ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Feb;21 (2):161-164
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# Influence of batroxobin on cerebral ischemia-reperfusion injury in gerbils[1]

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KEY WORDS cerebral ischemia; brain; adenosine triphosphate; free radicals; reptilase; maze learning; Gerbillinae

## ABSTRACT

**AIM**: To study the effects of batroxobin (Bat) on neurons survival, neurobehavioral test, ATP levels and hydroxyl radical outputs in hippocampus during forebrain ischemia-reperfusion in gerbils. **METHODS**: The forebrain ischemia was induced by occluding the bilateral common carotid arteries for 10 min in gerbils, and ATP levels and 2,3-dihydroxybenzoic acid (DHBA) outputs were assayed by HPLC. The neurons survival were assessed by histology, and behavioral tests of gerbils were assessed by open field test. **RESULTS**: The number of neurons survival in Ir at d 7 postischemic insult were (7 ± 4) % of sham-operated gerbils, much less than that in Bat (45 ± 16) %. The levels of explore activities of ischemic gerbils was 175 % and 159 % of sham-operated gerbils at d 3 and d 6 postischemic insult, much more than that in Bat (120 % d 3 and 140 % d 6). Hippocampal ATP levels in Ir were 64 % of sham-operated gerbils at reperfusion 60 min, much less than that in Bat Ⅰ and Ⅱ (82 % and 89 % respectively). The hippocampal 2,3-DHBA outputs in Ir increased by 4.5 folds of sham-operated gerbils at reperfusion 60 min, but the 2,3-DHBA outputs in Bat Ⅰ and Bat Ⅱ were only 2.6 and 2.4 folds respectively. **CONCLUSION**: Bat possesses the inhibitory effects on DND and OH· production following cerebral ischemia-reperfusion in gerbils.

## INTRODUCTION

After global ischemia, reperfusion is accompanied by a transient, diffuse increase in CBF followed by a protracted reduction in CBF, which could be caused by vasopasm, platelet aggregating rate and blood sludging and endothelial edema[1]. Batroxobin (Bat) is a newly thrombolytic and has the role of decreasing blood viscosity, plasma fibrinogen concentration and platelet aggregation rate, so we postulated that Bat could reduce cerebral ischemia-reperfusion injury. A brief period of forebrain ischemia results in neurons death in hippocampal CA1 subfield after 2-4 days reperfusion, and may take at least 7 d to be completed. This phenomenon is commonly referred to as delayed neuronal death (DND)[2]. Open field test is sensitive indices of hippocampal cell loss resulting from ischemia. Even following mild cases of ischemia, which have not resulted in detectable CA1 cell loss, deficits in spatial memory function were evident several days after ischemia[2,3]. It has been suggested that DND production after ischemia may be related to the increment of oxygen radicals, especially the hydroxyl radical (OH·), which is the most highly reactive radical among the reactive oxygen species (ROS)[4]. In this study, the effects of Bat on DND, open field test and OH· outputs of hippocampus following forebrain ischemia-reperfusion were observed.

## MATERIALS AND METHODS

Bat, 5 kBU·L$^{-1}$, purity >95 % [TOBISHI Pharmaceutical Co LTD (930498)]; ATP (Sigma); 2,3-dihydroxybenzoic acid (2,3-DHBA) (Sigma); All other chemicals were AR.

**Animal preparation**[4] Gerbils ($n$ = 32, Grade Ⅱ, Certificate № SUA95021, ♂, weighing 50-60 g) were randomly divided into 4 groups: sham-operated group (Sh), ischemia-reperfusion group (Ir), Bat group Ⅰ (Bat Ⅰ, 8 BU·kg$^{-1}$) and Bat Ⅱ (16 BU·kg$^{-1}$), and each group had 8 gerbils. Gerbils were anesthetized with sodium pentobarbital 45 mg·kg$^{-1}$ ip. Brief fore-

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ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Feb；21 (2)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

brain ischemia was induced by occluding the bilateral common carotid arteries for 10 min, then reperfusion was achieved by removal of vascular clamps[3]. Bat was injected ip at the onset of reperfusion. Salicylic acid reacts with hydroxyl radical (OH·) to produce 2,3- or 2,5-dihydroxybenzoic acid (2,3 or 2,5-DHBA) and a small amount of catechol. We only measured 2,3-DHBA as an indicator of OH·generation because it could be generated only by nonenzymatic hydroxylation, whereas 2,5-DHBA could be also generated by mixed function oxidasis[4]. Sodium salicylic acid 100 mg·kg$^{-1}$ was injected ip before 30 min of gerbil decapitation. Gerbils were killed after the 60 min reperfusion and brain was quickly removed from the skull. Then hippocampus was separated and one lateral was used to measure ATP levels, the other to 2,3-DHBA outputs.

