EFFECT OF CENTRAL MICROINJECTION OF CARBENOXOLONE IN AN EXPERIMENTAL MODEL OF FOCAL CEREBRAL ISCHEMIA

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ABSTRACT

Previous experimental studies have shown the protective effects of CBX on brain ischemic injures in global and *in vitro* models of ischemia. However, effects of CBX in temporary model of focal cerebral ischemia are not clear. Hence, the aim of this study was to investigate the effects of central microinjection of CBX on post-ischemic reperfusion injuries in a temporary model of focal cerebral ischemia.

Transient focal cerebral ischemia was induced in rats by 60 min middle cerebral artery occlusion (MCAO), followed by 23 h reperfusion. CBX was administered into the right ventricle at doses of 1, 12, 25, 50 and/or 100 µg/kg at the beginning of MCAO. Cortical and striatal infarct volumes and motor dysfunctions were assessed 24 h after MCAO.

Administration of CBX at doses of 1, 12, 25 and/or 50 µg/kg significantly reduced cortical infarct volumes by 35%، 49%, 41% and 43%, respectively ($P<0.001$). In addition, CBX only at dose of 25 µg/kg significantly reduced striatal infract volume and improved neurological dysfunctions ($P<0.01$). Our findings indicated that central microinjection of CBX has protective effect on against ischemic reperfusion injuries in a transient model of focal cerebral ischemia.

Keywords: Carbenoxolone, central injection, transient focal cerebral ischemia, rat.

INTRODUCTION

Carbenoxolone (CBX), a synthetic derivative of glycyrrhizinic acid, is currently used in the treatment of gastrointestinal ulceration (Turpie *et al*., 1965; Jellinck *et al*., 1993). Moreover, CBX has other pharmacological properties such as sedative and muscle relaxant (Hosseinzadeh & Nassiri, 2003), anticonvulsant (Nyikos *et al*., 2003; Bostanci *et al*., 2007), anti-inflammatory (Tangri *et al*., 1965), antioxidant activity (Haraguchi *et al*., 1998; Perez Velazquez *et al*., 2006), as well as inhibitory effects on voltage-gated calcium channels in retinal preparations (Vessey *et al*., 2004). In addition, this agent is widely employed as a mineralcorticoid agonist, inhibiting 11-beta hydroxysteroid dehydrogenase (Jellinck *et al*., 1993) and blocking gap junction communication in the various experimental studies (Davidson and Baumgarten, 1988; Goldberg *et al*., 1996; Bani-Yaghoub *et al*., 1999).

Early experimental studies have shown that CBX effectively decreases neuronal death in *in vitro* cerebral ischemia (Frantseva *et al*., 2002a) and brain trauma models (Frantseva *et al*., 2002b). In addition, a recent *in vivo* study showed that peripheral administration of CBX has protective effects on against ischemic reperfusion injuries in a global model cerebral ischemia (Hosseinzadeh *et al*., 2005). Likewise, another study demonstrated that systemic administrations of CBX immediately after induction of global ischemia (intrauterine hypoxia-ischemia) dramatically reduce long-term neuronal damage in rat pups (de Pina-Benabou *et al*., 2005). Furthermore, Velazquez and colleagues recently reported that intra-hippocampal injection of CBX significantly decreased cell death in a global model of cerebral ischemia (Perez Velazquez *et al*., 2006).

According to current literature, the effect of CBX on secondary ischemic damage in transient model of focal cerebral ischemia is not completely clear. On the other hand, some evidence have showed that CBX does not cross the blood brain barrier very efficiently (Zhang, 2002; Leshchenko *et al*., 2006). Therefore, the current study was performed to investigate the effects of central microinjection of CBX at various doses on cortex and striatum injuries and motor neurological dysfunctions in a transient model of focal cerebral ischemia.

MATERIALS AND METHODS

Animals

Male Wistar rats were obtained from breeding colony of Semnan University of Medical Sciences (SUMS), Semnan, Iran. They were housed in standard cages in a temperature (22-24ºC), humidity (40-60%), and light period (07.00-19.00 h) - controlled environment. All experiments were performed in conformity with SUMS research council guidelines for conducting animal studies.

