



Basic fibroblast growth factor alleviates brain injury following global ischemia reperfusion in rabbits

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Abstract: The aim of this study was to explore the protective effect of basic fibroblast growth factor (bFGF) on brain injury following global ischemia reperfusion and its mechanisms. Brain injury following global ischemia was induced by four vessels occlusion and systemic hypotension. Twenty-four rabbits were randomized into three groups: group A, only dissection of vessels; group B, intravenous infusion of normal saline after reperfusion for 6 h; group C, 30 µg/kg bFGF injected intravenously at the onset of reperfusion, then infused with 10 µg/(kg·h) for 6 h. Serum neuron specific enolase (NSE), S-100B, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-8 (IL-8) were measured before ischemia, 30 min after ischemia, 0.5, 1, 3, 6 h after reperfusion. Brain water content was determined and cerebral histopathological damages were compared. NSE and S-100B were increased 1 h after reperfusion and reached their peaks 6 h after reperfusion, but were much higher in group B than those in group C 3, 6 h after reperfusion. In groups B and C, TNF-α was increased after ischemia and IL-1 and IL-8 were increased significantly 0.5 h after reperfusion, then reached their peaks 6 h, 3 h, 6 h after reperfusion respectively. TNF-α and IL-8 at the time points of 1 h and 3 h and IL-1 at 3 h and 6 h in group C were correspondingly lower than those in group B. These indices in group A were nearly unchanged. There were less severe cerebral histopathological damages in group C compared with group B, but no difference in brain water content. It could be concluded that bFGF alleviates brain injury following global ischemia and reperfusion by down-regulating expression of inflammatory factors and inhibiting their activities.

Key words: Basic fibroblast growth factor, Brain injury, Global ischemia, Neuron specific enolase, S-100B protein, Tumor necrosis factor-α, Interleukin

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INTRODUCTION

With increasing public knowledge on cardiopulmonary resuscitation (CPR) and improvements in emergent early response, these have resulted in higher percentage of patients with out-of-hospital cardiac arrest (CA) surviving to admission (Bunch *et al.*, 2004). The hospital discharge rate is only 2%~14%, with most patients being still left with poor neurological outcome (Snyder-Ramos and Bottiger, 2003), due to persistent brain damage. Cerebral resuscitation

plays a key role during the CPR process and remains a challenge. Recent studies showed basic fibroblast growth factor (bFGF) had neuro-protective effect against focal ischemic, excitotoxic and traumatic brain injury (Dietrich *et al.*, 1996; Bethel *et al.*, 1997; Himmelseher *et al.*, 1997; Yoshimura *et al.*, 2003). However the effect on global cerebral ischemia-reperfusion injury, following CA and CPR, has not been investigated. By creating an animal model of global ischemia and reperfusion, we investigated effects of bFGF on serum neuron specific enolase (NSE), S-100B, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-8 (IL-8), in order to

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explore the potential neuroprotection of bFGF in cardiopulmonary resuscitation and the possible mechanisms.

