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ORIGINAL ARTICLE

Therapeutic time window for the neuroprotective effects of NGF when administered after focal cerebral ischemia

Ji-Ping Yang · Huai-Jun Liu · Hua Yang · Ping-Yong Feng

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Abstract In the present study, we evaluated the neuroprotection time window for nerve growth factor (NGF) after ischemia/reperfusion brain injury in rabbits as related to this anti-apoptosis mechanism. Male New Zealand rabbits were subjected to 2 h of middle cerebral artery occlusion (MCAO), followed by 70 h of reperfusion. NGF was administered after injury to evaluate the time window. Neurological deficits, infarct volume, neural cell apoptosis and expressions of caspase-3 and Bcl-2 were measured. Compared to saline-treated control, NGF treatment at 2, 3 and 5 h after MCAO significantly reduced infarct volume, neural cell apoptosis and expression of caspase-3 ($P < 0.01$), up-regulated the expression of Bcl-2 and improved functional recovery ($P < 0.01$). However, treatment at latter time points did not produce significant neuroprotection. Neuroprotection treatment with NGF provides an extended time window of up to 5 h after ischemia/reperfusion brain injury, in part by attenuating the apoptosis.

Keywords Focal cerebral ischemia · Nerve growth factor · Neuroprotection · Time window · Bcl-2 · Apoptosis

Introduction

Stroke is the third cause of death after heart disease and cancer and is the leading cause of severe disability in the developed countries. Thrombolysis by intravenous administration recombinant tissue plasminogen activator (r-tpA) is the only Food and Drug Administration (FDA)-approved treatment for acute stroke. However, fewer than 5% of stroke patients receive this therapy because of the narrow time window (<3 h) [1]. Furthermore, once reflow is established, reperfusion injury may limit the usefulness of intervention [2]. These observations suggest that there is a compelling need to develop treatments of ischemic stroke designed specifically to reduce neurologic deficits, for example, neuroprotection, which can target the vast majority of patients. Many neuroprotective drugs interrupting one or more of the pathway of ischemic cell death after stroke have been effective in reducing infarct volume in various animal models, but have failed to demonstrate effective in-phase III human studies. Several concomitant factors have likely contributed to these failures, including poor preclinical pharmacodynamic evaluations, especially narrow treatment windows [3]. Obviously, the development of preclinical testing must focus on these neuroprotective factors to improve opportunities for successful transitions from preclinical to clinical studies [4]. So, the therapeutic time window becomes an important parameter to study and evaluate the neuroprotective effect [5].

Nerve growth factor (NGF) is an evolutionarily conserved polypeptide neurotrophin, which plays a crucial role in the sympathetic and sensory nervous systems [6]. Recent studies have indicated that NGF can prevent the metabolic or excitotoxic injury of cultured hippocampal and cortical neurons [7, 8]. NGF has also been shown to stimulate neurite outgrowth in PC12 cells by activating either PI-3 K or PLC-γ [9, 10]. Furthermore, mitogen-activated protein kinase (MAPK), which follows the PLC-γ activation after the phosphorylation of Trk receptors by neurotrophins, is involved in the process of

J.-P. Yang · H.-J. Liu (✉) · H. Yang · P.-Y. Feng
Department of Medical Imaging,
The Second Hospital of Hebei Medical University,
215 West Heping Road, Shijiazhuang
050000, Hebei Province, China
e-mail: huaijun_hb@yahoo.com.cn

neuronal differentiation [11]. Exogenous NGF can protect cultured hippocampal neurons from excitotoxic damage induced by a high concentration of glutamate, hypoglycemia and iron-induced degeneration [7, 12–14]. In brain ischemia, NGF mRNA expression is up-regulated transiently and this seems to be protective for neurons [7, 13, 15]. Upregulation of NGF after injury to the central nervous system (CNS) is probably an endogenous protective mechanism to maintain neuronal survival in lesioned brain regions. Similarly, pretreatment with NGF or NGF administration in the early post-ischemic period decreases ischemia-induced brain infarction, brain edema, density of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) (+) neurons, and immunoreactivity of extracellular signal-regulated kinase (ERK) and caspase-3 in the ischemic cortex [7, 8, 15–22], which suggests that NGF may reduce ischemic insults by attenuating apoptosis and/or necrosis. However, this ultra-early administration is not practical in the clinical setting. Thus, there is an additional concern with later administration of NGF and few studies have assessed the effects of NGF when administered after the onset of ischemia. Therefore, in the present study, we investigated the therapeutic time window and molecular mechanisms for neuroprotective effects of NGF when administrated after focal ischemia injury in rabbits.

