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Neuropharmacology and analgesia

Anti-excitotoxic effects of cannabidiol are partly mediated by enhancement of NCX2 and NCX3 expression in animal model of cerebral ischemia

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Evans Blue Dye (PubChem CID: 24832074)
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ABSTRACT

Excitotoxicity and imbalance of sodium and calcium homeostasis trigger pathophysiologic processes in cerebral ischemia which can accelerate neuronal death. Neuroprotective role of cannabidiol (CBD), one of the main non-psychoactive phytocannabinoids of the *cannabis* plant, has attracted attention of many researchers in the neurodegenerative diseases studies. The present investigation was designed to determine whether cannabidiol can alleviate the severity of ischemic damages and if it is able to exert its anti-excitotoxic effects through sodium and calcium regulation. By using stereotaxic surgery, a guide cannula was implanted into the lateral ventricle. Cannabidiol (50, 100, and 200 ng/rat; i.c.v.) was administrated for 5 consecutive days. After pretreatment, the rats were subjected to 60 min of right middle cerebral artery occlusion (MCAO). After 24 h, neurological deficits score, infarct volume, brain edema, and blood–brain barrier (BBB) permeability in total of hemisphere, cortex, piriform cortex-amygdala, and striatum were assessed. The expression of Na^+/Ca^{2+} exchangers (NCXs) protein as an endogenous target in these regions was also studied. The present results indicate that administration of cannabidiol (100 and 200 ng/rat) in the MCAO-induced cerebral ischemia caused a remarkable reduction in neurological deficit, infarction, brain edema, and BBB permeability in comparison with the vehicle group. Up-regulation of NCX2 and NCX3 in cannabidiol-received groups was also observed. These findings support the view that the reduction of ischemic injuries elicited by cannabidiol can be at least partly due to the enhancement of NCX protein expression and its cerebro-protective role in those cerebral territories supplied by MCA.

1. Introduction

Inadequate brain perfusion is the primary cause of cerebral ischemia (Dirnagl et al., 1999). A condition which is responsible for 87% of all strokes (Donnan et al., 2008). Intriguingly, cerebral ischemia is associated with several kinds of abnormalities in individuals which are vulnerable to cerebral ischemia including sickle cell anemia, aneurysm, and patients with a history of heart attack. Therefore, prevention of ischemia and induction of ischemic tolerance are the main axes of stroke studies.

Excitotoxicity, calcium-overload, inflammatory, and neuronal death have been implicated as major ischemic injury mechanisms. During ischemia, high levels of glutamate, through activation of N-methyl-D-aspartate (NMDA) receptor, leads to excitotoxicity (Obrenovitch and Richards, 1995). Consequently, it causes the inordinate influx of extracellular calcium and dysregulation of cellular ion homeostasis (Dirnagl et al., 1999). Eventually, massive calcium accumulation induces cascade of events responsible for cellular death such as triggering protease, caspase, lipase, and nuclease (Bano and Nicotera, 2007). Anti-excitotoxic drugs, such as NMDA receptor antagonists, were used in clinical trials to ameliorate ischemic injuries (Mehta et al., 2007). However, unfavorable side effects were appeared by these medications (Liu et al., 2009). Hence, other strategies like increasing excitatory amino acid transporters (Bigdeli et al., 2008), suppressing glutamate release (Molina-Holgado et al., 2002), and decreasing intracellular calcium (Iuvone et al., 2004) has been suggested. Accordingly, treatments which improve ion imbalance by gathering excessive calcium ion can reduce infarction during cerebral ischemia.

A fundamental player against the progressive accumulation of intracellular sodium and calcium ions is Na^+/Ca^{2+} exchanger (NCX). NCX is a membrane antiporter that efflux calcium in exchange of sodium influx (Annunziato et al., 2004). It has been demonstrated that increasing of NCX activity promotes neuronal survival in cerebral ischemia (Pignataro et al., 2004b). On the other hands, the *Cannabis sativa* plant has been introduced as a constituent with high therapeutic properties for many years. Dominant characteristics of cannabidiol, the non-psychoactive phytocannabinoid of *cannabis*, which makes it a suitable candidate for the present study includes neuroprotective role

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(El-Remessy et al., 2006), anti-oxidant property (Hampson et al., 1998), anti-inflammatory effect (Esposito et al., 2007), and suppression of apoptosis (Castillo et al., 2010). Therefore, cannabidiol can influence the most important events leading to ischemic damages. Nevertheless, the mechanisms involved in cannabidiol-induced neuroprotection have not been fully explored. There are few reports regarding the effect of cannabidiol on the excitotoxicity phenomenon. In fact, cannabidiol has a dual role in the regulation of intracellular calcium. Under physiological condition, elevation of intracellular calcium by CBD occurs through intracellular calcium stores and voltage-gated calcium channels (Drysdale et al., 2006). Whereas, CBD maintains calcium homeostasis through mitochondrial NCX under high-excitability conditions (Ryan et al., 2009).

The main object of the present research is the examination of a possible candidate for ischemic tolerance induction. Despite NCX family importance in the maintenance of calcium homeostasis in physiological and pathophysiological conditions, the effect of cannabidiol on NCX expression in cerebral ischemia has not yet been fully investigated. This study is the first attempt to reveal this effect of cannabidiol.

2. Materials and methods

2.1. Animals and group assignment

Adult male Wistar rats (250–350 g) were housed under conditions of controlled temperature (22 ± 2 °C) and constant humidity, with 12 h light/dark cycle for all experiments. All experimental animals were managed in accordance with National Institute of Health and Guide for the care and use of laboratory animals (NIH Publications) revised in 2011, and the study was approved by the ethics committee of the Shahid Beheshti University of Iran (NO#920.667). We made every efforts to minimize the number of animals used for this study. Rats were divided randomly into 5 main groups (control, vehicle, 50, 100, and 200 ng/rat of cannabidiol) of 35 animals and a sham group with 29 animals. Each main group was subdivided to ischemia-operated (n=30) and intact (n=5) subgroups. Ischemia-operated subgroup was classified into smaller groups for evaluation of infarct volume (n=6), brain edema (total of hemisphere n=6; cortex, piriform cortex-amygdala, and striatum n=6), and blood-brain barrier (BBB) permeability (total of hemisphere n=6; cortex, piriform cortex-amygdala, and striatum n=6), separately. The animals of intact subgroup was also used for assessment of NCX2 and NCX3 expression levels in the cortex, piriform cortex-amygdala, and striatum (n=5). As well, five rats of ischemia-operated subgroup of control group was considered for evaluation of NCX2 and NCX3 protein expression. Furthermore, sham-operated group (n=29) was assigned for brain edema (n=12), BBB permeability (n=12), and NCXs expression (n=5) separately (Table 1). Seven days after stereotaxic surgery, treatment groups received cannabidiol (50, 100 and 200 ng/rat; i.c.v.) for 5 consecutive days. Ischemia-operated subgroups were subjected to 60 min of middle cerebral artery occlusion (MCAO). Twenty-four hours later, neurologic deficits scoring and then measurement of infarct volume, edema, and BBB integrity were performed separately. It should be noted that neurologic deficits scoring was carried out in all animals. In control group, all steps were similar to stereotaxic / ischemia-operated group without receiving any treatment. The sham-operated animals underwent the same surgery procedure of MCAO without introducing a filament. For western blot technique in intact subgroups, whole methodological strategy was similar to main groups without any surgery procedure.