Another 18 gerbils, which were used to histological study, also divided into Sh, Ir and Bat group. Bat was administered for 3 d after cerebral ischemia in a dose of 8 BU·kg$^{-1}$·d$^{-1}$ ip and the other groups were given 0.9 % NaCl 1 mL·kg$^{-1}$·d$^{-1}$ in the same way as Bat. To avoid the influence of different brain temperature on experimental results, the gerbils' brain temperature in this experiment was kept constantly at (37.0±0.2) ℃.

**Open field test**[3] Animals were tested in an open field maze (72 cm×76 cm×57 cm) to which they had not been exposed before ischemia or sham operations. Testing was carried out at 3 and 6 d postischemic insult. The floor of the maze was divided into 25 squares, and counted the total number of squares entered during each trial. All behavioral testing were carried out in a soundproofed room. Distinctive features of the room and lighting conditions were kept constant for the duration of the experiment.

**Histological examination**[3] Gerbils were anesthetized with sodium pentobarbital 45 mg·kg$^{-1}$ ip and then perfused with heparinized saline 15 mL followed by formalin 4 % 50 mL. Brains were stored in formalin until subsequently embedded in paraffin sectioned at 6 μm, and stained with haematoxylin and eosin. The number of remaining viable-looking neurons (distinct cell membrane and nucleus) was counted in hippocampal CA1 sector (100 μm×100 μm) at 1.7 mm posterior to bregma. Counts were summated over left and right hemispheres and expressed as a percent of normal (ie, Sh).

**Measurement of ATP** ATP was separated by reverse-phase high performance liquid chromatography (HPLC). The detection wavelength was 254 nm, and the column was a Lichrospher 100 RP-18. Mobile phase consists of phosphate buffer ($KH_2PO_4$, pH 6.5) 100 mmol·L$^{-1}$ and methanol 1.0 %, and the flow rate was 1 mL·min$^{-1}$. Hippocampus was homogenized in 0.1 mol·L$^{-1}$ $HClO_3$ with a polytron homogenizer. Homogenate was centrifuged (9000×$g$, 20 min, 4 ℃). A 10 μL supernatant was injected into HPLC to determine the ATP levels.

**Measurement of 2,3-DHBA**[4] 2,3-DHBA was specifically detected by HPLC coupled with electrochemical detection (ECD). The ECD system was set at +0.75 mv, and the column was a Lichrospher 100 RP-18. The mobile phase contained citric acid 0.03 mol·L$^{-1}$, acetic acid 0.03 mol·L$^{-1}$, and sodium azide 0.2 g·L$^{-1}$ (pH 3.6). The flow rate was 1.0 mL·min$^{-1}$. The hippocampus was weighed and homogenized in 3 volumes (V/W) of 3 % $CCl_3COOH$. The homogenate was centrifuged (9000×$g$, 15 min, 4 ℃), and 10 μL supernatant was injected into HPLC to measure the 2,3-DHBA outputs.

**Statistical method** Values are presented as $\bar{x} \pm s$, and compared by $t$-test.

## RESULTS

**Influence of Bat on open field test scores** Ischemic gerbils explored the open field more than sham-operated gerbils on all test days, and the activities of ischemic gerbils were increased by 175 % and 159 % of sham-operated gerbils at 3 and 6 d postischemic insult, respectively. Bat (8 BU·kg$^{-1}$) markedly inhibited this elevation, and the heightened levels of activity were only 120 % and 140 % (Tab 1).

