Exp Brain Res (1993) 95:388–396

Experimental Brain Research

Free radicals and brain damage due to transient middle cerebral artery occlusion: the effect of dimethylthiourea

Yoshihiro Kiyota, Kerstin Pahlmark, Hajime Memezawa, Maj-Lis Smith, Bo K. Siesjö

Laboratory for Experimental Brain Research, Experimental Research Center, University Hospital, S-221 85 Lund, Sweden

Received: 16 June 1992 / Accepted: 8 March 1993

Abstract. The objective of this study was to assess whether dimethylthiourea (DMTU), an established free radical scavenger, ameliorates ischaemic damage due to 2–3 h of transient middle cerebral artery (MCA) occlusion, induced by an intraluminal filament. A major point adressed was whether DMTU given before MCA occlusion only delayed the "maturation" of the damage, or if it had a lasting effect on infarct size. The end point was morphological, and either encompassed triphenyltetrazolium chloride (TTC) staining of tissue slices after 24 h or 48 h of recovery, or histopathological assessment of infarct size after 7 days of recovery. In a preliminary series of experiments, rats were subjected to 3 h of MCA occlusion, and infarct volume was assessed by TTC staining after 24 h of recovery. DMTU in a dose of 750 mg/kg reduced infarct volume by more than 50%. However, due to a high mortality rate, that protocol was not subsequently pursued. When the ischaemia duration was reduced to 2 h and the DMTU dose to 400 mg/kg, a similar amelioration of the tissue damage was observed. However, since DMTU reduced a spontaneous rise in body temperature to 39.0–39.5°C, DMTU-treated animals in the main series of experiments with 24 and 48 h of recovery were treated so that they had the same temperature rise as the saline controls. Under such constant temperature conditions, the effect of DMTU at 24 h of recovery was borderline ($P = 0.052$) and at 48 h it was nil. The lack of a lasting effect of DMTU was supported by the findings on evaluation of infarct area after 7 days of recovery. The results raise the important question whether DMTU, and perhaps other free radical scavengers, delay rather than ameliorate the ischaemic lesion developing after transient MCA occlusion.

Key words: Dimethylthiourea – Brain – Ischaemia – Middle cerebral artery occlusion – Rat

Correspondence to: K. Pahlmark

Introduction

Although it seems likely that free radicals contribute to ischaemic brain damage, particularly following long periods of ischaemia due to middle cerebral artery (MCA) occlusion, it has been difficult to obtain definite proof. In the gerbil, evidence has been reported that even brief periods of ischaemia with reperfusion leads to oxidative damage to proteins (Floyd 1990; Oliver et al. 1990). However, similar alterations have not been observed in rats (Folbergrová et al. 1993; Pahlmark et al. 1993). At present, therefore, the best evidence for participation of free radicals in the pathophysiology of ischaemic brain damage rests on the observations that ischaemic brain damage is ameliorated by drugs or enzymes whose best known (or only) effect is to scavenge free radicals. These drugs/enzymes encompass dimethylthiourea (DMTU) and allopurinol (Patt et al. 1988; Martz et al. 1989), as well as polyethylene glycol-conjugated superoxide dismutase (SOD) and catalase (Liu et al. 1989). Additional support has been obtained by the smaller infarcts observed after transient MCA occlusion in transgenic mice overexpressing human Cu-Zn SOD (Kinouchi et al. 1991).

In all of these studies, giving positive evidence of a free radical component in ischaemic damage, ischaemia was either permanent or relatively long-lasting (Liu et al. 1989; Martz et al. 1989; Patt et al. 1988; Kinouchi et al. 1991). Results obtained in rats suggest that free radical scavengers may reduce infarct size in permanently MCA-occluded animals by 30–35% (Liu et al. 1989; Martz et al. 1989). In theory, an even better protection could be obtained in reversible MCA occlusion, but extensive analyses only exist for gerbils (Patt et al. 1988).

Recent methodological developments allow reversible MCA occlusion by an intraluminal filament, introduced from the common carotid artery (Koizumi et al. 1986; Nagasawa and Kogure 1989; Zea Longa et al. 1989). We have recently modified this technique and assessed infarct size after various occlusion periods (Memezawa et al. 1992a,b). In the present study, we explored to what

extent DMTU ameliorates brain damage due to transient MCA occlusion. We specifically wished to study whether the drug merely delays infarct development, or whether the effects are sustained.

In the first part of the preliminary series of experiments, fasted rats pretreated with DMTU 750 mg/kg or saline were exposed to 3 h of MCA occlusion and allowed 24 h of recirculation. Infarct size in the DMTU group, assessed with triphenyltetrazolium choride (TTC), was reduced to less than 50% of control. Further experiments aimed for longer recirculation periods revealed a high mortality rate with the combination of 3 h of MCAO and a DMTU dose of 750 mg/kg. Furthermore, a spontaneous rise in body temperature, seen in saline controls during and after MCAO, was blunted by the DMTU treatment. The main experimental series were, therefore, performed with 2 h of MCA occlusion and a lower dose of DMTU (400 mg/kg). To explore the importance of temperature the DMTU-treated animals were warmed to the same body temperature as the controls spontaneously achieved.

The results of these experiments demonstrate that although DMTU seemed to ameliorate the lesions after 24 h, the drug did not reduce ischaemic infarction when the latter was evaluated after 48 h of recovery. Since these experiments suggested that DMTU only delayed the development of ischaemic infarction, an additional group was studied with 7 days of recovery. In this group the DMTU animals were not heated, so as to optimise conditions for amelioration. In spite of that, DMTU lacked effect on ischaemic infarction.

Materials and Methods

Experimental groups: preliminary series

Two separate series of experiments were carried out (Table 1). In the first groups (1 and 2), the effects of DMTU on infarction volume were studied following a 3 h period of MCA occlusion. DMTU (Janssen Chimica, Belgium) was dissolved in 0.9% saline to a final concentration of 100 mg/ml and administered i.p. in a dose of 750 mg/kg 1 h before MCA occlusion. Eleven rats each were treated with saline or DMTU and subjected to 3 h of MCA occlusion followed by 24 h of recirculation (group 1). One rat in each group died in the early recovery phase. The remaining animals were killed at 24 h of reperfusion and the infarct size of each brain was measured with the help of TTC staining. Fourteen additional rats (group 2) were divided into two subgroups of six and eight rats, treated with saline or DMTU, respectively, 1 h before a 3 h period of MCA occlusion. These rats were intended for histological evaluation after 7 days of reperfusion, but due to a high mortality rate, this line was not pursued. Instead, 36 untreated animals were added with different times of MCA occlusion (1, 2, or 3 h or permanent) to determine the mortality rate.

