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Capsaicin protects against ischaemia and reperfusion injury in the rat brain by decreasing oxidative stress and neuroinflammation

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Abstract

Capsaicin has been shown to exert neuroprotective effects. In this study, the effect of capsaicin in global cerebral ischaemia and reperfusion induced in the rat by transient occlusion of common carotid arteries was examined. Capsaicin was intraperitoneally (i.p.) given at doses of 1 or 2 mg/kg, 30 min prior to induction of brain ischaemia. The control group was treated with the vehicle. The lipid peroxidation biomarker malondialdehyde, nitric oxide and reduced glutathione brain levels were determined. Additionally, brain paraoxonase-1 activity, and the concentrations of glial fibrillary acidic protein, and interleukin-10 were measured and histopathological assessment of brain tissue damage was done. Results indicated that capsaicin significantly inhibited the ischaemia/reperfusion-induced increase in lipid peroxidation, nitric oxide and prevented the depletion of reduced glutathione. We further found that paraoxonase-1 activity and interleukin-10 concentration decreased significantly after ischaemia/reperfusion (I/R), which was prevented by treatment with capsaicin. Moreover, the increase in the level of glial fibrillary acidic protein induced by I/R was attenuated by capsaicin administration. The histopathological damage caused by ischaemia in cerebral cortex, hippocampus and substantia nigra was markedly improved by the higher dose of capsaicin. This suggests that capsaicin protects against the global ischaemia-induced neuronal damage by decreasing the level of oxidative stress and neuroinflammation.

Keywords: capsaicin; neuroprotection; brain; neurodegeneration; ischaemia-reperfusion; oxidative stress.

1. Introduction

Acute brain ischemia is the most common cause of stroke, and a leading cause of disability. The decrease in blood flow to brain tissues through a particularly narrowed blood vessel results in death of brain cells which depend on continuous supply of oxygen, and glucose for their survival [1]. Currently the treatment of acute ischemic stroke is dependent on restoration of blood flow with the use of thrombolytic agents, angioplasty, and operative revascularization [2]. During ischaemia, neuronal cell death occurs as a result of fall in energy production in the form of adenosine 5'-triphosphate (ATP), causing mitochondrial dysfunction and activation of the apoptotic pathway. The brain tissue with its high content of polyunsaturated fatty acids and low levels of antioxidants is particularly susceptible to free radical attack and oxidative stress may also contribute to neuronal damage during brain ischaemia [3,4]. Reactive oxygen species (ROS) can be produced in the ischaemic tissue by xanthine oxidase, induction of nitric oxide synthases (NOS) or cyclooxygenase, infiltrated neutrophils, and glutamate stimulation of *N*-methyl-D-aspartate receptors [1,5,6]. In the event of successful revascularization/reperfusion, there is marked increase in ROS generated as by-products of the reactions of arachidonic acid to yield prostaglandins and leukotrienes, owing to the increase in the availability of their substrate, free arachidonic acid [7]. ROS are also produced from such sources as dysfunctional mitochondria, xanthine oxidase

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converting hypoxanthine and O_2 into highly reactive superoxide and the reaction of nitric oxide and superoxide that generates peroxynitrite (ONOO–), a strong oxidant [2]. Other factors that make the brain tissue prone to increased ROS production are the presence of autoxidizable neurotransmitters and the redox active transition metal ions Fe^{2+} and Cu^{+} [8]. This increase in reactive oxygen and nitrogen species coupled with the already depleted cellular antioxidants during the ischaemic stage results in oxidative damage to membrane lipids, enzyme inactivation, DNA oxidation, ultimately leading to structural and functional neuronal damage [3,8].