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Middle cerebral artery occlusion

Middle cerebral artery occlusion (MCAO) was induced by intraluminal filament method as described previously (Vakili *et al*., 2007). Briefly, animals were anesthetized with chloral hydrate (400 mg/kg, ip) and the right common carotid (CCA) and external carotid artery was exposed. A nylon thread (3-0) was carefully inserted into the internal carotid artery and advanced towards the origin of the middle cerebral artery until a light resistance was felt. Such resistance was indication that tip of nylon thread was wedged at the beginning of anterior cerebral artery (20-22 mm from CCA bifurcation), resulting in occlusion of MCAO. After 60 minute of MCAO, reperfusion was accomplished by withdrawing the intraluminal filament. Animals were then recovered from anesthesia, and kept in single cages for 24 h. Rectal temperature was measured by a thermometer and maintained at 37 ± 0.5ºC throughout the experiment using an electrical blanket.

Experimental design and protocols

Animals were randomly assigned to control and five different treatment groups. Investigator who performed animal surgery was blinded to groups. The control group (n=8) was received saline (5 µl, icv) as the vehicle at the beginning of MCAO. Treatment groups received CBX (Sigma, Germany) at doses 1 (n=8), 12(n=8), 25(n=8), 50(n=8), 100(n=8), µg/kg icv at the beginning of MCAO.

For ICV injection, the animals were fixed in a stereotaxic apparatus, and a midline incision was made in the skin and then a small hole was induced in the cranial region. CBX in 5µl saline was injected using a Hamilton syringe, immediately after MCAO, into the right cerebral ventricle according to the following coordinates: bregma: AP -0.8 mm, L +1.6 mm (midline) and deep 3.4 mm form dura (Paxinos and Watson 1998).

Motor neurological deficits

Motor neurological examination was performed 24 h after MCAO blindly using a five-point scoring system as described previously (Vakili *et al*., 2005). The scoring is as follows: 0= normal motor function, 1=flexion of contralateral torso or forelimb upon lifting by tail or