In an additional group (group 3), we evaluated the protective effect of DMTU at a lower dose (400 mg/kg) and with a shorter period (2 h) of MCA occlusion. Rats were treated with saline ($n=9$) or DMTU ($n=8$) 1 h before a MCA occlusion. One of the saline-treated rats died within 24 h after ischaemia. The rats were sacrificed after 24 h of reperfusion and the brains were stained with TTC.

Experimental groups: main series

Since a significant reduction in rectal temperature was observed in the DMTU-treated rats in the preliminary experiments, in the subsequent experiments the animals given DMTU were warmed during the 2 h MCA occlusion period and the first 2 h of reperfusion to keep their body temperature at the same level as that of the saline-treated rats. After 24 or 48 h of reperfusion (groups 4 and 5), the rats were killed and their brains were stained with TTC. Since the results failed to show that DMTU ameliorated ischaemic damage after 48 h of recovery, one additional group was studied (group 6). In this, the rats were treated with saline ($n=5$) or DMTU ($n=6$), and subjected to 2 h of MCA occlusion. Postoperative temperature was not manipulated in the DMTU-treated animals. After 7 days of reperfusion, the animals were perfusion-fixed with phosphate-buffered formalin and their brains were histologically evaluated. In these animals infarct area at three different levels, rather than infarct volume, was assessed.

General operative techniques

Male Wistar rats (Mollegaard Breeding Center, Denmark) weighing 335–355 g were used for this study. The experimental procedures were approved by the Animal Use Ethical Committee of the University of Lund. The animals were fasted overnight, but had free access to water. Anaesthesia was induced by inhalation of 3.5% halothane (Halothane, ICS Chemicals, England) in N_2O: O_2 (70%: 30%), the halothane concentration being reduced to 1.5–2.0% during operation. The animals breathed spontaneously. A polyethylene catheter was inserted into a tail artery for blood pressure recording and blood sampling. A thermistor probe was inserted in the rectum

Table 1. The different experimental groups with DMTU-treatment

Experimental group	Number of rats		DMTU dose (mg/kg, i.p.)	Duration of ischaemia and reperfusion	Temperature control	Evaluation method
	DMTU	Saline				
Preliminary series						
Group 1	11	11	750	3 h MCAO + 24 h R	No	Histology (TTC)
Group 2	8	6	750	3 h MCAO + 1 w R	No	–
Group 3	8	9	400	2 h MCAO + 24 h R	No	Histology (TTC)
Main series						
Group 4	10	9	400	2 h MCAO + 24 h R	Yes	Histology (TTC)
Group 5	9	9	400	2 h MCAO + 48 h R	Yes	Histology (TTC)
Group 6	6	5	400	2 h MCAO + 1 w R	No	Histology (C + A)

MCAO, Middle cerebral artery occlusion; R, reperfusion; C + A, celestine blue/acid fuchsin staining; TTC, triphenyltetrazolium chloride

to monitor body temperature, which was maintainted at 38°C (the temperature of freely moving rats) during the operation with the help of a heated operating table. Body temperature, mean arterial blood pressure, arterial blood gases, pH and glucose concentration were measured just before, and 5 min after MCA occlusion, when the rats were still under anaesthesia, and also at the end of the period of MCA occlusion, as well as after 2 h of reperfusion, when the rats were awake.

Middle cerebral artery occlusion

Occlusion of the right MCA was performed by insertion of an intraluminal filament as described previously (Memezawa et al. 1992a). A surgical midline incision was made to expose the right common, internal, and external carotid arteries. The external carotid and the occipital arteries were ligated, and 0.15 ml of heparin (200 IU/ml) (Vitrum AB, Stockholm) was given through the tail artery. The common carotid artery was closed by a ligature, and the internal carotid artery was temporarily closed by a microvascular clip. A small incision was then made in the common carotid artery, and the MCA-occluding device was inserted into the internal carotid artery. The occluder filament was advanced to close the origin of MCA. The anaesthesia was discontinued, and the rats were returned to their cages after the operation. The animals were awake during the ischaemic period, and their behaviour was checked for neurological deficits. After the period of ischaemia, the rats were lightly reanaesthetised with halothane and the occluder filament was withdrawn to allow recirculation. The right common carotid artery remained occluded. This procedure permits reperfusion of the ischaemic focus (Memezawa et al. 1992b).

Triphenyltetrazolium chloride staining

The rats were anaesthetised by inhalation of 2.5% halothane and killed by decapitation. The brains were quickly removed and chilled in ice-cold saline for 10 min. Twelve 1 mm coronal slices were cut with a tissue slicer, beginning 1 mm posterior to the anterior pole, and the slices were immersed in a saline solution containing 1.0% 2,3,5-triphenyltetrazolium chloride (Sigma, St. Louis) at 37°C for 20 min (Bederson et al. 1986a). After staining, each brain slice was photographed with black and white film. The unstained area in each photograph was quantified from the developed film with a computer image-analyser (IBAS 2, Kontron Bildanalyse GMBH, Munich). The total infarct volume in cubic millimetres was determined by summing up the infarct areas of the 12 slices (Osborne et al. 1987).

Celestine blue/acid fuchsin staining

The rats were anaesthetised and tracheotomised under artificial ventilation with 1.5% halothane in N_2O: O_2 (70%: 30%). A thoracotomy was made and a cannula was inserted into the ascending aorta via the left ventricle. After a short flush with saline, the rats were perfusion-fixed with phosphate buffered 4% formaldehyde (pH 7.4), as described previously (Auer et al. 1984). Then the brains were removed, dehydrated, embedded in paraffin, sectioned coronally at 5 μm and stained with celestine blue and acid fuchsin. Brain sections were observed under the microscope, and the infarct area was sketched on copies from the rat brain atlas (Paxinos and Watson 1982). The infarct area at three different brain levels was quantified with help of the image analyser.

