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Effect of recombinant human erythropoietin on cerebral ischemia following experimental subarachnoid hemorrhage

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Abstract

Erythropoietin exerts a neuroprotective effect during cerebral ischemia. We investigated the effect of systemic administration of recombinant human erythropoietin in a rabbit model of subarachnoid hemorrhage-induced acute cerebral ischemia. The animals were divided into three groups: group 1, subarachnoid hemorrhage; group 2, subarachnoid hemorrhage plus placebo; group 3, subarachnoid hemorrhage plus recombinant human erythropoietin (each group, $n = 8$). Experimental subarachnoid hemorrhage was produced by injecting autologous blood into the cisterna magna. Treatment with recombinant human erythropoietin and placebo was started 5 min after subarachnoid hemorrhage and was continued every 8 h for 24 h. Before the animals were killed, erythropoietin concentration was measured in the cerebrospinal fluid. The rabbits were killed 24 h after subarachnoid hemorrhage and ischemic brain injury was histologically evaluated. In group 3, the concentration of erythropoietin in the cerebrospinal fluid was significantly increased and a significant reduction in cortical necrotic neuron count was also observed. These findings may encourage the use of erythropoietin in the treatment of cerebral ischemia that often occurs in the early stage of subarachnoid hemorrhage.

Keywords: Brain ischemia; Erythropoietin; Subarachnoid hemorrhage

1. Introduction

Acute cerebral ischemia, brain edema and cerebral vasospasm that often occur in subarachnoid hemorrhage are critical problems in the management of patients affected by ruptured intracranial aneurysms. So far, the literature has mainly dealt with many aspects of cerebral vasospasm, neglecting the therapeutic strategies to limit the ischemic brain damage which may occur, either in the early or late stage of aneurysmal subarachnoid hemorrhage. The mortality rate from subarachnoid hemorrhage is 45%, 25% of patients dying within 24 h (Broderick et al., 1994). The histopathological examination of brains of patients who died shortly after subarachnoid hemorrhage has shown extensive ischemic damage (Stoltenburg-Didinger and Schwarz, 1987). These findings suggest that acute cerebral ischemia after subarachnoid hemorrhage may contribute to early death. Acute cerebral ischemia, which occurs after subarachnoid hemorrhage, has largely been considered to be caused by luminal narrowing of the large extraparenchymal arteries (Weir et al., 1978). However, some authors report (Jakobson et al., 1990; Kelly et al., 1977) that angiographic vasospasm does not always correlate with early neurological symptoms, suggesting that other factors, such as the microcirculatory changes in intraparenchymal vessels (Kassel et al., 1985), must be considered together with the vasoconstriction of the major cerebral arteries as possible contributory causes in cerebral ischemia.

Ischemic brain injury is mainly mediated by excitatory amino acid neurotransmitters such as glutamate: the extracellular glutamate level increases rapidly after the onset of

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cerebral ischemia and in proportion to the severity of ischemia (Benveniste et al., 1984). In vitro and in vivo studies indicate that the hormone, erythropoietin, protects cerebral cortical neurons from *N*-methyl-D-aspartate (NMDA) receptor-mediated glutamate toxicity, which is believed to play an important role in neuronal death from ischemia (Morishida et al., 1997). Recent studies have documented the presence of erythropoietin and its specific receptors in the central nervous system, where astrocytes produce erythropoietin, and neurons express its specific receptors (Masuda et al., 1993, 1994; Morishida et al., 1996, 1997). Human erythropoietin is a glycoprotein growth factor which acts as the main regulator of erythropoiesis (Jacobson, 1992). In the fetus, it is produced by the liver, where erythropoiesis takes place (Zanjani et al., 1977). In adults, the kidney is the major source of erythropoietin production (Jacobson et al., 1957). Low oxygen tension stimulates erythropoietin production through an increase of its mRNA (Goldberg et al., 1988). A recent immunohistochemical study has shown a specific distribution of erythropoietin and its receptors in the developing human brain (Juul et al., 1999). These findings suggest that erythropoietin is important in neurodevelopment and may play a role in brain homeostasis later in life, functioning in an autocrine or paracrine manner.

Based on these findings and reports, which have demonstrated that erythropoietin has a protective effect in several types of microcirculatory dysfunction, such as ischemia or post-ischemic reperfusion of the splanchnic territory and cerebral district (Buemi et al., 1993, 2000b; Morishida et al., 1997), we have now evaluated the efficacy of recombinant human erythropoietin on acute cerebral ischemia following experimental subarachnoid hemorrhage in a rabbit model.