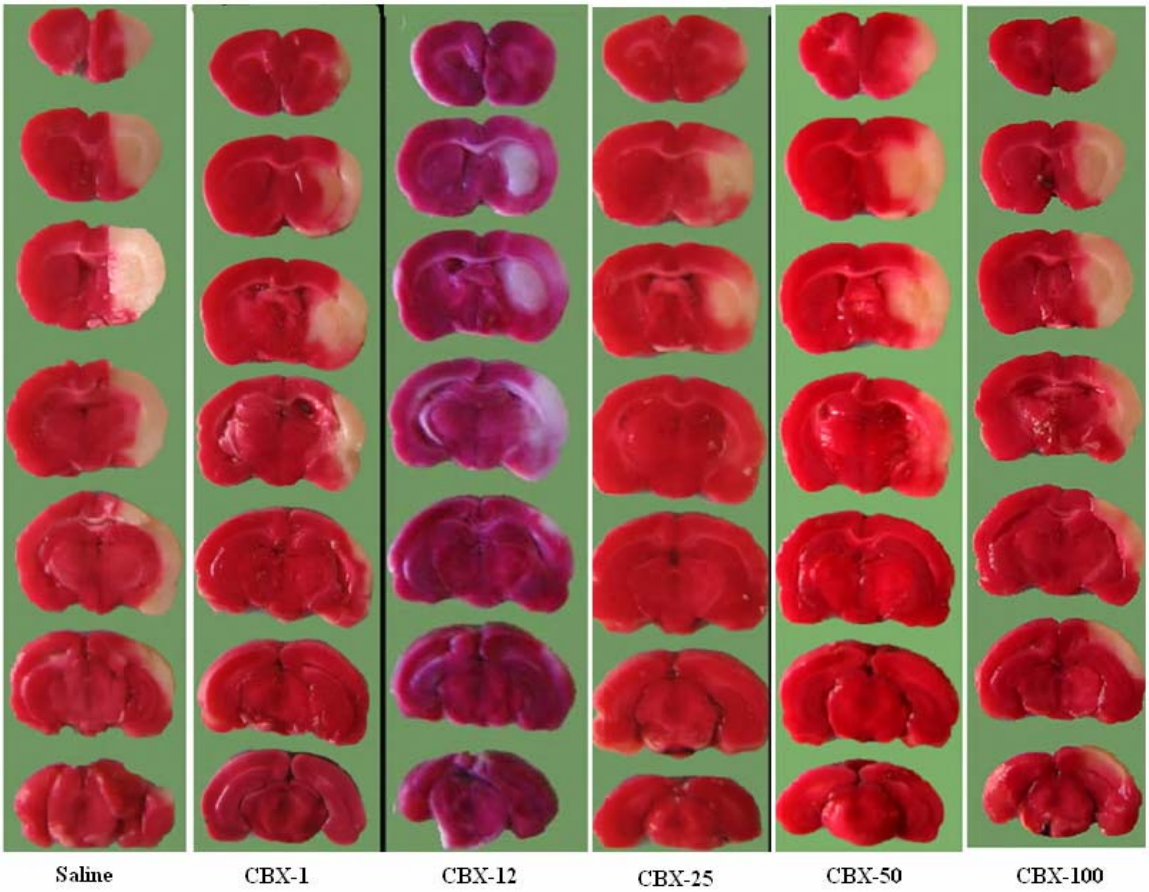

Fig. 1: Photographs illustrating in the seven coronal sections of the rat brain with TTC staining, after 60-min MCAO and 23h reperfusion, in which red color is normal area and white color is infarct area. Slices related to saline as control and carbenoxolone (CBX) treated groups at doses of 1 (CBX-1, n=8), 12 (CBX-12, n=8), 25(CBX-25, n=8), 50 (CBX-50, n=8) and/or 100(CBX-100, n=8) µg/kg icv. Colorless region corresponds to occluded MCA territory.

failure to extend forepaw when suspended vertically, 2=circling to the contralateral side but have normal posture at rest, 3=loss of righting reflex, and 4=no spontaneous motor activity.

Quantification of infarct volume

To calculation of infarct volume, 24 h after MCAO rats were deeply anesthetized and killed by cervical dislocation. Subsequently, their brains were removed and sectioned coronally into seven 2-mm thick slices using a Brain Matrix. Afterwards, the slices were immersed in 2% Triphenyltetrazolium chloride solution (Sigma, Germany), and kept in a water bath at 37°C for 15 minutes. The slices were then transferred to 10% buffered formalin (Merck, Germany). Twenty-four hours later, the slices were photographed using a digital camera connected to a computer (Cannon-Japan). Infarct areas were measured using an Image Analyzer Software (NIH Image Analyzer). The infarct volume of each slice was calculated by multiplying the infarct area of the slice by its thickness. The total infarct volume of each brain was calculated as the sum of the infarct volumes of the seven brain slices. The contribution of the edema to the infarct volume was corrected by following formula as previously described (Swanson *et al*., 1990):

Corrected infarct volume = Left Hemisphere size – (Right Hemisphere size – Measured Infarct size).

STATISTICAL ANALYSIS

Data are presented as Mean ± SEM. Infract size data were analyzed by one way analysis of variance (ANOVA) followed by Dunnet and/or Tukey test as post hoc analysis. Neurological scores and data from infarct area in each section of brain slices were analyzed by non-parametric Kruskal-Wallis ANOVA on Ranks followed by a Dunns test (SigmaStat 3.0, Jandel Scientific, Erkrath, Germany). Differences were considered significant at $P<0.05$.