Statistics

Statistical differences between saline- and DMTU-treated groups were determined using the two-tailed Student's *t*-test. $P<0.05$ was regarded as statistically significant. All values given are mean ± SEM

Results

Accuracy of middle cerebral artery occlusion

Rats in which MCA occlusion was successful showed typical behavioural signs when regaining consciousness, such as contralateral circling and walking to the contralateral side (Memezawa et al. 1992a). Also the DMTU-treated animals sedated by the drug showed such circling behaviour, although they had to be lightly manually aroused. Unilateral occlusion of the carotid arteries does not generate circling. Seventeen of the 154 rats operated in the present series did not show the typical behavioural signs during ischaemia or had subarachnoidal haemorrhage at the time of death; these rats were excluded from analysis.

Physiological parameters

The values of the physiological parameters in group 4 of the main series are shown in Table 2. Similar values, with

Table 2. Physiological parameters in experimental group 4 (2 h MCA occlusion and 24 h reperfusion with temperature control) measured at three different times

	5 min MCAO	2 h MCAO	2 h reperfusion
Saline ($n=9$)			
Rectal temperature (°C)	38.0 ±0.2	39.3 ±0.6	38.6 ±0.4
PCO_2 (mm Hg)	43.1 ±3.0	33.7 ±2.3	38.1 ±3.7
PO_2 (mm Hg)	109 ±7	86 ±7	80 ±8
pH	7.38±0.04	7.46±0.02	7.43±0.02
Blood pressure (mm Hg)	132 ±14		
Blood glucose (mmol/l)	4.7 ±0.7	5.0 ±0.8	5.1 ±0.7
DMTU ($n=8$)			
Rectal temperature (°C)	37.8 ±0.4	38.9 ±0.5	38.1 ±0.5
PCO_2 (mm Hg)	43.4 ±2.5	36.0 ±2.9	38.1 ±3.7
PO_2 (mm Hg)	107 ±7	80 ±7	81 ±5
pH	7.39±0.03	7.45±0.02	7.42±0.07
Blood pressure (mm Hg)	123 ±13		
Blood glucose (mmol/l)	5.0 ±0.5	5.7 ±0.6	5.6 ±0.6

the exception of temperature, were observed in all experiments. Rats were moderately hypercapnic (PCO_2 40–45 mmHg) during the operation, reflecting the effects of anaesthesia and the spontaneous ventilation. The other parameters were as are generally considered normal. No significant difference in physiological parameters between saline- and DMTU-treated groups was observed.

Preliminary series

Effect of DMTU in a dose of 750 *mg/kg.* Figure 1 shows coronal sections of rat brains stained with TTC after 24 h of reperfusion following a 3 h period of MCA occlusion. The contralateral hemisphere was darkly stained, indicating normal tissue. Portions of the ipsilateral cere-