**Influence of Bat on neurons survival** There

Tab 1. Influence of batroxobin on open field scores and neuronal survival after cerebral ischemia and reperfusion in gerbils. Bat was administered in dose of 8 BU·kg$^{-1}$·d$^{-1}$×3 ip and open field test was examined at 3 d and 6 d after cerebral ischemia-reperfusion. $n=6$. $\bar{x} \pm s$. $^{b}P<0.05$, $^{c}P<0.01$ *vs* Sh; $^{e}P<0.05$, $^{f}P<0.01$ *vs* Ir; $^{i}P<0.01$ *vs* Ir or Bat.

| | Open field test | | Neuronal survival |
|---|---|---|---|
| | 3d | 6d | 7d |
| Sh | 588±154 | 408±117 | – |
| Ir | 1034±271$^{c}$ | 648±153$^{b}$ | 7 %±4 %$^{i}$ |
| Bat | 706±184$^{e}$ | 574±127$^{f}$ | 45 %±16 % |

were few neurons survival in the hippocampal CA1 sector in Ir at 7 d postischemic insult, that is to say, forebrain ischemia 10 min induced severe cell loss (DND). However, the number of neurons survival in Bat was nearly 45 % of sham-operated gerbils, that means Bat obviously reduced the DND (Tab 1, Fig 1A, B, C).

**Influence of Bat on hippocampal ATP levels** Hippocampal ATP levels in Ir were 64 % of sham-operated gerbils at reperfusion 60 min. ATP levels in Bat Ⅰ and Ⅱ were 82 % and 89 % respectively, much more than that in Ir. However, the difference between Bat Ⅰ and Ⅱ was not significant (Tab 2).

Tab 2. Influence of batroxobin on hippocampal ATP levels and 2,3-DHBA outputs at the 60 min of reperfusion in gerbils. Bat was administrated in dose of 8 BU·kg$^{-1}$ (Bat Ⅰ) and 16 BU·kg$^{-1}$ (Bat Ⅱ) ip at the onset of reperfusion. $n = 8$. $\bar{x} \pm s$. $^cP<0.01$ *vs* Sh, $^eP<0.05$, $^fP<0.01$ *vs* Ir.

| | ATP/mmol·kg$^{-1}$ | 2,3-DHBA/mol·kg$^{-1}$ |
|---|---|---|
| Sh | **0.94 ± 0.19** | **0.21 ± 0.14** |
| Ir | **0.60 ± 0.09**$^c$ | **0.95 ± 0.25**$^c$ |
| Bat Ⅰ | 0.77 ± 0.10$^e$ | 0.55 ± 0.16$^f$ |
| Bat Ⅱ | 0.84 ± 0.17$^e$ | 0.51 ± 0.20$^f$ |

**Influence of Bat on hippocampal 2,3-DHBA outputs** Forebrain ischemia-reperfusion remarkably increased OH· production. The hippocampal 2,3-DHBA outputs in Ir increased by 4.5 folds of sham-operated gerbils after reperfusion 60 min, but the 2,3-DHBA outputs in Bat Ⅰ and Bat Ⅱ were only 2.6 and 2.4 folds respectively. The difference between Bat Ⅰ and Ⅱ was also not significant (Tab 2).