2. Materials and methods

Twenty-four male New Zealand white rabbits, weighing 3.2–3.9 kg, were used in this study. All procedures were approved by the Ethical Committee of the University of Messina. The animals were cared for in accordance with the guidelines for animal experiments at the University of Messina.

The animals were anesthetized by an intramuscular injection of a mixture of ketamine (40 mg/kg) and xylazine (8 mg/kg) and intubated. The central ear artery was cannulated to obtain 5 ml of autologous arterial blood. A 23-gauge butterfly needle was inserted percutaneously into the cisterna magna and autologous blood was slowly injected (within 1 min) to avoid a sudden rise in intracranial pressure. After the injection, the rabbits were positioned in ventral recumbence for 15 min to allow ventral blood-clot formation. The animals were then monitored during the next 24 h.

The rabbits were divided into the following three groups: group 1 ($n = 8$) subarachnoid hemorrhage; group 2 ($n = 8$) subarachnoid hemorrhage plus placebo; group 3 ($n = 8$) subarachnoid hemorrhage plus recombinant human erythropoietin. All injections were administered 5 min after the induction of subarachnoid hemorrhage (Buemi et al., 2000b), and were continued every 8 h for 24 h. All doses, placebo and recombinant human erythropoietin, were administered intraperitoneally. Rabbits in the subarachnoid hemorrhage plus placebo group (group 2) received the vehicle used for recombinant human erythropoietin administration (serum albumin 2.5 mg/ml, sodium chloride 5.84 mg/ml, sodium citrate 5.80 mg/ml, anhydrous citric acid 0.057 mg/ml, H_2O) at the dose of 1 ml/kg body weight (Buemi et al., 1993, 2000b). Rabbits in the subarachnoid hemorrhage plus recombinant human erythropoietin group (group 3) were given recombinant human erythropoietin at the dose of 209 000 000 pmol/kg (1000 IU/l). The dose of 209 000 000 pmol/kg was chosen according to a recent study on recombinant human erythropoietin pharmacokinetics in rabbits (Yoon et al., 1997) and was based on the results obtained in our previous study, which had shown that the dose of 209 000 000 pmol was effective to reduce the mortality rate and enhance the functional recovery in an experimental model of subarachnoid hemorrhage in rabbits (Buemi et al., 2000b).

Twenty-three hours post-subarachnoid hemorrhage, a 23-gauge butterfly needle was inserted into the cisterna magna and cerebrospinal fluid samples (1 ml) were collected from each animal in order to measure the cerebrospinal erythropoietin concentration by radioimmunoassay (Eckardt et al., 1988).

Twenty-four hours following subarachnoid hemorrhage, the rabbits were anesthetized and the central ear artery was catheterized for monitoring blood pressure and gas level. After obtaining satisfactory respiratory parameters, all animals were killed by the perfusion–fixation method (Arthur

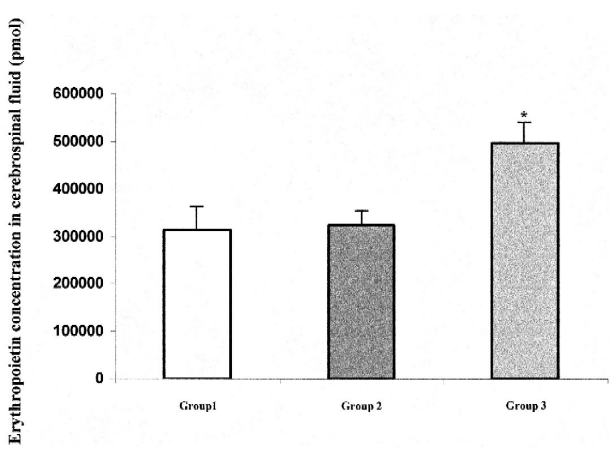

Fig. 1. Graph showing the mean erythropoietin concentration in cerebrospinal fluid which was quantified 23 h post-subarachnoid hemorrhage by radioimmunoassay. Erythropoietin concentration was significantly increased in the subarachnoid hemorrhage plus recombinant human erythropoietin group (group 3), compared with the subarachnoid hemorrhage (group 1), and subarachnoid hemorrhage plus placebo groups (group 2) ($^* P < 0.005$).