2.2. Drug administration

Cannabidiol (Tocris, UK) was dissolved in a mixture of dimethyl sulfoxide (10% DMSO) and phosphate buffer solution (90% PBS) (Shirazi-zand et al., 2013). A volume of 2 µl of cannabidiol solution at the doses of 50, 100, and 200 ng/rat; i.c.v. was injected into the right lateral ventricle of treatment rats (Shirazi-zand et al., 2013). The animals of vehicle group also received 10% DMSO (i.c.v.). Infusion of 1 µl solution continued for 60 s. Analysis between the control and vehicle groups was done because there is evidence about the possible neuroprotective role of vehicle (DMSO) (Di Giorgio et al., 2008).

2.3. Stereotaxic surgery

Animals were anesthetized and placed in a stereotaxic apparatus (Stoelting Instruments, USA). A midline incision was made, the skin was retracted and a hole in the position of the right lateral ventricle was created according to stereotaxic coordinates: AP,−0.58 mm posterior to the bregma; L, ± 1.4 mm from midline; and V,−1.6 mm relative to the skull (Paxinos et al., 1980). Sterile guide cannula (23 gauge) was implanted 1 mm above the injection position. The cannula was tightened to the skull with dental cement. After stereotaxic operation, animals were placed in the animal house for 7 days. Methylene blue solution (2 µl) was injected into the lateral ventricle of rat to verify the site of microinjection. After decapitation, the brain was removed and fixed in 10% formalin solution for 48 h. Then, sections were observed macroscopically to determine if the cannula had been correctly placed into the lateral cerebral ventricle.

2.4. Focal cerebral ischemia

At the fifth day, cannabidiol was injected to rats 30 min prior to MCAO surgery. Animals were anesthetized and the middle cerebral artery (MCA) was occluded by the intraluminal suture technique described by Longa et al. (Longa et al., 1989). Briefly, the right common carotid was exposed through a midline incision and the common and external carotids were ligated with a suture. A 3-0 nylon suture (Nylon, homemade), that had been rounded by heating and coated with poly-L-lysine (Sigma, USA) was introduced from the carotid bifurcation into the internal carotid artery until a mild resistance was felt (20 mm), thereby blocked the blood flow to the MCA. Reperfusion was achieved by withdrawal of the suture after 60 min of ischemia. Rectal temperature was monitored (Citizen-513w) and maintained at 37 °C by surface heating and cooling during surgery.

2.5. Neurobehavioral evaluation

24 h after MCAO surgery, neurological deficits were assessed by using eight tests. The sum of partial scores was calculated as the total

Table 1
Evaluation of ischemic tolerance in various experimental groups. CBD: Cannabidiol; MCAO: Middle Cerebral Artery Occlusion; BBB: Blood-brain Barrier; NCXs: Na^+/Ca^{2+} Exchangers.

Assessment	Control		Sham	Vehicle		CBD (50 ng/rat)		CBD (100 ng/rat)		CBD (200 ng/rat)	
	MCAO	Intact	MCAO	MCAO	Intact	MCAO	Intact	MCAO	Intact	MCAO	Intact
Infarction	+	–	–	+	–	+	–	+	–	+	–
Brain Edema	+	–	+	+	–	+	–	+	–	+	–
BBB	+	–	+	+	–	+	–	+	–	+	–
NCXS expression	+	+	+	–	+	–	+	–	+	–	+

neurologic score, with a maximum of 28 points and a minimum of 0 points in normal rats. In addition, the rats were assessed neurologically by following a chart that describes sensorimotor abilities and integration of rats (Reglodi et al., 2003). For instance, climbing, symmetry of muscle tone, and gait disturbance tests indicate motor ability of rats as well as limb placing is a suitable test for examination of sensorimotor integration.

2.6. Infarct volume

24 h after ischemia, rats were decapitated and the brains rapidly removed and cooled in saline at 4 °C for 15 min. Coronal sections with 2-mm thickness were prepared (Brain Matrix, Tehran, Iran). The slices were immersed in 2% 2,3,5-triphenyltetrazolium chloride solution(TTC) (Merck, Germany), and kept in a water bath at 37 °C for 15 min. These slices were then photographed separately using a digital camera (Nikon, D40x digital) connected to a computer. Unstained areas were defined as infarct and measured using image analysis software (Image J, version 1.46r). The infarct volume of each slice was calculated by multiplying the infarcted area of the slice by its thickness (2 mm). The total infarct volume of each brain was calculated as the sum of the infarct volume of the seven brain slices. The contribution of the edema to the infarct volume was corrected using the following formula (Swanson et al., 1990). Assessment of infarct volume in the cortex, piriform cortex-amygdala, and striatum regions was also done separately (Fig. 1).

$$\text{Corrected infarct volume} = \text{left hemisphere size} - (\text{right hemisphere size} - \text{measured infarct size}).$$

2.7. Cerebral edema

After killing the rats, brains were removed. The cerebellum, pons, and olfactory bulb were separated. Right and left wet weights (WW) were calculated separately. Subsequently, dry weights (DW) were assessed after 24 h at 120 °C. Brain water content (BWC) was calculated as [(WW−DW)/WW] ×100 (Bigdeli et al., 2007). In addition, brain edema was evaluated in the cortex, piriform cortex-amygdala, and striatum areas separately.