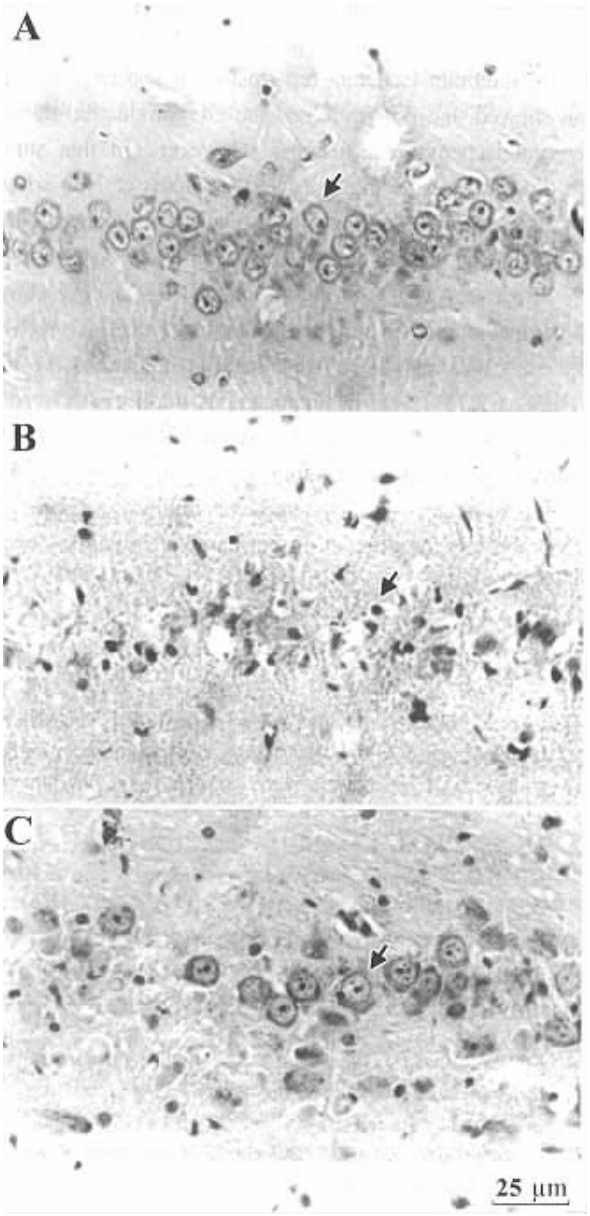


Fig 1. Hippocampal CA1 pyramidal cells d 7 after reperfusion following forebrain ischemia in gerbils. A: Sh, "→" showing the normal pyramidal neurons, ie. with well-defined nuclei (×400); B: Ir, "→" showing the pyknotic and dark neucleus and eosinophious cytoplasm (×400). C: Bat, Bat 8 BU·kg$^{-1}$ ip for 3 d following forebrain ischemia-reperfusion, "→" showing more normal pyramidal cells in Bat than that in Ir (×400). HE stain.

## DISCUSSION

The number of surviving neurons following 10 min forebrain ischemia in our study is close to the number of surviving neurons[3]. Our results showed that Bat (8 BU·kg$^{-1}$) markedly increased the numbers of surviving neurons, this result demonstrated Bat had the role of reducing DND.

Open field test is sensitive indices of hippocampal cell loss resulting from ischemia[3]. Our results also showed that Bat (8 BU·kg$^{-1}$) obviously reduced the heightened levels of activity in open field test following forebrain ischemia-reperfusion in gerbils, this result also demonstrated that Bat decreased DND after forebrain ischemia in gerbils.

OH· can oxidize essential cellular lipids, proteins,

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Feb；21 (2)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

and nucleic acids, leading to cell damage and ultimately to cell death[4,5]. Our study found that Bat (8 and 16 BU·kg$^{-1}$) significantly reduced the 2,3-DHBA outputs during forebrain ischemia-reperfusion in gerbils, this result showed that Bat decreased the OH· production during cerebral ischemia-reperfusion. However, in this study we did not measure the 2,3-DHBA outputs at 7 d, so we couldn't determine the positive relationship between the increment of OH· generation and DND.

Recovery of the concentrations of high-energy phosphate metabolites is a prerequisite for biological recovery[6,7]. Our results showed that Bat quickened the recovery of ATP levels in hippocampus during early reperfusion, and this role may be beneficial for recovery of neurons after cerebral ischemia.

In summary, Bat possesses the inhibitory effects on DND and OH· production during cerebral ischemia-reperfusion in gerbils.

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## 巴曲酶对沙土鼠脑缺血再灌注损伤的影响[1]

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**关键词**　脑缺血；脑；腺苷三磷酸；自由基；爬行动物酶；迷宫学习；沙鼠亚科

**目的**：研究巴曲酶对沙土鼠脑缺血再灌注(Ir)期间海马存活锥体细胞数目，神经行为学，海马 ATP 和羟自由基含量的影响. **方法**：阻断沙土鼠双侧颈总动脉造成前脑缺血 10 min. 高效液相测定海马 ATP 和 2,3-DHBA 的含量. **结果**：Ir 组沙土鼠脑缺血后 d 7 存活锥体细胞数(7±4) %少于 Bat 组的 (45±16) %. 在脑缺血后 d 3 和 d 6 时，Ir 组沙土鼠的探究活动次数多于 Bat 组的 120 %和 140 %. 在再灌注 60 min 时，Ir 组沙土鼠 ATP 水平低于 Bat Ⅰ和Ⅱ组(82 %和 89 %)，但 2,3-DHBA 的含量高于 Bat Ⅰ和Ⅱ组(2.6 和 2.4 倍). **结论**：巴曲酶具有减少脑缺血再灌注后延迟性神经元死亡和羟自由基产生的作用.

**（责任编辑　李　颖）**