors and transfer into target cells to perform their function [151]. MSC exosome is rich in angiogenic paracrine factors such as cadherin, EGFR, FGF, and PDGF, which indicated that MSC exosome may be a potential candidate for treatment of ischemic injury [152]. EVs released by neuronal system including NSCs, NPCs, astrocytes, microglias, and oligodendrocytes can be involved in neuronal development and regeneration both in physiological and pathological conditions [153–157]. Exosome-mediated intercellular communication was demonstrated between endothelial cells and human adipose-derived MSCs in angiogenesis. Further evidence showed that exosomal miRNA-125a could be the regulator transferred from MSCs into ECs and enhanced EC tube formation both in vitro and in vivo [158]. Endothelial

microparticle level correlated with endothelial injury and might be a marker of disease severity [159]. Systemic administration of exosomes from MSCs significantly improved functional recovery as well as neurogenesis in stroke rats [160], which provided a novel approach for stroke treatment. The underlying mechanism was considered as miRNAs, mainly miRNA-133b carried by exosomes, which subsequently regulate the neurite outgrowth and benefit neurite remodeling as well as functional recovery after stroke [22, 23]. Further study showed no differences in angiogenesis and neurogenesis between administration of MSCs and MSC-derived extracellular vesicles only [161]. Together, extracellular vesicles could be carriers or vehicles to transfer information that modulate recovery after cerebral ischemia.

21.3 Strategies for Improving Stem Cell-Based Therapy

21.3.1 Gene-Based Stem Cell Therapy

Combination of gene and stem cell therapy for stroke was considered as a novel therapeutic approach to enhance functional recovery except stem/progenitor cell-induced neurotrophic factors. Intravenous infusion of human MSCs with a nitric oxide donor (Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate promoted angiogenesis and neurogenesis after stroke [162, 163] and consequently improved functional recovery accompanied by enhancement of endothelial cell proliferation and the number of vessels [164]. Granulocyte colony-stimulating factor (G-CSF) was reported having the ability to induce proliferation of BM-derived cells. Administration of G-CSF combined with stem cell factors (SCF) to mice suffering MCAO increased angiogenesis and the number of newborn cells in the ischemic hemisphere, which suggested that G-CSF was an important recruiting factor to induce cells from BM into the circulation [165]. Intra-arterially administered autologous BM combined with an inhibitor of matrix metalloproteinase 1 and 2 (TIMP1 and TIMP2) improved NO bioavailability, decreased systemic oxidative stress, and showed a better neuroprotective effects [166]. Overexpression of the human anti-apoptotic gene bcl-2 in ESCs increased its survival rate and differentiation to improve the functional outcome [167]. Neurotrophic factor (NTF) was also an attractive therapeutic candidate. NTFs, such as BDNF, bFGF, GDNF, NGF, and TGF-β1, controlled the specific differentiation of neuronal and glial cells [168, 169]. N-acetylcysteine (NAC) was reported to protect ischemic injured brain effectively. Pretreatment with NAC increased the HIF-1a, erythropoietin (EPO), and glucose transporter (GLUT-3) to promote neuroprotection [170]. Combined treatment with MSCs and EPO increased both the number of BrdU-positive cells and BrdU-/NeuN-positive cells in the striatal IBZ, which showed a higher endogenous proliferation and differentiation accelerated by the co-administration [171]. The combination therapy of VEGF and stem cells showed therapeutic benefits in the model of stroke, mainly due to the promotion of proliferation, migration, and tube formation

[172, 173]. Intracerebral delivered netrin-1 could protect infarct tissue from apoptosis and stroke-induced degradation [174]. Blocking the expression of MMP-3 or MMP-9 in adult NPCs not only impaired the differentiation but also interfered the chemokine-induced cell migration, suggesting that MMPs mediated the neurogenic response to extrinsic signals [175]. SDF-1 upregulation could induce EPC migration after ischemic stroke, CXCR4 in neural stem cells, and EPCs can react with SDF-1, which suggested SDF-1 signals involved in EPC-mediated neuroprotection [30]. Administration of the adeno-associated virus (AAV) carrying SDF-1 gene showed a better prognosis after stroke [111]. And further study on SDF-1 gene therapy showed effectively protection of white matter and extending the therapeutic window [112].

21.3.2 Preconditioning for Stroke Treatment

Preconditioning means a brief subtoxic or sublethal insult before ischemia, such as hypoxic preconditioning, exposure to oxidative stress, and heat shock treatment to elicit protective response to ischemic or hypoxic injury known as cerebral "ischemic tolerance." Treatment of cardiac progenitor cells (CPCs) treated by hypoxic condition could increase CXCR4 expression and SDF-1 secretion, which enhanced cell survival and further benefited the CPC-based therapy in ischemic injury [176]. Hydrogen peroxide insult or oxygen-glucose deprivation preconditioned *h*ESC-derived neurospheres resulted in the increase of NPCs survival and differentiation, which were considered as an effective transplantation therapy [177]. Hypoxia-preconditioned senescent EPCs showed their regenerative potential, and it provided an in vitro expansion protocol for sustaining amount of functional EPCs in long-term culture [178]. Interleukin 6-preconditioned NSCs induced secretion of VEGF, which promoted angiogenesis, significantly attenuated infarct volume, and improved neurological performance [179]. Besides, pharmacological preconditioning such as isoflurane exposure 24 h before OGD significantly decreased the secretion of lactate dehydrogenase and induced the prolonged neuroprotection [180]. The precondition step could alter signaling reactions, molecular pathways, and possibly bring on genetic reprogramming at the level of neurovascular unit. These chronic adaptations suggested that sustained augmentation in resistance to ischemia had therapeutic potential [181].

21.3.3 Biomaterial-Based Strategy

Current cell therapy based on injection mainly including intravenous, intra-arterial, and intracerebral. Therapeutic cells were limited by the low survival rate; hence, the approach needed improvement to increase the efficacy of stem cells. Hyaluronan-based semisynthetic extracellular matrix (sECM) and HyStem® were available

commercial product to improve the survival rate of MSCs and iPS cells after injection [82, 182]. Transplantation of iPS-NPCs encapsulated in hydrogel to the stroke cavity of mice showed a better differentiation to neuroblast without disturbing the proliferation [82]. The study of cultured EPCs in 4% HA hydrogels provided a model system for bio-artificial stem cell niche. The encapsulated EPCs can be recruited from the niches with the aid of scaffold-dissolving enzymes mobilization to injury core for improving functional recovery after ischemic injury [183]. Fibrin blue used as biocompatible and biodegradable natural produce possesses an effective diffusion capacity; it is also a vehicle to deliver glial cell line-derived neurotrophic factors [78, 184]. The treatment of iPS cells combined with fibrin glue showed a better outcome in minimizing lesion area, functional recovery, and decreasing inflammatory events after MCAO. EPCs labeled with silica-coated super paramagnetic iron oxide nanoparticles (SiO4@SPIONs) were guided to ischemic brain hemisphere for functional recovery, which indicated that SiO4@SPION-labeled EPCs were effective in improving ischemic stroke therapy [185].

21.3.4 Stem/Progenitor Cell Combined Therapy

The mixture injection of stem/progenitor cells could increase the neovascularization after ischemic stroke. Since the CNS showed poor self-regeneration ability after injury, the co-transplantation may lead a synergistic work. Outgrowth endothelial cells (OECs) were considered as late population of EPCs. The combination of EPCs and OECs resulted in synergistic neovascularization and showed a better outcome compared to any single-cell-type transplantation after stroke [186]. A combination of EPCs and NPCs produced a significant reduction of brain damage and improvement of cognitive and motor function [187]. The co-administration of SMPCs and EPCs was demonstrated to induce the increase of angiogenesis and vascular remodeling in infarct areas and provide a mature vascular network for neuroblast survival [188]. The combination of EPCs and MSCs has already showed benefits including the formation of stable and mature blood vessels in clinical outcome [189]. As the concept of "neurovascular unit" emphasized the interaction between vascular and neural component, the co-transplantation of NPCs and VPCs provided the mutual support by each progenitor cell type and promoted the functional recovery significantly [72]. The improved outcomes represented a more effective therapeutic strategy for cell-based treatment of stroke.

21.4 Stem Cells Used in Clinical Trial

The clinical efficacy of stem cell transplantation was tested in various studies. Since the ethical issue, heterogeneity of donor cells, and unwanted immunological response limited the clinical translation, several ESC- and iPS cell-based clinical

trials have drawbacks. Despite the pluripotent cells could be produced from skin fibroblast of patients themselves, the source-dependent formation of teratomas in mouse brains was reported [190]. Meanwhile, neural precursor cells derived from these pluripotent cells showed a better outcome for stroke therapy without tumor formation [95, 191–193]. NSCs were thought to be a better choice for transplantation because of their stable expansion and robust engraftment. A retrospective study based on human umbilical cord blood mononuclear cells (HuCBCs) supported the safety of intravenous autologous HuCBCs infusion [47]. Because of easy collection, availability of autologous cell source, and lower tumorigenesis incidence, autologous bone marrow stromal cells (BMSCs) were one of the most promising cells for the clinical application [194]. Preclinical studies showed that modified bone marrow-derived mesenchymal stem cell (SB623) was associated with the increase of NSC migration and differentiation for ischemic recovery. The safety and clinical outcomes of SB623 cell transplantation were evaluated in a clinical trial, and the result showed improvement after ischemia in 12 months [195]. Data from pilot clinical trial PISCES have reported that the administration of modified human NSCs was acceptably safe and associated with neurological functional recovery [196]. Stereotactic implantation of cultured human neuronal cells into the patients' brain with stroke showed improvements in some patients but with no significant improvement in functional recovery in Phases 1 and 2 [197, 198]. Intravenously infused autologous BMSCs into chronic stroke patients for 1 year improved functional recovery, which showed that BMSC treatment was a feasible therapy [199]. Teratocarcinoma-derived Ntera2/D1 neuron-like cells (NT2N cells, also called hNT cells) showed the feasibility, safety, and tolerability after transplantation in Phase 1 and 2 stroke patients with motor infarction [200]. Although the safety and feasibility of neuron transplantation were tested, human neuronal cells implanted stereotactically into the patient's brain did not show significant benefit in motor functional recovery [198]. Intravenous administration of allogeneic MSCs from adipose tissue in patients with acute stroke was investigated for the safety and efficacy in the Phase 2 clinical trial and defined the criteria for the Phase 3 study [201]. EPCs were also considered as important participants of neovascularization in patients with acute ischemic stroke. Several clinical trials were underway to evaluate feasibility and safety of EPC-based therapy [NCT01468064, NCT01289795]. Strategies used for in vivo tracking of the grafted cells in CNS include optical imaging with high sensitivity, bioluminescence imaging by systemic injection of luciferase substrates, radiolabeling-based magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT) tracers, and positron emission tomography (PET) imaging [202–204]. Although stem cell-based therapy showed their promising perspective, many critical issues such as the selection of cell type, suitable time, and proper route to delivery as well as in vivo monitoring are all needed to be addressed for clinical application [205].

21.5 Conclusion and Perspective

Thrombolysis with tissue plasminogen activator (tPA) is the only FDA-approved treatment for ischemic stroke, but it is used in less than 5% of patients due to its narrow therapeutic time window [206]. Stem cell-based therapy showed its promising perspective and appeared to be one of the critical strategies for patients who are ineligible for tPA. Stem cells are appropriate vehicles for specific molecule delivery such as anti-inflammatory response and pro-angiogenic and pro-survival molecules [47]. Stem cells were encouraged to translate into clinical trials and the preclinical trials for stroke patients. Efficacy of these stem cells in clinical trials still needs to be improved. The optimal timing related with transient BBB leakage, route of injection, and dosage of stem cells needs to be determined [17]. Synergistic interaction of the neural and vascular system played critical roles in functional recovery due to its role of neurovascular protection [207]. Moreover, the endogenous response reveals an advanced therapeutic target for brain protection [208]. The underlying mechanism of cell therapy still needs to be discovered. With the discovery of molecule regulators such as paracrine factors, miRNAs, and microvesicles, further studies should focus on minimizing the risk and improving the benefit for the translation from preclinical to clinical trials.

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Chapter 22
Neuroprotective Strategies in Hemorrhagic Stroke

Nicolas K. Khattar, Brian J. Williams, Dale Ding, J. Marc Simard, and Robert F. James

Abstract Hemorrhagic stroke is a devastating disease that represents 10–15% of all strokes in the United States, with high rates of morbidity and mortality. Primary injury to the brain is caused by disruption of the neuronal network, while secondary injury correlates with neuroinflammation, cellular lysis, and perihematomal edema. An approach targeting primary injury involves the evacuation of the intraparenchymal hematoma, which has shown mixed results depending on the invasiveness of the approach. Various neuroprotective strategies have been employed to prevent neuronal damage. Both intraparenchymal and subarachnoid hemorrhages are associated with significant neuroinflammation. Anti-inflammatory pharmacological interventions, such as heparin and glyburide, show significant promise in decreasing the extent of the delayed neurological damage. Other strategies have focused on targeting mitochondria and the final steps of neuronal apoptosis with minocycline, which has showed significant promise in all stroke types. Hemorrhagic stroke remains a devastating disease, and more neuroprotective strategies are needed to maximize the available therapeutic interventions and their effectiveness.

Keywords Hemorrhagic stroke • Neuroprotection • Surgery • Pharmacological neuroprotection

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Abbreviations

ASTROH	Aneurysmal Subarachnoid Hemorrhage Trial Randomizing Heparin
BBB	Blood-brain barrier
CLEAR	Clot Lysis Evaluation of Accelerated Resolution of IVH
EGFR	Epidermal growth factor receptor
ENRICH	Early Minimally Invasive Removal of Intracerebral Hemorrhage
ET-1	Endothelin-1
EVD	External ventricular drain
GATA-1	Erythroid transcription factor
ICAM-1	Intercellular adhesion molecule-1
ICH	Intracerebral hemorrhage
IVH	Intraventricular hemorrhage
MAC	Membrane attack complex
MACH	Minocycline in Intracerebral Hemorrhage Patients
MISTIE	Minimally Invasive Surgery Plus rt-PA for Intracerebral Hemorrhage Evacuation
MMPs	Matrix metalloproteinases
MPO	Myeloperoxidase
rt-PA	Recombinant tissue plasminogen activator
SAH	Subarachnoid hemorrhage
SLEUTH	Stereotactic approaches associated with ultrasound-mediated lysis of the clot
STICH II	Surgical Trial in Lobar Intracerebral Hemorrhage
STICH	Surgical Trial in Intracerebral Hemorrhage
SUR1	Sulfonylurea receptor-1
TNF-α	Tumor necrosis factor-α
Trpm4	Transient receptor potential melastatin 4
UFH	Unfractionated heparin

22.1 Introduction

Despite tremendous advances in prevention and post-injury care, stroke remains the fifth leading cause of overall mortality in the United States [1]. According to the Centers for Disease Control and Prevention, in 2013, over 6.6 million individuals above the age of 20 were stroke survivors, with an annual incidence of nearly 800,000 [1]. The prevalence of stroke in the general population is estimated to be 2.6% [2]. It is currently estimated that by 2030, about ten million adults will have survived a vascular insult to the brain. Even though most strokes are ischemic in nature, hemorrhagic strokes still represent approximately 13% of all strokes [3]. The two most common subtypes of hemorrhagic stroke are intraparenchymal (10%) and subarachnoid (3%) hemorrhages [2]. Hypertension is, by far, the most

prominent cause of intracerebral hemorrhage (ICH), whereas ruptured aneurysms are the most common cause of subarachnoid hemorrhage (SAH). Less common causes of spontaneous ICH include amyloid angiopathy, vasculitis, arteriovenous malformations, arteriovenous fistulae, cavernous malformations, and hemorrhagic primary and secondary brain tumors [4–6]. In developing countries, ICH can account for up to 50% of all strokes, especially with the much higher prevalence of hypertension [4]. Anticoagulants, which have become widely used in the United States, are now associated with approximately 20% of hemorrhagic strokes [7]. Additionally, ICH locations are most commonly the basal ganglia, lobar, the cerebellum, and the pons in descending order [5]. ICH location is strongly associated with neurological outcomes (e.g., pontine hemorrhages cause significantly poorer outcomes than cortically based bleeds) [5, 8].

Many advances have been made in the realm of stroke prevention and treatment, and that have led the adjusted stroke-related mortality to significantly decrease in the past decade. However, data from various longitudinal studies shows that mortality from hemorrhagic strokes remains significantly higher than from ischemic strokes ranging from 10.5% to 46.8% [9, 10]. All-stroke mortality rates have declined over the past decade; however, the mortality rates for hemorrhagic strokes have not decreased to a similar proportion, although their incidence has decreased [10].

Prevention of neurological injury has been a top priority for neuroscience research since the turn of the century. Strategies for neuroprotection have been varied and include both pharmacological and interventional approaches. In this review, we highlight some of the most promising approaches in reducing the neurological sequelae caused by ICH-related primary or secondary injury.

22.1.1 Prevention Strategies for Primary ICH-Related Injury

Initial injury related to an expanding hemorrhage disrupts the cytoarchitecture of the surrounding parenchyma and traversing fibers [8, 11]. Elevated pressure due to increased occupancy of the cranial cavity by the hematoma can cause various sequelae, ranging from reduced blood flow and cerebral ischemia to brain herniation and death [11]. Subsequent injury also occurs from local mass effect of the hematoma against the surrounding brain, as well as delayed hemorrhage-induced toxicity. Removal of the blood products by surgical evacuation within the supratentorial space has not yet been proven to be successful in improving the rate of mortality or morbidity compared to medical management [12]. The management of intracerebral hematomas remains controversial, and the benefit of surgical evacuation has not been established [11]. The patients enrolled in Surgical Trial in Intracerebral Hemorrhage (STICH) and Surgical Trial in Lobar Intracerebral Hemorrhage (STICH II) underwent early craniotomy for evacuation of supratentorial hematomas but did not show any overall survival benefit possibly due to additional injury caused by the surgical procedure [8, 11]. Secondary brain injury has

been associated with the interaction of cytotoxicity, excitotoxicity, oxidative stress, and inflammation caused by red blood cell lysis, as well as release of hemoglobin and free radicals [12, 13]. Delayed evacuation of the hematoma could be beneficial to remove hemoglobin breakdown products, but no trial has not been able to prove that this improves patient outcomes.

Given the paucity of data showing a benefit from conventional craniotomy for ICH evacuation, minimally invasive surgery has been considered by a number of researchers. Auer et al. reported endoscopic evacuation of supratentorial intraparenchymal hematomas and showed significantly decreased mortality at the 6-month follow-up [14]. Wang et al. subsequently showed that endoscopic evacuation of intraparenchymal hematomas had significantly improved neurological outcomes at 14 days, lower mortality at 3 months, and better overall outcomes [15]. More recently, the Minimally Invasive Surgery Plus rt-PA for Intracerebral Hemorrhage Evacuation (MISTIE) II trial showed that minimally invasive clot removal significantly decreased perihematomal edema, with a trend toward improved outcomes [16, 17]. Minimally invasive evacuation of intraparenchymal hematomas associated with injection of recombinant tissue plasminogen activator (rt-PA) is associated with a significant mortality benefit, affirming the prior assumptions that stress from craniotomies may be detrimental to survival and overall outcomes [17]. Other studies have explored the use of focused ultrasound for clot lysis (SLEUTH trial) and showed comparable results to the MISTIE trial [18]. MISTIE III (NCT01827046) is a phase III, international multi-center randomized controlled trial with a recruitment goal of 500 patients that is seeking to show that greater ICH evacuation leads to decreased brain injury and improved overall neurological outcomes. Early Minimally Invasive Removal of Intracerebral Hemorrhage (ICH) (ENRICH; NCT02880878) is another phase III trial that is comparing minimally invasive parafascicular surgery using the BrainPath endoport system (NICO, Indianapolis, IN, USA) for clot evacuation within 24 h of symptom onset to best medical management.