Capsaicin (8-methyl-N-vanillyl-6-nonenamide) is the major pungent ingredient in a variety of hot green and red peppers of the plant genus Capsicum (family *Solanaceae*). Being the most preferred spice worldwide, pungent peppers constitute an important aspect of human food recipes as a food colorant, to add flavor to their cuisines, flavor fish soups, add pungency to meat dishes, fiery sauce etc. [9,10]. Capsicum contains up to 1.2% capsaicin [11]. Capsaicin stimulates and in large doses desensitizes a subset of primary afferent neurons with unmyelinated (C fibers) and thinly myelinated axons (Aδ fibers)[12,13]. Capsaicin sensitive neurons and their sensory nerve endings express/bear the capsaicin/vanilloid receptor or transient receptor potential cation channel vanilloid subfamily member 1 (TRPV1) [14]. TRPV1 is a nonselective cation channel, highly permeable to calcium that was cloned in 1997 from rat dorsal root ganglia [15]. TRPV1 functions as a polymodal signal detector that responds to various noxious chemical and physical stimuli including capsaicin and its potent analogue resiniferatoxin, noxious heat (> 43ºC), protons, bradykinins, and endogenous lipid ligands eg., anandamide, and *N*-arachidonoyl-dopamine [16]. Stimulation of TRPV1 on capsaicin-sensitive nociceptive nerve endings evokes the release of tachykinins and calcitonin-gene-related peptide (CGRP) that elicit tissue vascular responses [12,13]. In addition to peripheral tissues, TRPV1 was also detected in spinal cord and several regions of the brain eg., cerebral cortex, substantia nigra, striatum, dentate gyrus, thalamic nuclei, hypothalamus, and cerebellar cortex [17].

In present study, we investigated the ability of capsaicin, a selective TRPV1 agonist to modulate neuronal damage in the rat brain subjected to transient global cerebral ischaemia and reperfusion (I/R).

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 210-220 g were used in the study. Rats were obtained from the Animal House Colony of the National Research Centre, kept under temperature- and light-controlled conditions and given free access to standard laboratory rodent chow and tap water. The experimental procedures followed the Guide for Care and Use of Laboratory Animals by the U.S. National Institutes of Health (Publication No. 85-23, revised 1996) and the recommendations of the institutional Ethics Committee of the National Research Centre.

2.2. Chemicals and reagents

Capsaicin was purchased from Sigma-Aldrich (St Louis, MO, USA). Stock solutions of capsaicin (10 mg/ml) contained 10% ethanol, 10% Tween 80, and 80% saline solution. Capsaicin was further diluted in saline in order to obtain the necessary doses. The used chemicals and reagents in this study were of analytical grade and purchased from Sigma-Aldrich.

2.3. *Surgical procedure*

Global cerebral ischemia was induced by occlusion of both carotid arteries in rats anesthetized with thiopental sodium (20 mg/kg; i.p.). The common carotid arteries (CCAs) were exposed at the neck and ligated for 30 min using microvascular clips. Perfusion was then restored for two hours. The wound was then closed using silk sutures. Body temperature was maintained at 36.5 ± 0.5°C from the beginning of the procedure with the use of heating pad and respiration pattern monitored [18].

2.4. *Experimental groups*

Rats were randomly assigned into the following treatment groups (6 rats /group):
Group 1: Non-operated rats treated with the vehicle.
Group 2: Sham-treated: common carotid arteries exposed in the neck but not ligated and the wound was then closed using silk sutures.

Group 3: Rats received the vehicle and were subjected to cerebral ischemia- reperfusion injury (I/R).
Group 4: Rats treated with capsaicin at 1 mg/kg, i.p., 30 min before the induction of cerebral ischaemia.
Group 5: Rats treated with capsaicin at 2 mg/kg, i.p., 30 min before the induction of cerebral ischaemia.

At the end of the study, rats were euthanized by decapitation, their brains rapidly removed on ice-cold glass plate, and tissues stored at -80 °C until the time of biochemical assays. Representative brain samples were kept in 10% formol saline for the histopathological study.

2.5. Biochemical analyses

2.5.1. Lipid peroxidation assay

Malondialdehyde (MDA), a product of lipid peroxidation was determined in tissue homogenates according to the method of Nair and Turne [19]. In this assay thiobarbituric acid reactive substances (TBARS) react with thiobarbituric acid to form TBA-MDA adduct which can be measured colorimetrically at 532 nm.