RESULTS

Effects of CBX on post-ischemic reperfusion injuries

The total infarct volume in control animal group was 225±7 mm^3. Treatment with CBX at doses of 1 µg/kg (135±19 mm^3), 12 µg/kg (135±16 mm^3), 25 µg/kg (125±14 mm^3) and 50 µg/kg (157±23 mm^3) significantly reduced total infarct volume by 40%, 40%, 44% and 30%, respectively ($P<0.01$, figs. 1 and 2A).

Moreover, CBX at doses of 1 µg/kg (97±16 mm^3), 12 µg/kg (100±20 mm^3), 25 µg/kg (87±9 mm^3) and 50 µg/kg (110±17 mm^3) considerably reduced cortical infarct volumes in comparison with control group (171±6 mm3) (figs. 1 and 2B, $P<0.001$). CBX only at doses of 12 µg/kg (31±4 mm^3) and 25 µg/kg (32±6 mm^3) significantly reduced striatal infarct volumes in comparison saline group (54±6 mm^3) ($P<0.01$, figs. 1 and 2C). The higher dose of CBX (100 µg/kg) did not change infarct size (224±24 mm^3).

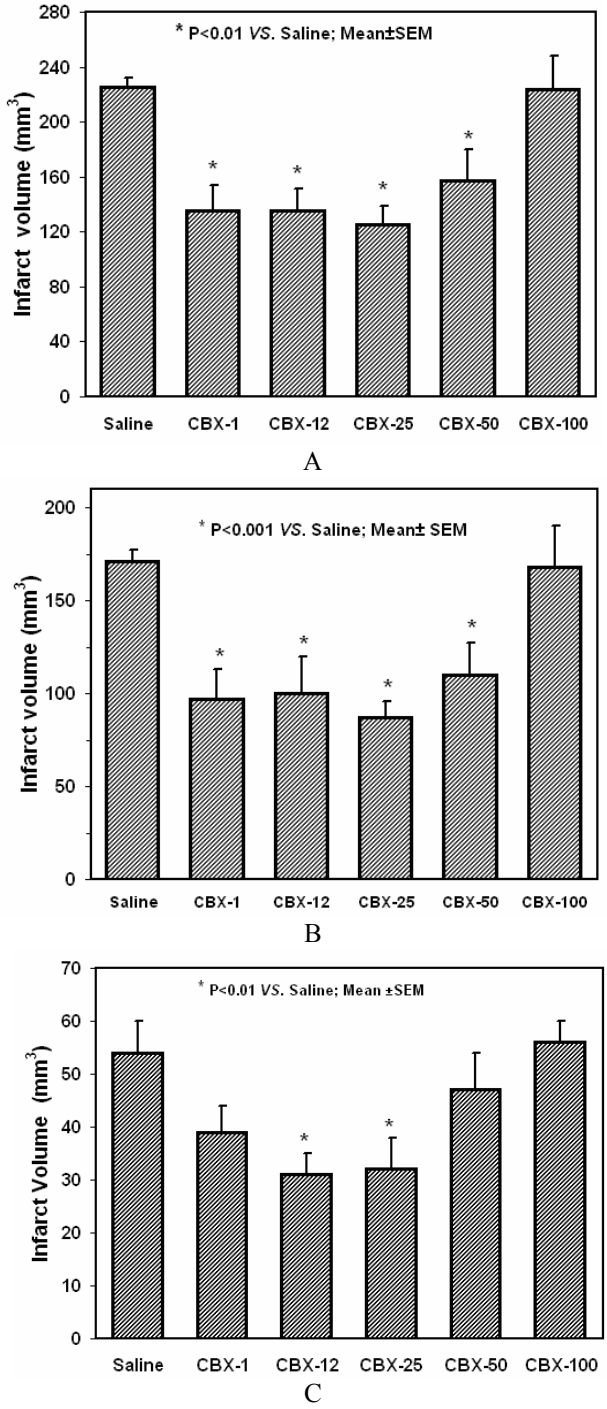

Fig. 2: Total (A), cortex (B) and striatum (C) infarct volumes in rats receiving saline (as vehicle) and/or CBX at doses of 1 (CBX-1, n=8), 12 (CBX-12, n=8), 25(CBX-25, n=8), 50 (CBX-50, n=8) and/or 100(CBX-100, n=8) µg/kg icv at the beginning of MCAO.