Intraventricular hemorrhage (IVH) occurs in around 40% of patients with ICH and is a significant negative predictor of neurological outcomes [19, 20]. The Clot Lysis Evaluation of Accelerated Resolution of IVH (CLEAR) trials have studied the use of rt-PA injection into an external ventricular drain (EVD) to promote dissolution of isolated IVHs; these studies have shown minimal improvements in mortality and overall outcomes [21, 22]. No study has definitively proven the efficacy of IVH evacuation in patients with ICH.

22.1.2 Prevention Strategies for Secondary ICH-Related Injury

Secondary injury in both SAH and ICH is due to various cellular and molecular processes. Neuronal injury after hemorrhagic stroke is a multifactorial process that involves activation of pro-inflammatory, hemolytic and procoagulant cascade

pathways. All of these processes disrupt the blood-brain barrier (BBB), promoting cerebral edema, exacerbating primary brain injury, and eventually leading to neuronal cell death. These effects do not occur immediately but rather develop hours to days following the ictus of ICH.

Mitochondria are the final effectors in the proapoptotic pathways [23]. Damage beyond a cell's capacity to repair usually starts a mitochondrial chain reaction that terminates in programed cell death (i.e., apoptosis) [24]. In perihematomas brain areas, significant hypoxia generally causes irreversible damage and leads to an expanding injury pattern. Tumor necrosis factor-α (TNF-α) initiates the signaling cascade of chain of proapoptotic caspases in the perihematomal brain, thereby causing disrupting various mechanisms necessary for cell survival [24, 25].

Neuro-inflammation has been negatively associated with overall outcomes following both SAH and ICH [26]. Animal studies have shown that neutrophil infiltration and microglial activation follow any intraparenchymal hemorrhage [27, 28]. In animal studies, microglia, which are already embedded in brain tissue, are generally the first cells to infiltrate the clot; their activation is seen as early as 1 h following the injury, peaks at 3–7 days, and persists for 4–5 weeks [29]. Microglia can secrete pro-inflammatory cytokines, chemokines, prostaglandins, proteases, and heme oxygenase I [30, 31]. These secreted molecules have been involved in both clot clearance and induction of further inflammation-mediated damage to the perihematomal brain [29]. Myeloperoxidase (MPO) and intercellular adhesion molecule-1 (ICAM-1)-positive cells, as well as microglia, have been identified in both the blood vessels surrounding an ICH and within the hematoma itself. Moreover, inhibition of microglial activation with tuftsin fragments resulted in decreased secondary brain injury in rat studies [32, 33].

Neutrophils are the first peripheral leukocytes to penetrate the hematoma, due to the disruption in the blood-brain barrier [34]. In rats, the neutrophil infiltration has also been associated with microglial activation [27]. Neutrophils secrete pro-inflammatory proteases and reactive oxygen species, allowing further disturbance of the BBB and exacerbating associated neuronal injury. In addition to their functional roles, leukocytes undergo apoptosis around 48 h after clot infiltration and, in the process, release pro-inflammatory, peroxisomal, and mitochondrial molecules which further promote neuroinflammation [34, 35]. In animal models, upregulation of all inflammatory markers has been observed in cases of hemorrhagic stroke, including but not limited to cytokines, TNF- α, interleukins, and matrix metalloproteinases (MMPs) 3 and 9 [36–38]. MMPs have been associated with various secondary brain injuries, but their role has not been established in a specific injury cascade [39].

Complement is tightly correlated with various immune reactions, including cell lysis and the inflammatory response [40]. Although excluded from the brain, the complement cascade is activated when blood infiltrates the brain parenchyma or the subarachnoid space [41]. Complement activation can allow for the modulation of cellular functions, such as the release of cytokines, reactive oxygen species, and matrix proteins [42]. These changes have been associated with perihematomal edema. The membrane attack complex (MAC) is significantly associated with

erythrolysis, leading to the release of both iron and hemoglobin, which causes further damage to the surrounding tissue. Mice deficient in the complement proteins have been found to have significantly decreased brain edema following induced hemorrhagic brain injury [43]. Multiple studies have shown that inhibition of certain elements of the complement system reduce hemorrhagic stroke-related injury [44, 45]. However, the long-term effects of complement inhibition in the brain remain unclear [44]. In rats, N-acetylheparin, a congener of heparin which lacks anticoagulant properties, has been shown to inhibit the complement cascade and decrease perihematomal edema, thereby preventing secondary neurologic injury [41].

Unfractionated heparin (UFH) is a commonly used anticoagulant for the prevention and treatment of deep venous thrombosis, pulmonary embolus, and other hypercoagulable conditions, with a good safety profile. UFH has also been shown to have neuroprotective properties in humans, especially in those with hemorrhagic strokes [46, 47]. UFH is a highly sulfated glycosaminoglycan polymer with the most negative charge of any biological molecule [48]. Inflammatory mediators generally are positively charged, and binding to heparin disturbs the biochemical and electrostatic microenvironment, effectively neutralizing these mediators [49]. Particularly in hemorrhagic stroke, heparin is able to bind to oxyhemoglobin and neutralize the toxic effects of free hemoglobin on the brain [50]. Additionally, heparin was found to decrease the expression of endothelin-1 (ET-1) mRNA, as well as the ET-1 promoter. Heparin also decreased expression of the erythroid transcription factor family (GATA-1), which is essential for ET-1 function in the endothelial cells [51, 52]. The potent vasoconstrictive effects of ET-1 are mediated in vascular smooth muscle cells through the epidermal growth factor receptor (EGFR) [53]. Heparin-binding epidermal growth factor, a ligand of EGFR, modulates its transactivation [53]. Binding of heparin to the ligand prevents EGFR receptor activation, thereby dampening any hemorrhage-induced vasoconstriction and further cerebral injury [53, 54].

Various studies have validated heparin's neuroprotective effects in hemorrhagic stroke. Animal studies of SAH administration of heparin significantly decreased the expression of cleaved caspase 3. The downregulation of apoptotic effectors reduces neuroinflammation, demyelination, and the overall burden of injury [55]. Human studies have investigated the role of enoxaparin (low-molecular-weight fractionated heparin) in aneurysmal SAH with mixed results [56, 57]. There was a statistically significant reduction in delayed ischemia and vasospasm in the enoxaparin group in one trial but no obvious benefit in another [56, 57]. A retrospective cohort study showed significant benefits of UFH in Fisher grade 3 aneurysmal SAH patients [47]. The patients in the heparin group had significantly less clinical and radiographic vasospasm, as well as a reduction in vasospasm-related infarction. Additionally, there were a significantly higher proportion of patients who were discharged home from the hospital, rather than being discharged to a rehabilitation facility [47]. Heparin has shown a lot of promise with regard to gross neurological outcomes, and it has also been associated with improved cognitive outcomes in SAH patients. Currently, the Aneurysmal Subarachnoid Hemorrhage Trial

Randomizing Heparin (ASTROH), a phase II multi-center randomized controlled trial, is studying the effects of low-dose intravenous heparin infusion on 90-day cognitive outcomes using the Montreal Cognitive Assessment. Enrollment is expected to be complete in December 2018 (NCT02501434).

The University of Maryland has shown, in animal studies, that the sulfonylurea receptor-1 (SUR1) membrane protein is significantly involved in cerebral edema following stroke [58]. This membrane protein binds pore-forming subunits in neuronal membranes and endothelial cells that make up the BBB to form ion channels [58]. In hemorrhagic strokes, SUR1 was demonstrated to bind to the transient receptor potential melastatin 4 (Trpm4) to form a Na^+ ion channel [59]. This SUR1-Trpm4 channel was found to be significantly overexpressed in human and animal brains after hemorrhagic stroke [59]. Na^+ ionic influxes cause Cl^- and H_2O movement as well, leading to neuronal edema and cytotoxic cell death. It was discovered that glyburide, a widely used antidiabetic drug, blocked the SUR1-Trpm4 channel, resulting in decreased neuronal cell death [60, 61]. Animal studies demonstrated that overexpression of the SUR1-Trpm4 channel was due to an upregulation of pro-inflammatory signaling (e.g., TNF-α and NF-κb) in the penumbra of hemorrhagic strokes [62, 63]. Glyburide decreased the overall inflammatory burden and prevented BBB breakdown, thereby protecting neurons from the sequelae of hemorrhagic stroke [62]. The Glyburide Advantage in Malignant Edema and Stroke (GAMES-RP) trial established the safety of glyburide in patients with large hemispheric ischemic strokes and showed the potential for intravenous glyburide (RP-1127; glibenclamide) to reduce inflammation and prevent neuronal damage [64]. Further study is needed in the form of a phase III trial to assess the efficacy of glyburide in both ischemic and hemorrhagic strokes.

All three basic mechanisms of cellular injury in the brain converge to mitochondrial dysfunction and neuronal apoptosis. Minocycline, a commonly used tetracycline antibiotic, has been shown to have significant neuroprotective effects by blocking the release of cytochrome c from mitochondria [65]. Cytochrome c release is one of the most delayed steps to initiating the proapoptotic signaling cascade. Minocycline has been found to have a neuroprotective effect on the penumbra of ischemic and hemorrhagic strokes [66]. Given its widespread availability and its excellent oral bioavailability, minocycline it is an attractive potential pharmacologic therapy for ameliorating or preventing neurological decline after ICH.

Recent studies of mice injected with autologous blood into the basal ganglia and treated with minocycline showed a significant decrease in all proapoptotic markers, especially caspase 3/8 and LC3B [67]. This effect is not limited to ICHs but also extends to SAH, where multiple reports have found minocycline to be a potent neuroprotective agent [68, 69]. Currently, minocycline is being investigated as a neuroprotective agent for hemorrhagic stroke in the Minocycline in Intracerebral Hemorrhage Patients (MACH) pilot study (NCT01805895). In addition to its role in the prevention of mitochondria-initiated neuronal apoptosis, minocycline has been shown in animal studies to be an inhibitor of microglial activation, as well as a general iron chelator [69].

22.2 Conclusion

Hemorrhagic stroke is a heterogeneous disease that continues to present significant challenges to physicians. Primary and secondary brain injuries caused by spontaneous ICH have different mechanisms of action with regard to neuronal damage. There are many different pharmacological and surgical approaches that have been employed in ICH patients with varying degrees of success, but a definitive therapy has yet to be established. Further research into novel neuroprotective strategies for hemorrhagic stroke needs to be conducted in order to find an intervention, medical therapy, or standardized management approach which is applicable to a broader patient population.

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Chapter 23
Improving Stroke Rehabilitation with Vagus Nerve Stimulation

Seth A. Hays

Abstract Stroke is a leading cause of neurological damage, with an estimated 795,000 cases reported in the United States each year. A large percentage of patients who suffer a stroke exhibit long-term impairments in motor function. Poststroke rehabilitation in part aims to promote adaptive changes in neural circuits to support recovery of function, but insufficient or maladaptive plasticity often limits benefits. Adjunctive strategies that support plasticity in conjunction with rehabilitation represent a potential means to improve recovery after stroke. Vagus nerve stimulation (VNS) has emerged as one such targeted plasticity strategy, providing phasic activation of neuromodulatory nuclei associated with plasticity. Repeatedly pairing brief bursts of VNS with motor training drives robust, specific plasticity in neural circuits. A number of studies in animal models of stroke and neurological injury demonstrate that VNS paired with rehabilitative training improves recovery of motor function. Moreover, emerging evidence from clinical trials indicates that VNS delivered during rehabilitation promotes functional recovery in stroke patients. Here, we provide a discussion of the existing literature of VNS-based targeted plasticity therapies in the context of stroke and outline challenges for clinical implementation.

Keywords Vagus nerve stimulation • Vagal nerve stimulation • Rehabilitation • Stroke • Neuroplasticity

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Abbreviations

BDNF	Brain-derived neurotrophic factor
DALY	Disability-adjusted life years
ET-1	Endothelin-1
ICH	Intracerebral hemorrhage
NMDAR	NMDA receptors
STDP	Spike-timing-dependent plasticity
TBI	Traumatic brain injury
VNS	Vagus nerve stimulation

23.1 Targeting Plasticity with Vagus Nerve Stimulation

Stroke is a leading cause of acquired disability, affecting 795,000 people in the United States each year [1]. The majority of cases lead to lasting impairments in upper limb function [2]. Physical rehabilitation is the most common poststroke and yields functional gains in some patients, but recovery is often incomplete, and many patients are left with chronic disability [2–4]. Networks of neurons in surviving peri-infarct regions, as well as the undamaged contralateral homotopic cortex and subcortical structures, demonstrate significant reorganization after stroke [5–8]. It is widely held that this plasticity in spared networks after stroke contributes to recovery of function [9]. As a result, a number of strategies have been developed to facilitate plasticity in these networks to potentially increase functional recovery. Recently, a novel implementation of vagus nerve stimulation (VNS) has emerged as a method to engage pro-plasticity neuromodulatory circuits during rehabilitation to improve recovery of motor function after stroke.

Vagus nerve stimulation is most widely recognized for its use as a means to reduce seizures and has been employed in over 80,000 epilepsy patients [10]. Implementation of VNS for epilepsy employs an open-loop stimulation scheme, in which patients typically receive 30 s trains of stimulation every 5 min [11]. A distinct closed-loop VNS strategy has been developed, referred to as targeted plasticity therapy, that uses short 0.5 s trains of VNS triggered to coincide with specific events during a training paradigm to direct plasticity specific to the paired event [12]. Targeted plasticity therapy is based on the premise that VNS engenders phasic release of neuromodulators, including acetylcholine and norepinephrine, that support plasticity [12–15]. These neuromodulators facilitate spike-timing-dependent plasticity in neural circuits concurrently activated by training [16, 17]. As a result, VNS facilitates robust, temporally precise enhancement of adaptive plasticity during rehabilitative training and improves recovery in a variety of models of neurological injury. Below, we outline studies examining VNS-based targeted plasticity therapy and discuss the future of this potentially transformative platform neuromodulation therapy to impact the treatment of stroke.

Interest in the vagus nerve as a means to facilitate neural plasticity stems from early studies that implicated the nerve in enhanced consolidation of memory [12]. Administration of a variety of chemical stimuli that do not cross the blood-brain barrier was found to enhance post-training memory retention in rats [18–23]. Vagotomy prevented this memory enhancement, suggesting that signaling via the vagus nerve may represent a peripheral means to alter circuits in the brain. In a series of seminal experiments, Clark and colleagues demonstrated that electrical stimulation of the vagus nerve immediately following training similarly enhanced memory in rats and humans, providing a direct link between activity in the vagus nerve and modulation of central nervous system function [24, 25]. Neural plasticity is the substrate for memory; thus, the enhancement of memory by VNS must be subserved by concurrent changes in neural circuits. Pioneering studies from Michael Kilgard and colleagues demonstrated that VNS paired with exposure to auditory stimuli drives robust, stimulus-specific plasticity in the auditory cortex of rats [26–29]. A matched amount of VNS delayed just 15 s after the presentation of the auditory stimulus failed to generate plasticity, supporting the notion that VNS drives precise temporal engagement of neuromodulatory systems to reinforce experience-dependent neural plasticity [26]. A subsequent study extended these findings to show that VNS paired with forelimb motor training drives robust, training-specific plasticity in motor cortex [30]. This enhancement of plasticity provided the basis for the development of VNS paired with rehabilitation as a targeted plasticity therapy.

23.2 Preclinical Studies on Targeted Plasticity Therapy

The ability of VNS to drive neuroplasticity in uninjured motor networks led to the prediction that VNS could potentially be used to enhance plasticity in conjunction with rehabilitation and support recovery of motor function after stroke. Khodaparast and colleagues tested this hypothesis using a rat model of ischemic stroke [31]. In an initial study, rats were trained on the bradykinesia assessment task, a quantitative measure of forelimb movement speed [32]. Once proficient, rats received injections of endothelin-1 (ET-1) into multiple locations in the motor cortex to engender an infarct that impaired function of the trained limb. One week after the induction of cortical ischemia, rats were assigned to receive rehabilitative training with or without the delivery of VNS on successful trials. In order to target plasticity to the trained movement, a short burst of VNS was temporally paired with forelimb movement on successful trials. This closed-loop training paradigm is identical to that used to enhance plasticity in motor cortex in uninjured animals [14, 30]. VNS was delivered during rehabilitative sessions over the course of 5 weeks. VNS paired with rehabilitative training significantly improved recovery of forelimb function compared to rehabilitative training without VNS. By the second week of therapy, performance of subjects that received VNS paired with rehabilitative training was indistinguishable from pre-lesion levels. This study provided the initial proof-of-concept demonstration that VNS paired with rehabilitative training may improve recovery after stroke.

A second study evaluated the ability of VNS paired with rehabilitation to improve recovery of forelimb strength, a main contributor to disability after stroke [33–35]. VNS paired with rehabilitative training significantly enhanced recovery of volitional forelimb strength compared to rehabilitative training without VNS [33]. The improved forelimb strength was observed for 1 week after the cessation of VNS, potentially indicating a long-term improvement. These lasting improvements in forelimb function are consistent with the notion that VNS facilitates plasticity to support recovery, rather than a short-term effect of VNS on excitability. All subjects that received VNS paired with rehabilitative training demonstrated a full restoration of forelimb strength to pre-lesion levels, while only 22 % of subjects that received rehabilitative training without VNS demonstrated a full recovery. Consistent with the previous study [31], no reduction in infarct size was observed in animals that received VNS, suggesting that VNS is not conferring a neuroprotective effect but rather improving recovery by enhancing plasticity. A follow-up study provided further evidence that VNS acts to improve stroke recovery by enhancing plasticity [36]. Using a similar design, the authors evaluated different stimulation paradigms of VNS on recovery of forelimb strength after stroke. A matched amount of VNS that is not paired with rehabilitative training is less effective in restoring function than VNS that is paired with rehabilitative training, suggesting that nonspecific effects of stimulation are unlikely to underlie VNS-dependent enhancement recovery. Moreover, delivery of sixfold more stimulation that was not precisely paired with forelimb movement during training resulted in significantly less recovery than VNS paired with rehabilitation. These findings highlight the importance of optimizing both timing and dose of stimulation for VNS therapy and indicate that closed-loop pairing of VNS with rehabilitative exercises is substantially more effective than open-loop delivery.

While these studies provide an initial proof-of-concept evaluation of targeted plasticity therapy, the animal models used in these experiments fail to incorporate several complicating factors observed in the clinical population of stroke patients, including advanced age and a long delay before onset of therapy. Advanced age is the leading non-modifiable risk factor for ischemic stroke [1]. Moreover, advanced age is associated with a reduction in neuroplasticity and worse poststroke outcomes; therefore, age-related decline in plasticity may reduce or block VNS-dependent enhancement of recovery [37–39]. Given the importance of advanced age in the context of stroke, a study evaluated whether VNS paired with rehabilitative training could improve motor recovery after an ischemic lesion to the motor cortex in rats aged at least 18 months [40]. VNS paired with rehabilitative training resulted in significantly greater recovery of forelimb function compared to equivalent rehabilitative training without VNS in rats aged at least 18 months. Moreover, the degree of forelimb recovery after stroke in aged rats receiving VNS paired with rehabilitative training was comparable to that observed in young rats with the same intervention [33]. These findings are consistent with studies suggesting that age alone is not a determinant in the benefits of rehabilitative therapies [41]. Corroborating previous findings, VNS did not reduce lesion size, suggesting that a neuroprotective effect cannot account for the improved recovery of function. No animal model captures

the complexity of advanced age in humans; however, this study provides preliminary evidence that advanced age does not preclude the effects of VNS therapy after stroke.

