The Neuroprotective Effects of Isosteviol against Focal Cerebral Ischemia Injury Induced by Middle Cerebral Artery Occlusion in Rats

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Key words
- isosteviol
- stroke
- rat
- ischemia-reperfusion
- NF-κB
- Bcl-2

Abstract

Occlusion of a cerebral artery impairs blood flow leading to neuronal death. Reperfusion of the tissue is associated with inflammation, increased reactive oxygen species, necrosis and apoptosis. Hence, damage to the brain will continue even after the blood flow is restored. Isosteviol has been demonstrated to have protective effects against ischemia-reperfusion (IR) injury in the rat heart and the current study was undertaken to determine whether it is also effective in preventing IR injury in the brain. Rats were divided into six groups: a sham-operation control group and 5 IR groups that were pre-treated with either isosteviol 5 mg·kg^{-1}, 10 mg·kg^{-1}, 20 mg·kg^{-1}, nimodipine 5 mg·kg^{-1}, or saline. Cerebral ischemia was induced for 2 hours. Twenty-two hours after reperfusion the rats were assessed for neurobehavioral deficit, infarct volume, histological changes, and malondialdehyde, superoxide dismutase (SOD), Bcl-2 and NF-κB levels in brain tissue. Pre-treatment with isosteviol reduced infarct volume, ameliorated cell death and infiltration of neutrocytes, improved neuro-locomotor activity, increased SOD activity, induced Bcl-2, suppressed lipid superoxidation and the expression of NF-κB, and therefore retarded necrosis and apoptosis of neurons and inflammation. These positive effects were dose-dependent with an isosteviol dose of 20 mg·kg^{-1}, thus being as effective as nimodipine.

Introduction

Stroke may result from a variety of causes that impair cerebral blood flow (CBF). Human cerebral ischemia most often results from a transient or permanent occlusion of the middle cerebral artery (MCA). A variety of animal models that mimic the pathological changes in the brains of stroke patients have been developed. Two widely employed models of ischemic brain injury are the transient global forebrain ischemia rat model and the focal cerebral ischemia rat model. The focal model can involve either permanent or transient occlusion of the MCA resulting in damage to the cerebral cortex and striatum in the hemisphere of the rat brain, with transient occlusion followed by reperfusion being generally accepted as the model that most closely mimics stroke in human patients [1], [2].

The options for the pharmacological treatment of stroke are still very limited. Thrombolytic therapy is aimed at restoring blood flow to the brain but carries with it the risk of hemorrhage and does not prevent the damage to the brain caused by reperfusion [3]. Treatments such as nimodipine, while having the ability to reduce infarct size and improve neurological outcomes in animal models, do not appear to be particularly effective clinically and there is currently no established treatment for cerebral ischemia [4].

Isosteviol (4α,8β,13β-13-methyl-16-oxo-17-norkauran-18-oic acid) is a beyerane diterpene obtained by acid hydrolysis of stevioside, a traditional non-caloric sweetener extracted from *Stevia rebaudiana* [5]. The plant belongs to the Asteraceae family and is known for its sweet taste and effects on the cardiovascular system in traditional medicines in South America [6]. Stevioside has been used commercially as a sugar substitute in South America, Southeast Asia, China and Japan for decades [7].

Several studies indicate that isosteviol possesses a variety of biological activities including antihypertension [8], antihyperglycemia [9], anti-inflammatory [10], antioxidation and potential antitumor effects [11].

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We have reported that pretreatment with isosteviol relieved ischemia-reperfusion (IR) injury in Langendorf perfused guinea pig heart *in vitro*, and reduced infarct volume of myocardium induced by occluding and re-opening the coronary artery in rat *in vivo* [12], [13]. However, whether isosteviol possesses similar effects on the brain has not been studied to date. This study was designed to demonstrate the effects of isosteviol on ischemia-reperfusion (IR) injury in rat brain induced by MCA occlusion–reperfusion.

