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Baclofen Is Cytoprotective to Cerebral Ischemia in Gerbils

Sumeer Lal,[1] Ashfaq Shuaib,[2,4] and Sadiq Ijaz[3]

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The release of the neurotransmitter, glutamate, and the activation of receptor operated calcium channels, may increase the degree of damage in ischemic brain tissue. Inhibition of excitatory neurotransmitters should therefore result in cytoprotection of ischemic brain tissue. In this study we evaluated the effect of baclofen, an inhibitor of presynaptic glutamate release, on ischemic gerbil cortex, hippocampus (CA 1 and CA4), striatum and thalamus. Histological evaluation was done in a blind manner in 4 groups (total 36 animals): a control group (9 animals) and three groups (27 animals) with varying doses of baclofen. For cerebral ischemia, we used single episode of five minutes of arterial occlusion of the carotid arteries. Baclofen in doses of 0, 25, 50, and 100 mg/kg were given to different groups five minutes prior to ischemic insult. This was followed by intraperitoneal injections given 24 and 48 hours after the initial insult. Statistically significant histological cytoprotection was demonstrated. Doses of 25 mg/kg appeared to demonstrate significant protection of the cortex ($p = 0.0002$), the CA1 and CA4 regions of the hippocampus ($p = 0.0004$ and 0.0001) respectively. At a dose of 50 mg/kg, significant cytoprotection was demonstrated at the hippocampus (CA1 and CA4 regions), in particular at the CA4 region ($p = 0.0029$). The 100 mg/kg dose appeared to have most significant protection at the CA1 and CA4 regions of the hippocampus (both $p = 0.0001$), striatum ($p = 0.0011$), and the thalamus ($p = 0.0008$). All statistical comparisons were done using non-parametric tests (Mann-Whitney U test). Our study demonstrates that baclofen is cytoprotective to ischemic neuronal cells, especially in the hippocampus. Clinically this may be beneficial to those patients with strokes or head injuries.

KEY WORDS: Baclofen; ischemia; gerbils; cerebral.

INTRODUCTION

The mechanisms of cellular damage in cerebral ischemia are becoming better understood and have received considerable attention (1). Increased intracellular calcium, oxygen free radical formation (2) and increased glutamate, or excitatory neurotransmitters, may play a role in the early stages of ischemic damage at the cellular level (3).

Sudden loss of cerebral blood flow is known to result in a depolarization of cellular membranes. This depolarization results in a marked increase in extra cellular glutamate levels, which in turn, leads to a build-up of intracellular calcium. This causes destruction of cell membranes and produces irreversible brain injury (2,4).

Antagonizing the release of glutamate has been attempted in many studies (5). Baclofen, B-(p-chlorophenyl)-y-aminobutyric acid, is a racemic mixture of D and L isomers. Clinically baclofen, an analogue to the neurotransmitter GABA, may inhibit the release of excitatory neurotransmitters that are responsible for cellular damage in ischemia. It is thought that baclofen may

[1] The Department of Medicine[2] and Cerebrovascular Research Labratory,[3] The Saskatchewan Stroke Research Centre, and the Division of Neurosurgery,[1] Department of Surgery, College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

[4] Address reprint requests to: Dr. Ashfaq Shuaib, Director, Saskatchewan Stroke Research Centre, Associate Professor, Dept. of Medicine, Royal University Hospital, Saskatoon, Saskatchewan, Canada. Ph: 306 966-8007 Fax: 306 966-8008

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depress depolarization by inhibiting glutamate, release (3,4,6–8).

In this study, we postulated that baclofen would be cytoprotective to neuronal cells, in keeping with the principles stated previously. The effect of baclofen on ischemic gerbil cortex, hippocampus (Ca1 and Ca4), striatum and thalamus was assessed histologically in four groups: a control group and three groups with varying doses of baclofen.

EXPERIMENTAL PROCEDURE

Male adult gerbils weighing 60–80 g (average-70 g) were used. All animals were housed under a controlled environment with 12 h light-darkness cycle and free access to food and water. Animals were in a vivarium for a minimum of five days and then randomly selected for the experiment involving baclofen. Animals were divided into four groups, a control group, and three groups receiving 25 mg/kg, 50 mg/kg, and 100 mg/kg of baclofen respectively, prior to the ischemic insult.