2.5.2. Nitric oxide assay

The level of nitric oxide, measured as nitrite, was determined using the Griess reagent. Nitrite, a stable end-product of nitric oxide, is used as an indicator of the production of nitric oxide. In this assay, nitrate is converted to nitrite by nitrate reductase. The Griess reagent then reacts with nitrite forming a deep purple azo compound. The absorbance is read at 540 nm using a spectrophotometer [20].

2.5.3. *Reduced glutathione assay*

Briefly, DTNB (5,5'-dithiobis (2-nitrobenzoic acid) or Ellman´s reagent is reduced by the free sulfhydryl group on GSH molecule to generate 5-thio-2-nitrobenzoic acid which has yellow color and can be determined by reading absorbance at 412 nm [21].

2.5.4. *Paraoxonase-1assay*

The arylesterase activity of PON-1 was determined by a colorimetric method using phenyl acetate as a substrate. In this assay, PON-1 catalyzes the cleavage of phenyl acetate resulting in phenol formation. The rate of formation of phenol was measured by monitoring the increase in absorbance at 270 nm at 25°C. The working mix consisted of 20 mMTris/HCl buffer, pH 8.0, containing 1 mM CaCl2 and 4 mM phenyl acetate as the substrate. Samples diluted 1:3 in buffer were added to the above mix and the changes in absorbance were recorded following a 20 s lag time. One unit of arylesterase activity is equal to 1 μmole of phenol formed per minute. The PON1 activity is expressed in kU/L, based on the extinction coefficient of phenol of 1310 M–1cm–1. Blank samples containing water were used to correct for the spontaneous hydrolysis of phenyl acetate [22].

2.5.5. *Quantification of brain interleukin-10*

Interleukin-10 (IL-10) was quantified with an enzyme-linked immunosorbent assay (ELISA) purchased from SinoGeneClon Biotech Co., Ltd., China.

2.5.6. *Quantification of glial fibrillary acidic protein*

Glial fibrillary acidic protein levels were quantified using an ELISA kit from Glory Science, Del Rio, TX, USA.

2.6. Brain histopathology

Brain samples were fixed in 10% buffered formalin, dehydrated in graded ethanol, and embedded in paraffin using standard procedures. Sections of 5 μm thickness were stained with hematoxylin and eosin (H&E) for histopathological examination using light microscope (Olympus Cx 41 with DP12 Olympous digital camera; Olympous optical Co. Ltd,Tokyo. Japan).

2.7. Statistical analyses

Results are expressed as mean ± SE. Data were statistically analyzed using one way analysis of variance (ANOVA) followed Tukey's multiple comparisons test for multiple group comparison. GraphPad Prism 6 for Windows (GraphPad Prism Software Inc., San Diego, CA, USA) was used. Statistical significance was considered at a probability value of less than 0.05 [23].

3. Results

3.1. Effect of capsaicin on ischaemia/reperfusion-induced biochemical changes

3.1.1. Lipid peroxidation

The MDA level in the I/R group was significantly higher by 41.2 % than in the sham-operated control rats (25.99 ± 0.53 vs. 18.41 ± 0.34 nmol/g. tissue). Rats treated with capsaicin prior to CCA occlusion exhibited significant decrease in brain MDA levels by 13.1% and 34.9% compared with the I/R control group (22.58 0.75 & 14.69 ± 0.46 vs. 25.99 ± 0.53 nmol/g. tissue) (Figure 1A).

3.1.2. Nitric oxide

In rats subjected to I/R, nitric oxide was increased by 65.7% in the I/R group compared with the sham control (36.75 ± 0.84 vs. 22.18 ± 0.61 µmol/g. tissue). After capsaicin treatment at 1 or 2 mg/kg, nitric oxide decreased by 14% and 29.3% (31.60 ± 0.56 & 25.98 ± 0.96 vs. 36.75 ± 0.84 µmol/g. tissue) (Figure 1B).

3.1.3. Reduced glutathione

I/R caused a significant decrease in brain GSH content by 23.8% compared with the sham control group (2.37 ± 0.08 vs. 3.11 ± 0.02 µmol/g. tissue). By contrast, there was increase in the level of reduced glutathione by 11.8% and 43.5% (2.65 ± 0.03 & 3.40 ± 0.1 vs. 2.37 ± 0.08 µmol/g. tissue) by capsaicin treatment at 1 and 2 mg/kg, respectively (Figure 2A).

