

# Effect of continuous infusion of asialoerythropoietin on short-term changes in infarct volume, penumbra apoptosis and behaviour following middle cerebral artery occlusion in rats

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## SUMMARY

1. Asialoerythropoietin (aEPO), a derivative of cytokine erythropoietin, has been shown to have neuroprotective effects without haematological complications when administered in single or repeated doses. The present study examines our hypothesis that aEPO may provide neuroprotection against programmed apoptotic cell death when administered in a continuous low dose.

2. Focal cerebral ischaemia was introduced by occlusion of the middle cerebral artery using a surgically placed intraluminal filament in young male Sprague Dawley rats (9 weeks old). After 90 min ischaemia, reperfusion was established by filament removal. Both study and control groups had implanted osmotic minipumps through which they received either aEPO (1  $\mu$ L/h; 20  $\mu$ g/kg per 24 h) or normal saline (1  $\mu$ L/h) for 4 days. On Day 4, infarct volume, the number of apoptotic cells and concentrations of activated caspase 3 and 9 were evaluated in the penumbra region.

3. Asialoerythropoietin was detected in the cerebrospinal fluid of the study group, whereas none was detected in the control group. Although there were no significant changes in haematocrit levels or behaviour scores (on Days 1 and 4) between the study and control groups, aEPO administration significantly reduced infarct volume in the study group compared with the control group (168  $\pm$  19 vs 249  $\pm$  28 mm<sup>3</sup>, respectively;  $P < 0.05$ ).

4. The number of terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL)-positive cells and the concentration of activated caspase 3 and 9 in the penumbra region were significantly lower in the study group compared with the control group.

5. In conclusion, our data suggest that aEPO provides a short-term, possibly histological, protection in young adult male rats when administered immediately after reperfusion.

**Key words:** apoptosis, asialoerythropoietin, infarct volume, ischaemia–reperfusion brain injury, minipump.

## INTRODUCTION

Acute cerebral ischaemia results in two major zones of injury: (i) the core of the injury zone; and (ii) the surrounding area, known as the ‘ischaemic penumbra’. The brain tissue in the core of the injury zone is fatally injured and subsequently undergoes necrotic cell death due to markedly reduced blood supply. The brain tissue in the ischaemic penumbra is less severely affected and is functionally silent as a result of reduced blood flow, but remains metabolically active with limited blood supply. The ischaemic penumbra is often targeted for neuroprotective intervention.<sup>1,2</sup> Apoptosis is not reversible; however, early intervention with a neuroprotectant, such as erythropoietin (EPO), may reduce the number of neurons that undergo apoptosis.<sup>3</sup> Extrinsically administered EPO crosses the blood–brain barrier by binding to EPO receptors expressed on the brain capillaries<sup>4</sup> and effectively protects neurons from ischaemic injury.<sup>5</sup> The neuroprotective effect of a single dose of EPO has been observed in the treatment of acute stroke patients; however, multiple doses may be required to maximally alleviate extended tissue damage by reducing programmed apoptotic cell death.<sup>6</sup> Multiple doses of EPO may be detrimental to stroke patients owing to increased red blood cell mass and hyperreactive platelets. For this reason, a new non-erythropoietic derivative of EPO, namely asialoerythropoietin (aEPO), has been developed.<sup>7</sup> Asialoerythropoietin does not induce thrombogenic complications, making it an ideal agent for neuroprotection. A recent study observed that aEPO provided similar neuroprotection to recombinant human (rh) EPO following acute ischaemia–reperfusion (I/R) brain injury in rats.<sup>7</sup> However, the effect of aEPO on programmed apoptotic cell death has not been investigated. To better understand the mechanisms underlying the neuroprotective effects of aEPO, a rat model of I/R brain injury was used in the present study to examine our hypothesis that continuous infusion of aEPO for a prolonged time may reduce I/R-induced increases in terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL)-positive cells and cells containing cleaved caspase 3 and caspase 9, which are correlated with delayed programmed cell death.

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## METHODS

### Animals

The proposed study was approved by the Institutional Committee for the Humane Use of Animals of SUNY Upstate Medical University and was performed in accordance with the guidelines established by the National Institutes of Health. Pathogen-free male Sprague-Dawley rats, between 250 and 275 g, were obtained from Taconic Farms (Germantown, NY, USA). Rats were acclimated for at least 7 days in a standard solid-bottom rodent cage with ambient temperature maintained at approximately 25°C and under a 12 h light–dark cycle. Animals were allowed free access to water and food.

### Preparation of aEPO

Asialoerythropoietin samples were generously provided by Marcel Leist (Lundbeck Pharma, Copenhagen, Denmark). Experimental aEPO was prepared as described previously.<sup>7</sup> Briefly, the sialic acid portion of erythropoietin (rhEPO; Dragon Pharmaceuticals, Vancouver, Canada) was digested with neuraminidase from *Streptococcus* (Seikagaku America, Rockville, MD, USA) at 37°C for 3 h. The protein fractions obtained from digestion with a neuraminidase enzyme were characterized before purification by both isoelectric focusing (IEF) gel analysis and protein acrylamide gel analysis. The digested product was purified using a cation-exchange liquid chromatography column.