Materials and methods

Animals

A total of 78 male New Zealand white rabbits weighing 2.5–3.0 kg were randomly divided into three groups: NGF-treated group ($n = 60$), saline-treated group ($n = 12$) and sham-operated group ($n = 6$). The rabbits were housed in separated cages and the room was kept at 24 ± 1°C temperature and 50–60% humidity, under a 12:12-h light/dark cycle and with access to food and water ad libitum. All experimental procedures were approved by the local animal care committee and carried out in accordance with the guidelines of the National Institutes of Health on animal care and the ethical guidelines for investigation of experimental pain in conscious animals. Anesthesia was induced with intravenous injection of 20 mg/kg pentobarbital sodium and, if necessary, maintained with a further 5 mg/kg. PE-50 polyethylene tubing was inserted into the left femoral artery for monitoring arterial blood gases, serum glucose and body temperature before, during and after operation. For pain relief, the surgical wounds were anesthetized in advance by using 2% lidocaine (0.1 ml).

Surgery

MCA occlusion

The model of intraluminal suture was used for induction of focal cerebral ischemia, as formerly described [22, 23]. Briefly, the left common carotid, internal carotid and external carotid arteries were exposed via a midline incision in the neck. Then the left common and external carotid arteries were ligated proximally (near the bifurcation) with 4-0 surgical sutures. A guide wire (RF SP26137 M, TERUMO, Japan) with a diameter of 0.53 mm was inserted into the internal carotid artery from the distal common carotid artery until the tip occluded the origin of the middle cerebral artery (MCA). The placement of guide wire in the MCA was confirmed by a contact X-ray. The guide wire was maintained in place for 2 h and then withdrawn to allow reperfusion. Magnetic resonance imaging (MRI) measurements were performed in all rabbits between 1.75 and 2 h after MCA occlusion (MCAO). Animals subjected to the same surgery without vascular occlusion served as sham-operated group ($n = 6$).

Study design

Rabbits received intracerebral microinjection of 50 μl NGF (16 μg/l) [22] at different time points, 2, 3, 5 or 8 h post-MCAO ($n = 15$ per time point) by the perifocal region route. Control animals received saline ($n = 12$) at 2 h after onset of MCAO. After 2 h of MCAO and 70 h of reperfusion, neurological deficits, infarct volume, neural cell apoptosis and expression levels of caspase-3 and Bcl-2 were measured.

MRI protocol

Diffusion weighted imaging (DWI) was conducted to document injury and to provide a guide for intercerebral injection and dissecting tissues from ischemic regions. All animals were imaged in a 1.5-T scanner (Toshiba Visart, Japan), with quadrature knee coil. DWI was performed using a spin-echo echo-planar imaging sequence [TR = 12,000 ms, TE = 108 ms, NA = 3, 2 different b values ($b = 0$ and $b = 900$ s/mm^2), FOV = 16.5 cm × 16.5 cm, matrix = 96 × 96, 5 slices, slice thickness = 5 mm]. If hyperintensity was observed in the MCA territory, apparent diffusion coefficient (ADC) maps were constructed by acquiring a set of five images with increasing diffusion gradient amplitude. The ADC-based measurements of the size of the ischemic core and penumbra regions were performed as follows: the ranges of the lowest ADC values and ADC values in the matching contralateral anatomic regions were derived from the ADC maps such that the

region with ADC values lower than the mean of the lowest ADC values plus 1 SD was referred to as the core [24]. The region with ADC values higher than the mean of the ADC values plus 1 SD in the core but lower than the mean of normal ADC values minus 2 SDs in that region was referred to as the ischemic penumbra [24].