Rehabilitative strategies have the highest potential to improve functional recovery when delivered early after stroke, and efficacy diminishes substantially with increasing time after stroke [42–45]. Moreover, an estimated 7.2 million patients in the United States are currently living with chronic poststroke disability, highlighting the need for interventions that are effective long after initial insult [1]. A study conducted by Khodaparast and colleagues sought to evaluate whether VNS paired with rehabilitative training would improve recovery in a model of chronic stroke [46]. When initiated on the seventh week after stroke in rats with stable, chronic deficits, VNS paired with rehabilitative training significantly enhanced recovery of forelimb function compared to rehabilitative training without VNS. The magnitude of recovery after chronic stroke appears similar to that observed after acute stroke, potentially suggesting that the efficacy of VNS paired with rehabilitative training does not substantially decline with time after stroke [31, 33, 36]. A matched amount of VNS that was not paired with rehabilitative training failed to improve recovery, highlighting the requirement for temporal association between rehabilitation and VNS and providing further evidence that VNS-dependent plasticity underlies recovery [36]. The ability of VNS paired with rehabilitative training to enhance recovery weeks after stroke indicates that VNS does not act by facilitating the action of pro-plasticity factors upregulated by stroke. Rather, consistent with the ability to drive plasticity in uninjured animals, VNS likely acts to improve stroke recovery by driving repeated, consistent activation of plasticity-enhancing neuromodulatory circuits to reinforce neural activity in rehabilitated neural circuits during training [12, 14, 30].

In addition to ischemic stroke, preclinical studies provide evidence that VNS paired with rehabilitation may improve recovery in a mechanistically distinct form of cerebrovascular injury. Intracerebral hemorrhage (ICH) is a devastating subtype of stroke characterized by bleeding in the brain parenchyma, resulting in a number of pathological sequelae that often damage cortical and subcortical structures [47, 48]. Although less prevalent, the disability-adjusted life years (DALY) lost to ICH are nearly double to that lost to ischemic stroke, highlighting the significant burden of this disease [49]. Like ischemic stroke, plasticity within spared circuitry is believed to support recovery after ICH [50–52]. Based on the notion that plasticity subserves recovery after ICH, a study evaluated whether VNS paired with rehabilitative training could improve recovery of forelimb function in a rat model of ICH [53]. VNS paired with rehabilitative training resulted in significantly improved forelimb function compared to rehabilitative training without VNS. However, unlike the complete recovery observed in models of ischemic stroke, VNS therapy after ICH failed to provide a complete restoration of forelimb function to pre-lesion levels [31, 33, 36]. In an attempt to further improve recovery, an additional cohort of rats received an alternative VNS paradigm utilizing approximately 40 % more stimulations paired with rehabilitative training. The additional VNS improved recovery compared to rehabilitative training alone, but did not result in greater improvements compared to the original VNS paradigm. Mechanistic studies evaluating the VNS-

dependent changes in motor networks after ICH may provide insight into whether the severity of the ICH lesion limits recovery or whether further optimization of stimulation parameters or rehabilitative regimen may promote greater recovery. Regardless, these findings represent a proof-of-concept demonstration that VNS paired with rehabilitative training may improve recovery after ICH.

Emerging evidence extends the efficacy of VNS paired with rehabilitative training beyond models of cerebrovascular injury. A recent study evaluated whether VNS paired with rehabilitative training could improve recovery of motor function in a controlled cortical impact model of severe traumatic brain injury (TBI) [54]. VNS paired with rehabilitative training significantly improved recovery of volitional forelimb strength compared to rehabilitative training without VNS after TBI. Enhanced recovery with VNS was observed without any reduction in lesion size, suggesting that VNS does not improve motor recovery through gross neuroprotection [31, 33, 53]. The flexibility to improve recovery of motor function in a range of models of brain damage opens the possibility for similar implementations of VNS to improve recovery in other neuromotor disorders. Plasticity in the central nervous system is believed to contribute to recovery after spinal cord injury and peripheral nerve injury; therefore VNS may represent a potential adjunctive strategy to enhance rehabilitation of motor function in these disorders.

23.3 Clinical Studies on Targeted Plasticity Therapy

Based on the preclinical evidence of VNS therapy in the context of stroke, Dawson and colleagues performed a first-in-human pilot study to evaluate VNS paired with physical rehabilitation in chronic stroke patients [55, 56]. The study included patients with moderate to severe arm weakness resulting from a stroke at least 6 months prior. Twenty patients were randomized to receive either rehabilitation alone ($n = 11$) or equivalent rehabilitation paired with VNS ($n = 9$) over the course of 6 weeks. VNS was delivered comparable to the closed-loop stimulation pairing paradigm used in the preclinical studies. The rehabilitative therapist pressed a button to trigger VNS coincident with upper limb movement during several typical rehabilitative exercises. As expected, no safety concerns were noted. A number of measures of upper limb function were also evaluated to assess efficacy of targeted plasticity therapy compared to standard rehabilitation. In the per protocol analysis, patients that received VNS paired with rehabilitation demonstrated a 9.6 point improvement in upper-extremity Fugl-Meyer (UEFM) score, significantly greater than the 3.0 point improvement observed in the rehabilitation-only group. Of the patients that received VNS paired with rehabilitation, 67 % exhibited a clinically meaningful improvement in UEFM scores (defined as an increase >6), compared to 36 % of patients that received rehabilitation only. Interestingly, corticospinal tract integrity and infarct volume were worse in VNS responders than rehabilitation-only responders, potentially indicating that VNS may yield benefits in patients that would typically be unlikely to respond to rehabilitation [57]. Together, the findings from

this study demonstrate that delivery of VNS paired with rehabilitation is safe and feasible in stroke patients and point to efficacy in enhancing recovery of upper-limb motor function.

An ongoing randomized, placebo-controlled, double-blind follow-up study provides additional evidence supporting the potential for VNS in stroke patients [58]. Preliminary results document a significant increase in UEFM score in patients that received VNS paired with rehabilitative training (+9.5) compared to patients that received rehabilitation alone (+3.8). The magnitude of increase in UEFM scores confirms the efficacy outcomes observed in the first-in-human study and provides further evidence that VNS paired with rehabilitation represents a potential strategy to enhance recovery of function after stroke. While these findings are encouraging, additional clinical evaluation in a large pivot trial is needed to definitively assess efficacy.

23.4 Considerations for Clinical Translation of Targeted Plasticity Therapy

A critical step in the translation of VNS-based targeted plasticity therapy for stroke is the establishment of stimulation parameters that yield maximal recovery. Targeted plasticity therapy utilizes stimulation of the left cervical branch of the vagus nerve via an implanted cuff electrode. At the cervical level, the vagus nerve is comprised of myelinated A and B fibers, as well as unmyelinated C fibers [59, 60]. There is considerably diversity in anatomical, morphological, and functional properties of the nerve from individual to individual [61–64]. The optimal fiber population needed to maximize VNS efficacy is largely untested. Based on the range of effective stimulation intensities, VNS-dependent engagement of neuromodulatory circuits and enhancement of plasticity are likely mediated by activation of low-threshold A-fibers [13, 14, 30, 31, 33, 36, 40, 46, 53, 54]. Higher VNS intensities recruit more vagal fibers and trigger greater activation of neuromodulatory nuclei, which may improve stroke recovery [13, 65–67]. However, studies examining VNS-dependent enhancement of memory retention and neural plasticity demonstrate that moderate intensity stimulation yields significant effects compared to lower- and higher-intensity stimulation [24, 25, 68]. The precise mechanisms that underlie this inverted-U response profile are not understood, but several explanations, including VNS-dependent activation of opposing neuromodulatory pathways or receptor desensitization, could account for the response [12]. Regardless of the underlying mechanism, the inverted-U response to VNS poses challenges for clinical implementation, as more stimulation does not necessarily equate to better efficacy. Moreover, natural variability in vagus anatomy and pharmacotherapies or comorbid conditions that perturb neuromodulatory pathways may influence the optimal parameters for individual patients. The fact that VNS enhances plasticity across a twofold range of intensities suggests that a potentially wide therapeutic range exists

[29]. Additionally, the clinical studies evaluating VNS therapy in stroke patients discussed above utilize a single set of parameters and observed significant recovery in most patients, supporting the notion that individual variability in response to VNS is unlikely to preclude benefits. However, it would be valuable to identify a biomarker that reports VNS-dependent activation to allow individual tailoring of stimulation levels for each patient. Additional studies to directly characterize the effects of varying stimulation parameters, including intensity, frequency, and pulse width, may be useful in defining parameters that maximize plasticity and recovery after stroke.

In addition to defining optimal stimulation parameters, one practical consideration related to the translation of targeted plasticity therapy is the importance of determining the therapy regimens to maximize recovery in a fixed amount of time. In a fixed amount of time with a therapist, a patient could either receive many VNS pairings on a limited number of rehabilitative exercises or alternatively a few pairings on a wider range of exercises. Insight into the appropriate trade-off between exercises can be gained by evaluating the generalization of VNS-dependent benefits from trained movements to similar untrained movements. In the initial clinical studies evaluating VNS therapy, patients that received VNS exhibited improvements in upper-extremity Fugl-Meyer scores despite not receiving VNS explicitly during Fugl-Meyer tasks. This provides preliminary evidence that the benefits of VNS paired with rehabilitation may generalize to similar upper-limb movements. However, considerably more detailed investigation of paradigms to maximize recovery in minimal rehabilitation time is warranted in order to increase the utility of target plasticity therapy for stroke patients.

The mechanisms that subserve the benefits of the targeted plasticity therapy after stroke are unknown, but VNS engages a variety of molecular and neuronal mechanisms that may enhance plasticity. In particular, VNS increases levels of brain-derived neurotrophic factor (BDNF), a key regulator of plasticity implicated in stroke recovery [69]. After stroke, treatment with BDNF increases functional recovery, whereas reduction of BDNF levels prevents the benefits of rehabilitative training [70, 71]. Similarly, VNS may increase BDNF levels during rehabilitation to facilitate recovery. In addition to influencing molecular signaling pathways, VNS induces changes in synaptic and intrinsic neuronal properties that may support recovery. VNS drives activation of the noradrenergic and cholinergic neuromodulatory systems, which act synergistically to alter spike-timing-dependent plasticity (STDP) properties in active networks [13, 15, 16, 72]. Indeed, the requirement of temporal association within 15 s between VNS and the paired stimulus to drive stimulus-specific plasticity aligns well with recent evidence indicating the time scale of neuromodulator action on STDP [17, 26]. VNS may also directly alter synaptic strength by regulating expression of NMDA receptors (NMDAR) [73]. Additionally, VNS-dependent modulation of CaMKII phosphorylation and Arc expression, both known to regulate spine turnover, may influence synaptic remodeling in motor networks with rehabilitation after stroke [74]. A clear delineation of the mechanisms engaged by VNS to promote adaptive neural plasticity in conjunction with rehabilitation may facilitate development of better therapeutic strategies.

Finally, in addition to the use of VNS for targeted plasticity therapy, a number of recent studies have demonstrated a neuroprotective effect of VNS when delivered shortly after induction of ischemia [75–77]. The differences in temporal requirements for neuroprotection, which is most effective within 30 min of ischemia onset, and for targeted plasticity therapy, which can be initiated months to years after injury but must be paired with rehabilitation to be effective, suggest that the therapeutic mechanisms engaged by VNS in each context are different. The complementary actions of each strategy may both be beneficial in the context of stroke, such that early VNS could be employed to limit progression of stroke damage and subsequent delivery of VNS paired with rehabilitation could be used to facilitate functional recovery.

23.5 Conclusions

Targeted plasticity therapy using VNS paired with rehabilitation has emerged as a potential strategy to enhance recovery of function after stroke. Future studies should focus on expanding the clinical evaluation of VNS therapy in a broad population of stroke patients. Moreover, efforts should be directed toward developing targeted plasticity therapies to treat other common poststroke deficits, such as delivering VNS during tactile stimulation for somatosensory impairments or pairing VNS with speech therapy for aphasia. Finally, optimizing delivery of the therapy and elucidating the neural mechanisms that underlie the benefits of targeted plasticity therapy would be valuable to facilitate clinical translation.

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Chapter 24
Post-stroke Motor Rehabilitation

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Abstract Despite great improvement in acute stroke management, a large number of stroke patients remain significantly impaired, which is a leading cause of disability in the world and a serious global health-care problem. Effective neurorehabilitation is critical in reducing disability after stroke. The multidisciplinary approach of incorporating expertise from physical therapy, occupational therapy, and speech therapy and cognitive rehabilitation is a routine application in clinical settings. Several large trials of rehabilitation practice and of novel therapies including virtual reality, stem cell therapy, and drug augmentation are in progress to explore the possibility for the future practice. In addition, there are numerous assistive devices available to stroke patients that can help them adjust to their new poststroke lifestyle. Here, we discussed potentially options of motor rehabilitation, including noninvasive brain stimulation, rehabilitation robotics, and other promising rehabilitation techniques, after stroke in clinic.

Keywords Outcome measures • Recovery • Rehabilitation • Robotics • Stroke • Therapeutic

Abbreviations

AHA	American Heart Association
ASA	American Stroke Association
BDNF	Brain-derived neurotrophic factor
CST	Corticospinal tract

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CT	Computerized tomography
DTI	Diffusion tensor imaging
EEG	Electroencephalograph
EMG	Electromyogram
fMRI	Functional magnetic resonance imaging
MEP	Motor evoked potentials
MRI	Magnetic resonance imaging
OT	Occupational therapy
PT	Physical therapy
tDCS	Transcranial direct current stimulation
TMS	Transcranial magnetic stimulation
UBS	Ultrasonic brain stimulation
VR	Virtual reality

24.1 Introduction

24.1.1 Importance of Stroke Rehabilitation

Stroke is the leading cause of adult disability in the world. Seventy to 80% of the stroke survivors show different levels of neurological impairments. To date, there are about 33 million stroke survivors worldwide. By 2030, there will be 77 million people living with stroke consequences [1]. As the most populous country, China has more than three million new stroke patients at a rate of approximately 9% rise each year, and more than ten million stroke survivors so far, based on the information from Chinese Center for Disease Control and Prevention. According to statistics, the annual economic loss of stroke in China is more than $10 billion.

The most common deficit after stroke is hemiparesis. More than 80% of stroke patients experience upper limb paresis [2]; 42–67%of patients suffer from dysphagia within 3 days after stroke [3]; 23–36%of patients are left with language impairments [4]; cognition impairments occur in 25–65% of survivors. Up to 33% of patients have depression, while more than 50% of survivors show apathy at 1 year after stroke [5].

Besides, more than 30% of stroke survivors manifest participation restrictions by 4 years after onset of stroke. Therefore, a stroke not only affects stroke victims themselves, a lot, but also affects their caregiver, who may develop emotional problems. Interestingly, clinical practices have documented that 80% of stroke survivors are able to regain walking ability, 30–40% of patients with upper limb paresis regain some dexterity after 6 months, 90% of them recover life self-care ability, and 20–66% survivors may return to their work after effective rehabilitation.

24.1.2 Guidelines for Adult Stroke Rehabilitation and Recovery

Several guidelines of rehabilitation were applied by the United States, Canada, Europe, Japan, etc. in which some are specifically for the recovery of ischemic stroke. Rehabilitation guidelines are an important educational tool for neurologists and rehabilitation physicians, since without communication and coordination, isolated efforts to rehabilitate the stroke survivor are unlikely to achieve their full potential. Currently, the American Heart Association (AHA) and the American Stroke Association (ASA) have established "guidelines for adult stroke rehabilitation and recovery." The guidelines emphasized that the stroke rehabilitation requires a sustained and coordinated effort from a large team, which includes the patients, family and friends, other caregivers, physicians, nurses, physical and occupational therapists, speech-language pathologists, recreation therapists, psychologists, nutritionists, social workers, and others. Communication and coordination in the team members are dominant in maximizing the efficiency of rehabilitation.

Importantly, early rehabilitation is greatly beneficial to stroke outcomes. Early mobilization became more prominent in the early 1990s, as clinical trial results showing marked reduction in death and disability in patients managed with early rehabilitation and mobilization when compared with general medical ward care [6]. It is recommended that medically stable patients could receive rehabilitation at 24–72 h after stroke onset as soon as possible [7]. The patients could receive a minimum of 3 h of direct task-specific therapy, 5 days a week, delivered by the professional stroke team, and the team should promote the practice and transfer of skills gained in therapy into the patient's daily routine [7]. A 6-month study in Canada showed that appropriate intensity and duration for patients received were an average of 37 min of active therapy from both PTs and OTs and 13 min from speech-language pathologists per day [8]. These results are consistent with those from other countries, in which patients receive less than 3 h of therapy per day within the first several weeks poststroke. Establishing safety criteria for early intervention is an important next step. Each patient has a different situation, and not all patients admitted with stroke should start out-of-bed activity or training within hours, or even days, of stroke onset. Currently, no clear safety guidelines have been provided to guide initiation or progress of treatment. When patients are no early deterioration, and signs of secondary intracerebral hemorrhage, acute coronary syndromes, or severe heart failure after ischemic or hemorrhagic stroke, they could receive rehabilitation therapy. Patients treated with r-tPA were excluded from earlier trials [9, 10]. Additional broad physiological safety criteria include systolic blood pressure between 120 and 220 mmHg and heart rate between 40 and 100 beats per minute [9]. The Canadian Best Practice Recommendations for Stroke Care in Stroke Rehabilitation update in 2013 emphasizes that the best practices in organizational structure could be divided into two parts: [1] organization of a stroke rehabilitation system for optimal service delivery (Table 24.1, Part 1) and [2] providing stroke rehabilitation to address physical, functional, cognitive, and emotional issues to maximize participation in usual life roles (Table 24.1, Part 2). This presents a best-practices approach to the rehabilitation continuum.

Table 24.1 Stroke rehabilitation best practice recommendations [7]

Part 1: Organization of a stroke rehabilitation system for optimal service delivery
1. Initial stroke rehabilitation assessment
(a). Triage for rehabilitation
(b). Criteria for inpatient stroke rehabilitation
(c). Initial stroke rehabilitation assessment
2. Inpatient stroke rehabilitation
(a). Early initiation of therapy
(b). Access to interdisciplinary team
(c). Stroke unit care
(d). Adequate intensity of therapy
(e). Task-oriented approach (specificity of tasks)
3. Community and ambulatory rehabilitation
(a). Early supported discharge
(b). Access to outpatient rehabilitation and recovery services
(c). Normalizing life/return to community
(i). Ongoing adaptive support programs
(d). Access to ongoing rehabilitation therapy beyond 3–6 months
(i). Wheelchair services, seating assessments, and other assistive device needs
4. Caregiver assessment and training
Part 2: Providing stroke rehabilitation to address physical, functional, cognitive, and emotional issues to maximize participation in usual life roles
Rehabilitation to improve upper extremity function
(a). Management of activities of daily living/self-care (ADLs)
(b). Management of shoulder pain
Rehabilitation to improve mobility and reduce falls (lower limb function)
Rehabilitation to reduce spasticity
Rehabilitation of swallowing and dysphagia
Rehabilitation to improve visual perceptual function
Rehabilitation to address central pain issues
Rehabilitation to improve communication and aphasia
Rehabilitation to improve cognition
Rehabilitation to improve psychosocial function
Major life roles
(a). Vocational rehabilitation
(b). Return to driving
(c). Relationships and sexuality

24.1.3 Recovery After Stroke

Stroke recovery is heterogeneous. The degree and speed of recovery mainly depends on the lesion site and area. In addition, other factors also affect stroke recovery, such as the victim's age and gender, personal motivation, and the medical and rehabilitative services provided. The recovery following stroke is a comprehensive process of spontaneous recovery and relearning, including restitution (functional recovery of the damaged penumbral neural tissue), substitution (reorganization of partly spared neural pathways near and remote from the infarct to relearn lost functions), and compensation (improvement of the difference between damaged skills and environmental needs) [11].

Researches considered that stroke recovery displays a nonlinear, logarithmic pattern with timing of intervention strategies [2]. In the first 3 months, neurological recovery benefits from both spontaneous mechanism and rehabilitation intervention. It was reported that 16–42% of progresses in the functional outcome could be attributed to spontaneous mechanism in the first 6–10 weeks after stroke [12]. Three months after stroke, the ability of independence in mobility, ADLs (activities of daily living), and social participation largely depended on learning process [7]. Although most studies reported that the great recovery potential happened for the first 3 months after stroke, there was evidence which confirmed that the recovery process could be sustainable for several years [13].