### Osmotic minipump preparation

Osmotic minipumps (Model 2001; ALZET Osmotic Pumps, Durect, Cupertino, CA, USA; length 3.0 cm, diameter 0.7 cm, weight 1.1 g) were individually filled and primed the day before the surgical procedure. The pumps were filled via a filling tube with 20 µg/kg laboratory produced aEPO (Lundbeck Pharma) dissolved in 200 µL vehicle (sterile saline solution; 0.9% NaCl). The flow moderator was placed into the filled pump and the pump was primed in normal sterile saline solution (0.9% NaCl) at 37°C overnight (approximately 12 h) in order to promote immediate pumping of aEPO after implantation.

### Model of I/R brain injury

Rats were anaesthetized by intramuscular injection of a rodent anaesthetic mixture containing ketamine and xylazine (150 : 30 mg/mL) at a dose of 0.6 mL/kg. Additional doses of the mixture were administered as needed to maintain anaesthesia.

Ischaemic injury was produced using a modification of the middle cerebral artery occlusion (MCAO) technique as described by Longa *et al.*<sup>8</sup> and in previous studies.<sup>9,10</sup> Briefly, a 1.5 cm longitudinal incision was made in the middle cervical skin. The left common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA) were exposed and isolated. The distal portion of the ECA was ligated using 4–0 silk sutures. A 4–0 silk suture was tied loosely at the origin of the ECA. The CCA and ICA were clamped temporarily using Schwarz microvascular clips. The MCA was occluded by inserting a 5 cm length of 4–0 monofilament nylon suture (with a rounded tip) via a small puncture. The skin was closed with 3–0 silk running sutures. After 90 min ischaemia, reperfusion was achieved by pulling the 4–0 monofilament nylon suture to the origin of the ECA. The rats were then randomly assigned to one of three experimental groups (i.e. sham ( $n = 3$ ), saline ( $n = 12$ ) or aEPO + saline ( $n = 10$ )). The sham-operated rats received the same experimental surgery without ligations, arterial cut or suture occlusion. Study and control rats received either aEPO (20 µg/kg per 24 h) or normal saline at a rate of 1 µL/h for 4 days. All animals showed infarction in the left parietal cortex and caudate putamen (MCA territory) on 2,3,5-triphenyltetrazolium chloride (TTC)-stained slides at the end of the experiments.

During anaesthesia, rectal temperature was maintained at  $37.0 \pm 0.5^\circ\text{C}$  using a thermostatically controlled heating pad. Following MCAO surgery, rats were positioned prone and a prefilled osmotic minipump was placed in

the subcutaneous pocket between the shoulder blades, as described previously.<sup>11</sup>

### Bodyweight changes after MCAO

Rats that appeared healthy and gained weight during acclimation were used in the study. Rats were weighed daily throughout the experiment.

### Determination of aEPO in the cerebrospinal fluid

At the end of the experiment, cerebrospinal fluid (CSF) concentrations of aEPO were determined. Under anaesthesia, CSF was collected according to Frankmann's technique:<sup>12</sup> the rat was placed on a stereotaxic frame, a 30 gauge needle connected to a 1 mL syringe was inserted into the cisterna magna through a midsagittal incision approximately 7 mm below the occipital crest and the CSF was collected. The CSF samples were centrifuged for 15 min at 700 *g* and frozen at  $-80^\circ\text{C}$  until assay with an ELISA kit (StemCell Technologies, Vancouver, BC, Canada).

### Determination of haematocrit

Haematocrit (Hct) levels were measured at the end of the experiment. Under anaesthesia, a bilateral thoracotomy was performed to expose the heart and 1 mL blood was collected from the left ventricle using a syringe. The blood was immediately placed in microcentrifuge tubes, samples were centrifuged at 1,500 *g* for 2 min at room temperature and the Hct determined.

### Neurological behaviour analysis

Twenty hours and then again 4 days after MCAO, the neurological status of each rat was evaluated according to a neurological grading score of 1–5 as follows:<sup>13</sup> 1, reflex folding of the contralateral paw over the chest; 2, weakened grip of the cage top; 3, circles to the right or left when placed on a clean counter surface; 4, moves only when stimulated; and 5, dead.

### Histological analysis

#### Brain staining with TTC

At the end of the experiments, anaesthetized rats were perfused via cardiac puncture with normal saline followed by 20 mL of 2% TTC solution. The brains were removed and then fixed in 10% formaldehyde before being divided into eight coronal sections and scanned optically. The size of the infarct area and the hemispheric area of each section were traced using computer-assisted colour image selection by Adobe PhotoShop 6.0 (Adobe System, San Jose, CA, USA). The area was measured and analysed using Scion Image 4.0.2b (Scion, Scion Corporation, MD, USA). The area of infarction and the area of both hemispheres were calculated (in  $\text{mm}^2$ ) by tracing the areas on a computer screen and the volume ( $\text{mm}^3$ ) was determined by integrating the appropriate area with the section interval thickness. To compensate for possible oedema on the ipsilateral side, the indirect method was used to calculate infarct volume according to Lin *et al.*<sup>14</sup> This method is based on the formula  $\text{RI} = \text{LT} - \text{RN}$ , where RI is infarct volume in the right hemisphere measured by the indirect method, LT is the total volume of the left hemisphere of the same brain and RN is the non-infarcted volume in the right hemisphere of the same brain.