3.1.4. Paraoxonase-1

I/R was associated with significantly lower brain PON-1 activity by 32% (5.96 ± 0.37 vs. 8.77 ± 0.33) compared with the sham control group. Compared with the I/R control group, capsaicin treatment at 1 and 2 mg/kg was associated with a significant increase in PON-1 activity by 29.9% and 28.9%, respectively (7.74 ± 0.18 & 7.63 ± 0.21 vs. 5.96 ± 0.37 kU/l) (Figure 2B).

3.1.5. Interleukin-10

I/R decreased IL-10 level by 24.5% to reach 23.02 ± 0.69 pg/ml compared with sham control value of 30.47 ± 0.65 pg/ml. IL-10 levels increased by 29.8% by treatment with capsaicin 2 mg/kg in comparison to the I/R control value (29.87 0.39 vs. 23.02 ± 0.67 pg/ml) (Figure 3).

3.1.6. Glial fibrillary acidic protein

In I/R control group, the level of GFAP was significantly higher by 180.1% compared with the sham control value (228.2 ± 5.62 vs. 81.46 ± 2.39 ng/ml). The increase in the level of GFAP induced by I/R was attenuated by 34.0% and 67.3% by capsaicin administration at 1 and 2 mg/kg, respectively (150.5 ± 7.31 & 74.72 ± 1.26 vs. 228.2 ± 5.62 ng/ml) (Figure 3).

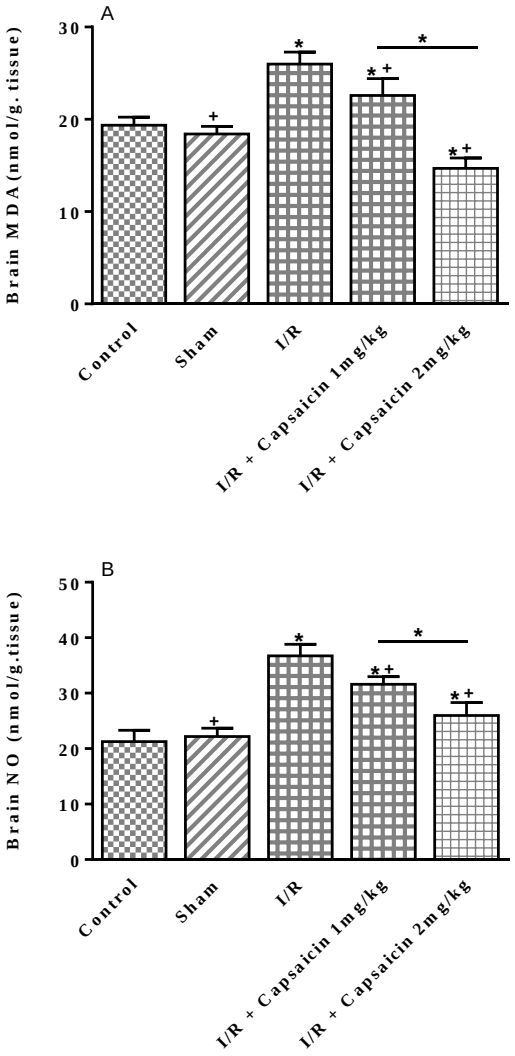

Figure 1. The effect of capsaicin on malondialdehyde (MDA), nitric oxide (NO) concentrations in the brain of rats subjected to ischaemia/reperfusion (I/R) injury. *:p<0.05 vs. sham control and between different groups as indicated in the graph. +: p<0.05 vs. I/R control. One way analysis of variance (ANOVA) & Tukey's multiple comparisons test.