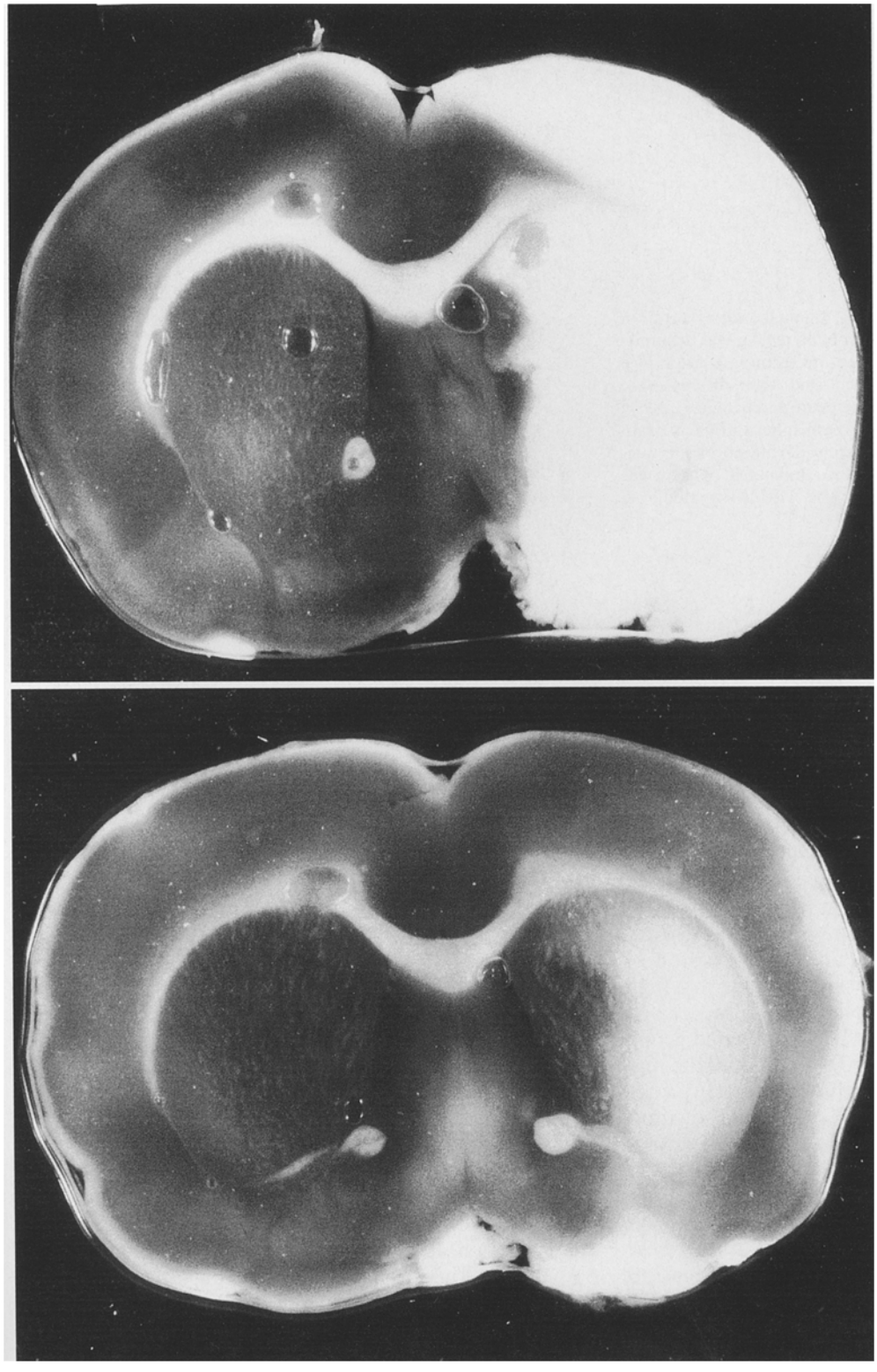

Fig. 1. Coronal section of rat brains obtained 24 h after a 3 h period of MCA occlusion. The sections were stained with TTC, and injured tissue is white. *Top*, Brain section from a rat treated with saline; *bottom*, brain section from a rat treated with DMTU (750 mg/kg, i.p.)

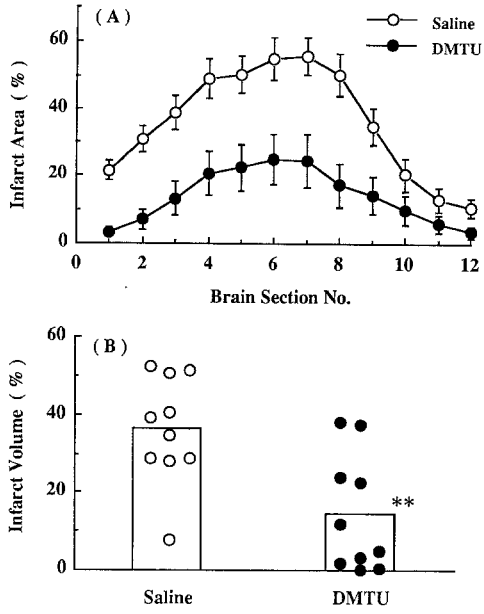

Fig. 2A,B. Ameliorative effect of DMTU against brain infarction after MCA occlusion. DMTU in a dose of 750 mg/kg was administered 1 h before a 3 h MCA occlusion period (group 1). After 24 h of reperfusion, the animals were killed and their brains were sectioned and stained with TTC. **A** The mean percentage ± SEM of the infarction area in the ipsilateral hemisphere of each brain section. **B** The mean percentage of infarction volume of the ipsilateral hemisphere, with individual values indicated. There was a significant difference between saline- and DMTU-treated rats (** $P < 0.01$)

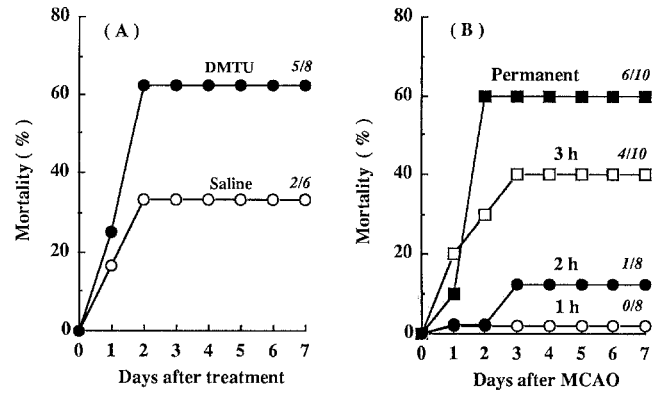

Fig. 3. A The cumulative mortality of rats subjected to 3 h MCA occlusion after treatment with DMTU at a dose of 750 mg/kg i.p. or saline 1 h before occlusion. Sixty-three percent of the DMTU-treated and 33% of the saline-treated animals died within 2 days. **B** The cumulative mortality rate after different duration of MCA occlusion: 1 h, 2 h, 3 h and permanent MCA occlusion. The animals were not treated with any drug. The number of dead animals/total number of experiments is indicated to the *right* of each curve

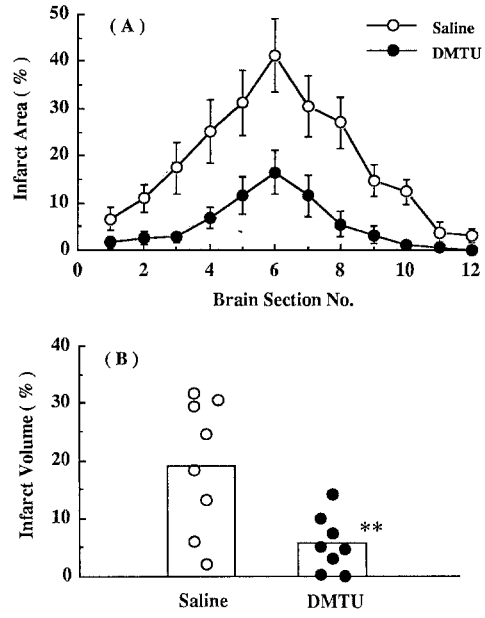

Fig. 4A,B. The protective effect of DMTU (400 mg/kg) compared with saline in rats subjected to 2 h MCA occlusion and 24 h of reperfusion (group 3). The mean infarct area (%) ± SEM and mean (*bars*) as well as individual (*symbols*) % infarct volumes measured with TTC staining are given in **A** and **B** respectively. There was a significant difference (** $P < 0.01$) between DMTU- and saline-treated animals

bral cortex and caudoputamen were not stained, demonstrating damaged areas in which the neurons and most of the glial cells had died (infarction). The largest infarct area, about 60% of the hemisphere, was observed in sections 6 and 7 (Fig. 2A). The infarct area was markedly reduced by DMTU treatment in each of the 12 brain slices examined (Fig. 2A). Futhermore, the total infarct volume was significantly reduced by more than 50% ($P < 0.01$); infarct volume was 36.3 ± 4.4% of the ipsilateral hemisphere in the saline-treated group, and 14.4 ± 4.8% in the DMTU-treated group (Fig. 2B).