#### Tissue preparation for immunohistochemical analysis

Brain samples were washed in phosphate-buffered saline (PBS) overnight to remove formalin residue. Samples were subjected to graded ethanol baths and xylene washes, placed in three steps of paraffin at 60°C under vacuum (2 h, 2 and overnight) and embedded. Tissue sections (5 µm) were cut on a microtome and placed on pretreated slides. The sections were deparaffinized in

xylene and hydrated through graded ethanol washes before being placed in a final deionized water wash for 1 min. Antigenic retrieval was performed using the heat treatment method as follows: slides were placed in a container and covered with 0.1 mol/L sodium citrate buffer, pH 6.0, and heated at 95°C for 5 min, followed by a fresh buffer wash, heating again to 95°C for 5 min, cooling and washing in deionized water at room temperature for 5 min.

#### *Haematoxylin and eosin staining*

Prepared slides were stained in haematoxylin for 5 min, rinsed in tap water and differentiated in acid alcohol for two to three quick dips. Slides were washed in tap water briefly and dipped in ammonia water (until bright blue; usually three to five dips). Slides were then washed in tap water for 10–20 min and stained with eosin for 5 min before being dipped in 95% ethanol (three to five dips), washed in absolute ethanol (twice for 5 min each time), washed in xylene (twice for 5 min each time) and mounted with permount and coverslips. Haematoxylin and eosin (HE) analysis was used for qualitative purposes.

#### *TUNEL staining*

The effects of aEPO in the penumbra area were evaluated using TUNEL staining (TUNEL Apoptag Peroxidase In Situ Apoptosis Detection Kit (S7100-KIT); Millipore, Billerica, MA, USA).

At the end of the experiments, anaesthetized rats were perfused via cardiac puncture with normal saline followed by 200 mL of 10% paraformaldehyde for brain fixation. Brains were isolated, dehydrated and embedded in paraffin. Brains were sectioned coronally (50 µm). The infarct area was defined on the basis of HE sections. There is a rim of eosinophilic cells just outside the infarct, which represents an area of selective neuronal cell death.<sup>15</sup> The area around the infarct showing TUNEL-positive cells was defined as the penumbra area. Sections were collected as soon as the infarct area appeared and one of every five sections was collected for TUNEL staining until the infarct area was no longer visible. The TUNEL measurements were performed systematically in different parts of the brain in the different experimental groups because of the smaller area of infarction. Because the area of the infarct varies, this strongly implies that the areas 'adjacent' to the infarct vary from animal to animal. This raises the possibility that the areas selected may not, in fact, be comparable. However, currently there is no good solution to this problem. In total, six to sections were collected per animal depending on infarct size. Cells were determined to be TUNEL positive on the basis of their colour (brown staining from light to dark) and morphology (apoptotic bodies, chromatin condensation and membrane blebbing).

#### *Cleaved (activated) caspase 9 staining*

Formalin-fixed paraffin sections were cut at 4 µm and dried overnight at room temperature. Sections were deparaffinized with two changes of xylene (10 min each), hydrated through descending grades of ethanol to deionized water and placed in Tris-buffered saline (TBS)/Tween for 5 min. Following microwave antigen retrieval in AR-10 (Tris) buffer (BioGenex, San Ramon, CA, USA) slides were immunostained on the DakoCytomation Autostainer (DakoCytomation, Carpinteria, CA, USA). Endogenous peroxidase was blocked with 3% aqueous hydrogen peroxide, followed by blocking of non-specific protein binding with DakoCytomation Protein Block, Serum Free for 10 min. Rabbit polyclonal antibody to active/cleaved caspase 9 (IMG-5705; Imgenex, San Diego, CA, USA) was used at a dilution of 1 : 1000 overnight at 4°C. The antibody was detected with DakoCytomation EnVision + Anti-Rabbit HRP-Labelled Polymer for 30 min at room temperature with DakoCytomation DAB+ as the chromogen. Sections were counterstained with modified Mayer's haematoxylin, dehydrated through graded alcohol, cleared with xylene and mounted with resinous mounting medium. Cleaved caspase 9-positive and -negative cells were counted in designated, predetermined areas of the penumbra region of injury using only the visual field immediately adjacent to the infarction area by an investigator blinded to the treatment group. Data were used for quantitative analysis.

#### *Cleaved (activated) caspase 3 staining*

Mounted slides were warmed and deparaffinized (three washes in xylene for 10 min each time) and hydrated in graded ethanol washes to deionized water (5 min each). Antigen retrieval was performed using the heating method, with sections steamed in 0.1 mol/L citrate buffer, pH 6.0, for 25 min and then cooled to room temperature for 20 min. Tissues were washed in PBS for 10 min, followed by quenching of endogenous peroxidase activity with a 3% H<sub>2</sub>O<sub>2</sub> wash for 10 min. Tissues were then washed in three times in PBS (5 min each), followed by blockade of non-specific protein binding with 10% normal goat serum, 2% bovine serum albumin (BSA) and PBS for 30 min at room temperature. Tissues were then incubated in avidin for 15 min, followed by incubation in biotin for 15 min and then incubated at room temperature with primary antibody (polyclonal cleaved caspase 3) for 60 min. Negative controls were incubated without the primary antibody. Following four PBS washes (5 min), tissues were incubated with the secondary antibody (biotinylated anti-rabbit IgG) at room temperature for 30 min. Tissues were again washed four times with PBS (5 min each) and then incubated with Vector ABC (elite Kit; Vector Laboratories, Burlingame, CA, USA) for 20 min at room temperature. Following four final washes in PBS (5 min each), sections were incubated in a diaminobenzidine (DAB) substrate for visualization of the peroxidase reaction. Sections were washed in deionized water (5 min), counterstained and dehydrated (in graded ethanol; 5 min each step). Sections were then washed in xylene three times (5 min each) and coverslipped with permount. Cleaved caspase 3-positive and -negative cells were in designated, predetermined areas of the penumbra region of injury using only the visual field immediately adjacent to the infarction area by an investigator blinded to the treatment group. Data were used for quantitative analysis.