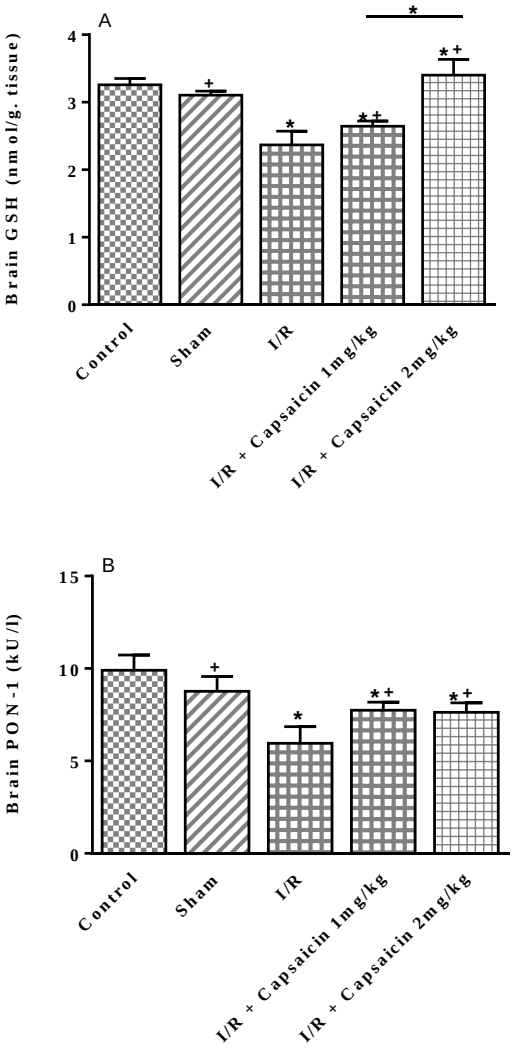

Figure 2. The effect of capsaicin on reduced glutathione (GSH) and paraoxonase-1 (PON-1) activity in the brain of rats subjected to ischaemia/reperfusion (I/R) injury. *:p<0.05 vs. sham control and between different groups as indicated in the graph. +: p<0.05 vs. I/R control. One way analysis of variance (ANOVA) & Tukey's multiple comparisons test.

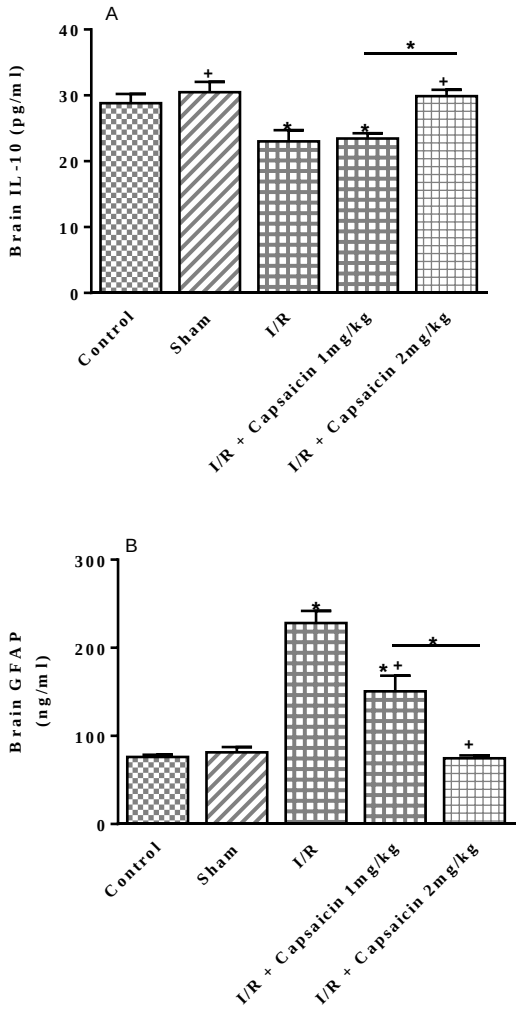

Figure 3. The effect of capsaicin on brain interleukin-10 (IL-10) and glial fibrillary acidic protein (GFAP) in rats with ischaemia/reperfusion (I/R) injury. *:p<0.05 vs. sham control and between different groups as indicated in the graph. +: p<0.05 vs. I/R control.