24.1.4 Rehabilitation Assessment After Stroke

Stroke severity assessment and prognosis prediction are essential for choosing optimal rehabilitation methods, which are very important contents of rehabilitation medicine. The National Institutes of Health Stroke Scale [14, 15] and the Canadian Neurological Stroke Scale [16, 17] have been earlier established and are reliable and validated evaluating tools to measures neurological recovery. The evaluation included consciousness, comprehension, speech, and motor function (face, arm, and leg) for a total score of 11 if the person scores normal on all the items. This initial stroke severity assessment should be performed in the first 24 h after onset of stroke. To determine the effectiveness of rehabilitation, the rehabilitation training program should be based on scientific targeting part of body and well planned. Rehabilitation training starts with the initial evaluation, which has always been the first step in the entire rehabilitation process. Accurate evaluation provides value reference data for the clinical rehabilitation therapy. The movement parameters in the corresponding rehabilitation training process, and the clinical experience of rehabilitation physicians could form database of an expert knowledge bank. To explore the relationship between the training parameters and the effect of treatment with rehabilitation, a quantitative real-time assessment of rehabilitation effect is important. Based on a

large number of clinical practices, physicians could select the movement parameter as the rehabilitation evaluation index and then establish updated scientific rehabilitation evaluation mechanism.

24.1.5 Prognosis Prediction After Stroke

Prognosis prediction helps in developing rehabilitation plans and informing patients and relatives. Clinical and electrophysiological assessments and neuroimaging tools, such as computerized tomography (CT), magnetic resonance imaging (MRI), and diffusion tensor imaging (DTI), are usually used to predict prognosis to some degree. But until now, there is no effective biomarker to predict prognosis of patients with stroke.

The use of clinical assessment has been well studied for years. In 2004, Weimar et al. developed two predictable models and externally validated that age and NIHSS scores within 6 h after stroke onset could predict ischemic stroke outcomes [18]. Beebe JA et al. recruited 33 stroke patients and found that the range of activity of the shoulder and hand within 1 month after stroke onset can be used to predict upper limb function at 3 months [19]. Another study included 188 stroke victims, showing that finger extension and shoulder abduction within 72 h after stroke onset could predict functional recovery of the hemiplegic arm at 6 months [20]. Clinical assessments are simple and easily done at bedside. However, the prediction remains poor in severe impairment patients.

It was reported that motor evoked potentials (MEP) induced by transcranial magnetic stimulation (TMS) have a strong correlation with stroke prognosis [21, 22]. The absence of MEP in the first hours after stroke onset is associated with poor functional outcome at 1 year [23], while a relatively high amplitude of MEP on the affected hand is a sign of good functional outcome [24]. Using MEP to predict stroke outcome is controversial. Evoked potentials are highly sensitive, but the specificity is relatively low. MEP absence does not mean poor functional recovery [25]. In addition, this tool is not good and applicable in clinical practice. In many places, TMS is not ready for bedside use. Furthermore, it is difficult for patients to keep sitting and keep their head still for 10–15 min, especially for severe impairment patients.

Infarct volume, collateral circulation, functional activity, and nerve conduction tract detected by neuroimaging tools can also be used to predict stroke outcomes. Vogt Get al. analyzed more than 1800 patients with ischemic stroke and conclude that initial lesion size (determined by CT or MRI) within 72 h after ischemic stroke is a strong and independent predictor of outcome at 3 months [26]. Another study including 66 patients showed that the infarct volume (calculated through diffusion-weighted MRI) within 36 h of stroke onset combined with clinical assessments (age and NIHSS score) has a better functional prediction at 3 months than any of the individual factors alone [27]. In most studies, majority of infarcts from anterior circulation were analyzed. The results may not be applicable to posterior circulation, in which small infarct can cause severe functional impairment [28].

In addition, a study including 111 patients who suffered from ischemic stroke showed that collateralization (angiography by CT or MRI) within 6 h after thrombolysis in acute ischemic stroke is a sign of favorable outcome [29]. The intact corticospinal tract (CST) is injured after stroke. Assessment of CST is another predictor to stroke prognosis. A retrospective study including 20 stroke patients found that CST apparent diffusion coefficient which decreases in less than 6 h following stroke is associated with poor motor outcome at 3 months [30]. Consistently, another study further proved that early ADC changes in motor structures predict outcome of acute stroke better than lesion volume [31]. DTI is a new way to describe the brain structure and is considered as a promising prognostic biomarker for motor recovery after stroke [32, 33]. However, task-related fMRI depends on the intact CST, and it is difficult to carry out in acute stroke, especially for patients with complete hemiplegia or unilateral neglect.

Function of the upper limb is fundamental for ADL. Statistically [11], dexterity of 71% of patients with mild to moderate upper limb paralysis has been restored, but 60% of severely affected patients cannot achieve some dexterity recovery in 6 months. Moreover, only 5% of patients who suffered from complete paralysis can reuse their arms. Theses years, a predicted potential for upper limb recovery (PREP) algorithm has been well established and developed [34, 35]. The algorithm suggests sequential tests of bedside assessment and neurophysiological and neuroimaging assessments, which provide a useful method for stroke prognosis.

24.2 Various Brain Stimulation Techniques for the Stroke Rehabilitation

It has a potential to enhance neuroplasticity during stroke rehabilitation, which supports the recovery of motor and other impairments. Invasive brain stimulation requires surgery, which limits its clinical application to some extent. Noninvasive brain stimulation techniques including transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS) are widely used in stroke rehabilitation, and these application ranges have been extended from poststroke motor dysfunction to aphasia, dysphagia, cognition, depression, etc. An emerging brain stimulation technology-ultrasonic brain stimulation, has recently been shown to be capable of noninvasively stimulating brain activity, and could be used for stroke rehabilitation.

24.2.1 TMS and tDCS

TMS could change the membrane potential of neural cell in the cortex. The induced current affects the metabolism and neural activity of the focal brain, causing a series of physiological and biochemical reactions. tDCS is a technique using a constant

weak polarizing current to the cerebral cortex, which modulates neural cell membrane potential, leading to either hyperpolarization or depolarization [36]. Unlike TMS, tDCS plays its role of modulating neural activity, not stimulating neural firing. In general, low-frequency (1 Hz) stimulation or cathodal stimulation diminished cortical excitability, whereas high-frequency (3–10 Hz) stimulation or anodal stimulation increased the excitability. According to the "interhemispheric interaction" theory, stroke induces the imbalance of interhemispheric motor interactions, leading to decreased motor activity in the lesioned hemisphere and excessive motor activity in the contralesional hemisphere [37]. Given this, applying high-frequency stimulation and anodal stimulation to the remaining motor cortex in the lesional hemisphere or low-frequency stimulation and cathodal stimulation to the contralesional motor cortex may improve neurological function after stroke.

In 2005, Drs. Mansur [38] and Takeuchi [39] reported, respectively, that low-frequency repetitive TMS stimulation on the contralateral primary motor cortex enhanced the hand function of stroke patients in double-blind studies, with significant improvement of hand movement reaction time and nine-hole plate results. In 2007, Boggio et al. confirmed that tDCS could also promote motor function recovery. They found that either anodal stimulation of tDCS on the lesional hemisphere or cathodal stimulation of tDCS on the contra lesional hemisphere could improve hand motor function of stroke patients [40]. Moreover, studies showed that the effects on cortical excitability induced by TMS or tDCS last beyond the intervention [41], which may result in long-lasting neurobehavioral remodeling. At present, TMS and tDCS have been well studied; based on a sufficient amount of evidence ($n > 500$), TMS and tDCS were considered to be valuable for improving upper extremity motor function after stroke. Meanwhile, basic researches also revealed that TMS and tDCS could alter the production of different neurotransmitters and neuromodulators [36] and modulate neurogenesis, synaptic plasticity, and immune response in stroke animals, which may change the neuroplasticity after brain ischemia.

24.2.2 Ultrasonic Brain Stimulation (UBS)

TMS and tDCS do not require invasive procedures which have promising characteristics in brain stimulation but lack spatial specificity and penetration depth. Ultrasound is a mechanical pressure wave, which can be transmitted into tissues in either pulsed or continuous wave forms and can influence physiological activity through thermal and/or nonthermal mechanical/biological mechanisms [42]. Noninvasively ultrasonic stimulation is safe and economic and is deep enough to reach deep tissues. More importantly, it offers a higher targeting specificity of about 2 mm. Compared to electrical, magnetic, photonic, or chemical methods, the potential for ultrasound stimulation on the brain has been largely ignored. Recent studies have demonstrated that ultrasonic brain stimulation regulates neural activity in different brain regions, in both normal human and animals, as well as promotes neurogenesis.

The effect of ultrasonic stimulation on neural activity has been well studied. Applying a 0.5 MHz focused ultrasound to the primary somatosensory cortex of human, Legon et al. demonstrated that it can significantly modulate somatosensory evoked potentials and improve sensory discrimination ability [43]. Another research from the same team showed that focused ultrasonic energy can change EEG oscillatory dynamics through mechanical effect [44]. The study used a MRI guidance method to apply the low-frequency ultrasound on the visual cortex and found that ultrasonic stimulation generated specific visual evoked potential that was similar with those from photic stimulation [45].Transcranial ultrasound on the prefrontal cortex also relieves pain and bad mood in patients with chronic pain [46]. Animal experiment reported that ultrasound can modulate the nerve excitability of sciatic in frog, cortical excitability in cat and rabbit, spinal cord excitability in cat, and hippocampal excitability in rodents. In addition, low-intensity and low-frequency ultrasonic stimulation could induce hippocampal slices of mouse brain to produce action potential and synaptic transmission [43, 47].

Ultrasonic stimulation also has the potential to alter neurogenesis. Using transcranially focused ultrasound to stimulate mice brain, Scarcelli et al. found that the number of neural stem cells and newborn neurons was significantly increased in the hippocampus [48]. Pulsed ultrasound brain stimulation was also reported to upregulate brain-derived neurotrophic factor (BDNF), which is known to enhance neurogenesis [42]. In vitro experiments showed that low-intensity ultrasound could promote neural stem cells derived from pluripotent stem cells to differentiate into neurons and astrocytes [49] and also promote the neural stem neurons from rate embryo to release NO, which is considered as a neuronal maturation-related protein [50].

Taken together, these data suggest that ultrasonic brain stimulation regulates neural plasticity, which may provide a promising brain stimulation method for nerve repair and functional recovery after stroke.

24.3 Therapeutic Rehabilitation Robotics

Rehabilitation robots are constituent of the robot technology, computer network control technology, digital image processing technology based on virtual reality technology, and medical technology. Rehabilitation robots can be used for training and disabled acquired immune deficiency syndrome (AIDS) in stroke patients. In general, the robot has two outstanding features in the application: one is to replace the human work and reduce labor intensity and the other is to extend human ability. Advantages of medical robots can be included in the following areas: (1) to be able to work with high precision, long time, and high strength; (2) to perform fine surgery in a small space, that is, the advantage of minimally invasive medical direction; (3) the front-end perception, the middle intelligent decision, the back-end execution, and control; (4) the clinical adaptability is strong; and (5) to greatly reduce the difficulty of operation.

Rehabilitation robotics is a combination of industrial robot and rehabilitation training. Rehabilitation robot techniques are involved in many areas including mechanical engineering, electronic engineering, biomedical science, artificial organs, automation technology, artificial intelligence, and sensor technology. The current novel rehabilitation robot is the combination of robot and computer as a new therapeutic tool to improve the efficiency of clinical rehabilitation. At present, the rehabilitation robot has been widely applied, which has not only promoted the development of rehabilitation medicine but also led to the development of new technology and new theory.

The development of rehabilitation robot began in the 1980s in the United States, Britain, and Canada. It was in a period of comprehensive development after 1990. Upper limb rehabilitation robot was first developed by Lum et al. for stroke patients. This system was called "hand-object-hand" system [51–53]. Rehabilitation manipulator hand is the most advanced manipulator hand, which was based on mobile robot manipulator. The manipulator is mounted on the mobile robot or auto or semi-autonomous vehicle, which is suitable for a large population of patients. Tachi et al. developed a mobile robot rehabilitation MELDOG laboratory, as seeing eye dogs to help the blind operation and handling object task [54].

The technical characteristics of rehabilitation robot from the clinical application should mainly focus on:

(1) The system design, which includes two aspects: design for mechanics and system architecture. The mechanical design should consider patients with the disease characteristics, functions, training mode, safety and comfort, etc. Improving the type of training action, increasing range of motion, and actively exploring the application of new material technology could make rehabilitation more fit with the biomechanical requirements, not only to wear more comfortable, but also improve the therapeutic effect.
(2) Rehabilitation robot movement efficacy is largely depending on the driving mode. The driving mode current has motor drive, pneumatic drive, hydraulic drive, and pneumatic muscles. Motor drive is widely used rehabilitation robotic device, which is easy to control, high precision, fast response, convenient use, signal monitoring, transmission and processing convenience. The pneumatic-driven or oil-driven robot can be also used in the rehabilitation, but they are relatively less used due to its limitations.

Due to changes of muscle tension/muscle strength, muscle spasm and other kinetic parameters of the system load could occur, which could cause the control system to be unstable and even induce secondary injury. Therefore, to ensure the control system stability and safety, detection technology and control algorithm is extremely important. In addition, based on the biological signal control, the rehabilitation robot movement in the process and processing method of EMG characteristics, measurement of EEG signals, the accuracy of BCI system, and communication speed control should be further explored.

A worldwide accepted classification for rehabilitation robotics is lacking. However, various types of supportive/assistive and/or resistive robotic and virtual reality-enhanced devices can improve outcomes of patients with neurologic disor-

ders [55]. The most popular used motor rehabilitation robots are upper limb, lower limb, and hand. The most promising approaches using rehabilitation robotics are task-oriented, based on current concepts of motor control/learning and practice-induced neuroplasticity. Based on the evidence in neurologic populations, virtual reality-enhanced robotics could be integrated with current concepts in rehabilitation shifting from an impairment-based focus to inclusion of more intense, task-specific training for patients with upper extremity disorders, specifically emphasizing the wrist and hand [56]. Clinical application of a task-oriented approach may be accomplished using commercially available haptic robotic device to focus on training of grasp and manipulation tasks.

24.4 Therapies Based on Mirror Neuron System

Mirror neuron is known for its activation with or without overt movement. Taking advantage of this physiological feature, therapies based on mirror neuron system including action observation, motor imagery, and mirror therapy are used to help rebuild motor function despite impairments. Now they are considered as promising therapeutic approaches to promote cortical plasticity and functional recovery in stroke patients [57, 58].

24.4.1 Action Observation

Increasing clinical studies proved the positive role of action observation on the rehabilitation of upper limb function poststroke [59, 60]. Using fMRI, researchers found that action observation could activate the bilateral motor- and motor-related brain areas in stroke patients, thus helping them to improve motor function recovery [61]. Another study observed that action observation can enhance the effects of motor training by modulating motor memory formation in stroke patients at chronic stage [62]. A small study including 37 stroke patients at the chronic stages showed that action observation plays an important role in motor learning of the hemiplegic arm after stroke [63]. Besides, motor observation could also improve gait and balance dysfunction of stroke patients [64, 65].

24.4.2 Motor Imagery

Just like action observation, motor imagery could also activate the motor system in the brain without motor execution. fMRI analysis indicated that motor imagery is closely related to the recovery of subcortical stroke [66, 67]. Evidences supported that motor imagery training could enhance upper limb motor function in patients

with chronic stroke [68–70] and even improve hand function in stroke patients with 2 years of motor impairments [71]. However, a randomized controlled sequential cohort study including 121 stroke patients found no therapeutic benefit of motor imagery on upper limb after subacute stroke [72]. Moreover, motor imagery also has therapeutic effects on gait and balance impairments [73–75] of stroke patients.

Both motor imagery and action observation can elicit similar brain activity in the motor system. They have been traditionally regarded as two isolated techniques to improve motor rehabilitation over the last 20 years. In recent years, research turned to combining the two techniques to explore the potential effect on motor recovery. Neuroimaging studies demonstrate combined therapy induced stronger cortical activity than either motor imagery or action observation application independently [76, 77], which provides a more effective method for motor recovery after stroke.

24.4.3 Mirror Imitation

Motor imitation consists of sequential stages of action observation, motor imagery, and motor execution. Rehabilitation therapies based on motor imitation require patients to imitate action from visual perception. Data showed that the imitation of hand shape could induce activation of premotor and parietal cortices. Particularly, the frontal region seems to play an important role in imitation of goal-oriented tasks [78]. Researchers also found that the basic brain circuitry under lying motor imitation coincides with that circuitry active during motor observation [79]. At present, motor imitation methods are in lack of evidence in promoting motor recovery after stroke and thus need to be further explored.

24.4.4 Mirror Therapy

Mirror therapy, also called mirror visual feedback, was originally applied to phantom limb pain in 1995 [80]. In 1999, mirror therapy was reported for the first time to be beneficial for motor functional recovery following stroke. Since then, the application field of mirror therapy is gradually transferred to the poststroke limb rehabilitation. Mirror therapy training is beneficial for stroke recovery in acute, subacute, and chronic poststroke phases. Randomized controlled trials showed that mirror therapy could improve the motor recovery of hand [81], upper extremity [82], and lower extremity [83] in poststroke patients.

Mirror therapy exerts an important influence on the motor network. This therapy remodels the motor system by functionally connecting movement to the ipsilateral sensorimotor cortex [84]. Previous studies suggested that mirror therapy could activate primary motor cortex of the stationary hand [85, 86] of stroke patients. Subsequent studies proved that primary motor cortex plasticity, especially its excitatory connections, is an essential therapeutic mechanism of mirror therapy [87].

Data also showed that mirror therapy influenced the excitability of the transcallosal pathway [88]. Evidences showed that mirror therapy training not only enhanced the excitability of the ipsilateral primary motor cortex to project the untrained hand/arm but also increased the ipsilateral projections from the contralateral M1 to the untrained/affected hand [89]. Lately, two randomized controlled trials reported that mirror therapy could improve unilateral neglect after stroke [90, 91]. Mirror therapy is simple, and easy to carry out, proving to be a valuable therapy for motor rehabilitation after stroke.

24.5 Virtual Reality Application in Stroke Rehabilitation

Virtual reality technology is a kind of computer simulation system. It generates multimodal, interactive, and realistic three-dimensional environments, which makes users to be immersed in a virtual world. At present, virtual reality technology has been widely applied to many fields including medicine, entertainment, interior design, military and aerospace, etc. From the early twenty-first century, studies began to examine its possible effectiveness on stroke rehabilitation [92]. Many studies showed that virtual reality facilitated functional recovery of the hand [93] and upper limb [94–96]. In most studies, the number of recruited patients is small. The results of these small trials must be reproduced in further large studies. On the other side, evidences demonstrated that virtual reality training has a small positive effect over conventional therapy [97, 98]. Future strategies may need to combine virtual reality with other rehabilitative interventions to promote motor recovery after stroke.

24.6 Stem Cell Therapy

Stem cell therapy for stroke is proved to be safe and effective in animal models of stroke. People have high hopes for stem cell therapy in stroke, because stem cells are capable of not only differentiating multiple cell types but also of secreting different kinds of cytokines, which could promote both neurogenesis and angiogenesis. A number of animal studies revealed that stem cell therapy has long-term positive effects on neurological recovery after stroke.

The success of animal experiments promotes basic research translation into clinical application. In 2000, Kondziolka et al. published the first trial of stem cell therapy for stroke patients [99]. They transplanted NT2N cells into the basal ganglia of 12 patients with 6 months to 6 years after stroke onset. After 1 year, neurological improvements compared to baseline was observed in all patients. Moreover, fluorodeoxyglucose uptake increased at the implant site in six patients after 6 months of treatment. The following phase 2 study also observed neurological function recovery in some patients [100]. Both the two studies indicate the safety and feasibility of

stem cell transplantation for stoke patients. Another study found that the transplanted NT2N cells can survive for more than 2 years after treatment [101].

In the past few years, there are several other researches of stem cell therapy which have been published. As autologous cells, mesenchymal stem cells are most commonly used. Studies have shown that transplanted mesenchymal stem cells into stroke patients intravenously [102, 103] or intra-arterially significantly improve stroke outcome [104]. These studies further confirmed the safety of stem cell transplantation, even though no significant improvement has been observed in most studies. Only two studies showed improved neurological function. For patients with severe or chronic (months or years after onset) stroke, stem cell therapy is still a promising method to promote functional recovery.