MATERIALS AND METHODS

Animals and model

Cerebral ischemia-reperfusion injury following cardiac arrest was simulated by four vessels occlusion (2 carotids and 2 vertebral arteries) and systemic hypotension (Pomfy and Franko, 1999). Twenty-four healthy rabbits (supplied by Experimental Animal Center, Medical Institute of Zhejiang Province) with mean body weight of 2.6 kg (2.2~3.0 kg), regardless of gender, were randomized into three groups: group A ($n=8$), subjected to dissection of four vessels without occlusion; group B ($n=8$), who underwent dissection and occlusion of vessels, infusion of normal saline (NS); group C ($n=8$), who underwent dissection and occlusion of vessels, infusion of bFGF. The rabbits were anesthetized by slow intravenous injection of Diazepam (4 mg/kg), Haloperidol (0.8 mg/kg) and Fentanyl (16 $\mu\text{g}/\text{kg}$). The femoral artery was cannulated and connected to a transducer. Direct arterial blood pressure together with electrocardiograph (ECG), rectal temperature and partial pressure of end tidal carbon dioxide ($P_{\text{ET},\text{CO}_2}$) were monitored using a multiple-parameters monitor (MB-526, USA). Rectal temperature was kept at about 38.5 °C with a heating system. Tracheotomy was performed and the rabbits were intubated, ventilated with an DH-150 animal ventilator (Medical Instrument Factory of Zhejiang University, China). Ventilator settings were adjusted to maintain $P_{\text{ET},\text{CO}_2}$ at 40 mmHg. Both common carotids and vertebral arteries were dissected and clamped, while hypotension was induced by exsanguinations via femoral arteries, mean blood pressure was kept at 50%~60% of basal value. After 30 min, the four vessels were unclamped and blood was transfused rapidly. In group B, a bolus of 5 ml NS was given intravenously, followed by a continuous infusion of NS at a rate of 5 ml/(kg·h). In group C, it was replaced by a bolus of 30 $\mu\text{g}/\text{kg}$ bFGF (Recombinant bovine basic fibroblast growth factor, 36 micrograms/ampoule, Changchun Changsheng Gene Pharmaceutical Co. Ltd., China), then 10 $\mu\text{g}/(\text{kg}\cdot\text{h})$ infused continuously, dissolved in the same volume of NS.

Sample preparation and measurements

Three milliliters of blood samples were taken before occlusion of vessels (basal time), at the end of ischemia (0 h), 0.5, 1, 3, 6 h after reperfusion respectively and the same volume of NS was infused after sampling. All blood specimens were processed to serum, centrifugated at 3000 rpm for 10 min at room temperature, then stored in aliquots at -80 °C until batch evaluation. Serum NSE, S-100B, TNF- α , IL-1 and IL-8 were measured by enzyme linked immunosorbant assay on Opsys MR microplate reader (Dynex Inc., USA), according to the operating instructions. Kits for rabbit NSE and S-100B were supplied by Lifekey BioMeditech Inc. (USA), and those for TNF- α , IL-1, IL-8 by Sigma Inc. (USA). The animals were sacrificed by exsanguination 6 h after reperfusion. The right hemispheres were sampled and kept at 100 °C in a drier for 12 h, then the brain water content was determined by the difference between dry and wet weight. The left hemispheres were stored in 10% formalin. Histopathological sections were performed routinely, stained with hematoxylin and eosin (HE).

Statistical analysis

All data were expressed as mean \pm standard deviation. Differences between groups or different time points were compared using one-way ANOVA and Tukey test (SigmaStat 2.03 software package was used). Correlation between serum NSE and S-100B was analyzed with linear regression. A P value of <0.05 was considered as statistically significant.

RESULTS

General animal characteristics

There were no differences in body weight, sexual ratio, and basal values of rectal temperature, arterial blood pressure and heart rate between the three groups. Compared with group A, arterial blood pressure in groups B and C increased rapidly after occlusion of four vessels, and was maintained at half of the basal value during exsanguination, decreased sequentially after reperfusion, ameliorated gradually after transfusion, then decreased again 1 h after reperfusion. Blood pressure at different time points in group C seemed slightly lower than that in group B,

but the differences were not statistically significant.

Changes in serum NSE and S-100B

As shown in Fig.1, serum NSE and S-100B in group A at basal time were $(2.51 \pm 0.67) \mu\text{g/L}$, $(21.53 \pm 3.84) \text{ng/L}$ respectively, remaining unchanged throughout the study. There were no differences between serum NSE and S-100B in groups B and C from those in group A at the first three time points. One hour after reperfusion, serum NSE and S-100B increased, then reached their peaks 6 h after reperfusion. Serum NSE and S-100B in group B were much higher than those in group C 3 h, 6 h after reperfusion. There was significant positive correlation between serum NSE and S-100B ($r=0.736$, $P<0.001$).