Materials and Methods

Chemicals

Stevioside was purchased from Stevioside Sweetener Factory, Nankai University (Tianjin, China). Isosteviol was obtained by the acidic hydrolysis of stevioside according to procedures previously described in the literature [5]. The structure of the yielded isosteviol was confirmed by nuclear magnetic resonance spectroscopy, infrared spectroscopy and determination of melting point (212 °C). The purity of the isosteviol (99 %) was determined by high-performance liquid chromatography. Isosteviol was dissolved in normal saline for administration to the rats. Nimodipine (purity 99.5 %) was provided by Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China) and also dissolved in normal saline for administration. Triphenyltetrasodium chloride (TTC) was purchased from Sigma.

Animals

Adult male Sprague-Dawley rats (250 to 300 g, 10 weeks old) were purchased from the Experimental Animal Center of the Nanjing Medical University. The rats were kept at a constant room temperature of 22 °C under 12 h light-dark cycle and free access to tap water and standard food. Rats were randomly divided into six groups (n = 30): a sham-operation (SO) control group and 5 IR groups. The IR groups included an IR vehicle control, isosteviol low (Iso L + IR, 5 $mg \cdot kg^{-1}$), middle (Iso M + IR, 10 $mg \cdot kg^{-1}$) and high (Iso H + IR, 20 $mg \cdot kg^{-1}$) dose group respectively, and nimodipine (nimodipine + IR, 5 $mg \cdot kg^{-1}$] as a positive control group. The vehicle, isosteviol or nimodipine was pre-administrated intravenously through the left femoral vein. Of the 30 rats in each group, 24 rats were used for assessment of neurobehavioral deficit and infarct volume (n = 8), determination of malondialdehyde and superoxide dismutase in brain tissue (n = 8), and general histological examination (n = 8); the remaining 6 rats were used for immunohistochemical testing for Bcl-2 and NF-κB.

Preparation of cerebral ischemia models

All experimental protocols were approved by our institutional Committee on Animal Research. The rats were anesthetized with chloral hydrate (300 mg/kg) injected intraperitoneally (*i.p.*). Cerebral ischemia models of rats were prepared using the intraluminal filament technique, as described in detail previously [14]. Briefly, a midline longitudinal incision of approximately 2.0 cm was made in the cervical area of the rat. The right common carotid artery (CCA), external and internal carotid arteries were exposed. Twenty minutes after the injection of test compounds or vehicle, a nylon monofilament (diameter: 0.28 mm) coated with silicone resin/hardener mixture was inserted into the internal carotid artery for a length of 17 – 20 mm from the CCA bifurcation until a slight resistance was felt. At this point, the filament blocked the origin of the MCA and occluded blood flow from the internal carotid artery. Two hours after the induction of ischemia, the filament was slowly withdrawn by 10 mm, causing a re-opening of the MCA and starting the reperfusion process. The incision was closed and the rat was then returned to the cage and monitored until fully recovered from the anesthesia. The reperfusion was maintained for 22 h. The rat was allowed free access to food and water. In sham–operated rats, the carotid arteries were surgically exposed, but no filament was inserted. During surgery, the body temperature of the rat was monitored using a rectal thermometer and maintained at 37 ± 0.5 °C using a lamp.

Assessment of neurological deficits

Neurological deficits in the rats were determined after 22-hour reperfusion. Neurological findings were scored on a 5-point scale in which 0 is for no neurological deficits, 1 for failure to extend left paw fully (mild neurological deficit), 2 for circling to the left (moderate neurological deficit), 3 for falling to left (severe neurological deficit), and 4 for failure to walk spontaneously and/or unconsciousness [15]. Except for the SO rats, any rat without neurological deficits after 22-h `reperfusion' was considered as an unsuccessful model and excluded from the study.