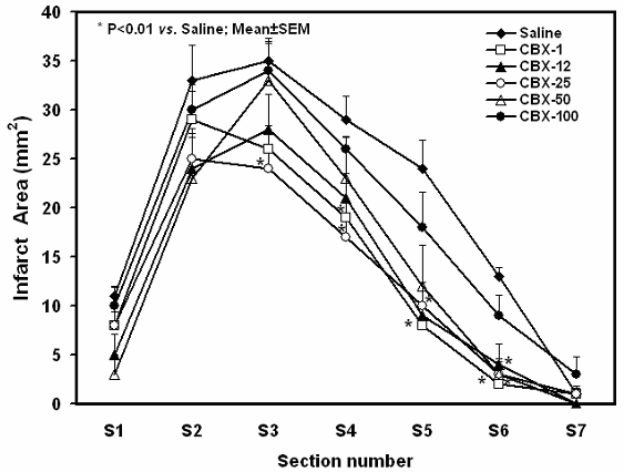

Fig. 3: Infarct areas for 7 coronal sections from anterior to posterior in rats receiving saline or CBX at doses of 1 (CBX-1, n=8), 12 (CBX-12, n=8), 25(CBX-25, n=8), 50 (CBX-50, n=8) and/or 100(CBX-100, n=8) µg/kg icv at the beginning of MCAO.

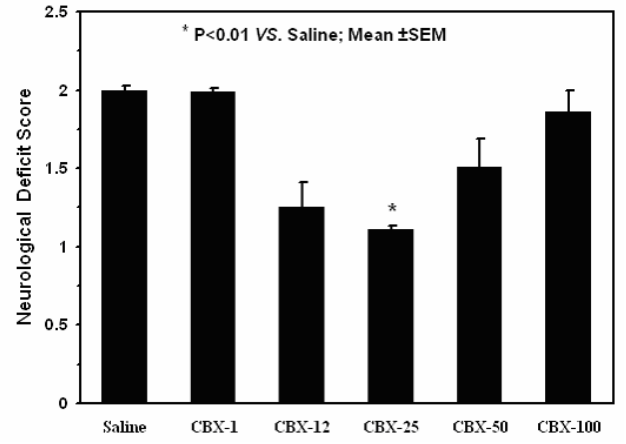

Fig. 4: Neurological deficit scores of rats receiving saline or CBX at at doses of 1 (CBX-1, n=8), 12 (CBX-12, n=8), 25(CBX-25, n=8), 50 (CBX-50, n=8) and/or 100(CBX-100, n=8) µg/kg icv at the beginning of MCAO.

Treatment with CBX mainly at doses of 1 to 50 µg/kg markedly reduced infarct area in sections of 3–6 when administrated immediately after induction of cerebral ischemia (figs. 1 and 3), in the posterior part of the MCA territory where cortical, i.e., penumbral, tissue predominates.

Effects of CBX on motor neurological deficits

In the saline group, the motor neurological deficits score was 1.99±0.04 at 24 h after MCAO. Administration of CBX significantly improved motor neurological deficits only at dose 25 µg/kg (1.38±0.18) (P<0.01). While, CBX did not significantly change the neurological deficits score when it was injected at doses of 1 µg/kg (1.98± 0.03), 12 µg/kg (1.25±0.16), 50 µg/kg (1.50±0.18) and 100 µg/kg (1.86± 0.14) (Fig. 4, p>0.05).