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Chapter 25
The Role of TMS for Predicting Motor Recovery and Outcomes After Stroke

Cathy M. Stinear and Winston D. Byblow

Abstract Transcranial magnetic stimulation (TMS) is a safe, non-invasive technique for studying the human motor system. It can be used to evaluate primary motor cortex (M1) function after stroke, by stimulating the ipsilesional M1 and recording motor-evoked potentials (MEPs) from the paretic limbs. In this chapter, we first outline the measures of M1, intracortical and interhemispheric function that can be made with TMS. The presence or absence of MEPs is the simplest and most reliable measure that can be made with TMS. In general, patients in whom TMS can elicit MEPs from the paretic limbs make a better motor recovery and experience better functional outcomes than those patients without MEPs. We provide an overview of recent research showing that MEP status is a particularly useful biomarker for patients with initially severe motor impairment. The limitations and potential benefits of MEP status as a biomarker for patient selection in stroke rehabilitation trials are discussed.

Keywords Stroke • Motor • TMS • Prognosis • Rehabilitation

Abbreviations

AMT	Active motor threshold
CST	Corticospinal tract
ECR	Extensor carpi radialis
EMG	Electromyography

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FM-UE	Fugl-Meyer Upper Extremity Scale
GABA	Gamma-aminobutyric acid
IHI	Interhemispheric inhibition
iSP	Ipsilateral silent period
M1	Primary motor cortex
MEP	Motor-evoked potential
MRC	Medical Research Council
MRI	Magnetic resonance imaging
MSO	Maximum stimulator output
NPV	Negative predictive value
PLIC	Posterior limb of the internal capsule
PPV	Positive predictive value
RMT	Resting motor threshold
SAFE	Shoulder abduction finger extension
SICI	Short-latency intracortical inhibition
SP	Silent period
TMS	Transcranial magnetic stimulation

25.1 Principles of Transcranial Magnetic Stimulation

Transcranial magnetic stimulation (TMS) is a safe, non-invasive and painless technique that can be used to investigate the excitability of the primary motor cortex (M1) and its descending pathways. TMS was first introduced by Barker and colleagues [1] to provide a safe and painless alternative to transcranial electrical stimulation, thus making the study of human motor cortex physiology more widely possible. With TMS a magnetic stimulus is applied via a coil (5–10 cm in diameter) through which a large but brief pulse of electrical current is passed from a high-voltage capacitor discharge system. With the coil held over the scalp, the discharge generates a brief magnetic field that induces electrical currents in the underlying tissues. These electrical currents can depolarise neurons in the cortex of the brain, causing them to fire.

A magnetic field of up to 2.5 Tesla can be generated in large circular coils. However, there is a trade-off between intensity and focality. Figure-of-eight coils consist of two smaller diameter coils wound together and provide more focal stimulation than circular coils. As well as type, size and orientation of the coil, the effects of stimulation are also dependent on whether the electrical pulse through the coil is monophasic or biphasic. Normally, coil orientation and waveform shape are set to induce posterior-anterior current in the brain which is optimal for M1 stimulation [2]. The interested reader can find further technical information, including safety procedures and contraindications for TMS, detailed elsewhere [3].

By using TMS, it is possible to probe various properties of the motor cortex and its descending pathways, including nerve conduction velocities, membrane

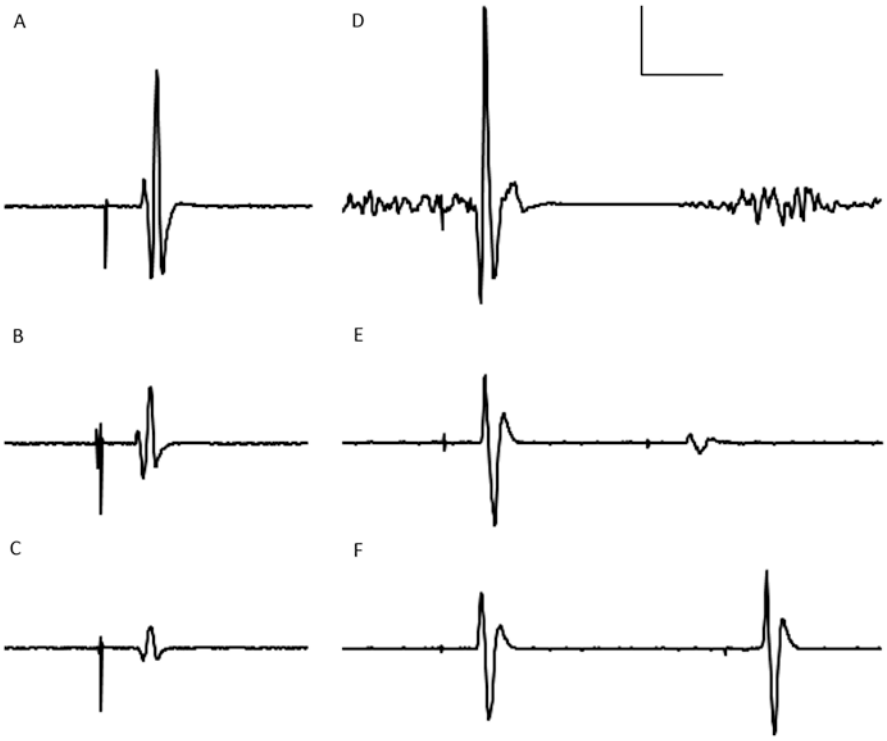

Fig. 25.1 Single- and paired-pulse TMS can be used to examine M1 cortical excitability, inhibition and disinhibition as shown in these EMG traces recorded from the first dorsal interosseous muscle. (**a)** MEP from single-pulse TMS at a stimulus intensity of 130% of RMT. (**b**, **c**) The MEP in panel (**a**) is suppressed by a subthreshold conditioning stimulus at 90% of active motor threshold delivered 2.5 ms (**b**) and 1 ms (**c**) prior to test stimulus. The decrease in MEP amplitude reflects synaptic $GABA_A$ receptor-mediated inhibition in M1 (2.5 ms) and extra-synaptic GABA-ergic tone (1 ms). (**d**) Silent period produced in the EMG trace of a preactivated muscle by single-pulse TMS at 130% RMT. The silent period reflects $GABA_B$ receptor-mediated inhibition in M1. (**e**, **f**) Suprathreshold conditioning and test stimulation (both at 130% RMT) elicit late intracortical inhibition (LICI) with an interstimulus interval of 150 ms (**e**) and late cortical disinhibition with an interstimulus interval of 250 ms (**e**) due to activation and then presynaptic inhibition of $GABA_B$-ergic neurons within M1. Calibration bars: 0.5 mV and 50 ms (Figure courtesy of Ronan Mooney)

excitability, intracortical inhibition and facilitation, interhemispheric transfer and central nervous system reorganisation. Most of these properties exhibit interesting dynamics after stroke as will be discussed below. A very brief description of the main measures of interest is provided here. Most measures are based on characteristics of the motor-evoked potential (MEP) observable in the EMG of the target muscle (Fig. 25.1). After stroke, it might not be possible to elicit a MEP due to a lesion affecting M1 itself, the descending white matter of the corticospinal tract (CST), or at the level of the brainstem. As such, the presence or absence of an MEP from TMS applied to the ipsilesional M1 early after stroke is informative about the

extent of stroke damage and recovery potential, as will be discussed in greater detail below.

Assuming MEPs can be elicited, the most common TMS measures are those which characterise the excitability of pyramidal neurons in M1 that form the CST. The MEP threshold is one such measure. Threshold depends on the excitability of neural elements including cortico-cortical axons, their synaptic contact with pyramidal neurons and the initial axon segments of the pyramidal neurons [4, 5]. The threshold can be determined with the target muscle at rest (RMT) or preactivated during slight voluntary contraction (AMT) and is defined as the minimum stimulator intensity required to consistently produce a MEP of a given amplitude (e.g. RMT = 50 uV; AMT = 100 uV; [6]). There are several methods to determine threshold including the relative frequency method, which is the most common, and adaptive methods which allow more rapid determination of threshold when using threshold tracking techniques [7]. Threshold is measured in units of stimulator output, normally expressed as a percentage of maximum (e.g. 50% MSO).

The size of the MEP (amplitude or area) for a given stimulus intensity can be used to track changes in corticospinal excitability over time. Stimulus intensity may be set relative to maximum stimulator output (e.g. 80% MSO) or threshold (e.g. 120% RMT), and stimuli are delivered at a single, optimal stimulation site. A stimulus-response curve characterises excitability by plotting MEP amplitude across a range of intensities from threshold to plateau. Although the curve is normally sigmoidal in shape, the slope of the linear region is often used as an index of corticospinal excitability [8]. In contrast, TMS mapping involves recording MEPs elicited by a constant stimulation intensity (e.g. 110% RMT) delivered at several stimulation sites. Map size (area, volume) and centre of gravity can be determined for a given muscle representation [9]. The added advantage of mapping is that it can be sensitive to shifts in representation position or area which may be evident after stroke [10]. The disadvantage is that it takes a considerable amount of time to perform, although recent advances with ‘rapid mapping’ make it more feasible for exploring spontaneous or treatment-related cortical reorganisation after stroke [11].

The cortical silent period (SP) is another measure that can be derived from single-pulse TMS, delivered while the target muscle is preactivated. This procedure involves suprathreshold TMS of the contralateral M1 (e.g. 80% MSO, 130% RMT) which is large enough to produce a period of EMG silence in the range of 150–250 ms, followed by the return of voluntary muscle activity. The duration of the SP is indicative of the excitability of $GABA_B$ receptor-mediated inhibitory function within M1 [12]. For some patients, the SP can be challenging or even impossible to obtain from stimulating the ipsilesional M1 because the patient is unable to voluntarily contract muscles on the paretic side [13].

A measure of transcallosally mediated inhibition can be obtained from suprathreshold single-pulse TMS of the M1 that is ipsilateral to the contracted muscle (iSP). The iSP is detected as a period of reduced EMG activity 30–70 ms poststimulus [14]. The iSP permits the examination of ipsilesional M1 function in more severely impaired patients, many of whom are unable to activate the paretic side

[15]. The iSP can be sensitive to post-stroke reorganisation that occurs with long-term motor practice [16].

Paired-pulse TMS is often used to investigate M1 intracortical and interhemispheric inhibition and facilitation, indicative of GABAergic and glutamatergic function within M1. Paired-pulse TMS involves delivering both a conditioning and test pulse in close succession (1–200 ms). Intracortical function can be assessed by delivering both pulses through the same coil placed over M1 and measuring the decrease or increase in size of the test MEP compared to that obtained with the test stimulus alone. Short-latency intracortical inhibition (SICI) is examined with a subthreshold conditioning stimulus and an interstimulus interval between 1 and 5 ms [17], reflecting $GABA_A$ receptor-mediated processes, which may be downregulated post-stroke [18]. Intracortical facilitation is examined with an interstimulus interval of 6–15 ms, with 10 or 15 ms intervals being most common, but this measure is not routinely investigated after stroke.

Dual-coil TMS can be used to examine interhemispheric inhibition (IHI) between M1s and provide a measure of transcallosally mediated inhibition similar to the ipsilateral SP measure. To examine IHI, each M1 is stimulated with a separate coil, delivering a suprathreshold pulse (e.g. 120% RMT). Inhibition of the test MEP is evident at short (10–15 ms) and long (40 ms) intervals, indicative of excitability of different populations of GABA receptor-mediated inhibitory neurons [19, 20].

Measures of functional connectivity between different cortical areas (e.g. premotor-motor, intrahemispheric or interhemispheric) can also be examined with dual-coil TMS. These approaches may reveal functional reorganisation after stroke [21], but to date, they have not been widely used, perhaps owing to the size of stimulating coils and difficulty in accurately targeting adjacent cortical regions.

25.2 TMS in Stroke

In general, greater interhemispheric asymmetry in measures obtained with TMS is related to worse motor performance at the time of testing, at the group level of analysis. It is well established that the ipsilesional M1 is less excitable than the contralesional M1 in patients with motor deficits after stroke [22–24]. This is reflected by higher resting and active motor thresholds, and smaller MEP amplitudes, compared to the contralesional M1 and to healthy controls [24]. As noted above, there is evidence of altered intracortical function, with reduced SICI in the ipsilesional M1 at the subacute stage of recovery [22, 24]. Conversely, studies of interhemispheric inhibition after stroke have produced more variable results. A recent meta-analysis did not detect any differences in the amount of IHI passed from the contralesional to ipsilesional M1 and vice versa nor in the amount of IHI produced by stroke patients and healthy controls [24]. Furthermore, this meta-analysis found no differences between the contralesional M1 and healthy controls on any TMS measure made at the subacute or chronic stage of stroke [24]. This indicates that the neurophysiological effects of stroke are most readily detected by TMS

measures of ipsilesional M1 excitability and SICI, rather than interhemispheric or contralesional measures. TMS measures made from muscles distal to the elbow produce very similar results [24]. The choice of target muscle is therefore less critical than using optimal TMS and EMG techniques.

As noted above, most TMS measures require a MEP to be generated, with the exception of MEP status. The use of TMS to evaluate ipsilesional M1 excitability and SICI is therefore limited to patients with a functional ipsilesional M1 and CST. In general, measurement error is lower, and reliability is higher for measures of corticospinal excitability obtained with single-pulse TMS, than for measures of intracortical function obtained with paired-pulse TMS [25–27]. This may be due to the fact that inhibition or facilitation measures are strongly affected by the test MEP amplitude, which may be compromised, or variable, or both after stroke. It remains to be determined whether paired-pulse inhibition and facilitation measures may be more reliable if obtained using modern threshold tracking techniques, which address the limitation of test MEP variability [28–30]. A further consideration is that TMS does not readily test the function of alternative descending motor pathways that may become more important for motor performance after stroke, such as the reticulospinal and rubrospinal tracts [31]. Finally, TMS can't be used with all patients due to contraindications, in the same way that not all patients can have an MRI scan. Despite these limitations, TMS can be used to make predictions for individual patients about motor recovery and outcomes after stroke. The most commonly used TMS predictor is MEP status, because it doesn't require a MEP to be generated and has good to excellent reliability [32].

25.3 TMS for Prediction After Stroke

It is important to note that we use the term 'predict' in the clinical rather than statistical sense. That is, we will discuss TMS measures made at one time point that can make a prediction about motor performance at a later time point. This is in contrast to TMS measures that are associated with motor performance at the time of testing.

TMS performed within the first few days after stroke can predict subsequent motor outcomes and motor recovery. Motor outcomes describe the level of impairment or function at a specific time point post stroke, regardless of performance immediately after the stroke. The disadvantage of measuring outcomes is that they provide no information about whether performance has improved, remained stable or deteriorated over time. This is overcome by measuring motor recovery, which is the change in motor impairment or function over time. Recovery can be quantified as the difference in a measure of motor performance between two time points, such as the difference in a clinical score obtained 1 week and 6 months after stroke. Recovery can also be expressed as a proportion of the available improvement, and this is described in further detail below.

25.3.1 Motor Outcomes

25.3.1.1 Upper Limb

The first reports of the prognostic value of MEP status at the subacute stage of stroke were published in the early 1990s. These early studies typically used MEP status as a predictor for upper limb motor outcomes and were systematically reviewed in 2002 [33]. This review of five studies including 255 patients concluded that the presence of MEPs in the paretic hand within 7 days of stroke predicted better outcomes for motor impairment and function. Motor impairment was evaluated with the Medical Research Council (MRC) grades, the Motricity Index and the Fugl-Meyer Upper Extremity (FM-UE) Scale, while function was evaluated with the Barthel Index [33]. This review also calculated odds ratios for studies with at least 50 patients and found that the odds of a good outcome for MEP+ patients ranged from 5.49 to 13.50 relative to MEP− patients.

These findings are supported by a more recent systematic review of 14 studies that included 480 patients [34]. The majority of studies evaluated MEP status in intrinsic hand muscles within 2 weeks of stroke and found that the presence of MEPs predicted better outcomes for both motor impairment and function. Motor impairment was typically evaluated with MRC grades, while function was most commonly evaluated with the Barthel Index [34]. None of the studies reviewed utilised a measure of functional outcome that was more specific to the upper limb, such as the Wolf Motor Function Test or Action Research Arm Test [35].

Positive predictive values (PPV) for MEP status range between 86% [36] and 93% [37], indicating that the presence of MEPs is a reasonably robust predictor of good upper limb motor outcome. Several authors have noted that MEP status is a particularly useful predictor for patients with initially more severe motor impairment [33, 38], with one study reporting a PPV of 100% in this subset of patients [37]. However, negative predictive values (NPV) for MEP status range between 35% [39] and 95% [38] demonstrating that the absence of MEPs does not necessarily mean a poor outcome.

MEP status primarily reflects the functional integrity of M1 and the CST and is not sensitive to the potential contributions of other areas of motor cortex and alternate descending motor pathways to motor outcomes. This may explain the lower NPV for MEP status. Patients who are initially MEP− may still have some capacity for recovery of motor output via cortical reorganisation and the upregulation of transmission via alternate ipsilesional motor pathways [31, 40, 41]. There is also some evidence for upregulation of transmission via uncrossed corticospinal projections after stroke [22]. This can be evaluated with TMS of the contralesional M1; however, the prognostic utility of ipsilateral MEPs at the subacute stage is currently unknown.

Some of the limitations of TMS as a predictor of motor outcomes can be overcome by combining TMS measures with other biomarkers. We have developed one approach to this, called the Predict Recovery Potential (PREP) algorithm [42]. This

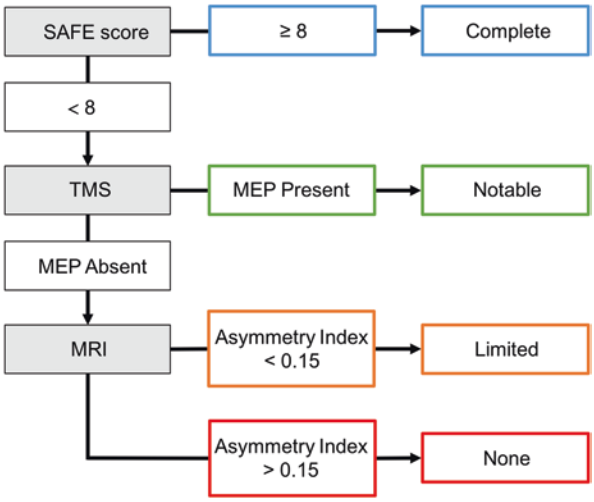

Fig. 25.2 The PREP algorithm combines clinical, TMS and MRI measures within the first few days after stroke to predict upper limb functional outcome 3 months later. The SAFE score is obtained by adding the MRC strength grades for paretic shoulder abduction and finger extension. If the score is 8 or more (out of 10) within 72 h of stroke symptom onset, the patient is likely to make a complete, or near-complete, recovery of upper limb function. If the score is less than 8 at 72 h post stroke, TMS is used to determine MEP status around 5 days post stroke. Patients in whom TMS can elicit a MEP in the paretic wrist extensors are MEP+ and are predicted to make a notable recovery of upper limb function. Patients who are MEP− proceed to an MRI scan 10–14 days post stroke. Diffusion-weighted imaging is used to calculate the mean fractional anisotropy of the posterior limbs of the internal capsules, and an asymmetry index is calculated. If the asymmetry index is <0.15, the patient is likely to have a limited recovery of upper limb function. If the asymmetry index is >0.15, the patient has essentially no potential for recovery of meaningful upper limb function (Adapted from Stinear et al. [43])

algorithm sequentially combines clinical, TMS and MRI measures to predict upper limb functional outcome for individual patients (Fig. 25.2). The PREP algorithm was initially developed with a sample of 40 first-ever ischaemic stroke patients [43] and has since been validated with a sample of 192 patients with first-ever or previous ischaemic or haemorrhagic stroke [44]. In brief, upper limb impairment is evaluated within 72 h of stroke symptom onset by grading shoulder abduction and finger extension strength with the MRC grades. The scores for each movement are summed to calculate a SAFE score out of 10. Patients with a SAFE score less than 5 at 72 h post-stroke are then assessed with TMS to determine MEP status. Those who are MEP+ are predicted to have a good functional outcome within 3 months. It should be noted that patients with a SAFE score of zero at 72 h post-stroke can be MEP+, indicating that their ipsilesional M1 and CST are functional despite their inability to produce voluntary muscle activity early after stroke. TMS is therefore essential for distinguishing between patients with initially severe motor impairment who have potential for a good motor outcome and those who do not. However, as described above, TMS of ipsilesional M1 does not evaluate all descending motor pathways,

and patients who are MEP− may still have potential for recovery of some upper limb function. For this reason, the PREP algorithm uses MRI to evaluate the structural integrity of the posterior limb of the internal capsule (PLIC). The mean fractional anisotropy is calculated for each PLIC and an asymmetry index calculated. Patients with an asymmetry index <0.15 are likely to have a limited recovery of upper limb function, while those with an asymmetry index >0.15 are likely to have none.