Determination of infarct volume in the brain

Infarct volume in the brain was evaluated using the TTC staining method [6]. Briefly, after 22-h reperfusion the rat was anesthetized by pentobarbital (60 mg/kg, *i.p.*) and killed by decapitation. The brain was quickly removed and placed in ice-cold saline for 5 min, and then cut coronally into 5 consecutive 2-mm thick sections. The brain slices were then immersed in 2 % TTC solution at 37 °C for 15 min where normal (non-infarct) tissue was stained red and necrotic or apoptotic (infract) tissue remained white. The stained brain sections were fixed in 10 % formalin overnight. Infarct area was measured using Medical Image Analysis System (BI-2000; Taimeng Science & Technology Pty Ltd). Infarct volume was calculated as the product of the infarct area and the thickness of the section and data were expressed by percentage of the infarct volume to the total volume of the brain.

Determination of contents of malondialdehyde (MDA) and superoxide dismutase (SOD) activity in brain tissue

After the reperfusion, the right hemisphere of the brain was weighed and homogenized with buffer (pH 7.4) consisting of 10 mM sucrose, 10 mM Tris-HCl and 0.1 mM EDTA, and then centrifuged at 3000 × *g* for 15 min at 4 °C. The contents of SOD and MDA in the supernatant of cerebral tissues were determined by the xanthine oxidase method [17] and thiobarbituric acid method [18].

Histological examination of brain tissue

Twenty-two hours after reperfusion of the brain, the rats were anesthetized with pentobarbital and killed by opening the chest. The brain was perfused with 200 mL of cold normal saline (4 °C), followed by 200 mL of 4 % paraformaldehyde solution through ascending aorta. The brain was removed and immersed in 10 % formaldehyde for 7 days and then embedded with paraffin. The tissue in paraffin was cut into 5 μm thick coronal sections and stained with hematoxylin and eosin. The morphological changes in brain tissue (in particular in cortical area and hippocampus CA1 region) were examined by microscopy.

Immunohistochemical examination for Bcl-2 and NF-κB

Bcl-2 and NF-κB in the brain tissue were examined by a streptavidin-biotin peroxidase complex immunohistochemical technique [19] using Bcl-2 and NF-κB assay kits (Zhongshan Gloden Bridge Biotechnology Co. Ltd). The rat brain was perfused with paraformaldehyde and sliced as described above. The sections were dewaxed in xylene for 2 × 10 min and hydrated through graded concentrations of ethanol (100%, 95% and 70%) for 3 × 5 min, followed by incubation in 3% hydrogen peroxide for 10 min to quench endogenous peroxidase activity and boiling in 0.01% citric acid buffer (pH 6.0) for 30 min for antigen retrieval. The sections were rinsed with distilled water for 15 min. All the above procedures were conducted at room temperature. The sections were then incubated at 4 °C overnight with the respective primary monoclonal antibodies such as mouse anti-NF-κB antibody and mouse anti Bcl-2 antibody (dilution 1 : 50 in 1% bovine serum albumin; Santa Cruz Biotechnology, Inc.). The next morning, the sections were warmed to 37 °C for 45 min and washed with phosphate buffer for 3 × 5 min, and followed by incubation for 30 min at 37 °C with secondary antibody (biotinylated goat anti-mouse immunoglobulins G; Santa Cruz Biotechnology). After being washed with phosphate buffer again, the sections were incubated with streptavidin-biotin peroxidase complex at 37 °C for 30 min. The sections was reacted with diaminobenzidine (Sigma) as a chromogen and counterstained with Mayer's hematoxylin. Negative controls were performed with 1% bovine serum albumin in phosphate buffer not containing the primary antibody. Then the sections were examined under light microscopy and the number of Bcl-2 or NF-κB positive cells per high power visual field (200 times) were counted in 10 different fields for each section in the area of the right (injured side) temporal lobe of the brain. Negative controls were prepared with absence of the primary antibody, and no staining was observed.

Statistical analysis

The data is expressed as mean ± standard deviation (SD). Differences between groups were examined using Wilcoxon test for the neurological deficit data and one-way analysis of variance (ANOVA) with Tukey's post-hoc test for all the other data. The relationship between infarct volume and neurological deficit score was analyzed by Pearson's correlation.