Although the objective of the experiments was to evaluate the effect of DMTU 7 days after a 3 h period of MCA occlusion, this could not be achieved due to a high mortality rate. Thus, two of six rats in the saline-treated group and five of eight rats in the DMTU-treated group died within 7 days (Fig. 3A). In order to analyse the reasons for this high mortality, we evaluated mortality rates in untreated animals subjected to 1, 2, and 3 h of transient, or to permanent, MCA occlusion (Fig. 3B). Permanent occlusion gave a high mortality rate, as did 3 h of transient occlusion. Following 1 h of occlusion, eight of eight animals survived and after 2 h of MCA occlusion seven of eight animals survived. On the basis of these results, we decided to change the occlusion time from 3 to 2 h of MCA. Furthermore, since DMTU in a dose of 750 mg/kg may exert unspecific effects, we decided to reduce the dose to 400 mg/kg.

Effect of DMTU at a dose of 400 *mg/kg.* Following an occlusion period of 2 h (group 3), infarction in the saline-treated group was observed in cortex and the lateral caudo-putamen. The largest infarct area was observed in section no. 6 (Fig. 4A), and the percentage of infarct volume in the ipsilateral hemisphere as evaluated after 24 h of recovery was 19.4 ± 4.1% (Fig. 4B). DMTU clearly reduced infarct size, since the percentage of infarct volume

was 5.5±1.7% in the DMTU-treated group, giving a significant difference ($P<0.01$) between saline- and DMTU-treated groups.

Body temperature

The results obtained in group 3 (2 h MCAO + 24 h R) in the preliminary series of experiments showed that body temperature increased in the MCA-occluded, freely moving animals and that DMTU blunted this temperature response. Since the normal temperature of the rat is around 38°C, the preocclusion temperature was held at that value. However, a spontaneous increase in body temperature to 39.0–39.5°C was observed in the saline-treated groups at the end of 2 h ischaemia (Fig. 5A). During the reperfusion period body temperature gradually recovered to normal levels. In the preliminary 2 h occlusion group, in which body temperature was not controlled, the temperature of the DMTU-treated group did not increase during ischaemia (38.3±0.5°C), and these animals showed a significantly lower ($P<0.05$) temperature than the animals in the control group after 2 h of reperfusion; the temperatures in the control and DMTU groups were 38.6±0.5°C and 37.0±0.6°C, respectively. However, no significant difference between the two groups was observed after 24 h of reperfusion (Fig. 5A). In the next two groups, the DMTU-treated rats were temperature controlled during ischaemia and the first 2 h of the reperfusion to achieve the same temperature as in the control animals (Fig. 5B).

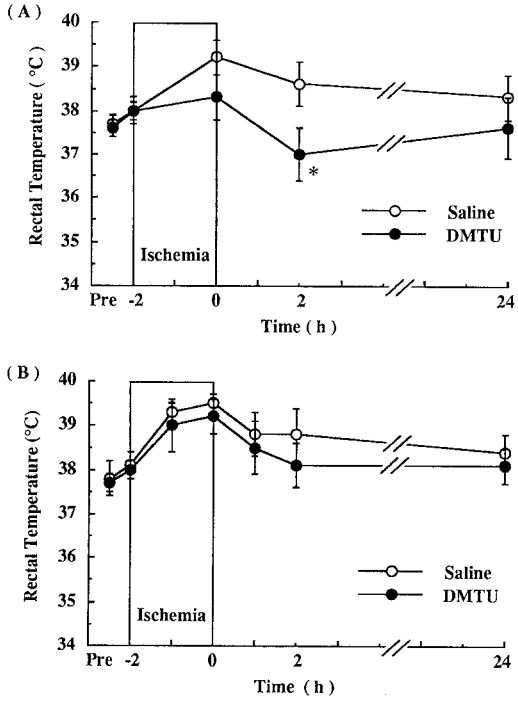

Fig. 5A,B. The effect of DMTU on rectal temperature during and after MCA occlusion. Rectal temperature was measured immediately before (*Pre*) and just after the MCA was occluded (–2), and at 0, 2 and 24 h of reperfusion. **A** The difference in rectal temperature between the DMTU- and saline-treated groups when body temperature was not controlled (group 3). There was a significant difference in temperature at 2 h of reperfusion (* $P < 0.05$). **B** Difference in rectal temperature when the body temperature was controlled with external heating in the DMTU-treated group during MCA occlusion and the following 2 h period of reperfusion (groups 4 and 5)

Main series

In group 4 of the main series, 24 h of recovery was allowed before TTC staining and, as stated above, the body temperature of the DMTU-treated rats was maintained at the same level as that of the saline-treated subjects. Infarction in the saline-treated group was almost the same as that in group 3 of the preliminary series (2 h MCAO + 24 h R). The largest area of infarct, 31.8±6.1%, was observed in section no.6 (Fig. 6A) and the percentage of infarct volume was 16.4±3.8% (Fig. 6B). DMTU seemed to reduce infarct size, since the percentage of infarct volume was 6.9±2.0%. However, the difference between saline- and DMTU-treated groups was of borderline statistical significance ($P=0.052$).

As discussed in the Introduction, the question arises whether the short-term effect of a drug represents only postponement of the final damage or a definite amelioration of the damage. For that reason, the effect of DMTU was also evaluated after a 48 h period of reperfusion (group 5). The other experimental conditions were the same as in group 4. Infarction in the saline-treated sub-

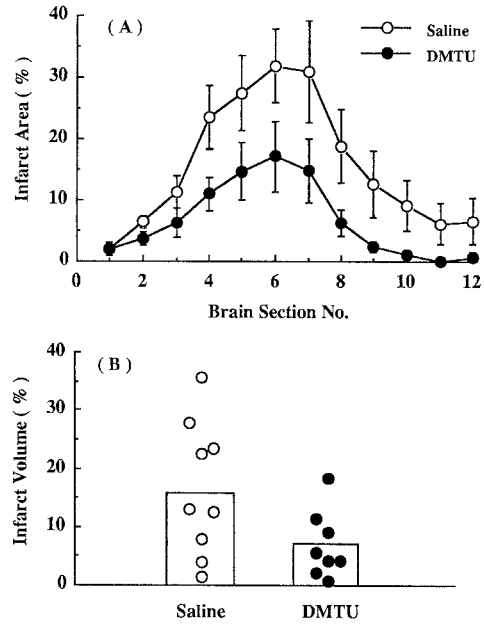

Fig. 6A,B. The results of experimental group 4, with the same dose of DMTU (400 mg/kg) and duration of ischaemia-reperfusion (2 h MCAO + 24 h re-perfusion) as in group 3 (Fig. 4). The only difference in the experimental procedure was that in group 4 the body temperature in the DMTU-treated group was controlled during MCAO and the first 2 h of reperfusion. **A** Mean % infarcted area ± SEM; **B** mean % infarct volume. Individual values are given as *symbols*. DMTU treatment showed protection but the difference was not significant ($P=0.052$)