#### **Data analysis**

Data are presented as the mean ± SEM and were analysed by paired Student's *t*-tests between groups. Analysis of variance was used to analyse the time-course data within groups. *P* < 0.05 was considered significant.

## **RESULTS**

#### **Determination of aEPO in the CSF**

In the present study, the existence of EPO in the CNS was verified by ELISA. After 4 days continuous administration of 20 µg/kg per day aEPO via an implanted osmotic minipump, the concentration of EPO in the CSF was 17 mU/mL, which was significantly higher than the 0.5 mU/mL aEPO in the CSF of rats administered normal saline.

#### **Effect of aEPO on bodyweight and Hct**

The body temperature for each rat was closely monitored and maintained within the normal range when rats were under anaesthesia on Day 1. Bodyweight loss was significantly greater in MCAO rats than in sham-operated rats. There was no significant difference in the Hct or in changes in bodyweight between the aEPO- and normal saline-treated groups (Table 1).

#### **Effects of aEPO on infarct volume**

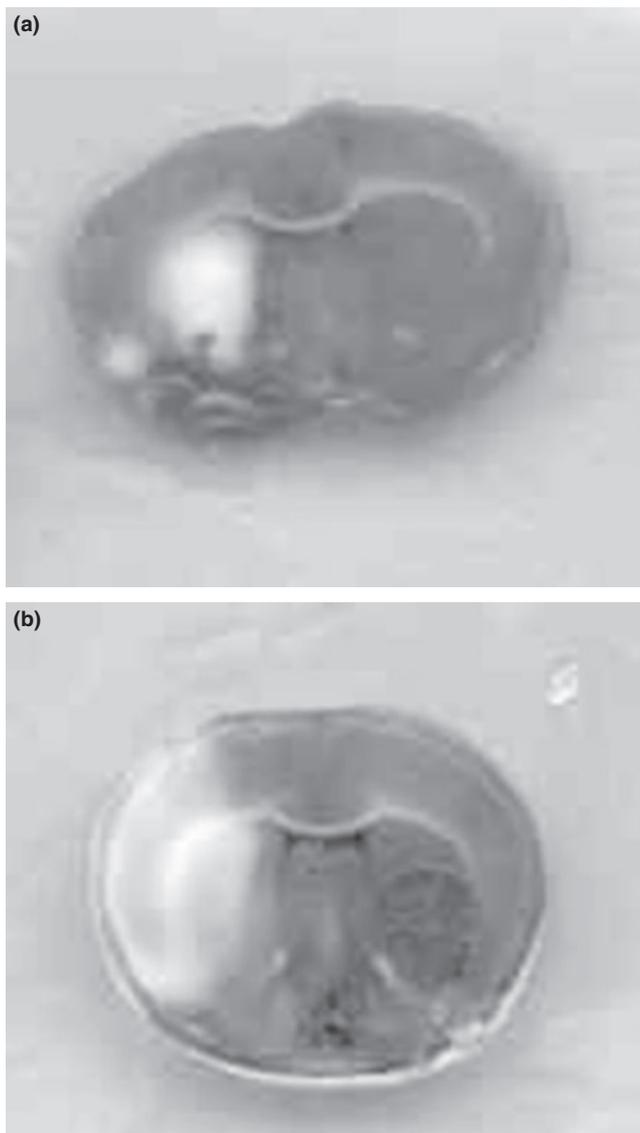
Continuous administration of aEPO significantly reduced MCAO-induced infarct volume evaluated on Day 4. The infarct area was significantly less in the aEPO-treated group than in the saline-treated group (168 ± 19 vs 249 ± 28 mm<sup>3</sup>, respectively; *P* < 0.05). The TTC-stained infarct volume is shown in Fig. 1a,b. Qualitative

**Table 1** Effects of continuous administration of asialoerythropoietin on bodyweight changes, haematocrit and rectal temperature (under anaesthesia)

	Bodyweight change (g)	Haematocrit (%)	Temperature (°C)
Control	-41.8 ± 5.8*	46.0 ± 0.5	36.5 ± 0.8
aEPO	-40.5 ± 6.9*	45.9 ± 0.5	36.2 ± 0.8
Sham	-18.0 ± 5.1	45.8 ± 0.6	36.7 ± 0.6