Administration

After the MRI examination, animals were placed in a stereotaxic frame, and a burr hole (<2 mm) was made at the left parietal skull 3 mm posterior from the infraorbital margin and 10 mm lateral to the midline till the dura mater. Our qualitative preliminary observations and previous research [22] suggest that a single dose of 50 μl NGF (16 μg/l) immediately after reperfusion by the route of perifocal region has protective effects, which could decrease immunoreactivity of ERK and caspase-3 in the ischemic cortex and hippocampus. So NGF was dissolved in PBS to prepare 16 μg/l as described previously [22]. Single intracerebral microinjection of 50 μl NGF was conducted directly into the left ischemic penumbra cortex over a 10-min time period at a depth of 6 mm from the skull surface using a stereotactic micromanipulator. The needle was retained in place for 5 min after each injection. The same volume of saline served as treatment for the saline-treated group (control). After injection, the defect of the skull was covered with bone wax and then the skin was sutured.

Neurological evaluation

Animals were examined for neurological function 24 and 72 h after the onset of occlusion. The neurological findings were scored according to Purdy scoring method [25]. Each animal was examined for motor function (score = 4), consciousness (score = 4), head turning (score = 1), circling (score = 1) and hemianopsia (score = 1). The lowest was 2 points, suggesting no neurological impairment; the highest was 11 points, suggesting that animals lost consciousness or died.

TTC staining

For quantitative infarct volume, rabbits were killed at 72 h of MCAO with overdosed barbiturate. After the skulls were removed, the brain was rapidly taken and cooled in cold saline for 10 min. The brains were then coronally sectioned into five 5-mm thick sections. The brain slices were incubated for 30 min in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C, and fixed by immersing in a 4% buffered formalin solution. Infarct size was quantified by using an image-processing software package (Beihang University, Beijing, China). To compensate for the effect of brain edema, the infarct volume was measured by a commonly used indirect method [26] [formula: infarct volume = contralateral hemisphere volume-(ipsilateral hemisphere volume-infarct volume)].

TUNEL assay

Neuronal damage was assessed by histological analysis of brain sections at 72 h of MCAO. The number of TUNEL-labeled cells in the penumbral cortex was counted on a computer screen grid from at least five random fields (×400) of each animal. Results were expressed as the average number of cells per field.

Flow cytometry analysis (percentage of apoptotic cells)

Flow cytometry was used to determine the extent of DNA fragmentation in cells extracted from the penumbral cortex. In separate rabbits ($n = 5$ per NGF-treated group, $n = 4$ saline-treated group, $n = 2$ sham-operated group), cortical tissue from the penumbral cortex was dissected immediately after killing. The cells in this tissue were dissociated by repeated aspiration in saline using a glass micropipette. Then a flow cytometer was used to calculate the cell apoptotic rate as reported by others [22, 27]. Absorbance ratio was measured at 488 nm of wavelength.

Immunohistochemistry

For histological examination, a total of 26 male New Zealand white rabbits ($n = 5$ per NGF-treated group, $n = 4$ saline-treated group, $n = 2$ sham-operated group) received pentobarbital sodium anesthesia and then were transcardially perfused with phosphate-buffered 4% paraformaldehyde. Five coronal paraffin sections (6 μm thick each) per animal corresponding to the same places in MR images were cut and processed for immunohistochemistry. The sections were washed in PBS, incubated in 3% H_2O_2 in PBS for 10 min, in blocking solution (10% goat serum in PBS) for 30 min at room temperature, and with rabbit polyclonal anti-caspase-3 (SC-7272) and anti-Bcl-2 (SC-7382, Wuhan Boshide Inc. Wuhan, China, 1:75) at 4°C overnight. Sections were washed and incubated. The reaction was stopped with DAB. The mean optical density values of the positive expression level of caspase-3 and Bcl-2 of five non-replicated fields were measured with the Meta Morph microimage analysis system under 400-fold light microscope.

Statistical analysis

All measurements in this study were performed blindly. Results were expressed as mean ± SD. Histological

outcome measures were compared using one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons. Nonparametric ANOVA on ranks was used to compare total neurological scores among groups. Results were considered significant at $P < 0.05$.

Results

Infarct volume

A total of 26 rabbits ($n = 5$ per NGF-treated group, $n = 4$ saline-treated group, $n = 2$ sham-operated group) were used for TTC analysis. No cerebral infarct was seen in the sham-operated rabbits. At 72 h of MCAO, a significant reduction of infarct volume was found when NGF was administered at 2, 3 or 5 h after MCAO, compared to the saline-treated group ($P < 0.01$, Fig. 1a). There was no significant difference between treatment with NGF 8 h post-MCAO and saline-treated control ($P > 0.05$, Fig. 1a).