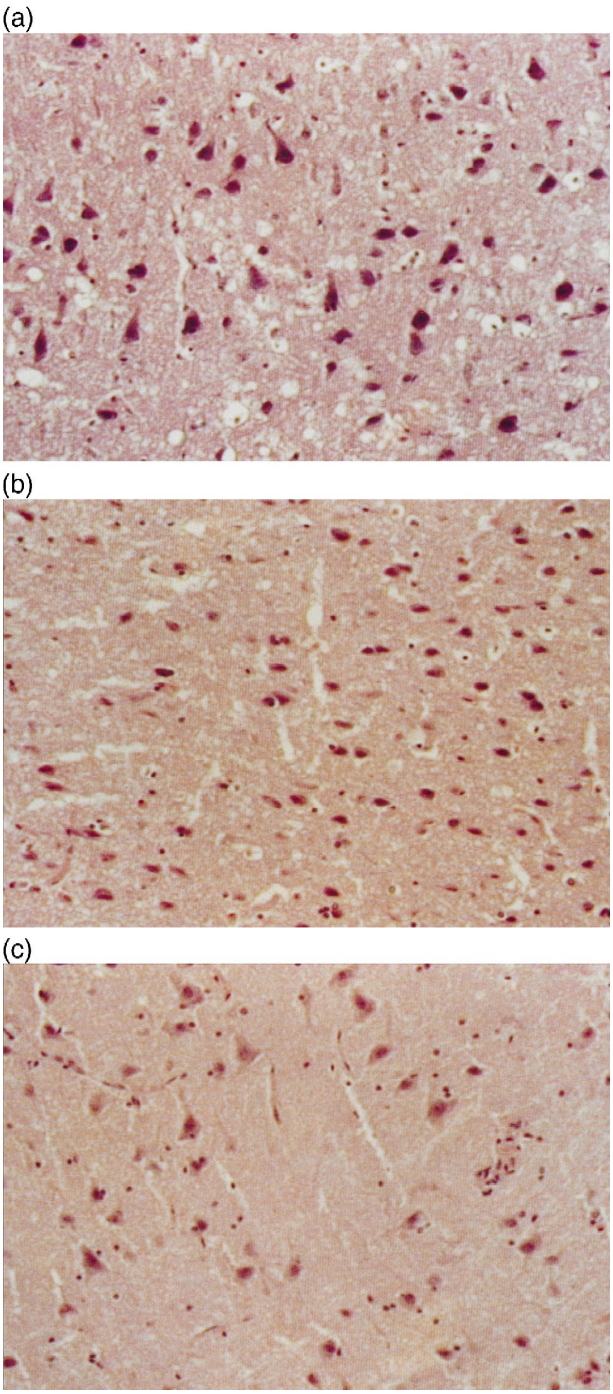

Fig. 2. Paraffin-embedded sections (stained with hematoxylin and eosin) obtained from each treatment group (original magnification ×920), 24 h after the induction of subarachnoid hemorrhage. The cortical parenchyma of the subarachnoid hemorrhage (a) and subarachnoid hemorrhage plus placebo (b) groups contains a high frequency of necrotic cortical neurons. Sections of subarachnoid hemorrhage plus recombinant human erythropoietin (c) contain fewer damaged neurons compared with those from the other groups.

et al., 1997). The brains were then removed and coronally sectioned into 2-mm thick slices, starting at the bregma and continuing posteriorly to include the cerebellum (Ireland and MacLeod, 1993). The slices were then placed in a freezing microtome and sectioned at 10–25 μm. The slices were stained with hematoxylin and eosin in order to assess the number of ischemia-induced damaged neurons. The number of eosinophilic neuronal profiles containing pyknotic nuclei per high-power microscopic field (100 ×) were counted in five randomly selected sections of the lateral cortex obtained at several coronal levels posterior to the bregma. Each microscopic field corresponded to approximately 1.6 mm^2.

2.1. Statistical analysis

Values for erythropoietin concentration in cerebrospinal fluid were expressed as means ± SEM. Student's *t*-test and Pearson's correlation coefficient were used for statistical analysis. Differences were considered significant at a P value of less than 0.05.

Data obtained from the analysis of the frequency of damaged cortical neurons were expressed as means ± SEM and compared by the Kruskal–Wallis one-way analysis of variance by ranks.

3. Results

In the cerebrospinal fluid, the mean concentration of erythropoietin was 313 500 pmol in the subarachnoid hemorrhage group (group 1), while 323 950 pmol was found in subarachnoid hemorrhage plus placebo group (group 2). An increase in erythropoietin concentration was found in subarachnoid hemorrhage plus recombinant human erythropoietin group (group 3), in which the mean value observed was 497 420 pmol ($P < 0.005$).