Results

Pretreatment with isosteviol reduced the volume of infarction in a dose-dependent manner from 5 to 20 mg/kg, with significant decrease of the infarct volume at 10 and 20 mg/kg dosing groups compared to the IR control group. The effect of isosteviol at 20 mg/kg was equivalent to that of nimodipine at 5 mg/kg (**Fig. 1**, **Table 1**).

Pretreatment with isosteviol also reduced neurological scores in a dose-dependent manner with significantly improved neurological appearance in the isosteviol 10 and 20 mg/kg groups compared to the IR control group (**Table 1**). The neurological

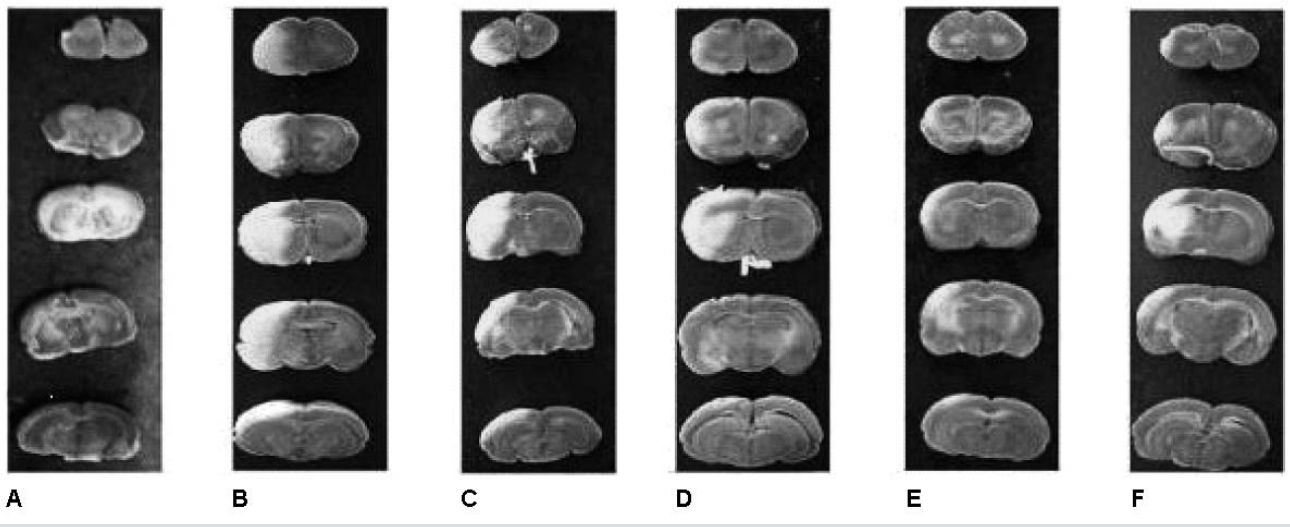

Fig. 1 The effect of isosteviol on the infarct volume in the right hemisphere of the brain injured by ischemia-reperfusion induced by occlusion (2 h) and reopening (22 h) of the MCA. Normal (non-infarct) tissue was stained red and necrotic or apoptotic (infract) tissue remained white using TTC staining method. A: SO control, B: IR control, C: Iso L + IR, D: Iso M + IR, E: Iso H + IR, F: nimodipine + IR.

Group (n = 8)	Infarct volume (%)	Neurological deficit score
SO control	0 ± 0	0 ± 0
IR control	36.6 ± 2.9	3.2 ± 0.5
Iso L + IR	35.3 ± 3.1	2.9 ± 1.2
Iso M + IR	29.1 ± 3.6*	2.4 ± 0.9*
Iso H + IR	24.5 ± 2.6**	1.9 ± 0.8**
Nimodipine + IR	26.1 ± 4.2**	2.0 ± 0.8**

Data are expressed as mean ± SD. * $p < 0.05$, ** $p < 0.01$ compared to the IR control group

Table 1 The effect of isosteviol on the infarct volume in the brain and neurological deficit score in rats with the right middle cerebral artery occlusion

deficit scores were positively correlated with the infarct volume of the brain in different groups of rats ($r^2 = 0.933$, $p < 0.01$).