Neurological deficits

No neurological deficit was seen in sham-operated rabbits. When tested at 24 or 72 h of MCAO, saline-treated rabbits displayed a severe neurological deficit. NGF treatment 2, 3 and 5 h post-MCAO produced significant improvement in neurological score as compared to saline-treated control (Fig. 1b, $P < 0.01$). There was no significant difference between treatment with NGF 8 h post-MCAO and saline-treated control (Fig. 1b, $P > 0.05$).

Flow cytometry analysis showing percentage of apoptotic cells

A total of 26 rabbits ($n = 5$ per NGF-treated group, $n = 4$ saline-treated group, $n = 2$ sham-operated group) were used for flow cytometric analysis. Cell apoptosis was characterised by a diploid nucleoliform peak (AP peak). Quantitative analysis of cell apoptosis could be performed according to the scale for which AP peak accounted. Apoptosis was rare in sham-operated group. At 72 h of MCAO, the percentage of apoptotic cells in the penumbral cortex markedly increased in saline-treated group than that in sham-operated group ($P < 0.01$, Fig. 2). The percentage of apoptotic cells in the penumbral cortex significantly reduced in NGF treatment 2, 3, and 5 h post-MCAO than in saline-treated group ($P < 0.01$, Fig. 2). There was no significant difference between treatment with NGF 8 h post-MCAO and saline-treated control ($P > 0.05$, Fig. 2).

TUNEL-positive cells

A total of 26 rabbits ($n = 5$ per NGF-treated group, $n = 4$ saline-treated group, $n = 2$ sham-operated group) were used for TUNEL staining. The number of TUNEL positive was rare in the sham-operated group. At 2 h of MCAO and 70 h of reperfusion, the number of TUNEL-positive cells in the penumbral cortex significantly decreased after treatment with NGF 2, 3 and 5 h post-MCAO compared to the saline-treated group (7.6 ± 1.5, 11.0 ± 2.9, 17.8 ± 2.4 vs. 32.8 ± 2.6, both $P < 0.01$). There was no significant difference between treatment with NGF 8 h post-MCAO and saline-treated control (31.0 ± 2.9 vs. 32.8 ± 2.6, $P > 0.05$).

The expressions of caspase-3 and Bcl-2

In the sham group, weak caspase-3 immunoreactivity was detected. The expression of caspase-3 in the penumbral cortex markedly increased in saline-treated rabbits. Treatment with NGF 2, 3 and 5 h post-MCAO produced significant reduction in expression of caspase-3 as compared to saline-treated control ($P < 0.01$, Fig. 3). There was no

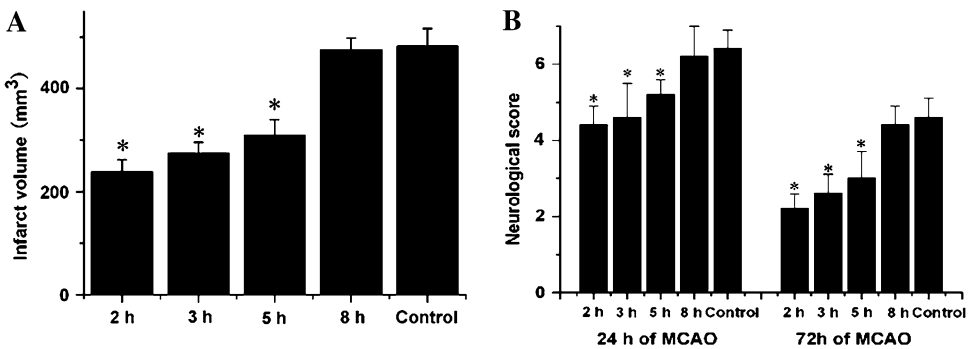

Fig. 1 Quantitative effects of NGF on infarct volume (**a**) and neurological function (**b**) after MCAO (Mean ± SD). **a** NGF treatment 2, 3 and 5 h post-MCAO produced significant reduction in infarct volume as compared to saline-treated control at 24 or 72 h of MCAO. There was no significant difference between treatment with NGF 8 h post-MCAO and saline-treated control. **b** NGF treatment 2, 3 and 5 h post-MCAO significantly improved neurological recovery as compared to saline-treated control at 24 or 72 h of MCAO. There was no significant difference between treatment with NGF 8 h post-MCAO and saline-treated control; *versus MCAO group, $P < 0.01$