Fig. 1 shows a graphical representation of the mean erythropoietin concentration in cerebrospinal fluid.

Both subarachnoid hemorrhage and subarachnoid hemorrhage plus placebo groups contained a high frequency of damaged cortical neurons, which were detected in the

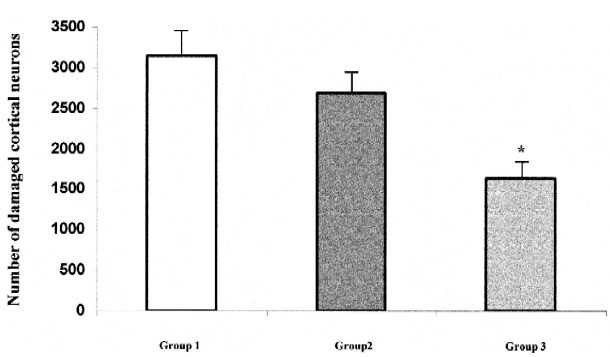

Fig. 3. Bar graph of means ± SEM showing the number of ischemia-induced damaged cortical neurons from each group which was detected histologically 24 h following subarachnoid hemorrhage. Animals treated with recombinant human erythropoietin (group 3) presented with a significant decrease in the frequency of necrotic neurons compared with the subarachnoid hemorrhage (group 1) and subarachnoid hemorrhage plus placebo groups (group 2) ($^{*}P < 0.01$).

lateral cerebral cortex (Fig. 2a,b). Analysis of the subarachnoid hemorrhage plus recombinant human erythropoietin group (Fig. 2c) demonstrated a significant decrease in the frequency of damaged neurons compared with the other groups ($P < 0.01$).

Fig. 3 shows the total number of damaged cortical neurons for the three groups.

4. Discussion

Aneurysmal subarachnoid hemorrhage causes brain injury either acutely or as a result of delayed cerebral vasospasm. Despite recent progress in the understanding of the pathogenesis of delayed cerebral vasospasm, few studies have been reported on subarachnoid hemorrhage-induced acute cerebral ischemia. So far, few therapeutic strategies have been assessed to treat this neglected aspect of aneurysmal subarachnoid hemorrhage.

Acute cerebral ischemia, which often follows subarachnoid hemorrhage, has been attributed to a decrease in cerebral perfusion pressure (Fisher, 1975; Weaver and Fisher, 1994). However, experimental studies demonstrate that cerebral perfusion pressure does not drop to the point of perfusion arrest (Dorsh et al., 1989; McCormick et al., 1994), suggesting that the decrease in cerebral perfusion pressure does not fully account for the acute ischemic brain damage after subarachnoid hemorrhage.

An unfavourable clinical course following subarachnoid hemorrhage is usually attributed to luminal narrowing of large extraparenchymal arteries. However, this phenomenon does not always explain the diffuse brain ischemia and altered cerebrovascular reactivity that frequently follow subarachnoid hemorrhage (Shigeno et al., 1982; Smith, 1963).

Based on this evidence, other mechanisms, such as cerebral microcirculatory dysfunction, may be involved. Morphological studies have demonstrated constriction and impaired perfusion of the intraparenchymal vessels following experimental subarachnoid hemorrhage. In particular, Hart (1981) and Wiernsperger et al. (1981) have documented the constriction of intraparenchymal vessels following cisternal injection of blood. Asano and Sano (1977) and Nagai et al. (1976) have also reported microcirculatory disturbances in experimental models of subarachnoid hemorrhage. Several theories have been proposed to explain this phenomenon, and the main question which arises is whether the constriction of the intraparenchymal vessels is due to active or passive mechanisms. The reduction in capillary diameter may be passive, being due to the fall in perfusion pressure gradient distal to arterial vessels spasm. However, as well as involving the extraparenchymal large vessels, the constriction could be determined by spasmogenic substances deriving from subarachnoid clots which penetrate via the Virchow–Robin spaces (Hart, 1981; Wiernsperger et al., 1981; Cardoso et al., 1985).

Ischemic brain damage is mediated in part by excitatory amino acid neurotransmitters, such as glutamate (Benveniste et al., 1984; Drejer et al., 1985).