The content of MDA in brain tissue from the right hemisphere was significantly increased in the IR control group as compared to the sham control group (**Table 2**). Pretreatment with isosteviol at 10 and 20 mg/kg significantly reduced the MDA content compared to the IR control group. In contrast, the activity of SOD was decreased significantly in the IR control group compared to the SO control group. When compared with the IR control group, pretreatment with isosteviol at 10 and 20 mg/kg significantly enhanced the activity of SOD in the brain tissue.

Brain tissue from the IR control rats exhibited a necrotic and apoptotic lesion and inflammatory changes involving both the cortical and subcortical regions of the right hemisphere. This was characterized by a decreased number of neurons and a number of cellular necrotic and apoptotic changes in nuclei including very dark staining, pyknosis, fragmentation, dissolution and disappearance. Disarrangement of neurons, infiltration of neutrocytes, cellular swelling and vacuolation were also observed. The necrotic, apoptotic and inflammatory changes in the brain tissue in the isosteviol pretreated rats were less severe than for the IR control rats, with significant improvement in 10 and 20 mg/kg dosing rats, which is similar to that for nimodipine rats (**Fig. 2**).

There were no Bcl-2 positive cells observed in normal tissue of temporal lobe of the brain (SO control). However, the expression of Bcl-2 was considerable when the tissue was injured by IR. This expression was further enhanced by pre-treatment with isosteviol (20 mg/kg, *i. v.*). In the meantime, there was a minimal expression of NF-κB in normal tissue of the temporal lobe of the brain (SO control). The expression of NF-κB extensively increased in the brain tissue injured by IR. In contrast to Bcl-2, this increased expression of NF-κB was attenuated by pre-treatment with isosteviol (20 mg/kg, *i. v.*). These results are summarized in **Fig. 3**.