2.8. Blood-brain barrier integrity

Evans Blue dye (EBD, Sigma Chemicals, USA) extravasation was used for evaluation of BBB permeability. Briefly, 4 ml/kg of 2% EB solution was injected intravenously 30 min after MCAO. Transcardial perfusion with 250 ml saline was accomplished on anesthetized rats 24 h after ischemia to replace intravascular EBD with saline. After decapitation, the brains were removed and the hemispheres separated and weighed. Two hemispheres were separately homogenized in 2.5 ml phosphate-buffered saline to extract the EBD. Then 2.5 ml of 60% trichloroacetic acid was added to precipitate protein and mixed by vortex for 3 min. The samples were placed at 4 °C for 30 min and centrifuged for 30 min at 1000 rpm. Measuring of EBD absorbance in the supernatant was carried out by using spectrophotometer at 610 nm (Perkin-Elmer, Illinois, USA). EBD concentration was expressed as μg/g of brain tissue through the standard curve (Bigdeli et al., 2007). BBB integrity was also evaluated in the cortex, piriform cortex-amygdala, and striatum areas separately.

2.9. Western blot analysis

The expression of NCX2 and NCX3 proteins was evaluated by western blotting technique following sample extraction and SDS-PAGE. The NCX2 and NCX3 expression levels were assessed in the cortex, piriform cortex-amygdala, and striatum separately. Each tissue sample was homogenized at 4 °C for 1 min in lysis buffer. Tissue extract was centrifuged at 12,000 rpm at 4 °C for 20 min. The supernatant was gathered as the tissue extract. After evaluation of protein concentration in every tissue extract by Bradford assay, sample buffer was added to aliquots of tissue extracts. Samples were heated at 100 °C for 5 min. Proteins were separated by SDS-PAGE (8% gel). Blotting was performed by semi-dry type blotting (BIORAD). The blots were blocked with 2% non-fat dry milk in Tris buffer saline in 0.1% Tween 20 at 4 °C for 75 min, and incubated for 18 h with polyclonal rabbit antibody to

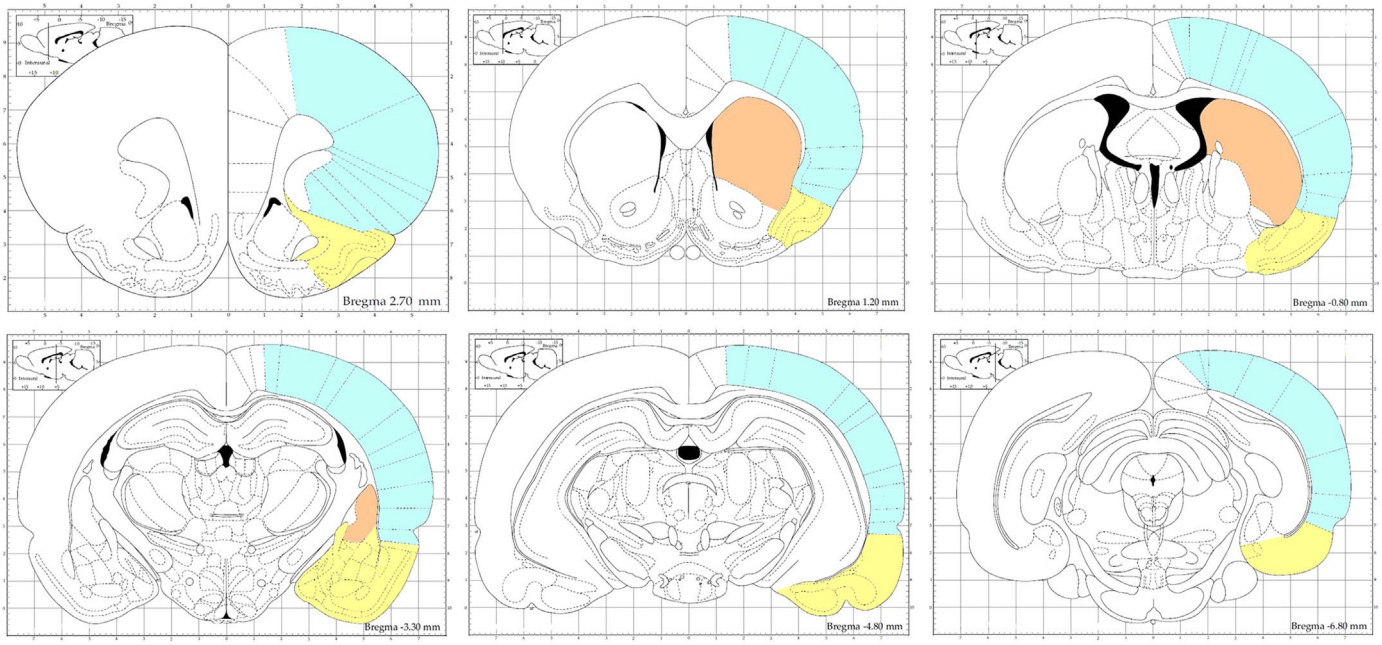

Fig. 1. Coronal schematic sections show the anatomic location of rat brain regions analyzed for infarction volume, brain edema, BBB integrity and NCXs expression. Brain levels are indicated by distance from bregma. Coronal rat brain atlas diagrams (Paxinos et al., 1980) corresponding to the middle of each 2 mm slice. Selected areas was shown in the diagram of the right hemisphere. These areas include cortex (blue), piriform cortex–amygdala (yellow), and striatum (orange).(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

NCX2 (1:500 dilution) (LS-C8A2, LifeSpan, BioSciences, United State), NCX3 (1:500 dilution) (SLC8A3, LifeSpan BioSciences), and rabbit antibody β-actin (1:1000 dilution) (Santa Cruz) separately, followed by secondary anti-rabbit antibody (1:500 dilution) (Santa Cruz) for 90 min. NCX2 and NCX3 immune-reactive proteins were detected with advanced chemiluminescence (Enhanced Chemiluminescence, Amersham Biosciences) and film exposure. The intensity of the blots was calculated by an image analysis system (Image J, version 1.46r).

2.10. Statistical analysis

Neurological deficit scores were compared using non-parametric Kruskal-Wallis analysis of variance followed by the Dunn test (SPSS v22.0). Infarct volume, brain edema, EBD extravasations, and data from NCX2 and NCX3 assays were analyzed by using two-way analysis of variance (ANOVA) (SPSS v22.0 post hoc LSD). Data were expressed as mean ± S.E.M. $P < 0.05$ was considered significant.