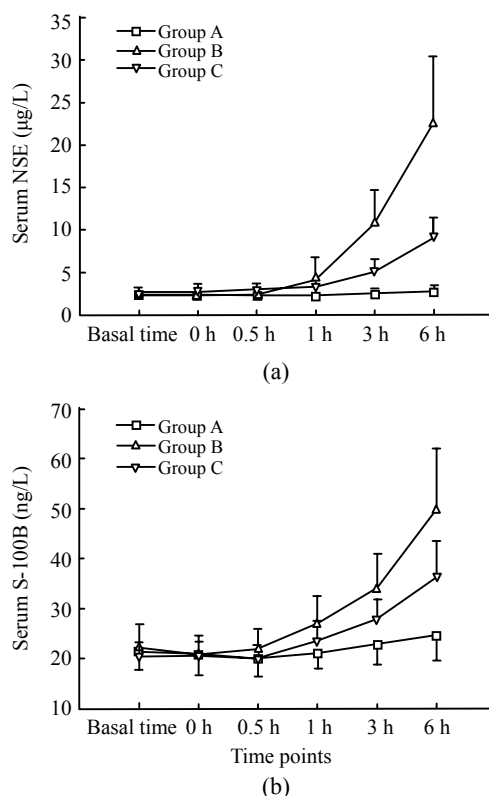


Fig.1 Dynamic changes of serum NSE and serum S-100B in three groups (a) Serum NSE at the time points of 3 h, 6 h in group C were significantly less than those in group B ($q=6.701$, 8.358 ; both $P<0.001$); (b) Serum S-100B at the time points of 3 h, 6 h in group C were significantly less than those in group B ($q=3.582$, 4.379 ; $P=0.049$, 0.015)

Changes in serum TNF- α , IL-1, IL-8

Details are shown in Fig.2. There were no differences for serum TNF- α , IL-1, IL-8 between the three groups at the basal point. Compared with that at

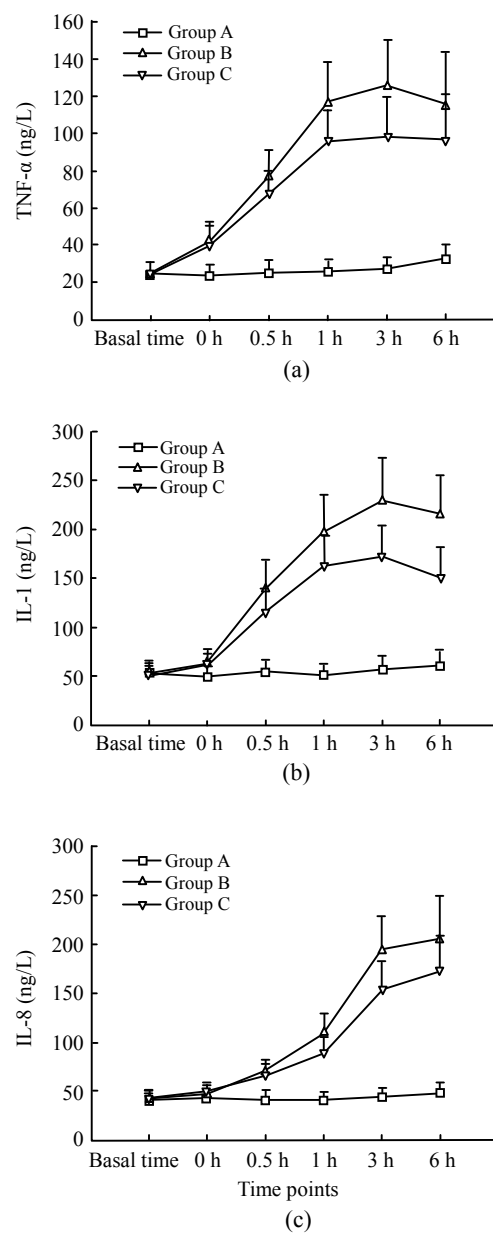


Fig.2 Dynamic changes of serum TNF- α (a), IL-1 (b), IL-8 (c) in three groups. Compared with that at basal time point, TNF- α in groups B and C increased significantly 30 min after ischemia (0 h) and reached its peak 3 h after reperfusion; these were correspondingly much higher than those in group A; TNF- α at time points of 1 h, 3 h in group B were significantly higher than those in group C ($q=3.665$, 4.158 ; $P=0.043$, 0.021), whereas there was no difference at other time points. IL-1 and IL-8 in group B and C increased significantly 0.5 h after reperfusion and reached their peaks 3 h, 6 h after reperfusion respectively, and were much higher than those in group A; IL-1 at time points of 3 h, 6 h in group B were significantly higher than those in group C ($q=5.168$, 5.938 ; $P=0.004$, 0.001); IL-8 at time points of 1 h, 3 h in group B were significantly higher than those in group C ($q=3.852$, 4.317 ; $P=0.033$, 0.016)