3.2. Effect of capsaicin on ischaemia/reperfusion-induced brain histologic damage

The histopathological changes induced by I/R and the effect of capsaicin treatment are shown in Figs. 4, 5 & 6. Following I/R, neurodegenerative changes were those of shrunken neurons with dark small nuclei in the cerebral cortex, flattened neurons with deeply colored nuclei and decreased thickness of the granular cell layer in hippocampus. Moreover, in the substantia nigra, the pigmented neurons were reduced in size and showed degranulation and karyolysis. By

contrast, these histopathological changes were substantially prevented by capsaicin at 2 mg/kg.

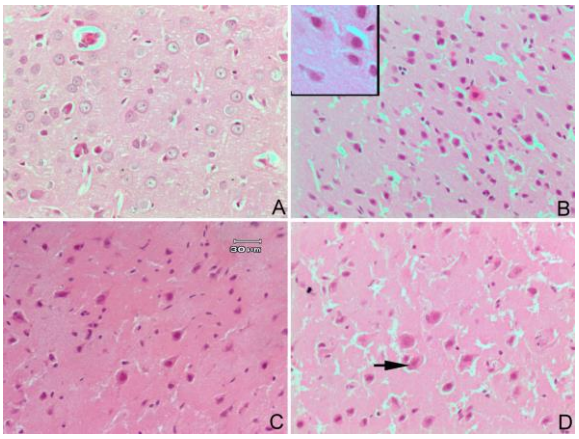

Figure 4. Representative photomicrographs of Hx & E stained sections of cerebral cortex tissue from: (A) Vehicle control: shows the normal appearance of normal neurons. (B) Brain ischaemia: shows a large number of neurons with dark small nuclei (arrow) if compared with normal neurons. (C) Brain ischaemia + 1 mg/kg capsaicin: shows many neurons with small dark nuclei are still appearing. (D) Brain ischaemia + 2 mg/kg capsaicin: shows that most neurons regained their normal size, although their nuclei show karyorrhexis (arrow).

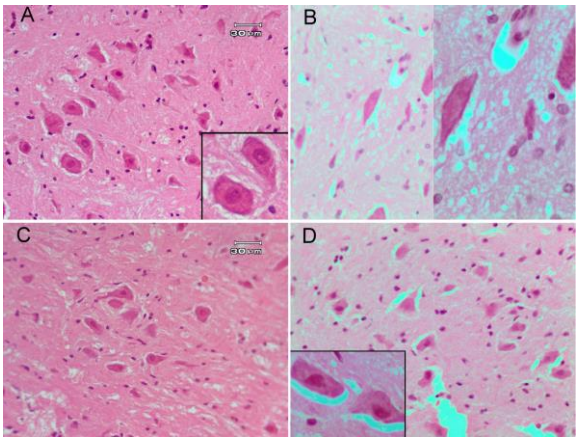

Figure 6. Representative photomicrographs of Hx & E stained sections of substantia nigra from: (A) Vehicle control rat: shows the normal shape of the pigmented neurons in this area with granules in their cytoplasm. (B) Brain ischaemia: shows reduced size, degranulation and karyolysis of the pigmented neurons. (C) Brain ischaemia + 1 mg/kg capsaicin: shows slight increase of neurons size & number. (D) Brain ischaemia + 2 mg/kg capsaicin: shows increase size & number of pigmented neurons that regained granulation.

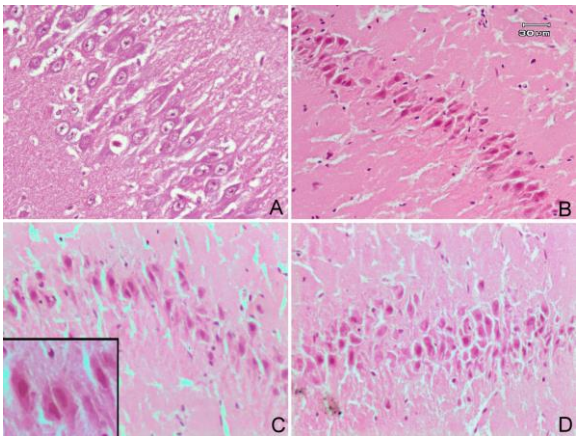

Figure 5. Representative photomicrographs of Hx & E stained sections of hippocampus tissue from: (A) Vehicle control rat: shows the normal structure of this area. (B) Brain ischaemia: shows reduced granular cell layer thickness. Most of the neurons are flattened with deep colored nuclei. (C) Brain ischaemia + 1 mg/kg capsaicin: shows reduced thickness of granular layer and neurons with small dark nuclei. (D) Brain ischaemia + 2 mg/kg capsaicin: shows mild amelioration in the form of increased thickness of granular layer, although many cells are still with deep colored nuclei.