3. Results

3.1. The effect of CBD on neurological deficits scores

The effect of neuroprotective agents on sensorimotor ability has increasingly become important in stroke studies. 24 h after ischemia, the total neurologic deficits score was 20.86 ± 1.93 in the vehicle group. Administration of CBD at dose of 100 ng/rat improved neurologic outcome significantly (8.8 ± 1.88, P=0.004). The beneficial effect of CBD at dose of 200 ng/rat was manifested by reduction of neurologic deficits in comparison with the vehicle group (15.46 ± 2.15, P=0.01), whereas CBD at a dose of 50 ng/rat (19.53 ± 2.35) did not change the neurological scores (Fig. 2). However, the average score of sham-operated rats was very low, with significant difference from MCA-occluded animals in all signs (P=0.004). Difference between the vehicle and control groups was not significant (Table 2).

3.2. The effect of CBD-induced protection on infarct volume

The total infarct volume in the vehicle group was 212.13 ± 20.2 mm^3. Administration of CBD at doses of 100 and 200 ng/rat resulted in a significant reduction of infarct volume (77.43 ± 9.7 mm^3, P<0.001 and 111.44 ± 12.1 mm^3, P=0.01, respectively). Moreover, dose of 50 ng/rat CBD did not modify infarct size in comparison with the vehicle group. As well, cerebral infarction in the cortex was decreased at doses of 100 and 200 ng/rat CBD significantly (32.95 ± 7.2 mm^3, P<0.001 and 50.21 ± 10.3 mm^3, P=0.02, respectively) compared with the vehicle group (100.52 ± 6.8 mm^3). Reduction in infarct volume exerted by two doses of CBD (100 and 200 ng/rat) was also seen in the striatum (21.74 ± 5.7 mm^3, P<0.01 and 32.51 ± 7.4 mm^3, P=0.03, respectively) when was compared with the vehicle group (59.67 ± 7.4 mm^3). Between experimental groups, no significant difference in piriform cortex-amygdala area was observed. Furthermore, there is no significant difference between the vehicle and control groups (Fig. 3).

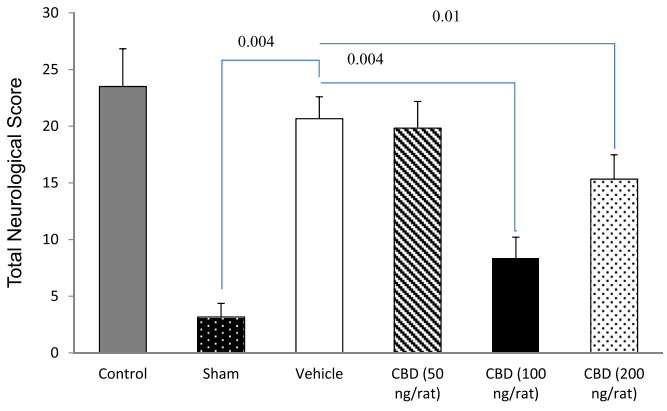

Fig. 2. Effect of cannabidiol (CBD) at doses of 50, 100, and 200 ng/rat on total neurological deficits score at 24 h after ischemia induction in rats. Cannabidiol in a dose-dependent response improved neurological deficits significantly. Values are expressed as the mean ± S.E.M. (n=10). P<0.05 compared with vehicle-treated group (Non-parametric Kruskal-Wallis analysis)..

3.3. Dose-dependent effect of CBD on brain edema

The effect of CBD on brain water content which is an indicator of brain edema was also considered. Brain edema as a major ischemic injury is associated with increasing BBB permeability. Focal cerebral ischemia significantly increased the brain edema in MCAO-operated groups in the ipsilateral hemisphere, in comparison with contralateral hemisphere ($P < 0.05$). However, this difference between two hemispheres was not significant in 100 and 200 ng/rat CBD groups as well as sham-operated group. Brain edema in three regions of the ipsilateral hemisphere was detected to increase in compassion with sham-operated group ($P < 0.05$). In this study, administration of 100 and 200 ng/rat of CBD significantly decreased brain edema of infarcted hemisphere (79.66 ± 0.40%, P=0.002 and 80.58 ± 0.20%, P<0.01, respectively) compared with the vehicle group (81.82 ± 0.30). It should be noted that the anti-edematous effect of CBD in the cortex area was observed only at dose of 100 ng/rat CBD (71.41 ± 0.51%, P=0.001). CBD did not alter the brain edema in piriform cortex-amygdala area. On the other hand, 100 and 200 ng/rat of CBD groups illustrated a significant effect on the decrease of brain edema in the striatum (71.56 ± 0.66%, P<0.01 and 72.2 ± 0.25%, P=0.04, respectively) in comparison with the vehicle group (73.02 ± 0.22%). Analysis between the control and vehicle groups showed no significant difference (Fig. 4).

3.4. Dose-dependent effect of CBD on blood-brain barrier integrity

The effect of CBD on BBB permeability is shown in Fig. 5. MCAO surgery increased the BBB permeability in the ipsilateral hemisphere in comparison with contralateral hemisphere significantly ($P < 0.05$). The BBB permeability in three regions of the ipsilateral hemisphere was shown to augment in compassion with sham-operated group ($P < 0.05$). In the vehicle group, Evans Blue concentration in the ischemic and the non-ischemic hemispheres was 10.55 ± 0.59 and 5.18 ± 0.46 μg/g tissue, respectively. Administration of CBD at doses of 100 ng/rat (right hemisphere=6.67 ± 0.44, left hemisphere=3.01 ± 0.44, $P < 0.01$) and 200 ng/rat (right hemisphere=7.93 ± 0.47, left hemisphere=3.16 ± 0.38, P=0.03) attenuated BBB disruption significantly in comparison with the vehicle group. This effect was not observed at the lower dose of CBD (50 ng/rat). Meanwhile, Evans Blue extravasations in the cortex and striatum areas were reduced by a dose of 100 ng/rat CBD (1.12 ± 0.43, P=0.01 and 2.59 ± 0.80, P=0.01, respectively) compared with the vehicle group (4.03 ± 0.78 and 2.89 ± 0.58, respectively), while CBD did not change BBB permeability of the piriform cortex-amygdala area. Moreover, CBD at a dose of 200 ng/rat significantly decreased brain Evans Blue concentration in the striatum (2.80 ± 0.50, P=0.04). Difference between the vehicle and control groups was not significant (Fig. 5).