basal time point, TNF- α in group B and C increased significantly 30 min after ischemia (0 h) and reached its peak 3 h after reperfusion; these were correspondingly much higher than those in group A; TNF- α at time points of 1 h, 3 h in group B were significantly higher than that in group C, while there were no difference at other time points. IL-1 and IL-8 in groups B and C increased significantly 0.5 h after reperfusion and reached their peaks 3 h, 6 h after reperfusion respectively, and were much higher than those in group A; IL-1 at time points of 3 h, 6 h in group B were significantly higher than those in group C; IL-8 at time points of 1 h, 3 h in group B were significantly higher than those in group C.

Brain water content

Brain water content was significantly higher in groups B and C ($80.19\% \pm 0.70\%$, $80.45\% \pm 0.30\%$) as compared with $78.49\% \pm 0.59\%$ in group A ($F=6.519$, $P=0.006$; $q=3.610$, 4.933 and $P=0.047$, 0.006 respectively). But there was no difference between groups B and C ($q=1.322$, $P=0.625$).

Cerebral histopathological damages

As shown in Fig.3, neurons in group A had normal sizes, clear contour and axons, intact organelles in group B, neurons were shrunken and deeply

stained, had unclear structures of organelles and significantly increased gap around neurons and microvessels. Damages of cerebral microscopic structures in group C were less severe than those in group B. Neurons had nearly normal sizes, slightly blurry contour and organelles, had less increased gap around neurons and microvessels.

DISCUSSION

Complete cerebral ischemia could not be achieved by simplex occlusion of two common carotids and two vertebral arteries in the animals (Furlow, 1982). When this combined with systemic hypotension induced by exsanguinations and reperfusion, changes of blood flow in the entire organism and brain following cardiac arrest and resuscitation are well simulated. So it may be accepted as an ideal animal model for cardiopulmonary cerebral resuscitation. Compared with the lower mean arterial blood pressure (35~40 mmHg) induced during ischemia in one published study (Wang *et al.*, 2001), about 50%~60% of basal values (50~60 mmHg) was maintained in the present experiment. In a preliminary study, we found that animals could hardly survive for more than 3 h with such low blood pressure