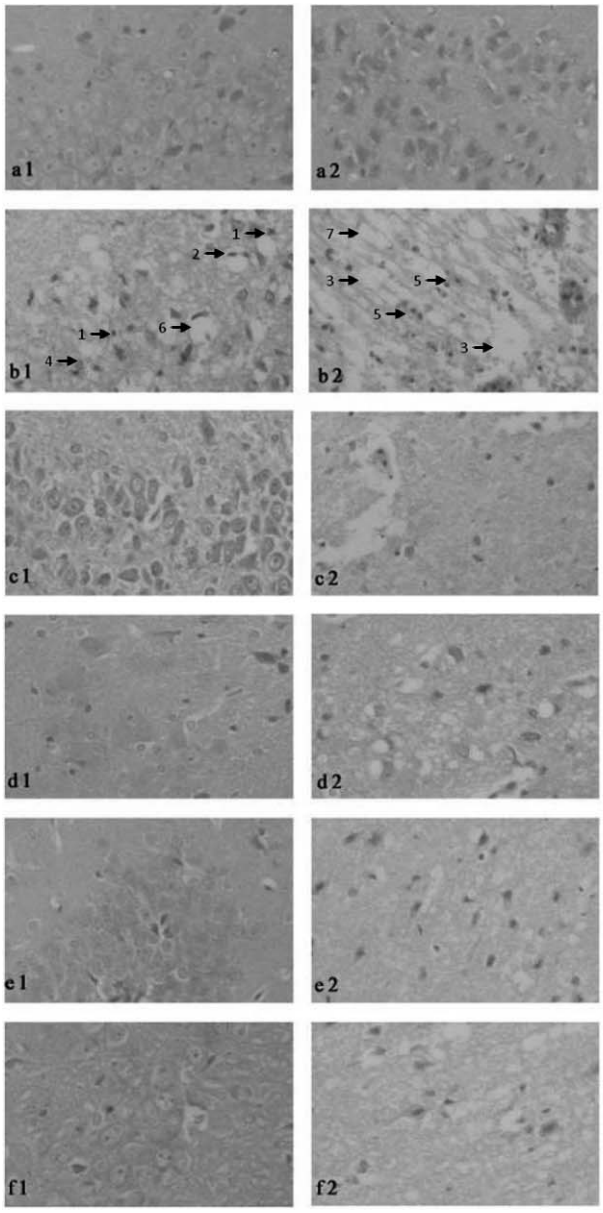

Fig. 2 The effect of isosteviol on histological appearance in the cerebral CA1 region (a1–f1) of hippocampus and cortex (a2–f2) in the right hemisphere of the brain injured by ischaemia-reperfusion induced by occlusion (2 h) and re-opening (22 h) of the MCA. The tissue sections were stained with hematoxylin and eosin and shown as high power visual field (200 times). a: SO control, b: IR control, c: Iso L + IR, d: Iso M + IR, e: Iso H+IR, f: nimodipine + IR. 1→ dark staining and pyknosis of nuclei; 2→ fragmentation of a nucleus; 3→ dissolution and disappearance of nuclei; 4→ disarrangement of neurons; 5→ infiltration of neutrocytes; 6→ cellular swelling; 7→ cellular vacuolation.

Discussion

The results confirm that pre-treatment with isosteviol is able to reduce infarct volume, cell death and inflammation in the rat brain following ischemia-reperfusion. This is consistent with our previous findings of anti-ischemia-reperfusion injury effects of isosteviol on guinea pig and rat hearts [12], [13]. However, the doses used with relevant efficacy in the brain were 10 times higher than for the heart, suggesting that the brain-blood barrier may have an impact in limiting isosteviol distribution from blood into the brain tissue. The highest dose (20 mg/kg) used in the study resulted in best outcome against IR injury in the brain. Whether even a higher dose can yield a better effect warrants further investigation.

Group (n = 8)	SOD (U/mg protein)	MDA (nmol/mg protein)
SO control	621 ± 18	4.7 ± 0.4
IR control	433 ± 30##	10.5 ± 1.1##
Iso L+IR	448 ± 50	10.6 ± 2.0
Iso M+IR	512 ± 37*	8.6 ± 1.8*
Iso H+IR	551 ± 45*	7.8 ± 1.1*
Nimodipine+IR	537 ± 50*	7.8 ± 1.0*

Data are expressed as mean ± SD. * $p < 0.05$ compared to the IR control group; ## $p < 0.01$ compared to the SO control group.

Table 2 Effect of isosteviol on SOD activity and MDA content in the brain tissue injured by ischemia-reperfusion

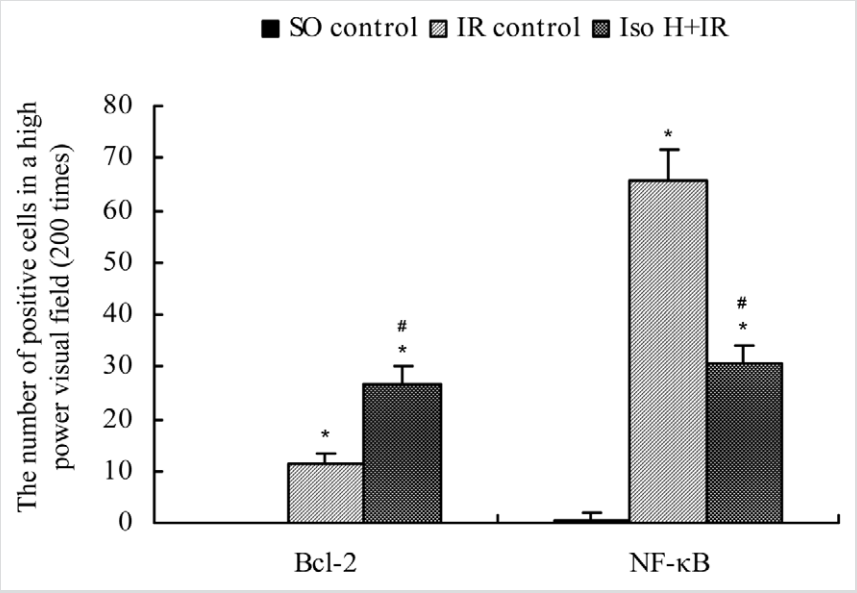

Fig. 3 The effect of isosteviol on the expression of Bcl-2 and NF-κB in the cortex tissue in the right hemisphere of the brain injured by ischaemia-reperfusion induced by occlusion (2 h) and re-opening (22 h) of the MCA. There was no Bcl-2 expression in the SO control rat brain. The data are presented as mean + SD (n = 6). * $p < 0.05$ compared to the SO control group; # $p < 0.05$ compared to the IR control group.