3.5. Effects of CBD-induced neuroprotection on NCX2 expression

Western blot technique shows that NCX2 protein was expressed in the cortex, piriform cortex-amygdala, and striatum. Regarding the expression of NCX2, there was no significant difference between control, MCAO-operated and vehicle–received groups. Furthermore, comparison between the sham-operated and control groups revealed no significant difference (data not shown). Nevertheless, the NCX2 expression in the cortex increased at a dose of 100 ng/rat CBD, when compared to the vehicle group (P=0.001). The remarkable difference was not observed in piriform cortex-amygdala and striatum. Therefore,

Table 2
The partial neurologic deficits scores in different experimental groups. This table indicates partial scores in six rats. CBD: Cannabidiol; MCAO: Middle Cerebral Artery Occlusion.

Groups	Rat	Motility	Sensory Function		Symmetry of Muscle Tone		Limb Placing		Biased Movement		Climbing/rat	Gait Disturbances	Postural Signs		Sum	Average
			Touching Reflex	Grasping Reflex	Grasping Strength	Lateral Resistance	Hind limb	Forelimb	Pushing Back	Pulling			Forelimb Flexion	Thorax Twisting		
Control(MCAO)	1	4	1	1	1	1	2	2	2	1	1	5	2	2	25	23.5
	2	4	1	0	1	1	1	1	1	1	1	4	2	2	20	
	3	3	1	1	2	1	2	2	2	2	1	3	2	2	24	
	4	4	1	1	1	1	2	2	2	2	1	4	2	2	25	
	5	4	1	1	2	1	2	2	1	2	0	3	2	2	23	
	6	4	1	1	2	1	1	1	2	2	1	4	2	2	24	
Sham	1	1	0	0	1	0	0	0	1	0	1	1	0	0	5	3.16
	2	1	0	0	0	0	0	0	0	0	0	0	0	0	1	
	3	0	0	0	1	0	0	0	1	0	0	1	0	0	3	
	4	1	0	0	0	0	0	0	0	0	0	0	0	0	1	
	5	2	0	0	1	0	1	0	1	0	0	0	0	0	5	
	6	1	0	0	1	0	0	0	1	0	0	1	0	0	4	
Vehicle	1	3	1	1	1	1	1	1	1	1	1	3	2	2	19	20.66
	2	4	1	1	0	1	2	2	1	1	1	5	2	2	23	
	3	4	1	0	1	1	2	2	1	1	0	4	2	2	21	
	4	4	1	1	1	1	1	1	1	1	1	3	2	2	20	
	5	1	1	1	1	1	2	2	1	2	1	1	1	1	16	
	6	4	1	1	1	2	2	2	1	2	1	4	2	2	25	
CBD(50 ng/rat)	1	3	1	1	1	1	1	1	1	1	1	4	2	2	20	19.8
	2	3	0	1	1	0	1	2	2	1	0	2	1	1	15	
	3	3	1	1	1	1	1	1	0	0	1	3	2	2	17	
	4	3	1	1	1	1	2	1	2	2	1	3	1	2	21	
	5	4	1	1	1	1	2	2	1	2	1	4	2	2	24	
	6	3	1	1	1	1	2	2	1	1	1	4	2	2	22	
CBD(100 ng/rat)	1	2	0	0	1	1	0	1	0	1	0	2	0	0	8	8.3
	2	2	0	1	0	0	0	0	0	0	0	3	0	1	7	
	3	3	0	1	0	0	1	1	0	0	1	3	1	1	12	
	4	2	0	0	0	1	0	0	0	1	1	2	0	0	7	
	5	2	0	1	0	1	1	0	0	1	0	2	1	1	10	
	6	1	0	0	1	0	1	0	1	1	0	1	0	0	6	
CBD(200 ng/rat)	1	3	1	1	0	1	1	1	0	1	1	3	2	2	17	15.5
	2	3	1	1	1	0	1	1	1	1	1	3	1	1	16	
	3	3	0	1	1	1	1	1	1	1	0	2	2	1	15	
	4	3	0	1	0	0	1	1	0	0	1	3	1	1	12	
	5	3	1	1	0	1	1	1	1	0	0	3	1	1	14	
	6	3	1	1	0	0	1	1	1	1	1	4	2	2	18	

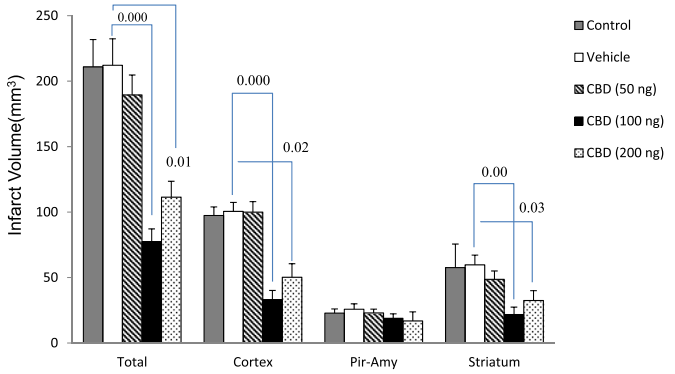

Fig. 3. The graph shows the effect of various doses 50, 100, and 200 ng/rat of cannabidiol (CBD) on MCAO-induced infarct volume in total, cortex, piriform cortex-amygdala (Pir-Amy), and striatum. Cannabidiol was continuously infused i.c.v. for 5 days before induction of ischemia. Total infarction volume significantly reduced at doses of 100 and 200 ng/rat of cannabidiol. The decreasing effect of cannabidiol at doses of 100 and 200 ng/rat was also observed in cortex and striatum. Each column represents the mean ± S.E.M. of the infarct volume (n=6). *P*<0.05 compared with vehicle-treated group (Two-way ANOVA test). The numbers above columns express *P* value.

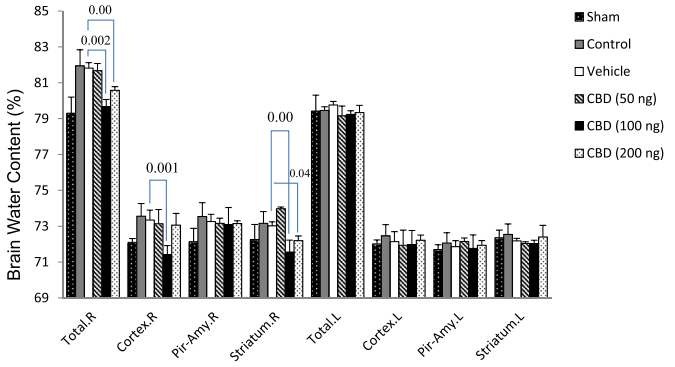

Fig. 4. Brain water content in various experimental groups including ischemic hemisphere (Total. R), non-ischemic hemisphere (Total. L), cortex (right and left), piriform cortex-amygdala (Pir-Amy) (right and left), and striatum (right and left) areas of sham-operated, control, vehicle, 50,100, and 200 ng/rat of cannabidiol (CBD) groups. Cannabidiol (100 and 200 ng/rat) statistically decreased brain edema of total infarcted hemisphere compared with the vehicle group. Dose of 100 ng/rat cannabidiol markedly alleviated brain edema in ischemic (ipsilateral) cortex and striatum. Values are expressed as the mean ± S.E.M. (n=6). *P*<0.05 (Two-way ANOVA test). The numbers above columns express *P* value.