The PREP algorithm capitalises on the high PPV for MEP status in patients with more severe initial upper limb impairment. The algorithm overcomes the low PPV for MEP status by using MRI to evaluate stroke damage to all tracts passing through the posterior limb of the internal capsule. Sequentially combining clinical, TMS and MRI measures is more efficient than obtaining all biomarkers from all patients. TMS is only required for approximately one third of patients, and MRI is only required for approximately one half of these [44]. Using TMS to evaluate patients with a SAFE score <5 efficiently leverages the high positive predictive value of MEP status, while using MRI in MEP− patients overcomes the lower negative predictive value of MEP status.

25.3.1.2 Lower Limb

Very few studies have evaluated the usefulness of TMS in predicting lower limb motor outcomes after stroke. This may be related to the technical challenges of eliciting MEPs from lower limb motor cortex. The location, orientation and relatively smaller surface area of the lower limb M1 representation make it difficult to stimulate with TMS. A double-cone stimulating coil and higher stimulus intensities are usually required, and MEPs are typically recorded from distal muscles such as tibialis anterior and abductor hallucis brevis [45].

Two studies have evaluated MEP status in tibialis anterior early after stroke. The first study of 38 patients found that the presence of MEPs within 10 days of stroke predicted recovery of ankle dorsiflexion, but not independent walking, 6 months post-stroke [46]. In contrast, a more recent study of 14 non-ambulatory patients found that those who were MEP+ within 4 weeks of stroke were independently walking 6 months post-stroke [47]. The relevance of lower limb MEP status to functional outcomes such as walking is therefore unclear. This may reflect the higher degree of redundancy in motor control of the lower limb compared to the upper limb. Upper limb function is heavily dependent on contralateral CST function, whereas the lower limbs receive descending commands from both hemispheres, and walking function is also supported by reticulospinal projections [48]. These important differences in neuroanatomy mean that MEP status determined with TMS of ipsilesional M1 may not be a strong predictor of subsequent lower limb impairment and walking outcomes.

25.3.2 Motor Recovery

As noted above, recovery is a dynamic process captured by a measure of change. Change in impairment can be measured with the upper and lower extremity portions of the Fugl-Meyer [49], which focus primarily on the presence of unwanted motor synergies which are common after stroke [50]. Recovery from impairment captures true biological recovery, whereas improvements in function can also reflect compensatory mechanisms. Selecting appropriate measures of recovery based on an intervention's mechanisms is crucial. A long-standing view is that the failure of many clinical trials of stroke rehabilitation may relate to the choice of outcome measures rather than the lack of efficacy of the study intervention [51]. In humans, almost all recovery from motor impairment occurs within the first 3 months of stroke, emphasising a time-sensitive period for spontaneous biological processes which give rise to recovery and the time-critical nature for interventions which may interact with it [52]. The dynamics of recovery from motor impairment are explored in further detail below.

25.3.2.1 Upper Limb

The dynamics of recovery from upper limb impairment have been the topic of considerable recent investigation since an original and noteworthy observation by Prabhakarahn and colleagues [53–59]. Within the first 3–6 months after stroke, a large subset of patients recover from upper limb impairment to a fixed proportion that is almost exactly 70% of the available improvement. That is, for this subset of patients, the change in UE-FM score (ΔFM) is proportional to the initial impairment (FM_{ii}) such that $\Delta FM = \beta \cdot FM_{ii}$, with β values approximating 0.7 within 95% confidence intervals [55, 58]. This '70% rule' has been observed in over 500 patients in countries with different rehabilitation services [53–56, 59], regardless of patients' age, gender, stroke type and therapy dose [55, 56, 58].

However, not all patients fit the 70% rule. Earliest studies suggested that initial impairment may determine whether or not recovery would be proportional, but the criterion FM-UE score has varied markedly between studies [53, 54, 60]. This matter appears to have been resolved by using TMS. Single-pulse TMS applied to the ipsilesional M1 within the first 2 weeks of stroke identified those patients who would fit the 70% rule. TMS was used to determine MEP status of the paretic wrist extensor muscle, extensor carpi radialis (ECR), using stimulus intensities of up to 100% MSO if necessary [55]. MEP status more accurately predicted which patients would fit the 70% rule than measures of initial impairment. Patients with MEPs (MEP+) could achieve a proportional recovery, *regardless* of initial impairment. MEP+ patients with initial FM-UE scores as low as 4 and 5 exhibited a proportional (70%) recovery, whereas MEP− patients did not even though some had higher initial FM-UE scores [55].

Presently it is not clear why the proportion of upper limb recovery from impairment is 70%, and not some other number, nor is it clear what intervention if any may help patients exceed 70% recovery [61]. Again, measures made with TMS have shed some light on these questions. Using TMS, we measured resting motor threshold from the ECR of both upper limbs between 2 and 26 weeks after stroke. Obviously, it was only possible to obtain RMT measures from the paretic upper limb in MEP+ patients. We were interested in the recovery of ipsilesional M1 excitability and so measured change in ipsilesional RMT (ΔRMT) relative to baseline, computed at 6, 12 and 26 weeks post stroke. Initial impairment of RMT was calculated as the difference between baseline ipsilesional RMT and contralesional RMT (which was stable and normal across the entire period). A near identical proportional relationship was observed in both ΔFM and ΔRMT. Ipsilesional RMT improved by approximately 70% of the available improvement. The ΔFM and ΔRMT data from the study are shown in Fig. 25.3. The RMT finding provides some insight into the potential neurobiological mechanisms of proportional recovery and led us to propose that there may be a consistent relationship between the volume of permanently damaged corticospinal axons and the volume of temporarily dysfunctional adjacent axons. If so it may be that initial impairment measured with the FM scale captures both permanent and temporary axonal dysfunction, MEP status is sensitive to the extent of permanent axonal loss, and RMT is sensitive to the recovery of temporarily dysfunctional axons [55]. These ideas warrant further investigation.

25.3.2.2 Lower Limb

Given the ubiquitous nature of proportional upper limb recovery, a similar proportional recovery dynamic may be expressed in other domains. Little is known at present, but there has been some evidence for proportional recovery from aphasia [62]. Only one study to date has demonstrated proportional recovery from lower limb impairment [57]. This study found that all patients followed the 70% rule, regardless of tibialis anterior MEP status and despite over half of the 32 patients being non-ambulatory at baseline. The lack of predictive power for MEP status may simply reflect the technical challenges associated with stimulating the lower limb motor cortex. Or it could mean that preserved ipsilesional corticomotor function is not essential for proportional recovery from lower limb impairment since neural pathways such as the reticulospinal tract provide greater redundancy in the control of the lower limb compared to the upper limb. Interestingly, similar to the upper limb and despite greater contributions of brainstem-mediated pathways, recovery from lower limb seems to be limited to 70%, but confirmation from larger samples is required.

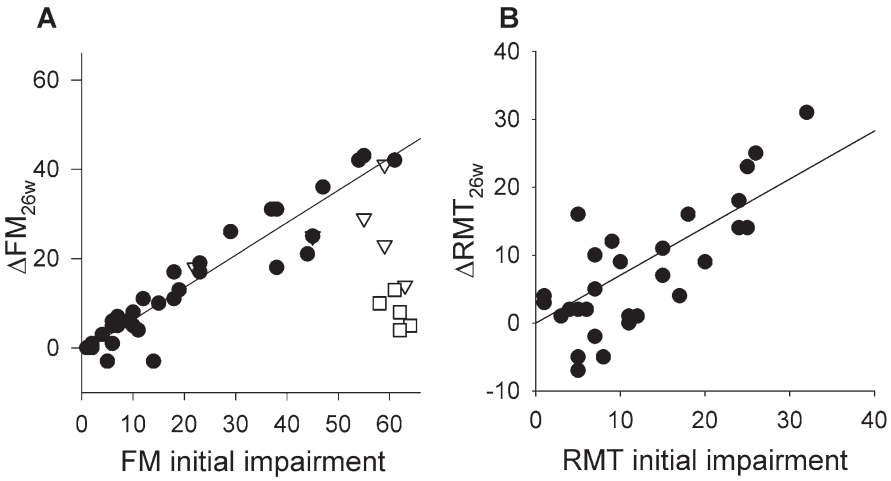

Fig. 25.3 Recovery from upper limb impairment and of ipsilesional M1 RMT is proportional to initial impairment for patients with MEPs (MEP+). (**a**) The recovery from upper limb impairment is reflected by change in FM-UE score between baseline and 26 weeks post-stroke (ΔFM_{26w}). Recovery is proportional to initial impairment, calculated as 66 minus baseline FM-UE score, for patients in whom TMS can elicit MEPs in the paretic wrist extensors (*filled circles*). The *line* represents the '70% rule'. Patients without MEPs (*open symbols*) make recovery between 0 and 70% of the available improvement (*triangles*) or make essentially no meaningful recovery (*squares*). Note that TMS is required to identify which patients with severe initial impairment will make a proportional recovery. (**b**). The recovery of ipsilesional M1 RMT is reflected by change in RMT between baseline and 26 weeks post-stroke (ΔRMT_{26w}). RMT initial impairment was calculated as the difference between ipsilesional RMT at baseline and the average contralesional RMT, which was stable. For these MEP+ patients, recovery of ipsilesional M1 RMT is proportional to initial RMT impairment, and the line represents the '70%' rule (Adapted from Byblow et al. [55])

25.3.3 TMS at the Chronic Stage

The previous section described the pivotal role that TMS can play in predicting whether a patient will experience proportional recovery at the subacute stage, when spontaneous biological recovery is evident. The logical implication of proportional recovery is that even patients who experience proportional recovery are left with some lingering impairment, precisely because motor impairment resolves incompletely, to 70% of the maximum possible. As such the majority of patients tend to benefit from goal-directed physical therapy which allows relearning presumably via mechanisms of neuroplasticity which facilitate adaptation and compensation. Recovery from motor impairment is complete at 6 months post stroke, and this time point is the most commonly accepted onset for the chronic stage. What further gains are possible at the chronic stage, and what role can TMS play in predicting responsiveness to therapy or motor practice?

In a small randomised controlled trial, we observed that MEP status was useful in determining response to daily upper limb motor practice undertaken by chronic

patients over a 1-month period. Immediately post intervention and at 1-month follow-up, patients with MEPs made larger gains on the hand and arm portion of the FM-UE assessment than patients without MEPs [63]. Subsequently other studies have also reported that patients with a functionally intact CST (having MEPs in the paretic hand or forearm) tend to make better gains in response to therapy or intervention at the chronic stage than those who do not [64, 65]. As noted in previous reviews, MEPs may reappear during recovery, but this does not always equate to clinical improvement at the chronic stage [66], and their late reappearance may have little predictive value [22, 33].

25.4 Conclusion

In light of the research described above, one can envisage several ways that TMS might contribute to translational stroke research. TMS provides researchers with a safe, non-invasive tool for evaluating motor system function in patients recovering from stroke, though it does have several limitations. Measures can be variable within subjects and over time and are more difficult to obtain for the lower limb. Furthermore, descending motor pathways other than the CST are not readily evaluated with TMS and may play important roles in both upper and lower limb recovery. Similarly, TMS sheds little light on the role of cortical areas other than M1 in recovery after stroke.

Despite these limitations, MEP status is probably the simplest and most reliable TMS measure and is a useful predictor of upper limb motor recovery and outcome. MEP status could be incorporated in the design of upper limb rehabilitation trials initiated in the first few days after stroke. Doing so would provide important information for patient selection and stratification, in addition to demographic and clinical measures already used. MEP status for patient selection could be particularly useful when recruiting patients with more severe initial motor impairment, as MEP+ patients are more likely to recover and achieve better outcomes than MEP− patients, despite similar baseline clinical scores. MEP status for patient selection might also be important in trials of interventions designed to enhance recovery from motor impairment. Matching treatment and control groups on MEP status, and therefore the potential for proportional recovery, could increase the trial's sensitivity to treatment effects. In addition to threshold measures, more sophisticated measures of intracortical and interhemispheric function may provide important mechanistic insights in longitudinal studies of motor recovery after stroke. These in turn could identify new therapeutic targets and biomarkers of treatment effects. The development of techniques to reduce the variability of TMS measures, and algorithms for combining MEP status with other biomarkers, will support greater use of TMS in stroke rehabilitation research.

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Chapter 26
Do We Have a Chance to Translate Bench-top Results to the Clinic Adequately? An Opinion

Kristine Edgar Danielyan

Abstract Animal models of ischemic, hemorrhagic stroke and transformation certainly have vivid importance for clinical studies and development of thrombolytic as well as neuroprotective drugs. Clear understanding of techniques for every type of stroke modeling highlights naturally impossible adverse effects of the surgery, which might greatly influence the interpretation of final experimental results. There are no stroke models that fully reflect human disease. Infarcts are relatively larger in experimental animals than in humans with strokes. The models are more analogous to massive hemispheric infarcts than to localized strokes such as those in the internal capsule. Every type of animal stroke model is a partial hallmark of clinical picture. Thus, knowledge about the variety of stroke models allows choosing the system, which will serves for testing drugs or compound, predicting effective doses, and evaluating possible adverse effects, pharmacokinatics. Clinical trials might be more informative and successful if benchtop results are clearly delineated and reflect treatment time window, mechanism, and doses.

Keywords Clinical stroke • Experimental stroke • Treatment • Time window • Dose

Abbreviation

ADMA	Asymmetric dimethylarginine
AF	Atrial fibrillation
AIS	Acute ischemic stroke
APP	Amyloid precursor protein
BBB	Blood-brain barrier
BFGF	Basic fibroblast growth factor

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CA	Cardiac attack
CBP	Central blood pressure
CIMT	Carotid intima-media thickness
CVR	Cerebrovascular reactivity
eNOS	Endothelial nitric oxide synthase
ER	Endothelial reticulum
ERO1	ER oxidoreductin 1
FAD	Flavin adenine dinucleotide
HI	Hypoxic-ischemic
HT	Hemorrhagic transformation
MABP	Mean arterial blood pressure
MAPK	Mitogen-activated protein kinase
MCA	Middle cerebral artery
NO	Nitric oxide
NOXs	NADPH oxidases
PDI	Disulfide isomerase
PMNs	Polymorph nuclear leukocytes
PYK-2	Protein tyrosine kinase-2
ROS	Reactive oxygen species
TIA	Transient ischemic attack
UA	Uric acid
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase
XOR	Xanthine oxidoreductase

26.1 Introduction

Animal models of ischemic stroke and hemorrhagic transformation certainly have vivid importance for stroke research and development of thrombolytic as well as neuroprotective drugs. Clear understanding of techniques for every type of stroke modeling highlights adverse effects of the surgery which might greatly influence on the interpretation of the final experimental results. There is no any stroke model which will fully reflect human disease. Clinical investigations evidenced the fact that infarcts are relatively larger in experimental animals than in human with strokes [1]. The models are more analogous to massive hemispheric infarcts. However, the percentile of stroke localization in the region of the MCA (middle cerebral artery) is very high in clinic, which is reflected by numerous models of focal cerebral ischemia.

Every type of animal stroke models is a partial hallmark of clinical picture. Thus, knowledge of the variety of stroke models allows choosing the system, which will

allow adequately testing drugs or compound, predicting effective dose, and evaluating possible adverse effects of predrugs, pharmacokinetically.

Generally, postsurgical parameters characterizing the severity of experimental stroke, which are infarction volume, neurology (behavioral tests), brain edema formation, dynamic methods, evaluating blood flow by laser, ultrasound Dopplers and MRI investigations are fully dependent on the environmental factors, such as animal care, genetic of animal spices, and environmental factors.

Another factor – age of the experimental animals – is a parameter in modeling of forebrain and focal ischemia in animals, which generally doesn't match with the clinical gerontological pictures. Generally, very young, healthy animals are used for stroke modeling; 95% of human strokes occur in the late middle age or in the geriatric age range and commonly are associated with one or more chronic diseases. Interaction between age-related changes in morphology, neurochemistry, and behavior on the ischemic cascade complicates the interpretation of mechanistic data, and pharmacological effects observed in younger animals may not necessarily translate to an older population [1, 2].

It was proven that mild hyperthermia during endarterectomy decreases the possibility of adverse postsurgical complication, which is reduction of the infarction volume and hemorrhage formation. These clinical observations for the first time were proven in experiments with rodents. Thus, constant physiological parameters and conditions of the surgery (cranial and rectal temperature [3], anesthesia, mean arterial blood pressure (MABP), blood gases) are the other important factors of stroke modeling. Even the speed of the vessel occlusion has an impact on the further developed severity of the infarction. The functional capacity of anastomoses depends not only on the site but also on the speed of vascular occlusion.

After abrupt occlusion, induced experimentally by clot embolism or mechanical obstruction, the maximal possible collateral blood supply is not immediately established because the vascular resistance of the small anastomotic vessels is much higher than that of the main supplying artery. However, if vascular occlusion develops slowly, collaterals may undergo arteriogenesis, i.e., an active outward remodeling of the vascular wall, leading to the increase in vascular diameter and conductance [4].

Of course the age of the patient, some genetic preconditioning, and social conditions are important parameters in the stroke research; however, in contrast to bench-top work, they cannot be regulated, controlled, and fully taken into account during clinical trials. The complexity of human disease diminishes possible positive effect of preclinical trials.

The scope of our chapter mostly includes description of the possible pitfalls of the experimental modeling techniques.

We also introduce the best pharmacological time frames for antioxidant, stimulating regeneration, and cell proliferation based on the evaluation of the mechanisms developing and promoting the exacerbation of the cerebral tissue damage.

26.1.1 Do We Need to Use the Nonhuman Primates in Our Research Instead of the Rodent Models?

The rodent genome is largely elucidated and serves as a basis to state about the strong similarity [5] with human. The mutations in rodents are useful to delineate human diseases and analyze direction which might be useful for the treatment of the pathologies [6].

Investigation of the stroke genes as well as the expression of them can't be fully understood without taking into the consideration the environmental factors having impact on their reflections.

Several important components of the neurological examination cannot be adequately tested in small animal models, and some components are inaccessible, such as language and other cognitive functions [7]. Also, experimental models of acute focal cerebral ischemia in subhuman primates and in larger mammals are probably similar to massive hemispheric infarcts in humans, particularly if the ischemia occurs within a closed skull containing cerebrospinal fluid. However, relevance to other kinds of ischemic strokes is limited [1].

Generally, all experimentally established widely used rodent models are divided into the focal and global ischemia types, whereas human strokes are characterized by the symptoms reflecting occlusion of the carotid, interior, exterior, middle, vertebrobasilar, spinal, basilar, and posterior arteries and by cerebellar, thalamic, and lacunar strokes [8].

Moreover, ratios of white matter to gray matter and the degree of microvascular collaterals are more comparable between baboons and humans but not between rodents and human.

Finally, humanized antibodies can be tested in primates and effective dosing determined with greater accuracy. Rodents in comparison with nonhuman primates can be investigated at a lower cost.

Numerous mechanisms individually might be investigated in rodents, including the participation of endothelial cells in the process of the ischemia development and exacerbation, microglia, angiogenesis, and involvement of the prooxidative as well as antioxidative compounds, such as xanthine oxidase, nitric oxide, glutathione, etc.