General anaesthesia (nitrous oxide, 1 l/min; oxygen 0.5 l/min; halothane 3%), was used five minutes after an intraperitoneal injection of baclofen was given. Scalp temperatures, monitored on all animals, were kept at 37.5, with a heat lamp, just prior to and during cerebral ischemia.

After the induction of anaesthesia, a midline neck incision was made and the carotid arteries were dissected from the surrounding tissues. Following this halothane anaesthesia was decreased to 1%. Application of aneurysm clips to the carotid arteries was then undertaken, with occlusion visually confirmed. After 5 minutes of continuous arterial occlusion, the clips were then removed. Flow was visualized in the carotid arteries, and the skin incision was closed with 2–3 interrupted silk sutures.

The animals were returned to the vivarium and for the next 2 days, at approximately the same time, were observed for 10 minutes and then given an intraperitoneal injection of baclofen. The dose given was the same as given prior to the ischemic event. Seventy-two hours after the 5 minutes of ischemia, the animals were euthanized with an overdose of sodium phenobarbital, perfused initially with saline (150 ml) and then were perfused with a 10% formalin/0.1 M phosphate solution. The brains were then removed and placed in a 30% sucrose/10% formalin solution until the brain fell to the bottom of the container. This indicated saturation with the sucrose solution. The sucrose solution was used as cryoprotector, its purpose being to replace any water molecules within tissues with sucrose molecules, thus protecting the tissue from damage from ice particles during cutting.

At this time the brain was cut for embedding. The embedding medium used was optimum cutting temperature compound (O.C.T). This medium provided by Miles Scientific consists of polyvinyl alcohol, benzalkonium chloride, polyethylene glycol, and distilled water. It provides for sectioning matrix for below −10°C, leaves no residue on the slides, and does no cause background staining. The tissue was embedded in O.C.T. in a vinyl cryomold, and then mounted on a cutting block. If tissue was not cut on that day, it was held in a −70°C freezer. When needed, or if it was cut on the same day, the tissue was taken to −20°C within the confines of the cryostat.

The tissue cut at −20°C at a thickness of 40 μ was floated in distilled water to remove the O.C.T., and then placed into distilled water in labelled tissue culture trays, with 25mm wells, that had been pretreated with 9% NaOH for at least two to three hours, and then thoroughly rinsed with distilled water. The sections were stained with Gallya's Silver Impregnation Method (9,10). If the staining was not done the same day, the sections were held, refrigerated, in tissue culture trays, the wells of which contain 10% formalin/0.1 M phosphate buffer. They would be held for two days maximum, and then placed into the rinsed wells of tissue culture trays that were pretreated with 9% NaOH. The same staining procedure was done after the sections were rinsed in three five minute changes of distilled water. Representative sections were cut through the cortex, striatum, hippocampus, and thalamus. The anatomy was identified using the Gerbil atlas of Loskota (11).

Neuronal damage was assessed with light microscopy. The scoring system used was modification of the Pulsinelli scoring system (1). Thus: 0, no damage; 1, <25% damage: 2, 25–75% damage; 3, >75% damage; and 4, infarction. Damage was assessed independently on both sides of the brain and all histological evaluation was done in a blind manner.

Statistical analysis was done using the Kruskal Wallis test and individual comparisons were made using the Mann-Whitney U test. Damage scores are means with standard error of the mean.

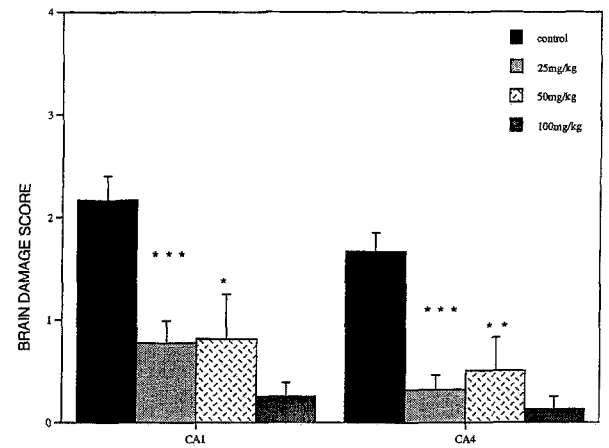
RESULTS

Prior to commencing the study, it was noted that many test animals could not tolerate anaesthesia followed by a dose of baclofen and many died even before carotid occlusion was complete. It was decided, therefore, to give the intraperitoneal baclofen five minutes prior to the induction of anaesthesia.