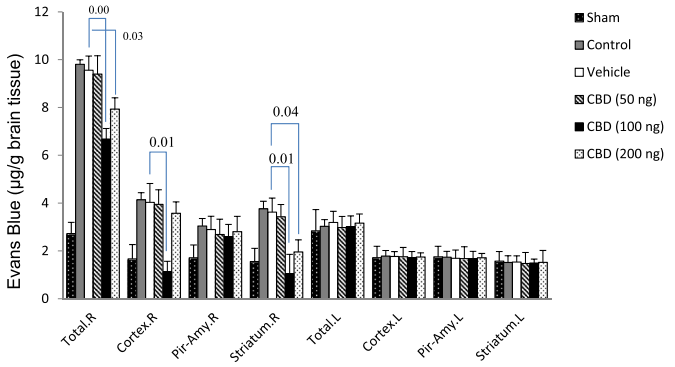

Fig. 5. Evans Blue extravasations in various experimental groups including ischemic hemisphere (Total. R), non-ischemic hemisphere (Total. L), cortex (right and left), piriform cortex-amygdala (Pir-Amy) (right and left) and striatum (right and left) areas of sham-operated, control, vehicle, 50, 100, and 200 ng/rat of cannabidiol (CBD) groups. Cannabidiol (100 and 200 ng/rat) significantly decreased BBB permeability of total infarcted hemisphere compared with the vehicle group. Tissue concentration of Evans Blue in the ischemic (ipsilateral) cortex and striatum were significantly lower in 100 ng/rat cannabidiol-treated rats at 24 h after MCAO. Values are expressed as the mean ± S.E.M. (n=6). $P < 0.05$, compared with the vehicle group (Two-way ANOVA test). The numbers above columns express *P* value.

it seems that CBD could cause an incremental effect in the expression of NCX2 protein in the cortex (Fig. 6).

3.6. Effects of CBD-induced neuroprotection on NCX3 expression

According to this study, NCX3 protein is expressed in the cortex, piriform cortex-amygdala, and striatum areas. Analysis between the sham-operated and control groups showed no significant difference (data not shown). As well, the similar results was observed between the control and vehicle-received groups. Based on these findings, induction of cerebral ischemia by MCAO surgery caused the significant reduction in NCX3 expression level in the cortex and striatum (P=0.01 and P=0.03, respectively). The present results expresses that NCX3 protein increased in the cortex area at doses 100 and 200 ng/rat of CBD-received groups in comparison with the vehicle group ($P < 0.01$ and P=0.001, respectively). In addition, NCX3 expression level in the striatum elevated in 100 ng/rat CBD group (P=0.05) (Fig. 7).

4. Discussion

The present results show that cannabidiol at the doses of 100 and 200 ng/rat improved neurological impairment induced by MCA occlusion. Moreover, cannabidiol at these doses reduced total infarct volume, brain edema, and BBB permeability. In the present study, the i.c.v. route of CBD administration has been done for the first time in the animal model of cerebral ischemia. The previous investigations were mostly focused on therapeutic approach, while induction of ischemic tolerance is an innovation in this study.

As similar studies have reported, the improvement of neurological deficits observed in CBD-received rats is attributed to attenuation of infarction as an indicator of neuronal death. In line with the present findings, CBD has been known to be protective against ischemic injuries through reduction of infarction (Braida et al., 2003; Mishima et al., 2005; Pazos et al., 2013). There is a direct relationship between extracellular glutamate levels and the severity of cellular loss (Fernandez-Lopez et al., 2005). We propose that CBD attenuates cellular death and the resulting infarction by decreasing excessive glutamate and calcium (Iuvone et al., 2004; Pertwee, 2004). This hypothesis is supported by other experimental studies in the animal model of cerebral ischemia which in them CBD reduced neuronal death by the decrease in glutamate release which is mediated by cannabinoid type 2, adenosine A_1, and serotonergic 5-HT_{1A} receptors (Castillo et al., 2010; Pazos et al., 2013). The previous report demonstrated that CBD prevents the increase of inducible nitric oxide synthase (iNOS) expression (Esposito et al., 2006). These findings can be related to the CBD-induced reduction of glutamate release due to the contribution of glutamate in iNOS induction during hypoxia (Martinez-Orgado et al., 2006). The other possibility is the effect of CBD on the decrease of tumor necrosis factor α (TNF-α) (Castillo et al., 2010). This is an expected effect, as TNF-α elevates glutamate release (Vesce et al., 2007). Based on these findings, CBD probably ameliorates infarction volume via reduction of glutamate and inflammatory cytokines. Consequently, a decline of infarction volume was observed in 100 and 200 ng/rat CBD-received groups in the cortex and striatum. Similarly, other studies confirmed the neuroprotective role of CBD in these areas (Hampson et al., 1998; Pazos et al., 2013). The possible mechanisms for the observed effects of CBD are augmentation of cerebral blood flow in the cortex and reduction of myeloperoxidase activity, an index of neutrophil accumulation, in the striatum (Hayakawa et al., 2007; Mishima et al., 2005). These findings were not unexpected because the cortex and striatum are brain regions that are supplied by MCA and are highly sensitive to ischemia.