The extracellular glutamate concentration in the central nervous system increases in specific cerebral regions and for a short period (Benveniste et al., 1984). A massive and sustained increase in the intracellular Ca^{2+} concentration evoked by activation of glutamate-induced NMDA receptors plays a critical role in determining the intracellular enzymatic cascade that ultimately causes neuronal death (Choi and Rothman, 1990). Extracellular glutamate levels increase rapidly after the onset of cerebral ischemia and in proportion to the severity of ischemia (Benveniste et al., 1984). Studies on subarachnoid hemorrhage in humans have demonstrated, using intracerebral microdialysis, variable increases in extracellular glutamate concentrations (Shimada et al., 1989). Such occurrences have been confirmed in experimental models of subarachnoid hemorrhage, in which extracellular measurement of glutamate levels in cortical regions, at various time points, has provided an indication of the severity and duration of ischemia during subarachnoid hemorrhage (Shimada et al., 1989; Choi and Rothman, 1990; Bederson et al., 1998).

Experimental studies have recently shown a neuroprotective effect of erythropoietin during cerebral ischemia. Morishida et al. (1997) cultured hippocampal and cortical neurons with glutamate, in order to assess the effect of erythropoietin on glutamate-induced neuronal death. Exposure of the cells to 1 mM glutamate for 15 min reduced the viable cells by 30% in hippocampal neurons, and by 40% in cortical neurons. Cultures were exposed to 3, 30 and 300 pmol of recombinant human erythropoietin. Erythropoietin prevented the glutamate-induced cell death in a dose-dependent manner in neurons cultured in its presence. Cultures with 3 pmol erythropoietin had a significantly increased number of viable cells compared with cultures without erythropoietin, and cultures with 30 pmol erythropoietin almost completely protected neurons from glutamate toxicity. Cells exposed to 300 pmol erythropoietin showed a protective effect similar to that observed with 30 pmol. The authors have suggested that a very short incubation period with erythropoietin (5 min or less) is sufficient to induce a neuroprotective effect against glutamate toxicity, although a relatively long incubation period (8 h) is required for the cells to become resistant to glutamate.

Sadamoto et al. (1998) have demonstrated that erythropoietin ameliorates place-navigation disability, cortical infarction and thalamic degeneration in permanently middle cerebral artery-occluded rats. Recombinant human erythropoietin was given by intraventricular infusion for 28 days at the dose of 0.2, 1, or 5 units/day. In this latter study, the dose of 5 units/day was effective to reduce the ischemia-induced cerebral damage. These authors, using in situ hybridization, have also shown that erythropoietin receptor-mRNA was upregulated in the periphery of the cerebrocortical infarct (so-called ischemic penumbra), sug-

gesting that an increased number of erythropoietin receptors in neurons facilitates erythropoietin signal transmission, preventing enlargement of the damaged area.

Sakanaka et al. (1998), in an experimental model of cerebral ischemia in the gerbil, demonstrated by using a lateral ventricular infusion of soluble erythropoietin receptor, that erythropoietin ameliorates neuron survival. Soluble erythropoietin receptor, an extracellular domain capable of binding with erythropoietin, forms a complex with erythropoietin which prevents its binding with neuronal erythropoietin receptors. In ischemic gerbils, the continuous infusion of soluble erythropoietin receptors caused a significant reduction in the erythropoietin response latency time and a significant decrease in neuron cell density when compared with those of vehicle-infused ischemic animals. The continuous infusion of erythropoietin at a dose of 2.5, 5 and 25 units/day for 7 days caused a significant increase in response latency time, preventing degeneration of ischemic neurons.

Koshimura et al. (1999) investigated the effect of erythropoietin on Ca^{2+} uptake, intracellular Ca^{2+} concentration, membrane potential, cell survival, release and biosynthesis of dopamine and nitric oxide production in differentiated PC12 neuronal cells. Cultures were exposed to $10(-12)$–$10(-10)$ M of recombinant human erythropoietin. The authors demonstrated that erythropoietin increased Ca^{2+} uptake, intracellular Ca^{2+} concentration and membrane depolarization in PC12 neuronal cells. Cultures exposed to erythropoietin showed an increase in number of viable cells, stimulation of mitogen-activated protein kinase activity, dopamine release and increased nitric oxide production. Based on these results, the authors suggested that erythropoietin stimulates neuronal function and viability via activation of Ca^{2+} channels.