A total of 47 animals were used. In the control group there was one death in the first twenty-four hour period after surgery. Two animals that received the 50 mg/kg dose in the first 24 hours died and 8 died that had received the 100 mg/kg dose (3 intraoperatively and 5 in the first 24 hours). Thus 36 animals were available for analysis (9 controls, 11 at 25 mg/kg, 8 at 50 mg/kg, and 8 at 100 mg/kg).

(a) Behavioral Change. All gerbils that received intraperitoneal baclofen, prior to and after cerebral ischemia, developed a temporary flaccid weakness of the hindlimbs.

It was noted that the controls developed a characteristic humped back posturing, with or without ptosis of an eyelid. These findings were found to resolve anywhere from one-half hour to twenty-four hours after the ischemic insult. The baclofen treated gerbils often did not demonstrate the humped back posturing, but did have the decreased tone of the hindlimbs, as already mentioned. Only one animal demonstrated hyperactivity after cerebral ischemia, and this was in the control group. No seizures were noted in any of the study animals.



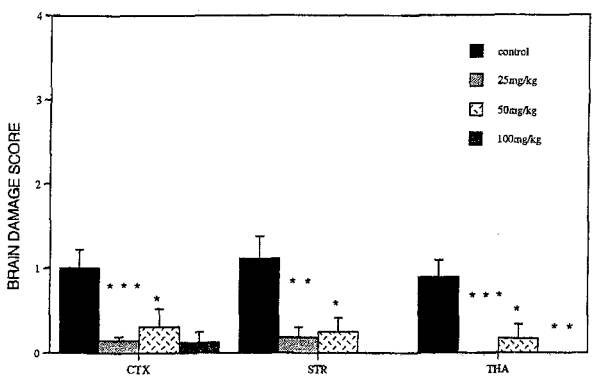


Fig. 1. and 2. These figures show the extent of neuronal damage in hippocampus CA1 and CA4 regions (Fig. 1) and the cortex, striatum and thalamus (Fig. 2) of the bacofen treated groups (n = 27) compared to the controls (n = 7). Damage scores are means with standard error of the mean. Statistical analysis was done using the Mann-Whitney U test.
CA1-CA1 region of the hippocampus
CA4-CA4 region of the hippocampus
CTX-Cortex
STR-Striatum
THA-Thalamus
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

(b) Histological Evaluation. The details of the neuronal damage in the control animals and the animals treated with baclofen are shown in Fig 1 and 2. Also see Table I.

Cortex. Control animals demonstrated damage in all histological areas examined. Cortical damage was evident in all but two controls, and the rest demonstrated mild to moderate damage. No infarctions were seen. Only one out of ten animals that received 25 mg/kg, demonstrated mild cortical damage. In the 50 mg/kg and 100 mg/kg groups, only two and one animal, respectively, demonstrated any cortical damage.

Hippocampus. In the controls maximal damage occurred in the hippocampus, but as the dose of baclofen increased, significant protection was demonstrated in the test groups. In the CA1 region, with the exception of one animal demonstrating mild cellular damage, all demonstrated moderate to severe damage. As the dose of baclofen increased in each test group, the same degree and number of animals with neuronal damage decreased significantly. This was especially true in those animals pretreated with the 100 mg/kg dose. Only three at 100 mg/kg dose, demonstrated mild degrees of neuronal damage. This damage was restricted to the neuropil (axons and dendrites). Only one animal had severe damage to CA1 region in the 50 mg/kg group.

Although damage to the CA4 region of the hippocampus was not as severe as in the CA1 region of the controls, the trend mentioned above continued. When compared only one animal given 100 mg/kg of baclofen demonstrated mild damage, while mild to moderate damage occurred in all the neuropils of the controls. Therefore, as the dose of baclofen increased, the damage in the CA4 region decreased.

Corpus Striatum. Histological evaluation of the striatum revealed that in the control group mild to moderate cellular damage occurred in seven of nine test animals. Only two animals in both the 25 mg/kg and 50 mg/kg group had mild cellular damage from cerebral ischemia. It was noted that no animal demonstrated any type of damage that could be ascertained with light microscopy in the 100 mg/kg group.