This is the first study to exhibit the dose–dependent effect of CBD on inhibition of brain edema and BBB permeability. Except Alvarez and colleague's study, no data regarding the anti-edematous activity of CBD in cerebral ischemia exists. In that study, the effect of CBD on the

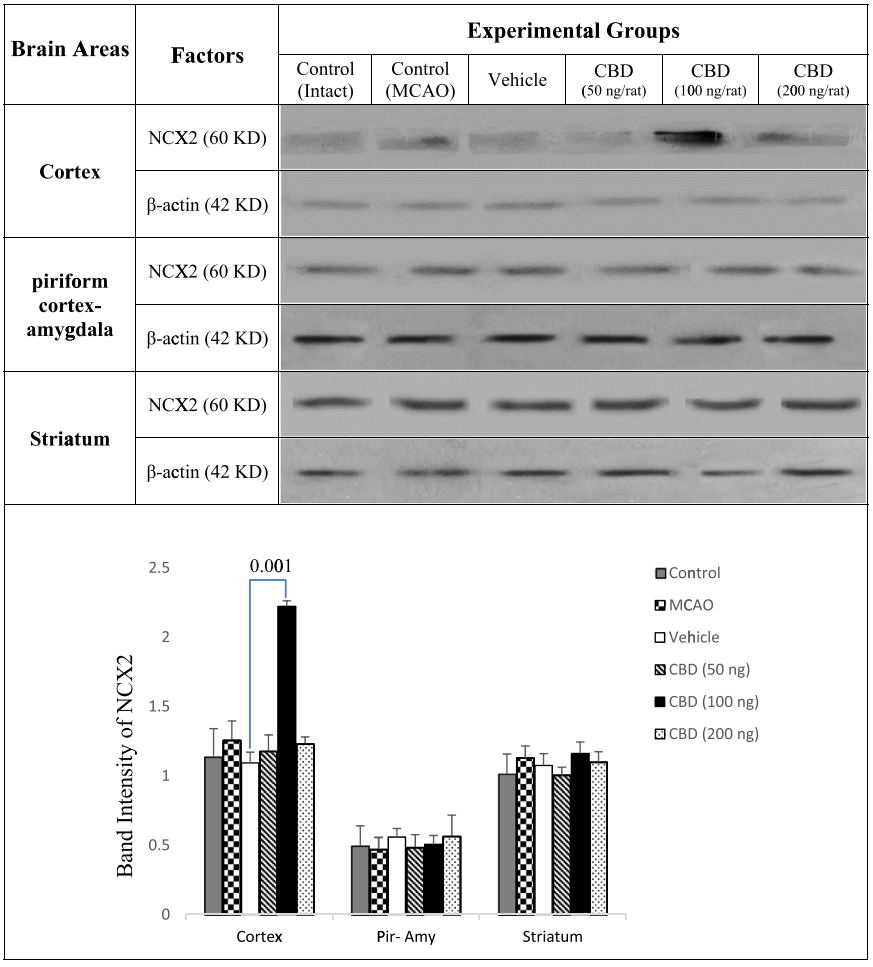

Fig. 6. Western blot of Na^+/Ca^{2+} exchanger type 2 (NCX2) protein in the right cortex, piriform cortex-amygdala (Pir-Amy), and striatum of the control(intact), control(MCAO), vehicle, 50, 100, and 200 ng/rat cannabidiol (CBD) groups. Analysis of NCX2 protein bands after normalization with β-action as loading control was carried out (n=5). The results showed augmentation of NCX2 expression in the cortex at a dose of 100 ng/rat cannabidiol. Quantification data used for this are mean ± S.E.M. of five different experiments. $P < 0.05$, compared with vehicle group (Two-way ANOVA Test). For final preparation of table, in some lines (NCX2 of cortex and striatum), the second and last columns were cropped and spliced with related bands.

increase of impedance, as an index for brain edema in EEG, was examined (Alvarez et al., 2008). The anti-edematous effects of CBD may be associated with reduction of intracellular calcium, interlukin_1, and NF-κB by CBD (Rama Rao et al., 2010; Ryan et al., 2009). We hypothesize that reduction of brain edema exerted by CBD might be related to an increase in BBB integrity. Disruption of the BBB is characterized by releasing excessive free radicals and pro-inflammatory mediators from injured tissues. These mediators elevate the expression of intercellular adhesion molecule 1(ICAM-1) and consequently promote the adhesion of leukocytes to endothelia cells, theirs migration out of vessels, and BBB breakdown (Yilmaz and Granger, 2008). The inhibitory effect of CBD on activity of pro-inflammatory mediators, ICAM-1 expression, and the leukocyte migration are the possible mechanisms involved in the increase of BBB integrity (Castillo et al., 2010; El-Remessy et al., 2006; McHugh et al., 2008). It is worth noting that dose of 100 ng/rat CBD indicated a notable decremental effect on brain edema and BBB permeability in the cortex and striatum, while dose of 200 ng/rat CBD only reduced these parameters in the striatum. Finding the optimal dose of medicine has outmost importance in therapeutic applications. The present study demonstrates that CBD at a dose of 100 ng/rat showed the best protective effect. Comparatively, other reports have confirmed a bell-shaped dose-dependent curve of CBD, with an optimal effect at 5 mg/kg i.p. per day (Braida et al., 2003; Gallily et al., 2015; Mishima et al., 2005).

Recently, researchers have concentrated to identify endogenous neuroprotective mechanisms as therapeutic targets and replace them due to the failure of the pharmacological components in clinical trials. NCX is one of the endogenous neuroprotective factors. Three isoforms of NCX family have been identified, so far. The NCX accelerates exchange of calcium and sodium (forward mode); alternatively, it can deliver sodium to extracellular space and transport calcium into the cell (reverse mode) (Molinaro et al., 2008). Our results show that NCX3 expression decreased in the cortex and striatum following MCAO. Expression of NCXs in the different brain areas seems to change after induction of ischemia (Pignataro et al., 2004a). In particular, down-regulation of three NCX isoforms in the ischemic core region was observed, while only NCX3 expression level was diminished in penumbra area. These findings could be attributed to the high activity of proteolytic enzymes during cerebral ischemia to cleave NCX proteins (Bano et al., 2005). Furthermore, our results prove for the first time that CBD upregulated the expression of NCX2 and NCX3 significantly in the cortex. Based on the related studied, NCXs are abundantly expressed in cortex and striatum (Cuomo et al., 2008; Molinaro et al., 2008) as the present data confirms. There is only one report regarding the effect of CBD on NCXs, in which restoration of intracellular calcium homeostasis is carried out through activity of mitochondrial NCX (Ryan et al., 2009). It is also relevant to mention that NCXs in penumbra operate via forward mode where there is still a persistence of ATPase activity. In this region, prevention of NCX action will inhibit calcium ion extrusion, thus will augment ischemic damages. By