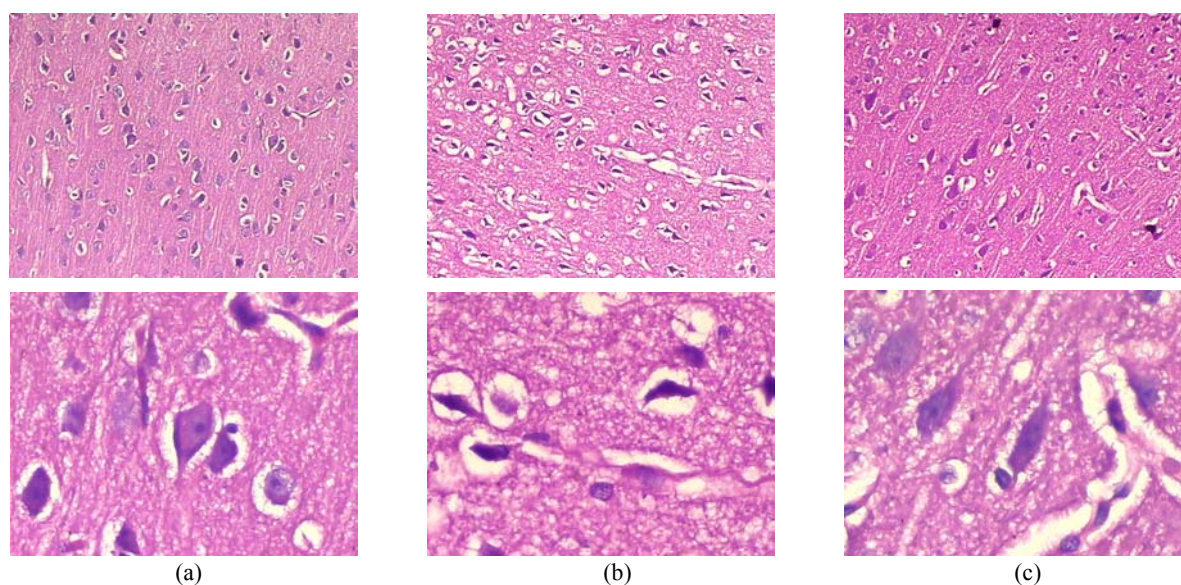


Fig.3 Cerebral histopathologica changes in 3 groups (Stained with HE; Upper, magnification of $\times 100$; Lower, magnification of $\times 400$) (a) In group A, neurons had normal sizes, clear contour and axons, intact organelles; (b) In group B, neurons were shrunken and deeply stained, had unclear structures of organelles and significantly increased gap around neurons and microvessels; (c) In group C, neurons had nearly normal sizes, slightly blurry contour and organelles, less increased gap around neurons and microvessels

during ischemia. Furthermore the rate and volume of exsanguination had to be well controlled to ensure a stable animal model.

Neuron-specific enolase (NSE) is an isoenzyme of the glycolytic enzyme enolase (2-phospho-D-glycerate hydrolase), a dimeric enzyme composed of 2 gamma-subunits with a molecular weight of 78 kDa and biological half-life of approximately 24 h (Tiainen *et al.*, 2003). It is nearly exclusively located in neurons, neuroendocrine cells (APUD cells) and neuroendocrine tumors, including small-cell lung cancer (Tiainen *et al.*, 2003). S-100B is a dimeric acidic calcium-binding protein with a molecular weight of 21 kDa and biological half-life of 0.5 h (Jonsson *et al.*, 2000), and is found especially in high concentrations in astroglial cells that are prevalent in the white matter of the brain (Mussack *et al.*, 2002). When the brain suffers from different insults leading to impaired cellular structure and blood-brain-barrier integrity, NSE and S-100B are released quite rapidly into the cerebrospinal fluid and then into the circulation. Increased serum levels are proportionally related to the extent of cellular brain injury (Martens *et al.*, 1998). So serum NSE and S-100B are sensitive and quantitative markers of parenchymal brain injury due to ischemia stroke, intracerebral hemorrhage, trauma and cardiopulmonary surgery (Mabe *et al.*, 1991; Skogseid *et al.*, 1992; Schaarschmidt *et al.*, 1994; Isgro *et al.*, 1997; Missler *et al.*, 1997; Elting *et al.*, 2000; Jonsson *et al.*, 2001). Cardiac arrest leads to a period of temporary systemic and global cerebral ischemia, while the following cardiopulmonary resuscitation causes reperfusion injury, mediated by a series of inflammatory responses (Jean *et al.*, 1998). As a consequence, the observed significantly elevated NSE and S-100B levels in the blood served as predictors of neurological outcome in several previous studies (Martens *et al.*, 1998; Schoerhuber *et al.*, 1999; Rosen *et al.*, 2001; Mussack *et al.*, 2002; Tiainen *et al.*, 2003).