Thalamus. Significant neuroprotection also occurred in the thalamus in the treated animals. Only two baclofen treated animals demonstrated any type of cellular damage, which was mild. Both were given the 50 mg/kg dose. In the control group, seven of nine animals demonstrated mild to moderate cellular damage in the thalamus.

DISCUSSION

In this study, we have demonstrated the significant cytoprotective effects of baclofen on ischemic gerbil brain. Baclofen appears to have the most statistically significant effects in the CA1 and CA4 regions of the hippocampus, as the pyramidal cells in these areas are the most vulnerable to cerebral ischemia (7). In addition to the damage done in the forebrain structures, damage was present in the cortex, striatum and thalamus of controls. Again baclofen demonstrated significant cytoprotection to the ischemic cells in these areas. It appeared that as the dose of baclofen increased, it accorded more protec-

Table I. Damage Scores Which are Means With Standard Deviation of the Mean

	Crt	CA1	CA4	Thalamus	Striatum
Controls	1 ±0.66	2.16 ± .71	1.72 ±.56	0.89±.60	1.11±.78
25 mg/kg	0.05 ± .15	0.8 ± .72	0.35 ±.46	0	0.2 ±.41
50 mg/kg	0.312± .59	0.812±1.25	0.5 ±.93	0.25±.41	0.25±.46
100 mg/kg	0.125± .35	0.25 ±.38	0.125±.35	0	0

Controls (n=9) and the baclofen treated groups (n=27).
CRT = cortex
CA1 = CA1 portion of the hippocampus
CA4 = Ca4 portion of the hippocampus

tion to the cells. In particular in the evaluation of the corpus striatum and thalamus. No animal with a dose of 100 mg/kg demonstrated histological damage in these areas.

Comparison of controls versus baclofen treated groups yielded statistically significant results, however; when the baclofen groups were compared, there was no demonstratable statistical difference noted. This study indicated that the optimal baclofen dose for cytoprotection in ischemic gerbil brain was 100 mg/kg. But as the dose of baclofen increased, the test animals sensitivity to anaesthesia may have become altered. This could be secondary to the intense muscle relaxant effects of the baclofen perhaps resulting in dysfunction of the diaphram and or other respiratory muscles. Many gerbils subjected to large doses of baclfen (50–100 mg/kg) and general anaesthesia did not tolerate the combination and died before appropriate evaluation could be completed.

We did not evaluate scalp temperatures in the post-ischemic period, as the teat animals were awake and not anaesthesized. Thus we have no evidence that the baclofen, given in the post-ischemic period, resulted in hypothermia thereby according some neuronal protection. It is however deemed unlikely that this mechanism played a role in cytoprotection.

Glutamate is an important factor in the genesis of ischemic injury of the brain. This has been supported by early studies that demonstrated surgical section of the glutamatergic afferents to the CA1 portion of the hippocampus protected the neurons from subsequent ischemic insult (1,12).

Under anoxic or ischemic conditions, glutamate, synaptically released, accumulates to neurotoxic levels. These levels are unknown. Acting at post-synaptic receptors, glutamate produces an influx of sodium and calcium followed by chloride. Immediate effects include cellular swelling, which can result in cell lysis. If the neuronal cells are not damaged immediately by the osmotic effects the cells are damaged slowly by the elevated intracellular calcium concentrations through proteolysis of neurofilaments, mitochondrial damage, and the breakdown of membrane phospholipids (6,13,14).

Baclofen is though to depress depolarization by inhibiting glutamate release, and thus protects against ischemic damage (6,7). Perhaps as the dose of baclofen is increased it produces a GABA-agonist effect that is cytoprotective. This much is however clearly evident, that baclfen is antiischemic and has a dose-related cytoprotective effect, as seen by histological evaluation. This antiischemic effect is due to a drug induced inhibition of excitatory transmitter release.

Future considerations would entail the use of baclofen in test animals after an ischemic insult, rather than before to assess neuronal protection. This could have potential benefits in the head injured and those with strokes. Also microdialysis evaluation of glutamate levels, as well as histological assessment, may be advantageous. Further studies in primates would be of great benefit.

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