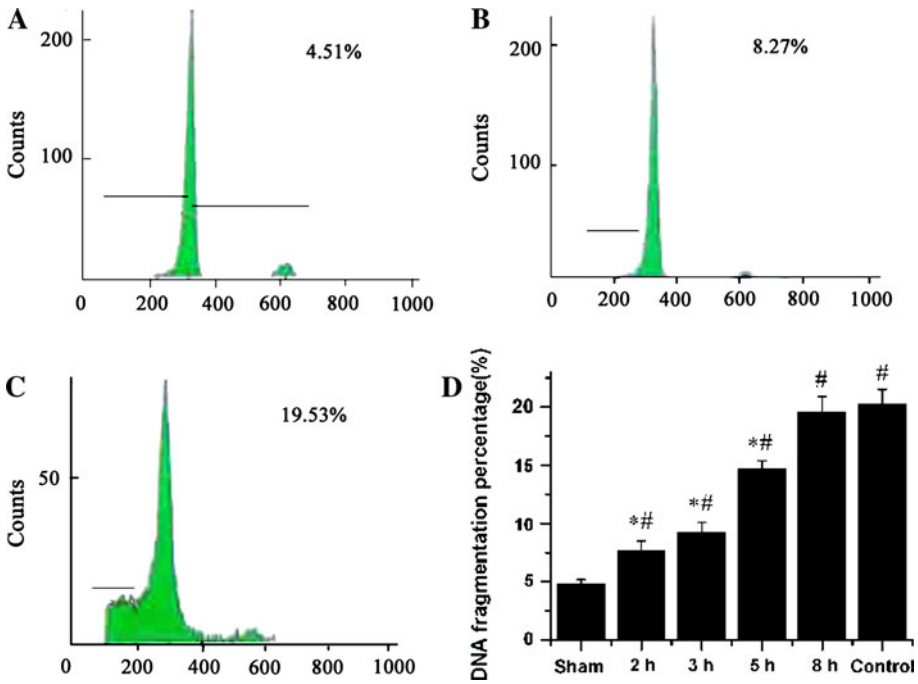

Fig. 2 Flow cytometry analysis showing percentage of apoptotic cells at 72 h of MCAO. Apoptosis was rare in the sham-operated group (**a**). The percentage of apoptotic cells in the penumbral cortex was significantly reduced with NGF treatment 2 (**b**), 3, and 5 h post-MCAO than that in the saline-treated group. There was no significant difference between treatment with NGF 8 h (**c**) post-MCAO and saline-treated control. **d** Quantification of the percentage of apoptotic cells in the penumbral cortex; *versus saline-treated group (control), $P < 0.01$. # versus Sham-operated group, $P < 0.01$

significant difference between treatment with NGF 8 h post-MCAO and saline-treated control ($P > 0.05$, Fig. 3).

In the sham group, no Bcl-2 immunoreactivity was detected in the cerebral cortex. The expression of Bcl-2 in the penumbral cortex markedly increased in saline-treated rabbits. Treatment with NGF 2, 3 and 5 h post-MCAO significantly increased expression of Bcl-2 as compared to saline-treated control ($P < 0.01$, Fig. 4). There was no significant difference between treatment with NGF 8 h post-MCAO and saline-treated control ($P > 0.05$, Fig. 4).

Discussion

The extent of the ischemic penumbra is critical to the success of any acute stroke therapy, including thrombolysis. Patients without a penumbra are unlikely to benefit from any acute tissue salvage therapies, as irreversible injury had already occurred [28]. Conversely, patients with penumbral tissue may benefit from tissue salvage therapies, regardless of the duration of symptoms [28]. A necessary prerequisite of the effective therapeutic strategy is the existence of functionally impaired but viable and potentially salvageable brain tissue (i.e., penumbra) [29].