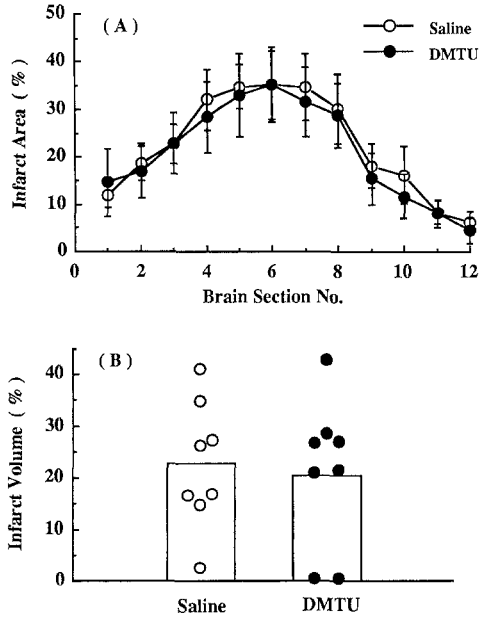

Fig. 7A,B. In group 5 the same experimental conditions as in group 4 were used (DMTU 400 mg/kg, 2 h of MCA occlusion and control of the body temperature in the DMTU-treated animals), but the reperfusion period was prolonged to 48 h. At this time there were no significant differences in infarct area or volume. **A** Mean percentage infarcted area ± SEM; **B** mean % infarct volume. Individual values are given as *symbols*

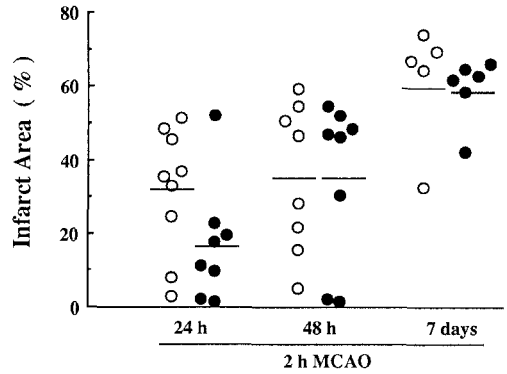

Fig. 8. After 2 h of MCA occlusion the increasing infarct size with increasing durations of reperfusion (24 h, 48 h with TTC method, and 7 days with celestine blue/acid fuchsin staining) demonstrated that a single dose treatment with DMTU 400 mg/kg only delayed the maturation. The DMTU-treated animals in the 7 days reperfusion group were not temperature controlled, hence their temperature was lower during and after MCA occlusion than in the other groups. The symbols represent individual infarct areas in percentage of the ipsilateral hemisphere in section 6. The mean value in each group is indicated by a *horizontal bar*. *Filled circles*, DMTU-treated rats; *open circles*, saline-treated rats. The data are from experimental groups 4, 5 and 6

group was a little more extensive than after a 24 h period of reperfusion (group 4); the infarct area in section no.6 was 35.2±7.1% and the percentage of infarct volume hemisphere was 22.6±4.3% (Fig. 7). However, there was now no difference between the saline- and DMTU-treated groups in infarct volume, the percentages of infarct volume being 22.6±4.3% and 21.1±5.1%, respectively (Fig. 7B).

Long-term recovery

Although the results of the main series suggest that DMTU postpones rather than ameliorates brain damage due to transient ischaemia, temperature represents a confounding factor. Therefore, and in order to evaluate the time factor, one group of animals was allowed a recovery period of 7 days, body temperature in the DMTU-treated animals not being raised. Thus, the results should be comparable to those illustrated in Fig. 4 (2 h MCAO + 24 h R, no temperature control) in which DMTU reduced infarct size by more than 50%. However, since TTC staining is not a reliable method of infarct assessment after such long periods of recovery, we used histopathology, and assessment of infarct area (at three different levels) rather than tissue volume.

Brain sections 4, 6 and 8 were chosen for histological evaluation, as those levels represented the main infarct area demonstrated in the TTC-stained sections (Figs. 4A, 6A, 7A). Wide-spread infarction was observed in the cortex and caudoputamen, similar to that shown in Fig. 1, suggesting that the results with the TTC staining and histopathology can be compared. The following conclusions are warranted. The saline-treated animals showed a much larger infarct area after 7 days of recovery than that observed after 24 h of reperfusion (group 3, saline-treated); the percentages of infarct area in sections 4, 6 and 8 were 25.2±6.7%, 41.3±7.8% and 27.0±5.4%, respectively, after 24 h of reperfusion (group 3) and 56.9±5.0%, 61.5±5.2% and 33.4±3.3% after 7 days of reperfusion (group 6). No effect of DMTU on brain infarction was observed after 7 days of recovery (Fig. 8).

Discussion

As stated in the Introduction, it is highly likely that free radicals contribute to the damage which is incurred after ischaemic episodes, particularly if the ischaemia is of long duration and followed by recirculation. The preliminary experiments supported this contention, since DMTU in doses of 750 or 400 mg/kg reduced infarct size to <50% of control, as measured after 24 h of recovery. However, there are clearly problems of interpretation. The first is that DMTU proved to blunt a rise in temperature which occurred spontaneously in the MCA-occluded, saline-injected animals. The second, and most important, result is that the effect of DMTU was no longer apparent after 48 h of recovery, and that similarly negative results were obtained when infarct area was evaluated after 7 days of recovery in animals in which the DMTU-treated ones were not heated. We will discuss the established effects of DMTU, and the possibilty that DMTU (and possibly other free radical scavengers) merely postpones ischaemic damage, not really preventing it.