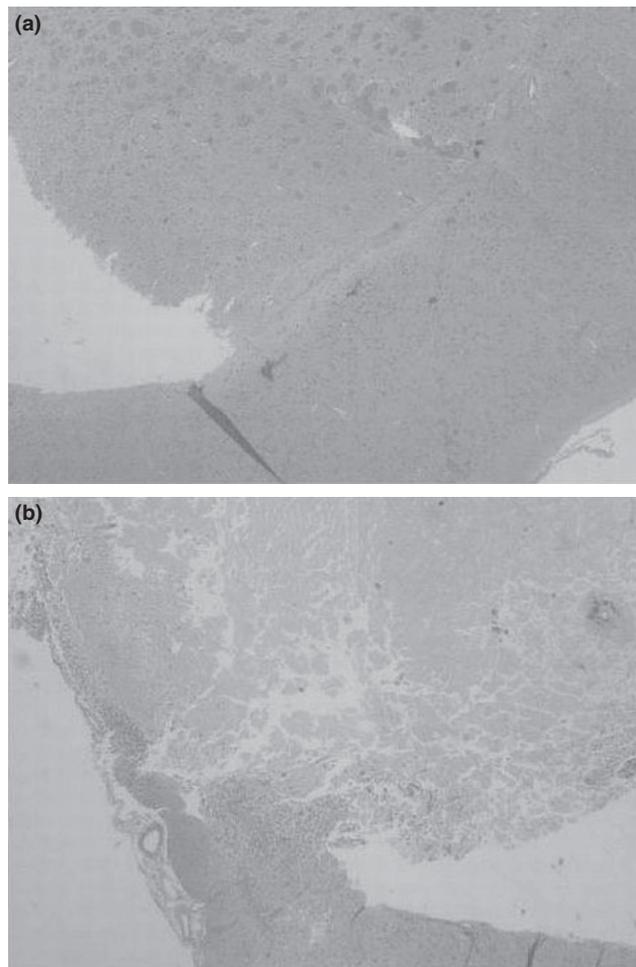
Data are the mean ± SEM. \* $P < 0.01$  compared with the sham-operated group.

aEPO, asialoerythropoietin.



**Fig. 1** Effects of asialoerythropoietin (aEPO) on brain infarction after infusion of 20 µg/kg per day aEPO for 4 days (a). (b) Control group (saline infused).

analysis of HE staining of brain samples demonstrated a significantly decreased amount of tissue damage and loss of nuclear definition in the aEPO-treated group compared with the saline-treated group (Fig. 2a,b).



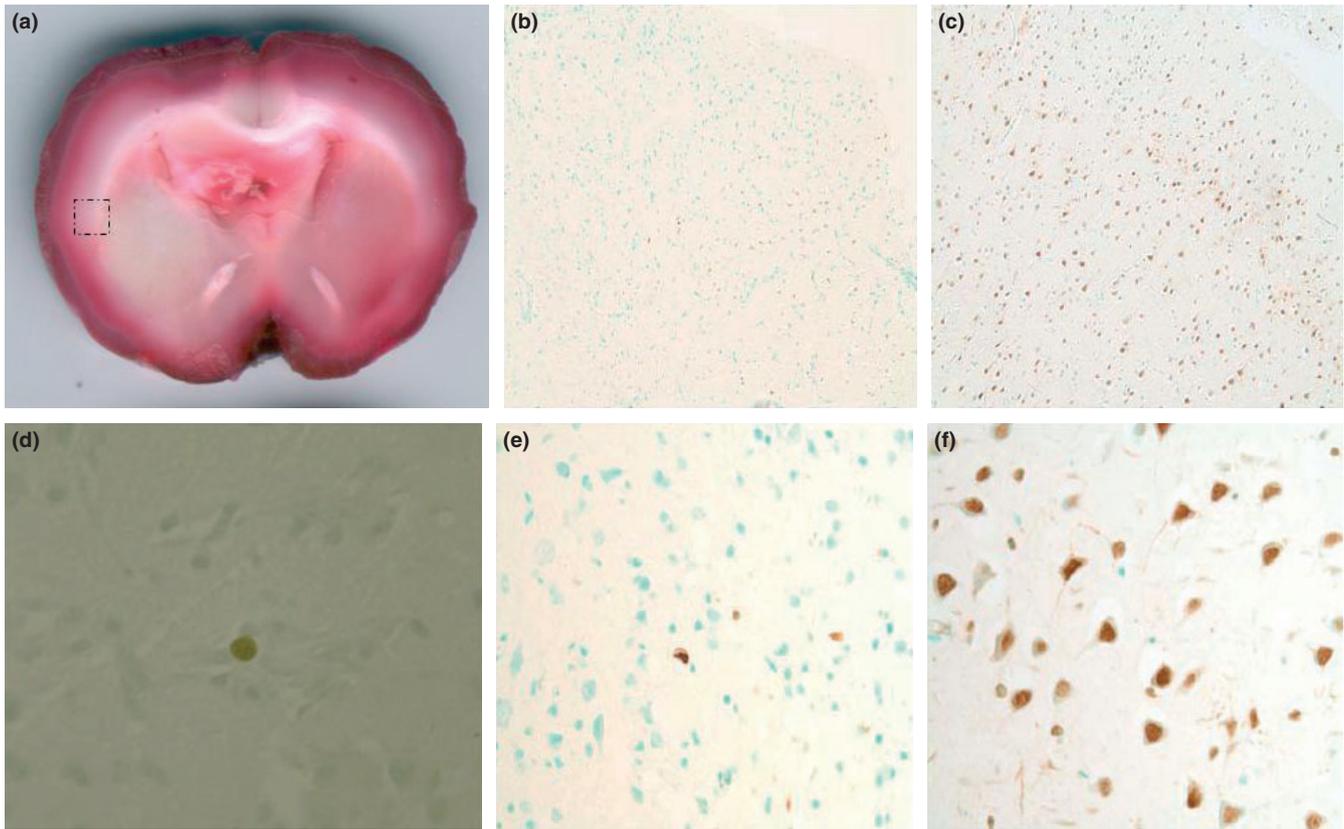
**Fig. 2** Haematoxylin and eosin staining of brain tissues from (a) asialoerythropoietin (aEPO)- and (b) saline-treated rats. The control group shows increased tissue damage and tissue fragmentation compared with the more intact tissues of the 20 µg/kg per day aEPO-treated group.