NGF is the prototype of the neurotrophin family of growth factor molecules [30–32]. NGF regulates the growth, development and plasticity of selective neuronal populations in the nervous system [33–36]. It acts through binding and activating specific cell surface receptors named trkA and p-75 [37, 38]. Neuronal NGF expression in vivo is markedly up-regulated by seizures, forebrain ischemia, marked hypoglycemia and tissue injury [28, 39, 40]. NGF promotes the survival of specific groups of neurons, both in vivo and in vitro [15, 41–46], and has a protective effect against delayed neuronal death (DND) after cerebral ischemia [15, 47].

The therapeutic time window of an agent interests people mostly, because increasing research has suggested that many neuroprotective agents had a special therapeutic time window. The failure of clinical trials with a number of neuroprotective drugs, which had previously shown dramatic efficacy in animal ischemia models, was due to inadequate investigation of the therapeutic time window [48]. Therefore, it is necessary to establish the time window for efficacy in animal models as a part of the evaluation of neuroprotection.

In the present study, NGF produced significant neuroprotection in focal cerebral ischemia, as evident from significant reduction in cerebral infarction and neurological deficits. The neuroprotection was found to be maximal in 2 h post-MCAO treatment groups. NGF treatment even up to 5 h post-MCAO produced significant reduction in infarct volume and neurological deficits. Treatment at latter time point (8 h post-MCAO) did not produce significant neuroprotection. Thus, we concluded that the therapeutic time window for NGF in rabbit focal cerebral ischemia model should be limited within 5 h after ischemia.

However, it is difficult to estimate the therapeutic time window in stroke patients from the animal data, but we can reasonably speculate that NGF may possess a considerably