DISCUSSION

Our data demonstrated that central administration of CBX at doses 1, 12, 25 and/or 50 µg/kg considerably reduce cortical infarct volume in a transient model of focal cerebral ischemic. Moreover, CBX only at doses of 12 and/or 25 µg/kg reduces the size of ischemic lesion in striatum. These results demonstrate that CBX-induced neuroprotection was mainly seen in the ischemic penumbra (cortex), a potentially salvageable tissue. Only a slightly protective effect was observed in the ischemic core (striatum). These findings are in agreement somewhat with a recent study showing that intra-hippocampal injection CBX for up to 30 min after the stroke injury considerably decreased neuronal cell death in a transient model of global cerebral ischemia in rats (Perez Velazquez *et al*., 2006). Furthermore, it has been reported that administration of CBX significantly decreases ischemic damage in the photochemical model of focal cerebral ischemia (Zhang *et al*., 2004), which support the results of the present study. Additionally, our findings are supported by the results of another study showing CBX neuroprotective effects in *in vitro* ischemic model (Frantseva *et al*., 2002b). In contrast, some studies have reported that CBX increased the neuronal vulnerability, when co-cultures of astrocytes and neurons were exposed to oxidative stress (Blanc *et al*. 1998) or to excitotoxic conditions (Ozog *et al*., 2002; Zundorf *et al*., 2007).

Moreover, the finding of the study demonstrated that CBX at dose of 100 µg/kg did not change cortical or striatal infarct volume. However, it is not clear why the higher dose of CBX did not alter of ischemic damage. There is some evidence indicating that exposure of rat aortic ring with CBX (50 or 100 µmol/L) for 24h leads to vasoconstriction and elimination of endothelium-dependent relaxation, while dose 10 µmol/L was ineffective (Ullian *et al*., 1996). CBX is highly bound to plasma protein, with a long half-life of about 19 h (Hayes *et al*., 1977). On these bases, we hypothesized that the direct delivery of CBX at dose 100 µg/kg into CSF may result in vasoconstriction, decrease of cerebral blood flow to high-risk area (penumbra zone), and causes protective effect of CBX dose not appear. It is remain to determine whether CBX has effects on brain vessel tone in *in vivo* experiments.

The mechanism underlying the anti-ischemic activity of CBX at low and middle doses are not clear in this study. However, it has established that astrocyte (Cotrina *et al*., 1998; Nagy and Li, 2000; Contreras *et al*., 2002) gap junctions remain open under ischemic conditions in *in vitro* experiment. Recently, several experimental studies

support a role for gap junctions channels in propagation and amplification of ischemic neuronal injury (Khan *et al*., 1996; Azzam *et al*., 2001; Frantseva *et al*., 2002a; Contreas *et al*., 2004, Perez Velazquez *et al*., 2006). It has been suggested that gap junctions may facilitate the spread apoptotic factors, ions and small potentially toxic molecules form dying cells of ischemic core to less injured neighbors, penumbra area, and cause enlargement of ischemic injuries during brain ischemia (Khan *et al*., 1996; Azzam *et al*., 2001; Contreas *et al*., 2004). On the other hand, several studies have shown that CBX effectively blocks gap junction communication mainly in *in vitro* experiments (Davidson and Baumgarten, 1988; Goldberg *et al*., 1996; Bani-Yaghoub *et al*., 1999). Thus, part of the neuroprotective effect of CBX observed in this study might be related to closing gap junction channels. In addition, some studies indicated that CBX reduced free radical overproduction during of ischemic, which, in part, may contribute to neuroprotective of CBX (Perez Velazquez *et al*., 2006).

Previously, it has been shown 3-6 days after ICV injections of CBX hypertension was induced in rat (Gomez-Sanchez, 1992). Therefore, one possibility is that the effects of CBX on brain ischemia observed in this study may result from changes in arterial blood pressure. However, this is unlikely because the present experiments were terminated 24 h after MCAO, a long time before increased in blood pressure reported by Gomez-Sanchez (Gomez-Sanchez, 1992).

Taken together, results of this study suggest that CBX may have biphasic effect on ischemic damage in transient focal cerebral ischemia, because at low and middle doses reduces but at higher dose did not alter infarct volume. Therefore, recommended dose of CBX carefully was chosen in experimental and clinical trial studies are concerned with cerebral ischemia.

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