4. Discussion

Cerebral I/R caused by stroke or cardiac arrest induces the release of ROS and pro-inflammatory cytokines, and causes widespread functional and structural neuronal injury in different brain regions. Free radical-mediated neuronal injury occurs during ischaemia but is aggravated by the marked increase in ROS generated in the reperfusion stage. Reactive oxygen species result in oxidative damage to cellular macromolecules, microvascular occlusion and trigger the expression of pro-inflammatory genes [6].The release of pro-inflammatory cytokines by derived phagocytic cells, T lymphocytes, natural killer cells and neutrophils contributes to brain inflammation [5]. Consistent with these studies, our results indicated that, transient global cerebral ischemia and reperfusion caused oxidative and inflammatory responses and neuronal degeneration in the rat brain. We also showed that the TRPV-1 agonist capsaicin given prior to induction of I/R inhibited oxidative stress evidenced by the alleviation in the increase in lipid peroxidation and nitric oxide and restoration of reduced glutathione brain content. Capsaicin also

prevented the inhibition of paraoxonae-1, IL-10 and the increase in the astrocytic marker GFAP parallel with substantial reduction in I/R–induced neurodegeneration in various regions of the brain.

The observed neuroprotective potential of systemically administered capsaicin is supported by a number of previous studies in which capsaicin given i.p. at 1 or 2 mg/kg, was shown to decrease the number of degenerated neurons in hippocampus, cerebral cortex and substantia nigra in experimentally-induced status epilepticus in the rat [24]. Subcutaneously injected capsaicin (0.01–0.6 mg/kg) also was reported to increase the number of survived neurons in the CA1 hippocampal region in global cerebral ischaemia in Mongolian gerbils [25]. Other researchers reported decreased brain infarct volume and functional recovery after intracerebral injection of capsaicin 1 or 3 nmol (0.3-1µg) into the peri-infarct area in rats with focal cerebral ischaemia [26]. Moreover, capsaicin (0.5 mg/kg, i.p.) rescued tyrosine-hydroxylase positive cells in substantia nigra of rats with experimentally-induced Parkinson's disease [27].

Although these studies show that capsaicin can reduce neuronal loss in ischaemic or toxic brain pathology, the exact mechanism that underlies such beneficial effect of capsaicin is not entirely clear. Capsaicin is lipophilic and readily crosses the blood brain barrier. Capsaicin was detected at ng/g concentrations in brain after intravenous injection of 2 mg/kg [28]. Such concentrations exert a powerful excitatory effect on peripheral sensory nerve endings [29,30] and can excite TRPV1 in brain [14] which in turn modulate of neural activity, release of synaptic neurotransmitters and/or neuropeptides eg., substance P, somatostatin and CGRP [31,32]. The latter is a potent vasodilatory substance in many vascular beds [33] and was shown to exert neuroprotective action in brain I/R injury, reducing brain oedema and size of infracted brain tissue [34]. Capsaicin can also stimulate abdominal vagal afferent fibers expressing TRPV1 receptors which are involved in immune-to-brain signaling [35,36]. Stimulation of the vagus nerve was shown to inhibit neuroinflammation by reducing brain levels of pro-inflammatory cytokines, and activated microglia and macrophages during sepsis [37,38]. Capsaicin might exert neuroprotective action by inducing hypothermia. Capsaicin given s.c. from 1 mg kg causes a fall of body temperature in mice with kainic acid-induced seizures parallel with a neuroprotective effect [39]. Stimulation of TRPV1 on capsaicin-sensitive nociceptive nerve endings evokes the release of their peptide content and among them somatostatin which reaches into the circulation and elicits systemic anti-inflammatory effects, the so called "systemic "sensocrine" function of neuronally derived somatostatin [14,40].