To identify the genes involved in stroke and stroke risk is important to eliminate variables dictated by the environmental factors, which are impossible to realize in human population. In order to process gene identification, animal models of stroke resemble an essential tool, which might be used to characterize and distinguish separate factors involved in complex of multifactorial diseases such as stroke.

More than 100 such studies have been reported to date, and notable successes have been achieved in elucidating the roles of the neuronal [9, 10], endothelial [11], and inducible [12] isoforms of nitric oxide synthase and of the cytosolic (CuZn) [13] and mitochondrial (Mn) forms [14] of the antioxidant defense enzyme superoxide dismutase in brain ischemia. Other studies of cerebral ischemia have used mutant mice to assess altered glutamate receptor subunit composition [15]; vascular adhesion molecules [16]; gene products related to nuclear damage, cell death and

survival, and apoptosis [17]; transcription factors and early-response genes [18]; cytokines [19]; apolipoprotein E [20]; and the role of endogenous tPA in the development of the brain infarction [21].

Cerebral ischemia models in rodents give impetus to the research in the field of stroke: it is more desirable and serviceable from ecological and ethical state of points. Small animal surgery is much faster and less expensive than in cats, dogs, and nonhuman primates. However, it is worth to mention that in comparison with human subjects, nonhuman primates, like other animal models, have several advantages: (1) constant environmental conditions can be maintained over long periods of time, greatly increasing the power to detect genetic effects; (2) different environmental conditions can be imposed sequentially on individuals to characterize genotype-environment interactions; (3) and complex pedigrees that are much more powerful for genetic analysis than typically available human pedigrees can be generated. Limitations of genetic research with nonhuman primates include cost and availability [22].

The best way to plan the preclinical studies is the evaluation of predrug impact in numerous stroke-related rodent models and transfer of the possible positively acting in vivo already evaluated compounds to the nonhuman primates testing conditions [23].

26.1.2 Pitfalls in Translation from the Bench to the Bedside

Numerous specialists discuss the issues related with the "getting results lost" in translation from the benchtop to the clinical practice [24–27]. Animal surgery is a highly complicated and time-consuming procedure. It is clear that lack of the knowledge as well as technical experience of the scientists might be the main reason for the failures of clinical trials.

However, clinical trials are not always organized appropriately because of wrong chosen predrug treatment time frame, doses, and frequency of the periods.

Robust animal models for ischemic stroke are now available. They may not faithfully reflect the pathophysiologic process, which leads to stroke, but most researchers agree that they are suited to the study of pathobiology that leads to the tissue damage once a major brain vessel is occluded [28–35], and the results might be applicable in the clinical settings if clinicians match the experimental time frames with the clinical pathophysiological stage of stroke.

The first pitfall, which is worthy to mention, is related with technical details and conditions of the benchtop research. One of the main parameters of stroke modeling is the infarction volume measurement, which generally might be performed in two routine histological ways: (a) H&E staining and (b) TTC staining. TTC staining is fast, not expensive, and easy to perform. However, it doesn't guarantee precise measurement of the infarction; it doesn't reveal the ischemic areas with totally no death territories in contrast to H&E staining. Utility of TTC staining instead of H&E might be the reason to overlook experimentally received positive or negative effects

of testable drugs or compounds. Also, the scanning of the stained brain slices, tracing, and calculation of infarction volume are not less important than histology by itself.

Approximately one third of human strokes are small-vessel lacunas, yet adequate animal models of lacunar stroke are lacking [36, 37]. According to the recent classification, lacunar infarcts are small subcortical infarcts that result from occlusion of a single perforating artery [38]. Several studies using diffusion-weighted MRI (dw-MRI) show that a small subcortical ischemic area corresponding the territory of a penetrating artery is the most common finding, being present in 84–94% of patients with a lacunar syndrome [39]. Despite the volume of infarction after lacunar stroke is negligible in comparison with widely utilized middle cerebral artery occlusion in experimental studies and in the clinic, symptomatic, lacunar infarcts present with severe lacunar syndromes, five of which are well documented: pure motor stroke, pure sensory stroke, sensorimotor stroke, ataxic hemiparesis, and dysarthria (i.e., clumsy hand).

On the other hand, small, TTC-stained, lacunar-like stroke results might not reflect the real clinical setting as the larger ischemic areas. Thus, the TTC staining in small animal species for visualization of 1–2 mm in diameter tissue damage is not realistic and might be the reason for generation of the pitfalls in benchwork with further translation of the non-accurate results into the clinical practice.

One of the most important pitfalls in the translational process from bench to bedside is that preclinical studies target the ischemic penumbra, whereas clinical trials do not. As Fisher [40], Baron [41], and others [42] have emphasized, the target of current neuroprotective therapy is the penumbra, an ischemic tissue that is functionally impaired but whose damage is potentially reversible [43, 44]. If reversible ischemic tissue is not present at the time of treatment, then neuroprotective therapy cannot be expected to work. The privileged opinion in the field evidences that the target for the treatment shouldn't be just cortical strokes but those with large volume of penumbra [40, 45, 46]. Patients with potentially salvageable penumbra tissue may be identified by functional neuroimaging [40, 46, 47]. According to PET studies by Heiss et al. [48] performed in patients within 3 h of acute stroke, the penumbra made up 18% (range, 8–34%) of the final infarct volume; 70% (range, 51–92%) was already critically hypoperfused, and 12% (2–25%) was sufficiently perfused. This observation implies that generally neuroprotective therapy might be applied to the small fraction of the infarct volume; however, there are patients with larger volume of penumbra and might benefit in the wider extent [46, 49]. The time frame of the treatment after stroke occurrence is generally within 3 h; however, recent PET study suggested that 45% of the final infarct (and in some patients, up to 85%) remained viable for up to 12 h [46, 50]. This investigation is evidencing about the prolonged treatment necessity with the neuroprotective compounds.

Also, in the experimental models for the evaluation of the neuroprotective compounds, the best models for testing the compounds are the temporal ischemia and the local injection of the neuronal death-inducing compounds such as kainite, hydrogen peroxide, etc.

Preclinical studies have demonstrated protection of gray matter, whereas clinical trials frequently enroll patients without specifying location of damage.

The role of neuroimaging becomes even more valuable for the immediate treatment or surgery after stroke as well as for further evaluation of the final efficiency of applied drugs: for the characterization of the targeted type of the brain tissue. The main concern is related to the fact that neuroprotective studies are focused on the treatment of the white matter, whereas the benchtop experiments mainly are targeting the gray matter [36, 51]. The brain of the rat contains greater proportion of the gray matter than white matter in comparison with the human brain [36] which might be the logical explanation for the failure of neuroprotective agents due to inability to preserve axonal viability [36, 37, 51]. The best models to overcome abovementioned problem are the intracranial injection of cell death-inducing agents.

Animal models are homogenous, whereas human disease is heterogeneous and might have several preconditionings.

One of the most important problems confounding the evaluation of the neuroprotective thrombolytic agents is the tremendous variability of human strokes and the difference between plasticity and general recovery processes in human stroke and experimental models. Benchwork during preclinical studies involves experiments relating middle cerebral artery (MCA) occlusion with controlled physiological parameters: oxygenation, cranial and body temperatures, glucose level, blood pressure, and interventional homeostasis representing parameters before, during, and after surgery [42, 52]. Experimental animals are healthy without other synchronal exacerbating diseases. In contrast, participants in clinical trials might suffer from multiple stroke types (pure subcortical white matter, cortical, and mixed cortical-subcortical strokes and, in some neuroprotective trials, both ischemic and hemorrhagic strokes). Moreover, there is a lack of standardized physiological parameters [42]. Comorbidities, old age, polypharmacy, recurrent ischemia, poor collateral circulation, and prior strokes are all related with the multiple variables of clinical stroke that may affect the severity of the developed ischemia or stroke, recovery and the treatment [53–55]. Hyperglycemia and other metabolic prognostic markers [56] may be particularly important variables to control or adjust future trials [57–59]. Carmichael thinks that instead of viewing stroke as a single disease entity, future trials should be more appropriately directed only to specific homogeneous stroke subtypes [60].

The best scenario to include multiple ethological parameters into the same investigation is the utility of the double and triple modeling systems. For instance, before stroke modeling, the diabetes mellitus pharmacological or genetic model might be initiated.

Preclinical studies have relied on infarct size to judge therapeutic efficacy, whereas clinical trials rely on behavioral outcomes.

Generally during animal experimental studies, reduction of the infarction size is the main parameter which evidences about efficiency of the therapeutic or prophylactic agents. In contrast, the clinical trials evaluate the efficiency of the experimental compounds referring to the neurological outcomes, mostly in the end of the third month [61, 62]. The antineutrophil adhesion agent Hu23F2G (LeukArrest) and the

glycine antagonist GV150526 (gavestinel; Glycine Antagonist in Neuroprotection study [63]) are several vivid examples to compare benchwork and the clinic. During the experimental work with animals, assessment of infarction volume was performed within hours of occlusion, while patients were evaluated [64] [65] a day or days later on for neurological and functional outcomes.

The battery of behavioral test should be applied after every benchtop stroke modeling to evaluate motor and cognitive impairments in rodents and other species.

Inconsistencies mentioned above indicate measurement of the infarction volume during the benchwork might not be enough and misleading for assessment of the testable efficiency compounds [65, 66]. Histological end points cannot be predictive for the assessment of the neuronal survival fate for the long period of time. In contrast, early behavioral assessment cannot be the hallmark for the improvement of the late histological pictures [65].

Moreover, some agents (e.g., basic fibroblast growth factor (BFGF), osteogenic protein-1) trigger functional improvement without affecting infarct size in animals, suggesting that they act by other mechanisms, e.g., enhancement of neural repair, rather than by neuroprotection [67, 68].

Motor, sensory and language recovery involves reorganization of particular parts of the brain after stroke: peri-infarct and ipsilateral connected cortical sites [37, 62, 69–71]. Improvement of the functions as a consequence of remapping in brain areas adjacent to stroke during successful rehabilitative therapies such as constraint-induced therapies [72, 73] goes far beyond studies of cell death and modeling neural repair in human stroke. This knowledge will require creation of the models of the stroke with defined location of the injury to identify the molecular and cellular events that produce reorganization and recovery in the spared circuits adjacent to the infarct.

Small size

Human strokes are mostly small in size. In large population studies and clinical trials, stroke volumes range from 28 to 80 mm^3 which is 4.5–14% in accordance with the volumetric measurements [74]. This relative size holds true in selected subsets of stroke, such as cardioembolic [75] or larger cortical-subcortical strokes [76]. Stroke with less severe symptomatology, and hence smaller stroke, would be more likely to lead to survival and hospital presentation [76] or inclusion in the trial design. Strokes with small volume are the most common types and might serve as the best target for neurotherapeutics. Larger strokes have worse functional recovery and more often present as malignant infarction. Malignant infarction comprises approximately 10% of all strokes and is a syndrome of large stroke producing progressive edema, arterial compression, and infarct expansion [77]. Treatment of malignant stroke subtype is almost ineffective, which leads to severe brain edema formation, herniation and death, and emerging craniotomy leading to the overall mortality up to 80% [77].

Despite the report about the approximate size of malignant stroke, which is about 50% of the ipsilateral hemisphere on imaging, a comparison of malignant infarction with estimated hemispheric size indicates that this type of stroke occurs when the

volume of the damaged tissue is greater than 39% of ipsilateral hemisphere, which is ten times larger than nonmalignant infarction [77].

Thus, the preclinical assessments of the compounds should include several stroke model types, including MCA occlusion, lacunar infarctions, and global ischemia models.

Brain collateral blood supply

Reperfusion of occluded vessel occurs in the organism in three main ways. First, the native hemostasis-supporting mechanisms lyse the forming clot in [78] 8% of all strokes as assessed by serial monitoring [78]. Second, collateralization occurs through the circle of Willis and leptomeningeal collaterals [79]. The field is full of controversy [80]. Third, leptomeningeal collaterals have been formally shown to provide peri-infarct blood flow and improved outcome in stroke [81].

Experimentally suggested time frames for the treatment with potential medicines of different mechanisms don't always reflect the chosen clinical settings. In experimental condition, generation of reactive oxygen species (ROS) initiated in microglia after focal temporal cerebral ischemia in mice (120 min) was prevented by antioxidants, which proved to be a protective tool [82]. In clinical settings, transferring of the experimental results might suggest fast utility of the antioxidant after plasminogen-activator-mediated or mechanical recanalization within 2–3 h after stroke.

The other exacerbating condition after stroke is the blood-brain barrier (BBB) opening because of the formation of cytotoxic and angio-determined edema. In accordance to the experimental results, this BBB opening has biphasic nature. It might be suggested that the early stage is determined by the angiogenetic type of edema formation, whereas the second type is the cytotoxic one.

After global ischemia in rats, induced by cardiac attack (CA), after occlusion of the cardiac vessels for 3.5–10 min, increase of BBB permeability within 2 h of ischemia was detected and lasted through the first post insult hour. The same scientific group states the BBB reopening was detected after 6 h again [83]. Similar results are presented after experiments with piglets. After CA induced by 9 min occlusion, fluorescein permeability was revealed 2 h after ischemia [83].

In another study in focal ischemia models in mice, BBB permeability was increased 1 h later after 2 h of MCA occlusion [84]. In Sprague-Dawley rats [85], penetration of Evans blue was detected 3 h after MCA occlusion. The most significant stage of BBB breakdown occurs in humans within 48–72 h postinfarction and is accompanied by increased brain edema volume [86]. One of the ongoing acute stroke treatment studies is designed to fulfill the BBB opening and vessel occlusion-dependent oxygen deprivation by the provision of the normobaric hyperoxia [87]. The design of the study includes treatment of the patients via mask from the first poststroke hour and continuation of the procedure during the following 4 h.

And again, authors haven't taken into consideration the experimental results, evidencing about double-faced opening of the BBB after stroke for the treatment with anti-edema formulations: during the first several hours and 9–24 h later.

26.2 Infiltration of the Leukocytes and General Activation of the Immune System

Another protective, meanwhile exacerbating mechanism of the stroke development is the penetration of the leukocytes via the adhesion receptors on the microvascular endothelium. Interestingly, this stage of the pathology with the following angiogenesis starts very early during the first few hours [88] in experimental settings.

Most of the data evidence that infections and inflammations influence on the development of the stroke. Particularly, pulmonary infections, which very often occur after stroke, might stand not just as a consequence of the vascular pathology but also serve as the basis for etiological triggering of the disease.

In accordance with one of the studies, where the authors were trying to delineate whether the generalized inflammation influences the development of stroke, the prevalence of prior infection was measured in stroke patients compared to neurological patient controls and was approximately threefold higher in patients the week prior to a stroke [89].

However, these data do not distinguish between leukocytes within the vasculature or parenchyma but do suggest that leukocytes accumulate after stroke in living patients [90]. Significant differences were noted between the chronic and acute stroke patients within the first week although asymmetry tended to persist for up to 5 weeks [90].

During the investigations of human ischemic stroke, polymorphonuclear leukocytes (PMNs) were observed in the infarcts with collaterals, embolic and lobar hematomas, but not in infarcts without collaterals. The authors stated that numbers of PMNs increased between 1 and 2 days, peaked at 3 days, and returned to baseline by 2 weeks. The peak increase for embolic and pale infarcts was about 5 cells/ml, while lobar hematomas peaked at 1,000 cells/ml [91]. Intravascular neutrophils were observed between 1 and 2 days after infarction and at later time points were observed infiltrating the parenchyma. They remained elevated weeks and months after infarction [92].

Similar results were observed in experimental settings. In mice, 24 h after 90 min focal ischemia, the elevated increase of the leukocytes was observed in the ischemic region [82, 93]. Regions of increased binding extended beyond the lesioned area identified by T1-weighted MRI and were observed between 5 and 53 days after injury [94].

Integrins and the receptors responsible for the binding and the transmission of the PML into the brain parenchyma appear within the first 24 h. P-selectin appears within 60–90 min, ICAM-1 within 4 h, and E-selectin 7 h after the onset of focal ischemia induced by the occlusion of the middle cerebral artery [95, 96].

An increase in P-selectin was also observed following permanent middle cerebral artery occlusion in rats, peaking between 2 and 8 h and returning to baseline by 1 day [89].

For anti-inflammatory investigations, the first 24 h of the treatment should be targeted intensively, and during the following month or two, the treatment should be continued with the decreased doses. The best anti-inflammatory treatment might be achieved after vessel reocclusion or surgical elimination of the developed hemorrhage or hemorrhagic transformation.

A new study designed to evaluate the impact of the neutrophils on the pathological processing of acute stroke is recruiting patients. The study is called "Impact of Neutrophil Activation in Acute Ischemic Stroke Patients Treated With Endovascular Therapy (NEUTROSTROKE)" [97]. According to the responsible specialist Dr. Laurence Salomon, inhibition of neutrophil recruitment several hours after the start of ischemia appears therefore too late to have a clinical relevance; thus, it is necessary to delineate time-dependent impact of neutrophils in acute ischemic stroke (AIS) and the predominant mediators in each time point to identify the appropriate therapeutic target and time window [97].

Clinical and experimental data suggest that neutrophil activation and extravasation are deleterious in AIS involving an increased risk of unfavorable outcome and hemorrhagic transformation (HT). However, clinical trials targeting neutrophil recruitment in AIS patients were negative, just because of the incorrect treatment time window and dosages.

In another clinical trial, called "Colchicine in Atrial Fibrillation to Prevent Stroke (CIAFS-1)," colchicine use is designed for the prevention of the stroke after atrial fibrillation (AF), which is the ethiological parameter for 20% of the stroke [98]. Prophylactic time treatment frame 3 months after AF is the perfect time frame for the visualization and detection of the results. However, it is necessary to mention that atherosclerosis, which is one of the leading ethological reasons for the stroke occurrence and might have an inflammation-related ethology by itself, is a chronic disease and might be developed during years or decades [99].

Recently completed clinical trial in Italy is evidencing about the positive outcome of the study with the treatment of the stroke patients with 80 mg of atorvastatin once per day. The authors state about the decrease of inflammatory plasma markers and improvement of the neurological deficit evidencing scores [100].

The "Vinpocetine Inhibits NF-κB-dependent Inflammation in Acute Ischemic Stroke" clinical trial was devoted to the investigations related to evidencing the role of NF-κB. Patients were receiving 30 mg of the drug by intravenous infusion once daily, for 14 consecutive days, beginning within 1 h after the baseline MRI and no later than 48 h after the onset of symptoms [101]. The results are not announced. However, intracellular mechanism, in this case mediated by NF-κB, might be initiated in 10–20 min if not faster, which means that the early several hours might be key points for the successful treatment.

Losmapimod inhibits p38 mitogen-activated protein kinase (MAPK), an enzyme which may play a central role in inflammation in the setting of heart attack. Inhibition of p38 MAPK may stabilize atherosclerotic plaques, reduce the risk of subsequent plaque rupture, indirectly improve vascular function and prevent subsequent thrombosis, and thus reduce infarct size and the risk of subsequent cardiac events [102]. "Clinical Outcomes Study to Compare the Incidence of Major Adverse

Cardiovascular Events in Subjects Presenting With Acute Coronary Syndrome Treated With Losmapimod Compared to Placebo (LATITUDE-TIMI 60)," a phase 3 clinical study, was initiated to evaluate the abovementioned medicine. The patients were taking losmapimod 7.5 mg twice daily. The patients had heart attack and were randomly assigned to receive 3 months of treatment with either losmapimod twice daily or placebo, which was administered in addition to the usual standard-of-care therapies for heart attack. Following the in-hospital period, subjects returned for outpatient visits at 4 and 12 weeks, as well as a follow-up visit at 24 weeks. The results are not published; however, by taking into consideration the fact that losmapimod for stroke treatment was used as the prophylactic agent and the mechanism has an intracellular cascade-based nature, there is a hope for positive clinical trial-related outcome/results [102].