#### Effects of aEPO on TUNEL-stained cell populations, an analysis of apoptotic cells

Figure 3a,d shows results of immunohistochemical TUNEL analysis in aEPO-treated and control groups, indicating apoptotic DNA fragmentation of tissue samples. Microscopic analysis (Fig. 3b,c) demonstrated a reduced number of TUNEL-positive cells, as indicated by the brown stain, in the aEPO-treated group compared with the control group. Following MCAO, I/R brain injury led to a TUNEL-stained area of 26.4 ± 17.4% of the total brain field. Continuous administration of aEPO significantly reduced the TUNEL-stained area to 3.8 ± 1.8% of the total brain field ( $P < 0.01$ ).

#### Effects of aEPO on caspase 9 and caspase 3

Immunohistochemical detection of apoptotic initiators and activated caspase 9 and 3 is shown in Figs 4 and 5. Quantitative analysis of the aEPO-treated group (Fig. 4a) compared with the control group (Fig. 4b) revealed a decreased number of cells containing activated, cleaved caspase 9 after continuous administration of aEPO. In the control group, 24.6 ± 2.1% cells were positive for cleaved caspase 9



**Fig. 3** Terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL) and 2,3,4-triphenyltetrazolium chloride (TTC) staining of brain tissues from asialoerythropoietin (aEPO)- and saline-treated rats. (a) Representative photograph showing a brain coronal section (TTC stain) located on the posterior bregma (0.48 mm). The dotted square indicates the area evaluated. A cell was considered TUNEL positive if it was stained brown (from light to dark) and if it exhibited apoptotic bodies, chromatin condensation and membrane blebbing (d). (b,c) Qualitative TUNEL image from the aEPO- (b) and control (c) groups, showing decreased positive staining after aEPO treatment (original magnification  $\times 10$ ). (e,f) Reduced quantitative TUNEL staining in the aEPO- (e) and control (f) groups (original magnification  $\times 40$ ;  $P < 0.01$ ).

compared with  $6.6 \pm 2.7\%$  cells in the aEPO-treated group ( $P < 0.001$ ). Qualitative differences between aEPO and control samples in the number of cells containing activated, cleaved caspase 3 are shown in Fig. 5. Quantitative analysis of cleaved caspase 3 staining demonstrated that fewer cells stained positive in the immediate penumbra region in the aEPO group compared with the control group (13.4 vs 17.7%, respectively;  $n = 3$  in both groups), although the difference did not reach statistical significance.

#### Effects of aEPO on neurological behaviour

The average neurological behaviour scores for the aEPO- and saline-treated groups improved significantly from Day 1 to Day 4 (both  $P < 0.05$ ). However, no significant differences were found in average neurological behaviour scores between the two groups on either Day 1 or Day 4.

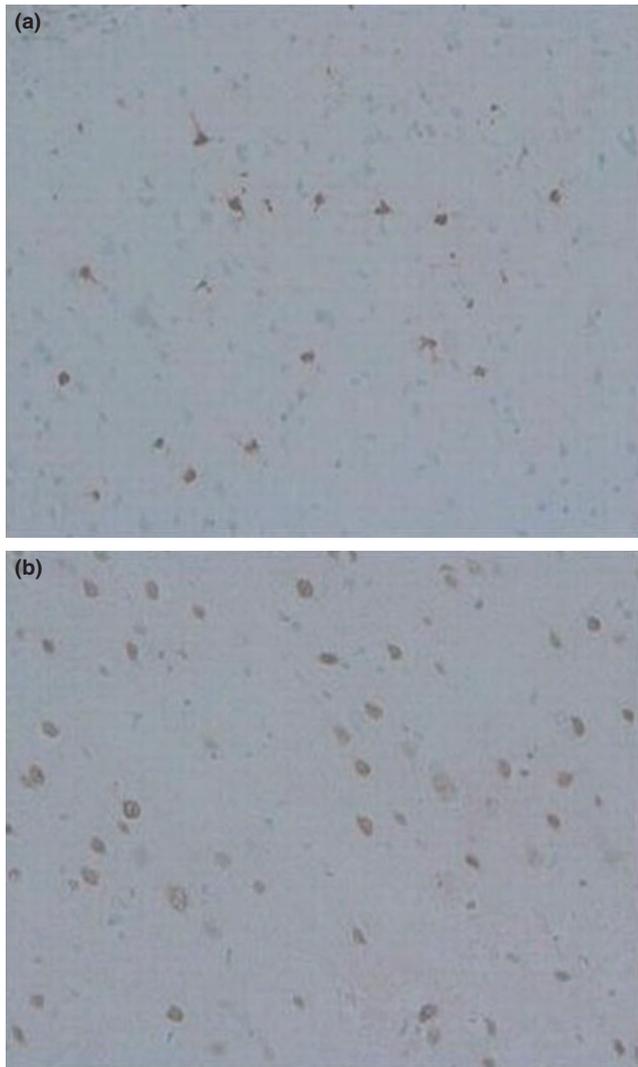
## DISCUSSION

The present study demonstrated that aEPO crosses the blood–brain barrier when administered at a continuous rate via subcutaneously implanted osmotic minipumps. The presence of aEPO in the brain significantly alleviated MCAO-induced I/R brain injury. However,

the presence of aEPO in the brain did not improve MCAO-induced behavioural outcomes.