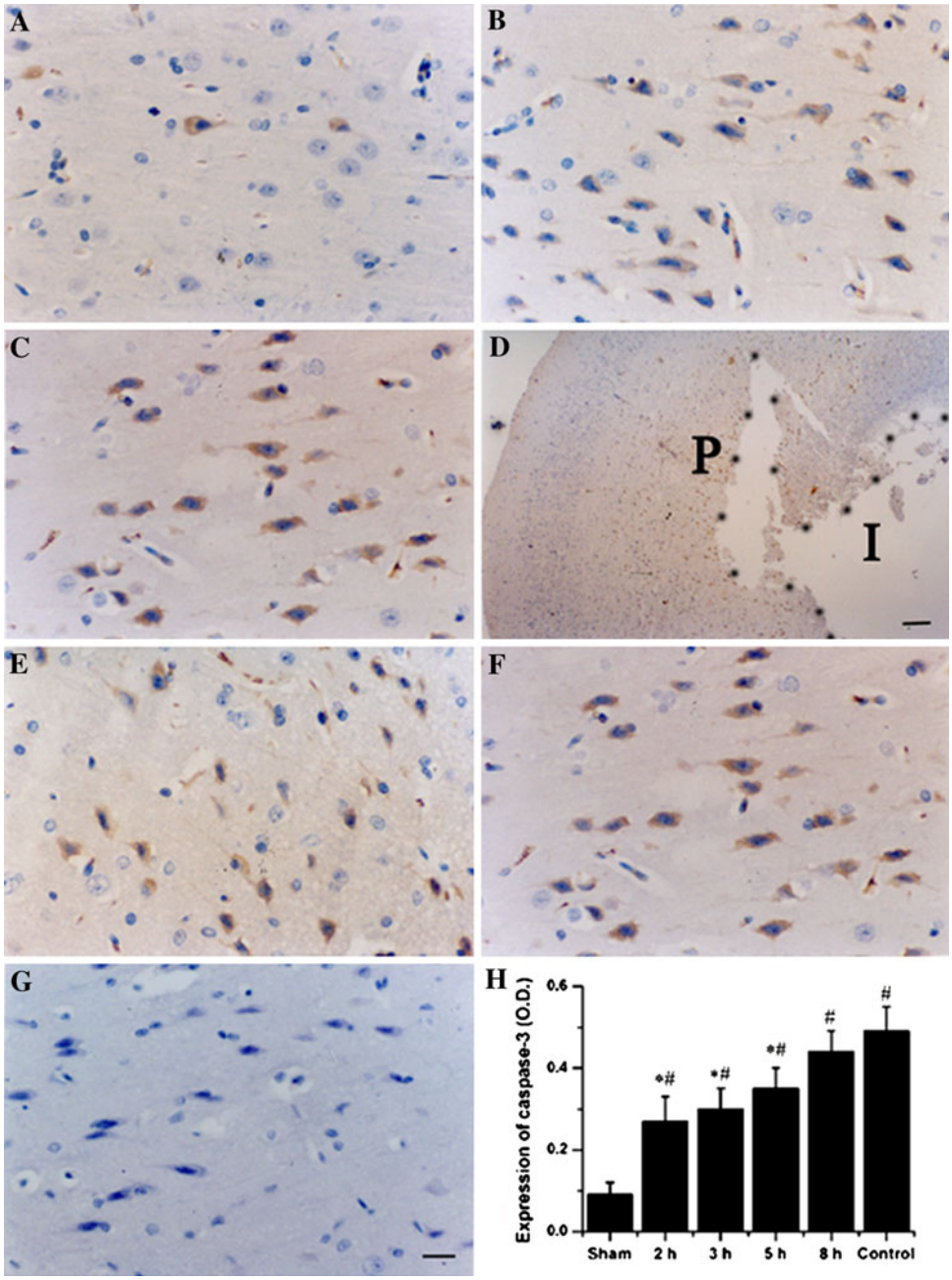

Fig. 3 Immunohistochemical staining for caspase-3 in the penumbral cortex after 72 h of MCAO. The photomicrographs showed caspase-3 expression in the penumbral cortex of animals that received NGF at 2 h (**a**), 3 h (**b**), 5 h (**c**) and 8 h (**d**, **e**) after MCAO and saline (**f**). **g** Microphotograph of negative control staining. **a**–**c** and **e**–**g** *Bar* 20 μm. **d** *Bar* 2 μm. H, Quantification of caspase-3 expression in the penumbral cortex after 72 h of MCAO; *versus saline-treated group (control), $P < 0.01$. *I* infarct area, *P* peri-infarct area. *Dotted lines* demarcate the infarct and peri-infarct areas

longer time window for successful therapeutic intervention in the clinical setting, according to recent studies hat suggest that the therapeutic time window might be wider in human strokes than in animal models [48, 49]. This implied that NGF might be of clinical value for the treatment of acute stroke.

In discussing the limitations of these studies using NGF for stroke, it is important to consider the animal models used and the timing and routes of drug delivery [50]. Experimental data from transient focal cerebral ischemia indicate that delayed reperfusion contributes to ischemic injury and increases the size of the ischemic lesion [51, 52]. Other experiments showed that mortality increased to 45% if reperfusion was initiated 120 min after MCAO [53]. The pathophysiological mechanism of injury from delayed reperfusion itself is not yet fully understood, but may include breakdown of the blood–brain barrier, increase of postischemic edema, secondary increase of excitatory amino acids, inflammatory reaction, and altered microvascular permeability and integrity [53–55]. In our study, NGF reduced significantly cerebral infarct volume when it was induced directly after a 120-min MCAO. NGF plays a key role in neuroprotection of CNS against ischemic damage. But its access to the CNS is restricted by poor