Cerebral ischemia-reperfusion is accompanied by enhanced formation of reactive oxygen radicals (ROS). These ROS can be scavenged by endogenous antioxidant enzymes, including SOD. Increased free radical formation coupled with a reduced antioxidant defence has been postulated to play a pivotal role in brain injury associated with stroke [20]. In the current study, SOD activity decreased and MDA content increased in the ischemia-reperfusion injured brain tissue; and pre-treatment with isosteviol increased SOD activity and decreased MDA content in the brain tissue injured by ischemia-reperfusion, suggesting that isosteviol may reverse the down-regulation of SOD and reduce lipid superoxidation level which may also contribute to its anti-ischemia-reperfusion properties.

The anti-inflammatory effect of isosteviol and related compounds has been shown in mouse ear edema induced by 12-*O*-tetradecanoylphorbol 13-acetate [10], [11]. In the current study, the number of infiltrated neutrocytes in the brain tissue decreased in the isosteviol pretreated rats, suggesting that the anti-ischemia-reperfusion effect of isosteviol may be partly due to its anti-inflammatory effect.

Bcl-2 is a member of a new category of proto-oncogenes that modulate apoptosis. Over-expression of Bcl-2 in cultured neural cells results in resistance of the cells to oxidative stress-induced apoptosis and transgenic mice over-expressing Bcl-2 exhibit increased resistance of hippocampal neurons to transient ischemic injury [21]. In addition, endogenous levels of Bcl-2 can promote survival in ischemic neurons after transient focal cerebral ischemia and suppression of Bcl-2 is known to worsen ischemic damage after temporary middle cerebral artery occlusion [22]. Bcl-2 may reduce the rise of mitochondrial Ca^{2+}, presumably by preventing opening of the mitochondrial transition pore, and inhibit cytochrome C release consequently blocking caspase activation and the apoptotic process [23]. The antiapoptotic property of Bcl-2 is also associated with its ability to suppress accumulation of ROS, and Bcl-2 may act locally in membranes to suppress lipid peroxidation [24]. Furthermore, Bcl-2 may interfere with the nuclear migration of the NF-κB [25].

NF-κB, an inducible dimeric transcription factor that belongs to the Rel family of transcription factors, is a main mediator of the cellular response to a variety of extracellular stress stimuli. NF-κB activated in neurons mediates infiltration of leukocytes into the brain parenchyma following focal ischemia [26], [27]. Activation of NF-κB increased the expression of the pro-apoptotic Bcl-xS, and nuclear localization of NF-κB after brain ischaemia was restricted to dying hippocampal neurons [28]. The response to hypoxia of the different subunits of NF-κB differed between different populations of neurons and led to regionally specific effects on the induction of Bcl-x [29]. Therefore, inhibition of NF-κB may be beneficial for reducing inflammation and apoptosis in IR injury [30].

In the current study, the expression of Bcl-2 in the cortex of the IR injured rat pretreated with isosteviol was increased compared to the IR control group. In contrast, the expression of NF-κB in the brain was attenuated in the isosteviol pretreated rats. As shown in **Fig. 2**, pretreatment with isosteviol was able to improve histological appearance of injured brain tissue, in which the number of surviving neurons was greater and the number of infiltrated neutrocytes decreased when compared to the controls. These effects may be due to isosteviol increasing the expression of Bcl-2 and decreasing the expression of NF-κB, retarding injury and inflammation in the brain tissue.

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