In the present study, serum NSE and S-100B began to increase 1 h after reperfusion, continued to rise and reached their peaks 6 h later in groups B and C. The good correlation between the two indices ($r=0.736$) indicated increases of serum NSE and S-100B occurred very early after global cerebral ischemia-reperfusion injury. In group C treated with basic fibroblast growth factor, changes of blood

pressure, heart rate and body temperature were similar to those in group B. But serum NSE and S-100B levels were lower at corresponding time points in group C, and with less severe cerebral histopathological damages. So it could be deduced that bFGF alleviated global cerebral ischemia-reperfusion injury, which was in accordance with previous findings showing that bFGF protected the brain from different insults. Himmelseher *et al.* (1997) found that bFGF increased cell survival and supported axonal re-elongations in adult hippocampal neurons in vitro when applied after axotomy. In cats undergoing left middle cerebral artery (MCA) occlusion for 4 h, intravenous bFGF at a rate of 5, 50, 250 $\mu\text{g}/\text{kg}$ per hour decreased injury volume of ipsilateral cerebral cortex (Bethel *et al.*, 1997). A continuous intravenous infusion of 45 $\mu\text{g}/(\text{kg}\cdot\text{h})$ bFGF for 3 h, beginning 30 min after fluid-percussion brain injury, reduced both the number of damaged neurons and the contusion volume in rats (Dietrich *et al.*, 1996). Brain water content was not decreased by bFGF in the present study, with results different from those of Liu and Zhu (1998) who found that bFGF minimized brain water content in rat following forebrain ischemia-reperfusion, which could probably be attributable to different animal models employed. In this study, the test animals underwent complete global cerebral ischemia and longer time of reperfusion, which led to much severer brain injury.

Inflammatory response plays a main causative role in cerebral ischemia-reperfusion injury, in which a variety of inflammatory mediators are involved. Increased levels of TNF- α are observed early in cerebral ischemia and reperfusion (Liu *et al.*, 1994; Buttini *et al.*, 1996). The sources of TNF- α under these conditions include activated macrophages/monocytes, neurons, astrocytes and microglia. TNF- α recruits and activates leukocytes, leading to increased leukocyte-endothelial adhesion (Fabry *et al.*, 1992; Liu *et al.*, 1994), which progressively induces expression of other cytokines, including IL-1, IL-6, IL-8 and so on, thus forming a positive feedback loop further exacerbating brain injury. Levels of TNF- α , IL-1 and IL-8 are positively correlated proportionably to neurons damage, brain edema and infarction extension, and can serve as good indicators of cerebral ischemia-reperfusion injury (Yamasaki *et al.*, 1995; Barone *et al.*, 1997; Yang *et al.*

al., 1997). Elevated serum IL-8 and TNF- α levels comprise the worse outcome in patients following out-of-hospital cardiac arrest (Ito et al., 2001). Blocking or countering their activities lead to decreased neutrophil infiltration, blood-brain barrier breakdown, infarct size and extent of injury (Kadoya et al., 1995; Matsumoto et al., 1997; Yamasaki et al., 1997; Yang et al., 1997). In the present study, serum TNF- α was increased 30 min after ischemia, continued to rise after reperfusion, concomitant with increased serum IL-1 and IL-8. In those treated with bFGF, serum TNF- α , IL-1 and IL-8 were significantly decreased 1, 3, 6 h after reperfusion, compared with those in group B. It may indicate that bFGF probably exerts its neuroprotective effect by interfering with the expression and functions of these cytokines. In rats undergoing superior mesenteric artery occlusion (45 min) and reperfusion (3 d), an intravenous administration of 4 μ g bFGF at the onset of reperfusion reduced serum tumor necrosis factor-alpha and free radicals, while protecting gut and liver against ischemia and reperfusion injury (Fu et al., 1997). bFGF could suppress apoptosis of L929 cells induced by TNF- α , which was mediated by activation of the MAPK (mitogen activated protein kinases) intracellular signaling pathways (Gardner and Johnson, 1996). bFGF also reduced IL-1 secretion (Bodo et al., 2000), and down-regulated expression of vascular cell adhesion molecules stimulated by TNF- α and IL-1, resulting in markedly decreased levels of leukocyte recruitment and adhesion (Kikuchi, 2000; Tromp et al., 2000), subsequently decreasing tissue injury.

CONCLUSION

The present study suggests that basic fibroblast growth factor alleviates brain injury following global ischemia reperfusion. And this is probably achieved by down-regulating expression of inflammatory factors and countering their activities to reduce inflammatory injury during reperfusion. It shows promising application in patients with cardiac arrest and deserves further investigations.

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