Recently, Bernaudin et al. (1999) showed the potential role of erythropoietin in focal permanent cerebral ischemia in mice. Focal ischemia was induced by permanent occlusion of the left middle cerebral artery. Mice were treated by intraventricular injection of recombinant mouse erythropoietin at the dose of 0.4 μg/kg, 24 h before operation and at the time of the occlusion. Histological analysis has shown a significant reduction of the infarct volume in animals treated with erythropoietin. In the same study, the authors assessed the efficacy of recombinant human erythropoietin to prevent neuronal death caused by exposure of neocortical neurons to 15 μmol/l NMDA for 24 h. This substance caused 50% cell death. When cells were pretreated for 24 h with recombinant human erythropoietin at a concentration of either 30 or 300 pmol/l, in a subsequent exposure to NMDA the 65–70% of cells were protected from damage.

We have recently reported that administration of recombinant human erythropoietin immediately after experimental subarachnoid hemorrhage is effective to reduce the mortality rate and ameliorates functional recovery (Buemi et al., 2000b). In particular, we have demonstrated that all rabbits (100%) treated with 1000 IU/kg of recombinant human erythropoietin survived for at least 72 h, while 42.9% of the subarachnoid hemorrhage plus placebo group, died within 72 h. An open-field test performed at 24, 48 and 72 h after subarachnoid hemorrhage showed an increase in locomotor activity at 72 h in the placebo-treated group, while no increase in locomotor activity was observed in rabbits treated with recombinant human erythropoietin. In animals treated with recombinant human erythropoietin, an increase of erythropoietin concentration in cerebrospinal fluid was detected compared to that in the other groups.

The results obtained in the present study, suggest that erythropoietin is effective to prevent brain ischemic damage following subarachnoid hemorrhage. Histological analysis performed 24 h following subarachnoid hemorrhage has documented a reduction in brain ischemic damage in animals given recombinant human erythropoietin. In particular, analysis of cortical neurons has shown that the subarachnoid hemorrhage plus recombinant human erythropoietin group, presented with a significant decrease in the amount of necrotic neurons compared with the subarachnoid hemorrhage and subarachnoid hemorrhage plus placebo groups. Our findings are in agreement with previous reports which showed a neuroprotective effect of erythropoietin on ischemia-induced neuronal cell death.

At present, the mechanism by which erythropoietin acts as neuroprotective agent in the central nervous system is not well understood. Several theories have been proposed. Morishida et al. (1997) and Koshimura et al. (1999) have suggested that, during ischemia, erythropoietin protects neurons from glutamate toxicity by activation of Ca^{2+} channels. Sakanaka et al. (1998) reported that erythropoietin, like the platelet-derived growth factor, may increase the activity of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase in neurons, protecting the brain parenchyma from ischemic damage.

Based on the evidence that erythropoietin stimulates the migration and proliferation of endothelial cells in vitro, Yamaji et al. (1996) suggested that erythropoietin could modulate angiogenesis in the ischemic brain by improving blood flow and tissue oxygenation in the border zone of the ischemic area. Erythropoietin may also reduce the brain ischemic area by protecting endothelial cells from apoptotic cell death (Bernaudin et al., 1999).

Recently, it has been reported that systemically administered recombinant human erythropoietin does not cross the blood–brain barrier (Buemi et al., 2000a). However, in the present study, we have observed that the concentration of erythropoietin in the cerebrospinal fluid, assessed 23 h before killing of the animals, was significantly higher in the subarachnoid hemorrhage plus recombinant human erythropoietin group than in the other groups. Based on the evidence that subarachnoid hemorrhage is followed by an impairment of the blood–brain barrier, our findings suggest that administration of recombinant human erythropoi-

etin increases the concentration of erythropoietin in the cerebrospinal fluid in the presence of blood–brain barrier dysfunction. This hypothesis is consistent with a recent report (Marti et al., 1997), which has shown a correlation between the concentration of erythropoietin in the serum and cerebrospinal fluid in patients affected by traumatic brain injury. Erythropoietin concentration was detected both in cerebrospinal fluid and serum, and the values obtained were compared with the degree of blood–brain barrier dysfunction. No correlation between the concentration of erythropoietin in the serum and cerebrospinal fluid was observed in the presence of a normal blood–brain barrier. However, when the blood–brain barrier was disturbed, the concentration of erythropoietin in cerebrospinal fluid was significantly increased.

In conclusion, our results suggest that administration of recombinant human erythropoietin significantly attenuates acute ischemic neuronal damage and increases the erythropoietin concentration in the cerebrospinal fluid by crossing the impaired blood–brain barrier following experimental subarachnoid hemorrhage.

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