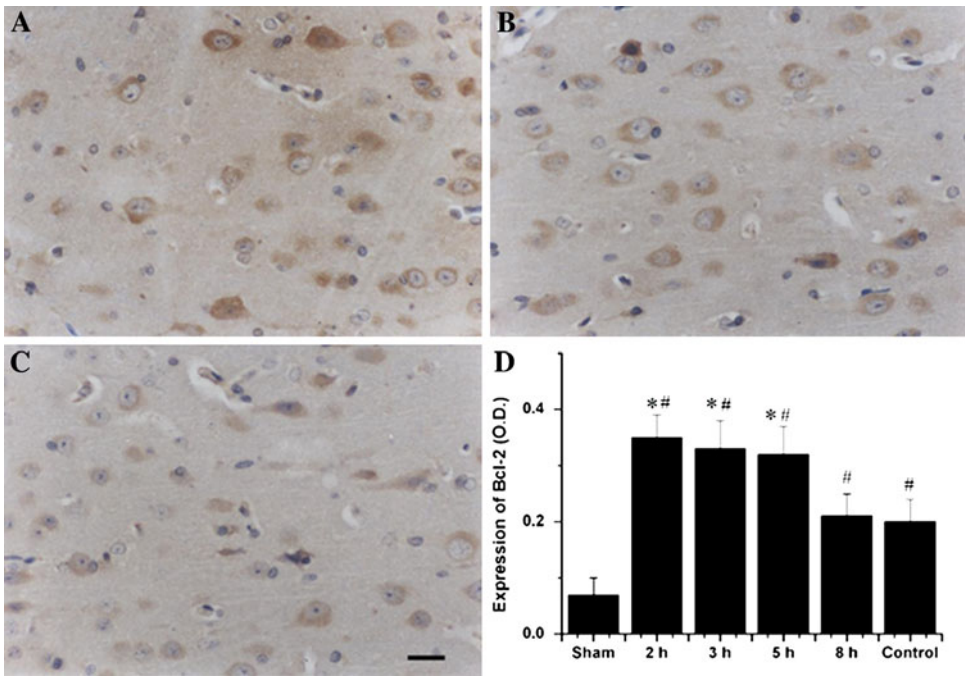

Fig. 4 Immunohistochemical staining for Bcl-2 in penumbral cortex after 72 h of MCAO. The photomicrographs showed Bcl-2 expression in the penumbral cortex of animals receiving saline (**c**) and NGF 2 h (**a**) and 8 h (**b**) after MCAO. *Bar* 20 μm. **d** Quantification of Bcl-2 expression in penumbral cortex after 72 h of MCAO; *versus saline-treated group (control), $P < 0.01$

blood–brain barrier (BBB) permeability because of its large molecular weight [56]. Besides that, peripheral adverse effects following systemic administration also limit the clinical use of NGF [57, 58]. Though intracerebroventricular administration or grafting of NGF-producing cells can be beneficial, it requires surgery with attendant risks of complications and high cost. Intranasal administration (IN) is a non-invasive method that can directly deliver neurotrophins to the CNS [59]. Previous studies [59, 60] have proved that intranasal NGF could bypass the BBB and elicit biological effects. However, the IN NGF concentration in the brain interstitium (perineural fluid that bathes the neuronal cell surfaces), the therapeutic effectiveness, adverse effects and toxicity need further study. So, in our study, we investigated the therapeutic time window for neuroprotective effects of NGF when administered intracerebrally through the perifocal region after focal ischemic injury in rabbits.

Apoptosis contributes to the development of neuronal ischemic infarction after both global [61] and focal [22, 62] ischemia. With more and more researches and understanding in morphology of apoptosis and its biochemical characteristics, we realize that apoptosis is an important form of cell death. Caspase family proteins are known to play a central role in the regulation of apoptosis. Caspase-3, a critical member of the caspase family, accelerates apoptosis and has been suggested as an apoptotic marker [63–65]. Bcl-2 is an integral membrane protein located mainly on the outer membrane of mitochondria. Accumulating evidence indicates that Bcl-2 provides protection against apoptosis by regulating the mitochondrial membrane potential and thereby blocking cytochrome c release into the cytosol [66, 67], where cytochrome c plays a key role in the initiation of apoptosis by activating caspase-3 [68]. Thus, Bcl-2 acts to inhibit cytochrome c translocation, thereby blocking caspase-3 activation and the apoptotic process.

The present study demonstrated that NGF inhibited the expression of pro-apoptotic proteins (caspase-3) and induced that of the anti-apoptotic protein (Bcl-2) in rabbit stroke model, thereby providing the molecular evidence for its neuroprotective activity. In addition, the single NGF administration showed clear neuroprotective and anti-apoptotic activity in rabbit stroke model. These results suggest that NGF showed its therapeutic effect on stroke via directly modulating cellular apoptotic process at the protein expression level.

In summary, NGF is highly efficient in reducing infarct volume and improving neurobehavioral outcome in transient MCAO within the first 5 h. The neuroprotective effect of NGF is time dependent with the best results occurring with administration of NGF as soon after the ischemic injury as possible. Although more studies need to be undertaken to better understand the effects evoked by NGF during neuroprotection, it can be concluded that the inhibition of activation of caspase-3 by increasing the level of anti-apoptotic Bcl-2 is one of the mechanisms underlying the anti-apoptotic effect of NGF on neurons.

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