The ability of systemically administered capsaicin to decrease brain levels of oxidative stress and neuroinflammation has been reported by several studies. In vitro, capsaicin (0.1–0.5 µg/ml) decreased UV-induced lipid peroxidation in the liposomal membrane [41], reduced oxidative injury (MDA and protein carbonyl) in human erythrocytes exposed to tert-butyl hydroperoxide [42]. In vivo, systemic administration of small doses of capsaicin (1.5 mg/kg, i.p.) resulted in increased brain reduced glutathione in lipopolysaccharide (LPS)-treated rats [43]. Capsaicin (1 or 2 mg/kg, i.p.) also alleviated increased lipid peroxidation, nitric oxide and increased reduced glutathione in brain of rats with status epilepticus [24] and its subcutaneous administration at 1 mg/kg, prevented the increase in brain TBARS and the decreased total antioxidant capacity in blood of mice with kainic acid-induced seizures [39]. Other studies reported decreased MDA in liver, lung, kidney, muscle and cardiac tissues as well as, plasma nitric oxide during sepsis following s.c. or i.p. injection of capsaicin at 1 or 3 mg/kg, respectively [44,45]. Capsaicin given via systemic routes at the small doses indicated above showed anti-inflammatory action being capable of inhibiting the increase in increase in the levels of pro-inflammatory cytokines IL-1β and TNF-α in brain tissue of kainic acid-treated mice [39] or concentration of IL-6 and TNF-α in plasma of septic rats [44]. In the present study, we further demonstrated significant decrease in anti-inflammatory cytokine IL-10 after ischaemia/reperfusion which was restored by capsaicin administration.

Paraoxonase-1 (PON1) is an esterase/lactonase which has the capacity to hydrolyze some organophosphorous insecticides, nerve agent, lipid hydroperoxides and many other xenobiotics. It is mainly synthesized in the liver and circulates in plasma bound to high-density lipoprotein particles [46].A decrease in enzyme activity has been found in

liver disease [47] and central nervous system disorders [48]. It is suggested that the enzyme has an important role in modulation of oxidative stress and inflammation. Mice deficient in this enzyme showed downregulation of antioxidant proteins eg., superoxide dismutase, DJ-1 and Park-7 [49]. Paraoxonase-1 inhibited the release of pro-inflammatory cytokines such as TNF-α and IL-6 as well as ROS from macrophages [50]. Meanwhile, PON-1 deficiency caused an increase in oxidative stress in serum and macrophages [51]. A previous study demonstrated that cerebral I/R injury results in decrease in enzyme activity in brain tissue [52]. Other studies reported decreased activity of the enzyme toxic and inflammatory pathologies affecting the brain [53,54]. In the present study, the decline in PON-1 activity after I/R injury was prevented by treatment with capsaicin. This could be due to a lower level of brain oxidative stress and increased availability of reduced glutathione after treatment with capsaicin, thereby, preventing the oxidation of the free sulfhydryl group of the enzyme.

Our results also indicated that, I/R caused marked increase in brain concentration of GFAP. The latter is a monomeric intermediate filament protein found exclusively in astrocytes being a major part of their cytoskeleton [55]. Astrocytes constitute the major cell type in the brain, providing structural and functional support to neurons. Glial fibrillary acidic protein is considered a marker for astrogliosis, a condition of astroglia cell activation, whereby astrocytes undergoes morphological changes and increase in size, number and GFAP expression in response to brain tissue injury [56]. The release of this protein from astroglia into the circulation correlates with the volume of brain lesions in patients with acute ischaemic stroke [57,58]. The present study reports the first time that the administration of capsaicin was able to inhibit the I/R-induced activation of glia cells and the increase in brain GFAP level.

5. Conclusions

In summary, the present study indicates that the systemic administration of a small dose of the TPRV1 agonist capsaicin exerts neuroprotective effects in a global transient I/R rat model. This effect involves inhibition of oxidative stress and neuroinflammation which is possibly mediated through the inactivation of astroglia.

Conflicts of interest

There are no conflicts to declare.

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