Before these issues are discussed, it seems justified to make a few remarks about the techniques used to assess

tissue damage. TTC staining was used in the studies with 24 and 48 h of reperfusion because the damaged areas can be clearly distinguished from normal tissue, and because it is technically easier and less time consuming than light microscopical evaluation of infarct volume. TTC, which has no colour per se, turns dark red when reduced by mitochondrial dehydrogenases (Bederson et al. 1986a). Normal tissues, which have intact dehydrogenases, are stained red while damaged areas remain unstained. In a pilot study, comparing TTC staining to celestine blue/acid fuchsin staining, we could demonstrate that unstained areas contained necrotic tissue, both neurons and glial cells being affected. Therefore, the unstained areas were evaluated as "infarction". We have also compared the size of infarct area with the two techniques after 24 h of permanent MCA occlusion, and found them to be of similar size (Memezawa et al. 1992a). Some previous studies also demonstrate a good correlation between infarct areas determined by TTC staining and those determined by hematoxylin/eosin staining (Bederson et al. 1986b). Some animals in this study showed no, or very small infarcts, but all of them had damage; reflected in a pale staining with TTC, interpreted as selective neuronal necrosis, with some cells (probably mainly glia) still viable. We only measured the completely non-stained area, and so a few animals had a value of zero.

DMTU – specific and unspecific effects

It is well known that DMTU, an established hydroxyl radical scavenger, ameliorates damage due to oxidative stress (Patt et al. 1988; Martz et al. 1989). Patt et al. (1988) demonstrated that, in gerbils with 3 or 6 h of carotid artery occlusion and 48 h of reperfusion, DMTU ameliorates tissue oedema and neurological deficits. Martz et al. (1989) subsequentely reported that DMTU reduced brain infarction after permanent MCA occlusion in rats. A dose of 750 mg/kg (i.p.) was used in these studies. This high dose, which gives a plasma concentration of about 5 mM, is usually tolerable, since DMTU has been reported not to be toxic below doses of 1000 mg/kg in rats (Fox 1984). Our results confirm this observation, at least in normal animals. In a pilot study, we checked the general behaviour of the animals given DMTU in a dose of 750 mg/kg, and observed only slight depression of behaviour ("sedation"), but no change in body temperature.

In the present experiments, we found that although DMTU in a dose of 750 mg/kg ameliorated the ischaemic damage caused by 3 h of MCA occlusion, as assessed by TTC staining after 24 h of recovery, it also increased mortality. At this dose (750 mg/kg) DMTU did not increase mortality in rats subjected to 15 min of forebrain ischaemia (Pahlmark et al. 1993). It is unclear how DMTU aggravated mortality in the present study, but 3 h of MCA occlusion by an intraluminal filament normally gives such an extensive brain lesion that survival is jeopardised, and in this precarious situation, DMTU may suppress restorative reactions sufficiently to reduce survival.

Our results reveal that MCA occlusion leads to a rise in body temperature, and that DMTU ameliorates this rise, but they give no hint to the mechanisms involved.

Postponement or prevention of ischaemic damage?

At least following global or forebrain ischaemia of brief to moderate duration ischaemic damage in several brain regions is conspicuously delayed (Kirino 1982; Pulsinelli et al. 1982; Smith et al. 1988). Such slow "maturation" of ischaemic neuronal damage suggests that damage due to transient MCA occlusion is not necessarily definitive after 24 h of recovery. Thus one can envisage that drugs, particularly if given in a single dose, could postpone the damage, albeit not preventing it from occurring. There are no precedents for this assumption. It is of interest, though, that Gotti et al. (1990) reported that amelioration of ischaemic damage due to permanent MCA occlusion in mice by *N*-methyl-D-aspartate (NMDA) antagonists was only observed if repeated doses were given over the first 18 h following the arterial occlusion. Similar results were reported by Dirnagl et al. (1990), who administered MK-801 to MCA-occluded rats.

Our results may be seemingly at variance with those showing a similar volume of infarction after 24 h of recovery as after longer periods (Isayama et al. 1991). However, such results do not exclude the possibility that drugs may postpone "maturation" of ischaemic damage beyond the 24 h point when the MCA occlusion is relatively short-lasting. It seems highly justified that all drugs are evaluated at "infinite" recovery periods, here considered in terms of 1-week recovery.

The role of temperature

Admittedly, our results after 7 days of recovery were obtained with a different histopathological technique, and with evaluation of infarct area rather than infarct volume. However, the results were so consistent that we submit that DMTU, even in a situation when temperature was allowed to fall during and immediately after ischaemia, failed to exert a lasting amelioration of infarct size. It is clear that a moderate reduction of temperature per se reduces brain damage due to ischaemia (see Busto et al. 1987; Minamisawa et al. 1990a), and also that drugs with allegedly protective effects against ischaemia may at least in part act by lowering body temperature (Buchan and Pulsinelli 1990; Corbett et al. 1990; Kuroiwa et al. 1990). It is equally well established that moderate hyperthermia has adverse effects on the ischaemic brain (Dietrich et al. 1990; Dietrich et al. 1991; Minamisawa et al. 1990a,b). These results raise the question whether the hyperthermia arising as a result of the MCA occlusion with an intraluminal filament aggravates the ischaemic damage, e.g. explaining the large infarcts and the high mortality observed after occlusion periods of 3 h, or longer.

In conclusion, important evidence for a participation of free radicals in the events leading to ischaemic brain damage rests on results which reflect the ameliorating

effects of free radical scavengers. In this respect, the present results are disconcerting in the sense that the ameliorating effect of an established free radical scavenger (DMTU) seems, at least in part, secondary to a depression of body temperature; furthermore, since the effect was no longer apparent after 48 h or 7 days of recovery, the results raise the question of whether some drugs, including free radical scavengers, merely postpone the ultimate damage incurred. This question can only be answered by additional experiments in which temperature is accurately controlled, repeated doses are given of the drug tested and ischaemic damage is assessed after long recovery periods.

Acknowledgements. This work was supported by the Swedish Medical Research Council grant B91-14X-00263-27A, the United States Public Health Service grant 2.RO1 NS-07838-22, and the Medical Faculty, University of Lund. The skilful technical assistance of Lillemor Lindeström is gratefully achnowledged.