Loss of bodyweight is common after surgery.<sup>16</sup> In the MCAO model, permanent ligation of the ECA reduces blood perfusion to the tongue, salivary glands, face and mastication muscles necessary for proper food ingestion, all of which can further decrease food intake, resulting in greater weight loss.<sup>17</sup> In the present study, there was no difference in weight loss between the aEPO- and saline-treated groups 4 days after MCAO, suggesting that continuous infusion of aEPO has no significant effect on food intake and bodyweight changes.

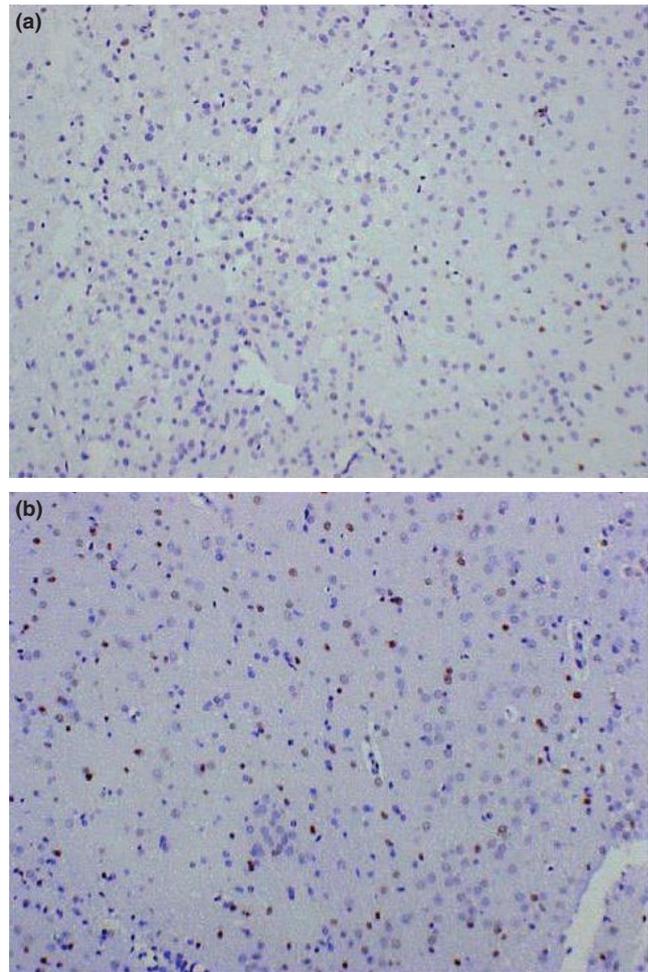
In clinical studies of ischaemic brain injury, repetitive doses of rhEPO have been administered to achieve the highest possible neuroprotection, especially against delayed cell death.<sup>2</sup> Because rhEPO has a haematopoietic function, repeated dosing with rhEPO may increase blood Hct, leading to thrombic aggregations, and decrease the potential neuroprotective effect.<sup>18</sup> A recent study showed that repeated injection of aEPO biweekly for 5 weeks did not increase haemoglobin concentrations in mice.<sup>7</sup> In the present study, continuous administration of aEPO for 4 days did not increase the Hct, providing further evidence that aEPO does not have a haematopoietic effect. Therefore, aEPO may be preferable in a clinical setting when repeated dosing is necessary.



**Fig. 4** Immunohistochemical analysis of activated caspase 9 in brain tissues from (a) asialoerythropoietin (aEPO)- and (b) saline-treated rats. Caspase 9 activation was significantly reduced in the aEPO-treated group compared with the control group 4 days after the ischaemic insult.

Wang *et al.*<sup>19</sup> administered aEPO before ischaemic injury as a preconditioning technique and observed significant improvement in viable neuronal counts and neuropathology scores 5 days after injury. In another study, aEPO was administered immediately following reperfusion after MCAO and its beneficial effect demonstrated 24 h after insult (a marked reduction in the infarct area).<sup>7</sup> The results of the present study suggest that aEPO may be administered continuously via an implanted pump for extended periods of time to achieve greater neuroprotective effects after ischaemic insult.