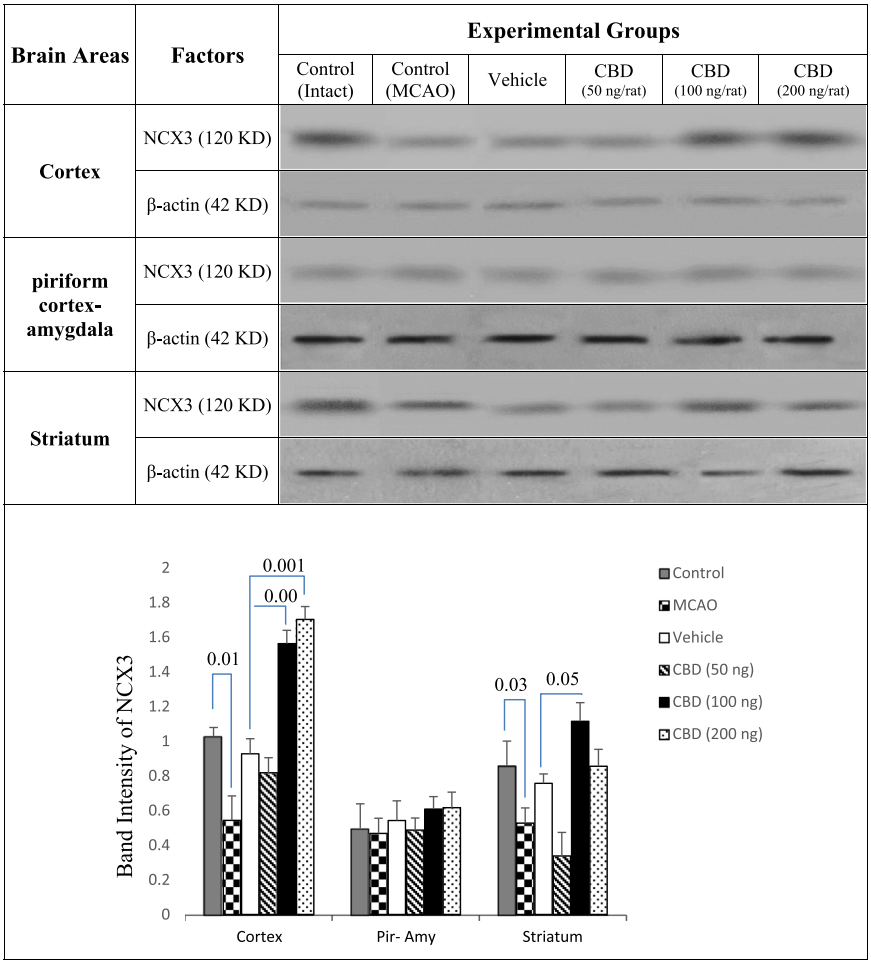

Fig. 7. Effects of various doses of cannabidiol (50, 100, and 200 ng/rat) on Na^+/Ca^{2+} exchanger type 3 (NCX3) protein expression in assessed cortex, piriform cortex-amygdala (Pir-Amy), and striatum areas of right hemisphere. Western blot and its densitometric analysis of NCX3 protein after the administration of cannabidiol (CBD) was accomplished. Overexpress of NCX3 protein was observed in the cortex region at doses of 100 and 200 ng/rat of cannabidiol. All the data was expressed as the means ± S.E.M. of five different experiments and were normalized based on β-actin levels (n=5). $P < 0.05$, compared with vehicle group (Two-way ANOVA Test). For final preparation of table, the second column was cropped and replaced with related band (MCAO).

contrast, impairment of Na^+/K^+ ATPase action occurs in the ischemic core because of remarkable depletion of ATP. Therefore, there is a massive accumulation of intracellular sodium that triggers NCX to work in the reverse mode, inhibition of NCX in the core can further exacerbate the necrotic lesion (Pignataro et al., 2004a). Generally, the cortex is defined as the ischemic core region. Probably, depletion of ATP and involvement of mitochondrial NCX in the maintenances of calcium homeostasis can result in the further efficacy of CBD in the cortex than the striatum (Pignataro et al., 2004a; Ryan et al., 2009).

There is a possibility that reduction of ischemic injuries elicited by CBD can be at least partly due to overexpression of NCXs. Our results are well in agreement with other experimental studies implying preconditioning with normobaric hyperoxia and ischemic preconditioning protect from brain against ischemia by up-regulation of NCXs (Mohammadi and Bigdeli, 2013; Pignataro et al., 2013). Exclusively, the previous reports have reported that upregulation of NCX1 and NCX3 is directed to neuroprotective effects in cerebral ischemia (Annunziato et al., 2007; Pignataro et al., 2011, 2004b), whereas suppression of NCX activity by pharmacological agents extends cerebral damages (Pignataro et al., 2004b). The neuroprotective mechanism of NCX in cerebral ischemia has partly been determined but the exact mechanisms remained to be fully characterized. In this respect, it has been reported that in the early phase of cerebral ischemia, lack of ATP and failure of Na^+/K^+ ATPase increase intracellular sodium and activate the reverse mode of NCX. This efficient action of NCX prevents sodium overload and neuronal death. Conversely, in the later phase of ischemia, intracellular calcium accumulation happens and forward mode of NCX can preserve neurons against neurotoxicity (Amoroso et al., 1997). It should also be stated that NCX3 unlike other isoforms in the absence of ATP can maintain intracellular calcium homeostasis which highlights its high potency in neuronal protection during cerebral ischemia (Secondo et al., 2007). Similarly, activation of NCX3 interrupts the extension of ischemic injury in those cerebral territories supplied by the MCA as our results exhibited. Accordingly, NCX3 with its exclusive capabilities emerges as a novel target for the therapeutic application in cerebral ischemia (Molinaro et al., 2008).

In spite of a large amount of data describing the significant neuroprotective property of CBD in neurodegenerative diseases, mechanism of action of cannabidiol in cerebral ischemia has yet remained elusive. To understand this, the present study investigated the effects of pre-treatment of cannabidiol on the major processes of ischemic injury specially excitotoxicity. Overall, based on these findings, CBD-mediated neuroprotection in experimental cerebral ischemia is at least partly associated with the modulation of calcium pathway especially plasma membrane NCX pathway. One of the aspects of this study is the use of CBD as a preventive neuroprotection in individuals who are at high risk of cerebral ischemia. There are still many unanswered questions about the protective mechanisms of suggested intervention. Thus, more research is required to develop these observations.

Conflict of interest

The authors declare that they have no conflict of interest.

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