References

Auer RN, Olsson Y, Siesjö BK (1984) Hypoglycemic brain injury in the rat. Correlation of density of brain damage with the EEG isoelectric time: a quantitative study. Diabetes 33:1090–1098

Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM (1986a) Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke 17:1304–1308

Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H (1986b) Rat middle cerebral artery occlusion: evaluation of the model and development of a neurological examination. Stroke 17:472–476

Buchan A, Pulsinelli WA (1990) Hypothermia but not the *N*-methyl-d-aspartate antagonist, MK-801, attenuates neuronal damage in gerbils subjected to transient global ischemia. J Neurosci 10:311–316

Busto R, Dietrich WD, Globus MY-T, Valdés I, Scheinberg P, Ginsberg MD (1987) Small differences in intraischaemic brain temperature critically determine the extent of ischemic neuronal injury. J Cereb Blood Flow Metabol 7:729–738

Corbett D, Evans S, Thomas C, Wang D, Jonas RA (1990) MK-801 reduced cerbral ischemic injury by inducing hypothermia. Brain Res 514:300–304

Dietrich WD, Busto R, Valdes I, Loor Y (1990) Effects of normothermic versus mild hyperthermic forebrain ischemia in rats. Stroke 21:1318–1325

Dietrich WD, Halley M, Valdes I, Busto R (1991) Interrelationships between increased vascular permeability and acute neuronal damage following temperature-controlled brain ischemia in rats. Acta Neuropathol 81:615–625

Dirnagl U, Tanabe J, Pulsinelli W (1990) Pre- and post-treatment with MK-801 but not pretreatment alone reduces neocortical damage after focal cerebral ischemia in the rat. Brain Res 527:62–68

Floyd RA (1990) Role of oxygen free radicals in carcinogenesis and brain ischemia. FASEB J 4:2587–2597

Folbergrová J, Kiyota Y, Pahlmark K, Memezawa H, Smith M-L, Siesjö BK (1993) Does ischemia with reperfusion lead to oxidative damage to proteins in the brain? J Cereb Blood Flow Metab 13:145–152

Fox RB (1984) Prevention of granulocyte-mediated oxidant lung injury in rats by a hydroxyl radical scavenger, dimethylthiourea. J Clin Invest 74:1456–1464

Gotti B, Benavides J, MacKenzie ET, Scatton B (1990) The pharmacotherapy of focal cortical ischaemia in the mouse. Brain Res 522:290–307

Isayama K, Pitts LH, Nishimura MC (1991) Evaluation of 2,3,5-triphenyltetrazolium chloride staining to delineate rat brain infarcts. Stroke 22:1394–1398

Kinouchi H, Epstein CJ, Mizui T, Carlson E, Chen SF, Chan PH (1991) Attenuation of focal cerebral ischemic injury in transgenic mice overexpressing CuZn superoxide dismutase. Proc Natl Acad Sci USA 88:11|158–11|162

Kirino T (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 239:57–69

Koizumi J, Yoshida Y, Nakazawa T, Ooneda G (1986) Experimental studies of ischemic brain edema. 1. A new experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. Jpn J Stroke 8:1–8

Kuroiwa T, Bonnekoh P, Hossman K-A (1990) Prevention of postischemic hyperthermia prevents ischemic injury of CA1 neurons in gerbils. J Cereb Blood Flow Metab 10:550–556

Liu TH, Beckman JS, Freeman BA, Hogan EL, Hsu CY (1989) Polyethylene glycol-conjugated superoxide dismutase and catalase reduce ischemic brain injury. Am J Physiol 256: H589–H593

Martz D, Rayos G, Schielke GP, Betz AL (1989) Allopurinol and dimethylthiourea reduce brain infarction following middle cerebral artery occlusion in the rat. Stroke 20:488–494

Memezawa H, Minamisawa H, Smith M-L, Siesjö BK (1992a) Ischemic penumbra in a model of reversible middle cerebral artery occlusion in the rat. Exp Brain Res 89:67–78

Memezawa H, Smith M-L, Siesjö BK (1992b) Penumbral tissues salvaged by reperfusion following middle cerebral artery occlusion in rats. Stroke 23:552–559

Minamisawa H, Smith M-L, Siesjö BK (1990a) The effect of mild hyperthermia and hypothermia on brain damage following 5, 10, and 15 minutes of forebrain ischemia. Ann Neurol 28:26–33

Minamisawa H, Mellergård P, Smith M-L, Bengtsson F, Theander S, Boris-Möller F, Siesjö BK (1990b) Preservation of brain temperature during ischemia in rats. Stroke 21:758–764

Nagasawa H, Kogure K (1989) Correlation between cerebral blood flow and histologic changes in a new rat model of middle cerebral artery occlusion. Stroke 20:1037–1043

Oliver CN, Starke-Reed PE, Stadtman ER, Liu GJ, Carney JM, Floyd RA (1990) Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. Proc Natl Acad Sci USA 87:5144–5147

Osborne KA, Shigeno T, Balarsky AM, Ford I, McCulloch J, Teasdale GM, Graham DI (1987) Quantitative assessment of early brain damage in a rat model of focal cerebral ischemia. J Neurol Neurosurg Psychiatry 50:402–410

Pahlmark K, Folbergrová J, Smith M-L, Siesjö BK (1993) Effects of dimethylthiourea on selective neuronal vulnerability in forebrain ischemia in rats. Stroke 24:731–737

Patt A, Harken AH, Burton LK, Rodell TC, Piermattei D, Schorr WJ, Parker NB, Berger EM, Horesh IR, Terada LS, Linas SL, Cheronis JC, Repine JE (1988) Xanthine oxidase-derived hydrogen peroxide contributes to ischemia reperfusion-induced edema in gerbil brains. J Clin Invest 81:1556–1562

Paxinos G, Watson C (1982) The rat brain in stereotaxic coordinates. Academic, New York

Pulsinelli WA, Brierley JB, Plum F (1982) Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol 11:491–498

Smith M-L, Kalimo H, Warner DS, Siesjö BK (1988) Morphological lesions in the brain preceding the development of postischemic seizures. Acta Neuropathol (Berl) 76:253–264

Zea Longa E, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20:84–91