Caspases play an essential role in the ischaemic injury induced programmed cell death cascade. There are two types of apoptotic caspases: initiator (apical) caspases (e.g. caspase 9) and effector (executioner) caspases (e.g. caspase 3). Initiator caspases cleave inactive proforms of effector caspases, thereby activating them. Effector caspases, in turn, cleave other protein substrates within the cell, resulting in apoptosis. The initiation of this cascade is regulated by caspase inhibitors.<sup>20</sup> A recent study has suggested that, in an experimental traumatic brain injury model, the neuroprotective effects of EPO may



**Fig. 5** Immunohistochemical qualitative imaging of activated caspase 3 in brain tissues from (a) asialoerythropoietin (aEPO)- and (b) saline-treated rats. Decreased staining for activated caspase 3 was seen in the aEPO-treated group compared with the control group.

be mediated via modulation of gene expression (i.e. enhanced expression of anti-apoptotic genes and inhibition of pro-apoptotic genes).<sup>21</sup> The results of the present study provide further evidence that the neuroprotective effect of aEPO involves an inhibitory effect of ischaemia-induced activation of caspases, especially initiator caspase 9. The present study, as well as previous studies, suggest that aEPO may have a neuroprotective action against both necrotic cell death<sup>7,22,23</sup> and apoptotic cell death<sup>24–26</sup> in ischaemic brain injury.<sup>7,19</sup>

Recent *in vitro* studies suggest that the neuroprotective effects of EPO may be dose related, following a bell-shaped curve.<sup>5,25,27,28</sup> Erbayraktar *et al.*<sup>7</sup> reported that single i.p. injections of aEPO in the range 5–50 µg/kg provided similar neuroprotective effects compared with rhEPO; specifically, a single i.v. bolus of 44 µg/kg quantitatively reduced infarct volume by approximately 50% 24 h after MCAO. The present study has shown that aEPO does reach the brain under conditions of continuous administration of aEPO via subcutaneously implanted osmotic minipumps. Due to the short plasma half-life of aEPO, the use of minipumps may be useful in delivering aEPO into the brain over an extended period of time to maximize the neuroprotection afforded by the compound. Future studies are needed to examine the neuroprotective effects of different doses and duration of administration of aEPO in ischaemic brain injury.

One limitation of the present study is that only motor function was evaluated. Neurological behaviour scores improved significantly from Day 1 to Day 4 after MCAO. This result is in agreement with those of previous studies, namely that there are significant signs of neurological recovery for up to 7 days after MCAO.<sup>13</sup> Conversely, the improved neurological scores in the present study were similar in the aEPO and control groups following MCAO. Based on our data, aEPO may not be able to improve MCAO-induced behavioural dysfunction. As Robinson *et al.* pointed out,<sup>29</sup> the major limitation of the rat model of cerebral ischaemia is the lack of an accurate method with which to assess neurological dysfunction after cerebral infarction has been produced. The present study suggests that the five-point scale used to assess neurological behaviour in the present study may not be sensitive enough to detect discrepancies created by the infusion of aEPO. It has been suggested that in addition to motor abnormalities, a variety of behavioural and learning functions should be evaluated to distinguish differences between aEPO and control rats.<sup>29,30</sup>

A second limitation of the present study is that brain temperature was not further monitored when rats recovered from the surgical procedure. As pointed out by DeBow and Colbourne,<sup>31</sup> postischaemic temperature often changes unintentionally as a result of putative neuroprotective drugs, the anaesthetic used, the ischaemia model and perhaps the method of measuring temperature. Body temperature, either hypothermia or hyperthermia, can significantly affect the overall severity of MCAO-induced brain injury in animal models,<sup>32,33</sup> suggesting that prolonged temperature measurement and regulation are essential in studies of ischaemia. It is not known whether administration of aEPO affects brain temperature. Although all rats were kept in the same environment in the present study, the possibility that secondary brain temperature changes may confound outcome cannot be ruled out. An implantable telemetry temperature probe may be used in future studies.

A third limitation of the present study is that aEPO administration started almost immediately after reperfusion. This is essentially not possible in the treatment of clinical stroke. A recent study suggests that rhEPO and carbamylated erythropoietin (CEPO) may have anti-inflammatory and anti-apoptotic effects even with administration 6 h after embolic MCAO.<sup>34</sup> In order to evaluate the possible clinical benefit of aEPO in human stroke patients, a further study is needed to examine the effect of delayed treatment with aEPO.

A fourth limitation of the present study is that only young male rats were investigated. The incidence and prevalence of ischaemic stroke differ between males and females.<sup>35</sup> Female rats exhibit better outcomes after ischaemic brain injury, possible due to the neuroprotective and blood flow-preserving effects of oestrogen.<sup>35,36</sup> In addition to endogenous neuroprotection from oestrogen, emerging data suggest that biochemical pathways involved in hypoxic or ischaemic cell death may have a differential effect in males versus females.<sup>37</sup> The neuroprotective effects of aEPO in female rats warrant further investigation.

Another limitation of the present study is that TUNEL-stained cell populations and cells positive for cleaved caspase 3 and 9 were evaluated in the areas 'adjacent' to the infarct area. Because the area of the infarct varied from animal to animal, the areas 'adjacent' to the infarct also varied. This raises the possibility that the areas selected may not, in fact, be comparable. However, there currently is no good solution to this problem.

Finally, as highlighted by DeBow *et al.*,<sup>38</sup> long-term follow up is essential for any study of neuroprotection. Future studies should focus on the long-term neuroprotective effects of aEPO in I/R brain injury.

In conclusion, our data suggest that aEPO provides short-term, possibly histological, protection in young adult male rats when administered after reperfusion. The reduced apoptotic markers indicate a possible neuroprotective effect of aEPO against apoptotic cell death. However, our study suggests that aEPO may not have beneficial effects on behavioural outcomes. Theoretically, aEPO may be more clinically advantageous than rhEPO because it maintains its neuroprotective effect without thrombogenic potential, especially if repeated dosing is necessary.

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