Extensive literature investigations shows that lycopene, resveratrol, and soy isoflavones are key ingredients in diets that have long been known to reduce the risk of heart attack and stroke. However, they are normally poorly absorbed (not "bioavailable") [103]. The investigators of another study in the UK use in the clinical trial the unique forms of lycopene, soy isoflavones, and resveratrol, which were easily absorbed and used by the body [103]. The 12-month study had a prophylactic nature of the treatment, and the results haven't been published yet.

26.2.1 Microglial Activation or Injection as the Positive, Preventing Ischemia Development Tool

Studies indicate that elimination of endogenous microglia during stroke worsened the ischemic injury [104, 105]. Thus, microglia may play different roles at different times (early versus late stages) following stroke. Nevertheless, considerable evidence implicates microglia as potential targets in ameliorating the effects of ischemia [106].

Using 2 h transient occlusion model, injection of microglia within an hour after onset of experimental ischemia improved neuronal survival [107] and behavioral function [108]. Similarly, when microglia were injected into brains 4–7 days prior to experimental global ischemia, functional deficits in neurons were prevented [109]. Moreover, an increased number of surviving neurons was observed following transient global ischemia in gerbils when microglia were introduced as late as 24 h after ischemic onset [110]. Furthermore, when human microglia were transplanted into rats subjected to transient focal ischemia as late as 48 h after the insult, protection was still observed, including enhanced secretion of neurotrophic factors, reduction of apoptotic cells, and functional recovery in motor, sensory, reflex, and balance tests [111].

In another study, 48 h later after ischemia, injection of HMO6 human microglial cells reduced ischemic deficits and apoptotic events in stroke animals [111]. Detection was performed 3 days later after 2 h occlusion. Microglia, prophylactic,

or late injection had positive effect [107] in transient global ischemia model in gerbils when microglia were introduced as late as 24 h before ischemic onset [110]. Positive effect was seen 2 h as well as 3 and 7 days later on. When the spinal reflex was absent, both common carotid arteries were exposed through a ventral cervical incision and occluded with aneurysm clips for 5 min. The intra-arterial injection of microglia protects hippocampal CA1 neurons against global ischemia-induced functional deficits in rats as shown in results, 48 and 96 h later [109].

Microglia is very similar by its function with the immune system representing, first sanitary help reflecting cells type – macrophages. The role for the elimination of the dead cells or hemoglobin in the brain parenchyma is essential after stroke. However, these cells might have a deleterious effect in the late stages of pathology.

26.2.2 Microglial Activation as the Negative, Exacerbating Effect for the Ischemia Development

Tomimoto et al. presented data evidencing cytokines were co-expressed mostly in the microglia/macrophages and in a few astroglia in the brains with acute cerebral infarction and cerebral hemorrhage. In cases with cerebral infarction, they were observed as early as 33 h after the onset of the illness and persisted for up to 40 days after the onset. In one patient with cerebral hemorrhage who survived for 4 h, the cytokine-immunoreactive glial cells were confined to the margins of the hematoma. In contrast, cytokine-immunoreactive glias were distributed diffusely in one patient with cerebral hemorrhage who died 12 days after the onset of the illness. Labeling these cytokines was weak in the glial cells of control brains and those with neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and multiple system atrophy, insofar as there were no concomitant acute CVD foci [112].

Interestingly, after 90 min of middle cerebral artery occlusion and 3 days after microglia inhibition by minocycline, positive improvements were detected [93].

In one of the other studies, Yrjänheikki et al. [113] proved tetracyclines inhibit microglial activation and are neuroprotective in global brain ischemia in 6 days' time frame [113]. The major finding in this study is that treatment with minocycline and doxycycline provides a significant neuroprotection against global brain ischemia even when the treatment is started after the ischemic insult. The finding is in agreement with previous studies showing that doxycycline protects against mesenteric [114] and hepatic [115] ischemia-reperfusion injury.

While this study was in progress, Clark et al. [116] reported that doxycycline treatment reduces ischemic brain damage in transient focal brain ischemia when the treatment is started before ischemia. The presented results show that minocycline is even more potent neuroprotective compound than doxycycline and that minocycline blocks activation of microglia; the cells believed to have an active role in brain inflammatory and degenerative processes [113].

26.2.3 Astrocyte Damage

In the experimental settings, authors demonstrate that at 24 h after induction of ischemia, axonal damage determined by amyloid precursor protein (APP) immunohistochemistry was reduced by 70% in the transiently occluded animals from that in permanently occluded animals [117]. The other authors present different time points for axonal damages: increased in 40 min [118] [119], at 24 h [117, 118, 120], and at 24 h [117, 121] and decreased at 1 to 2 weeks [121]. Experimental results are evidencing about the possible benefit of early neuro- or glia-protective compound use for prevention of the cell death.

26.2.4 Generation of Free Radicals

ROS generation is a vitally important process. The formation of reactive free radicals exacerbates the pathology after stroke.

The reason for the formation of free radicals might be the peroxidation of the membrane-associated lipids or the robust excretion of the radicals by the macrophages as well as the other element of blood.

Oxidative stress is determined as a misbalance between the formation of ROS and the utilization of the last one. Generation of ROS in the normally functioning cells occurs in the organelles, as it was suggested previously, and transfers into the cytoplasm. Overwhelming amount of ROS might trigger the nonreversible cell death.

It is supposed that mitochondria are the major reservoirs for ROS generation in most mammalian cells.

The respiratory chain is mainly localized in the inner membrane of the mitochondria, and it is proved that complexes I and III are the main responsible components of the chain for the production of free radicals [122–125].

It is necessary to mention that 25% of free radical formation occurs because of the protein folding in the endothelial reticulum (ER) [126]. Protein disulfide isomerase (PDI) and ER oxidoreductin 1 (ERO1) are two major enzymes responsible for oxidative protein folding in ER as well as for catalyzing disulfide bond formation, isomerization, and reduction. In the process of oxidative protein folding, PDI receives electrons through catalyzing disulfide bond formation and is converted to the reduced form, which then transfers electrons to ERO1 to recycle itself [127]. The synthesis of the ROS by PDI/ERO1 is a regulated process, which might be controlled by the supply of flavin adenine nucleotide for ERO1 [128]. Because the folding and misfolding process of the protein is highly energy dependent, the depletion of ATP might trigger the formation of ATP in mitochondria and, consequently, the formation of ROS [129].

The leakage of Ca^{2+} ions into the cytoplasm also might trigger the production of ROS in mitochondria [129, 130].

NADPH oxidases (NOXs) are the key regulators in the pathological processes such as ischemia-reperfusion, diabetes, neurodegenerative diseases, and atherosclerosis, as well as vessels related with other diseases [131–134]. The basic catalytic subunit of NOXs contains a C-terminal dehydrogenase domain featuring a binding site for NADPH and a bound FAD, as well as an N-terminal domain consisting of six transmembrane alpha helices that bind two heme groups. Once activated, cytosolic NADPH transfers its electrons to FAD, which in turn passes electrons sequentially to the two hemes and ultimately to molecular O_2, forming superoxide anion (O^{2-}) [133, 135]. Oxidants from various sources may affect the translocation of important regulatory subunits of NOX2 (and NOX1), p47phox, by oxidation of thiol groups in PKC with subsequent phosphorylation and translocation of these subunits to the membrane causing activation and superoxide formation by NOX2 (and NOX1). Likewise, those oxidants cause oxidation of thiols in protein disulfide isomerase (PDIox) leading to the association with p47phox (maybe also NOXO1 or NOXA1), translocation of this dimer to the membrane, and activation of NOX2 (and NOX1).

Endothelial nitric oxide synthase (eNOS) redox switches might be based on S-glutathionylation, PKC- and protein tyrosine kinase-2 (PYK-2)-dependent phosphorylation, oxidative BH4 depletion, disruption of the zinc-sulfur cluster as well as asymmetric dimethylarginine (ADMA) synthesis/degradation processes, all of which contribute to the regulation of its enzymatic activity (GSH, glutathione; GSSG, glutathione disulfide) [136].

Since AT-II-induced oxidative stress is largely due to activation of mtROS formation [137–139], the conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) under chronic AT-II treatment or increased levels of this vasoconstrictor (e.g., in diabetes or hypertension) could be mainly driven by mtROS formation. A direct proof for the interaction between mtROS and XO activity was based on the improvement of cardiac complications and XO activation in a model of heart failure by the therapy with the mitochondria-targeted antioxidant mitoquinone [140, 141].

Hemoglobin and myoglobins might serve as a source for the formation of free radicals. Reduction of nitrite to ·NO under hypoxic conditions serve as a putative autoregulatory mechanism for capillaries and muscle [142]. Nitric oxide synthases also might serve as a source of ·NO [142].

The other enzyme, which will be highlighted in the frames of this chapter, is xanthine oxidoreductase (XOR). Under normal circumstances, most amount of this enzyme exists in the form of NAD-dependent cytosolic dehydrogenase (XDH).

XO as well as the XDH are two enzymes responsible for the last steps of purine metabolism and hydroxylation of a wide variety pyrimidine, pterin, and aldehyde substrate. XOR has been isolated from a wide range of organisms, from bacteria to man. All of these proteins have similar molecular weights and composition of redox centers [143, 144]. The mammalian enzymes, which catalyze the hydroxylation of hypoxanthine and xanthine, the last two steps in the formation of urate, are synthesized as the dehydrogenase form. XDH exists mostly as such in the cell but can be readily converted to oxidase form, XO, by oxidation of sulfhydryl residues or by proteolysis. XDH shows a preference for NADH reduction at the flavin adenine

dinucleotide (FAD) reaction site, whereas XO fails to react with NADH and exclusively uses dioxygen as its substrate, leading to the formation of superoxide anion and hydrogen peroxide [144]. The active form of the enzyme is that of a homodimer of molecular mass 290 kDa, with each of the monomers acting independently in catalysis. Each subunit contains one molybdopterin cofactor, two spectroscopically distinct [2Fe-2S] centers, and one FAD cofactor. The oxidation of xanthine takes place at the molybdopterin center (Mo-pt), and the electrons thus introduced are rapidly distributed to the other centers by intramolecular electron transfer [145]. The full amino acid sequences of XOR enzymes from various sources have been deduced by sequencing of respective cDNAs or genes. They all consist of approximately 1,330 amino acids and are highly homologous with bovine milk enzyme (1,332 residues) showing 90% sequence identity to the human liver enzyme (1,333 residues) [146]. Also, it is necessary to mention that main cellular localization of XOR is cytoplasm.

Evidences exist that allopurinol, a xanthine-oxidase inhibitor, reduces delayed cell death in animal models [147] of perinatal asphyxia and in human patients with other forms of organ reperfusion injury. Thus, allopurinol pretreatment suppresses generation of superoxide anion radicals, making XO the main enzyme responsible for the oxidative damage caused after oxidative stress, early inflammation, and endothelial injury [148]. By the other group of authors, it was demonstrated that XO and cyclooxygenase are mostly responsible for postanoxic damage of the brain [149]. Also, it was demonstrated that hydrogen peroxide damage is mediated through the activity of the XO in cerebellar granule neurons obtained from 8-day-old Sprague-Dawley rat pups [150]. By the other work, it was shown that superoxide anion generated by the XO as well as singlet oxygen initiated the apoptosis-like cell death, whereas hydrogen peroxide was generated because of the activity of the glucose oxidase and glucose deprivation in neuronal cell culture initiated necrosis [151].

It is documented also that allopurinol is very well tolerated and attenuates the rise in ICAM-1 level after stroke. Also, it was shown that uric acid level was decreased, making allopurinol a desirable drug for the treatment after stroke [152]. On the other hand, there is a work, evidencing about the irrelevance of the XO [153] as well as the nitric oxide synthase with the processes of the protection against TNF-alfa-induced inflammation in the brain vasculature [154]. The work delineated the interaction between the free radical formation (mediated due to the presence of XO and xanthine in the medium) and IL-1 as well as the inhibition of PA-1. This work does demonstrate the probability of no-reflow phenomenon dependence after ischemia-reperfusion on the xanthine oxidase activity [155] in cardiac microvascular endothelial cells. Neutrophils contribute to ischemic brain injury in adult animals. The role of neutrophils in perinatal hypoxic-ischemic (HI) brain injury is unknown. It was shown that allopurinol reduces neutrophil accumulation after tissue ischemia and is protective against HI brain injury [156].

Most of the vasculature diseases related with the ROS generation are treated with the antioxidants, which are not too effective (vitamins C and E and β-carotene). However, it is necessary to mention that these compounds are able to remove

chemically excessive amount of ROS from the model systems of vascular diseases [157].

Explanations why vitamin E is ineffective during the clinical trials are perfectly suggested and described by Drummond et al. [158]. In accordance to him, the first most important reason why the clinical trial failed is the wide diversity of the diseases of the patients involved, particularly vascular diseases, and also the application of the vitamins was too late for the initiation of significant protection.

Along with the existing known antioxidants, we are proposing, proving, and stating about the antioxidant abilities of pyridoxine, one of the subcomponents of vitamin B complex. We were also able to prove that pyridoxine, by suppressing the XO activity, might influence the proliferative activity of the human brain-derived primer cell culture [159].

During our own experiments, we have determined the growth of the cells as well as their death in the presence of single components of vitamin B complex: nicotinamide, riboflavin, pyridoxine, and thiamine number of the cells in the field: (1668,50 ± 189,51; 1738,33 ± 196,60; 2556,17 ± 355,68; and 2179,00 ± 223,55, respectively). As a positive control in these experimental series, BFGF was used. It was clear from the experiments that in comparison with the two types of negative controls (number of the cells on the 1st (820,14 ± 50,07) and 4th (1562,94 ± 146,45) days as well as positive control – growth of the cells in the presence of BFGF (2131,08 ± 144,59)), the most effective components of vitamin B complex were pyridoxine and thiamine after calculation of the number of cells after 12 days of growth. In comparison with the nicotinamide and riboflavin, these two components vividly have increased the growth of the cells even on the 4th day [159].

During the next set of the experiments, we evaluated the influence of the vitamin B complex subcomponents on the activity of XO. Percentile of inhibition/non-inhibition of all subcomponents of vitamin B complex, thiamine, pyridoxine, riboflavin, and nicotinamide (−201,39 ± 32,76; 160,00 ± 60,00; −120,91 ± 39,091; and −152,73 ± 107,27, respectively) was in statistically significant way different from the control sample (31,0343 ± 6,9222, $p < 0{,}05$), which is the percentile of inhibition of XO in the presence of allopurinol and absence of subcomponents. The most prominent inhibitor of XO was pyridoxine.

Our previous results [160] indicated that the early inhibition of the XO in the human brain-derived cell culture utilizing allopurinol [161, 162] was initiating the increase in the number of the cells in comparison with the later stage of inhibition.

We also showed that similar to allopurinol, addition of pyridoxine into the cell medium during the early stages of the treatment initiates the increase in the number of the cells, whereas in the late stages, that process was suppressed.

Along with the existing systems of ROS generation, we are highlighting the role of XOR in processes of ROS generation and cell proliferation. As the final enzyme of purine catabolism, XOR is able to stand as regulating enzyme, and its inhibition by allopurinol as well as by newly delineated native compound, pyridoxine, might initiate neurogenesis and cell proliferation after stroke.

As the antioxidant, we propose utility of XO inhibitors in the early stages of ischemic stroke. As the proproliferative compounds, we suggest utility of XO inhibitors during the late stages with 1–2 years of continuation.

26.3 Why Clinical Trials Fail?

Time and dose are two parameters for treatment of diseases, as was stated in the twelfth century by Paracelsus.

By taking into the consideration all the abovementioned mechanisms, any compound targeted to prevent the pathology-based particular mechanism should be administered timely. The time of treatment should match with the time frame of the targeted developing pathology-based mechanism.

Allopurinol as the compound preventing formation of free radicals based on the inhibition of XO was passing the clinical trials in several different countries.

One of the trials included patients with the ischemic stroke or transient ischemic attack (TIA). Central blood pressure (CBP) and carotid intima-media thickness (CIMT) were measured in that particular study as the parameters for evaluation of the stroke occurrence. In accordance to the authors, allopurinol lowered CBP and reduced CIMT progression at 1 year compared with placebo in patients with recent ischemic stroke and TIA [163].

The other group of scientists in their early studies showed that allopurinol use might prevent the increase of ICAM-1 levels after stroke [152] and stimulate improvements in cerebrovascular function in those with type 2 diabetes [164]. In their report, 50 patients were enrolled with recent (within 6 months) subcortical stroke and evaluated the effect of a 3-month course of 300 mg allopurinol once daily vs. placebo on cerebrovascular reactivity (CVR) [165]. According to the authors, no any benefit was registered.

According to Dawson's opinion, many previous studies have investigated the effect of short-duration treatment and even single dose of allopurinol treatment and have consistently revealed benefit on measures of vascular function [166]. The scientist thinks it is of interest that two of the three published studies [167–169] testing a longer-duration treatment have failed to yield benefit. Any beneficial effects of XO inhibition on the vasculature may be short-lived and bypassed by other sources of oxidative stress. It may also be that upregulation of XO occurs with time, meaning any improvements may not be sustained for a prolonged period. He also thinks that uric acid (UA) levels were not elevated in population and studies in the setting of heart failure suggest that the vascular effects of allopurinol are demonstrable only where XO activity is upregulated and UA levels are increased [170], although the effects may not be mediated by UA reduction per se [171].

Dawson with colleagues led the clinical trial regarding type 2 diabetes and basal nitric oxide (NO) activity impact on the development of the stroke. They have investigated whether decrease of XO activity by allopurinol during 2 weeks will be beneficial as the treatment of the cerebrovascular pathology.

Dawson et al. concluded XO inhibition with allopurinol in the group consisting of 14 patients improves cerebral NO bioavailability [164].

In our own experimental investigations, early inhibition of XO activity after intracranial hydrogen peroxide injection in rats was diminishing BBB damage [172].

Our investigations are evidencing that XO inhibition is vital for the prevention of ROS generation and brain parenchyma damage.

In the earlier studies, Muir with colleagues [152] performed the clinical study regarding the influence of allopurinol on the upregulation of the inflammatory activity of the organism, particularly ICAM-1. The group of investigation included 50 patients and the treatment period lasted for 6 weeks. Participants were treated with high (300 mg once a day)- or low (100 mg once a day)-dose allopurinol, and they investigated the levels of uric acid and circulating inflammatory markers after ischemic stroke. Obtained results were promising: allopurinol might serve as the "preventive measure after stroke."

Khan with colleagues led a small clinical double-blind trial with evaluation of the augmentation index in 30 patients with high blood urate. The investigation lasted 8 weeks with the 300 mg dose once per day treatment scheme [173]. Investigators concluded that allopurinol has beneficial effects on augmentation index, a validated measure of vascular function.

Most of the clinical trials were led with the short-duration treatment of allopurinol for stroke patients.

To clarify whether the XO inhibitor – allopurinol – might have a positive impact on the development of the stroke, a new clinical trial is organized called "Xanthine Oxidase Inhibition for Improvement of Long-term Outcomes Following Ischemic Stroke and Transient Ischemic Attack (XILO-FIST)" [174]. Results haven't been presented yet.

Thus, analyzing the involvement of XO in the pathophysiology of the stroke, and taking into the consideration that the enzyme inhibitor suppresses the ICAM-1 initiation and appearance, we can conclude that it is one of the most desirable targets for the prevention of ROS formation in the macrophages and for decreasing the initiation of ICAM-1 on the surface of the endothelial cells.

Thus, allopurinol should be used during the first several days after stroke when the immune system is aggressively attacking the neuronal parenchyma of the brain.

Also, in our long-term animal experiments with XO inhibitors and KI-67 marker utility, we concluded that the enzyme might be involved into the processes of neurogenesis (results are not published yet). Thus, poststroke allopurinol treatment during 1–2 years after stroke might initiate regenerative processes and improve the state of the patients with lacunar strokes as well as with the larger stroke types.

We have analyzed only one compound and only one enzyme involved in two different poststroke pathological processes. However, before any clinical trial, to prevent negative and not objective results, the time, targeted mechanism and its development time period, stage of the pathology, as well as the doses of the compounds should be carefully analyzed and planned.

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