Abstract

Background: Hydroxy safflor yellow A (HSYA) has been translated clinically for cardiovascular diseases. HSYA is also greatly acknowledged for its protective effects against cerebral ischemic-reperfusion (I/R) injury. Although the precise mechanism of cerebral I/R injury is not fully understood, oxygen-derived free radicals and mitochondrial permeability transition pore (mPTP) opening during I/R injury are widely recognized as an important contributor to neuronal injury. Thus, we speculated that the neuroprotective effects of HSYA against cerebral I/R injury may be associated with mPTP modulation.

Methods: Induction of I/R injury was achieved by 60 min of middle cerebral artery occlusion, followed by reperfusion for 24 h. For behavior and cognitive assessment, neurological scoring (NSS), rotarod, and Y-maze task were performed. Oxidative damage was measured in terms of markers such as malondialdehyde, reduced glutathione, and catalase levels and cerebral infarct volumes were quantified using 2,3,5-triphenyl tetrazolinium chloride staining. I/R injury-induced inflammation was determined using tumor necrosis factor-α (TNF-α) levels.

Results: Animals exposed to I/R injury showed neurological severity, functional and cognitive disability, elevated oxidative markers, and TNF-α levels along with large infarct volumes. HSYA treatment during onset of reperfusion ameliorated performance in NSS, rotarod and Y-maze and attenuated oxidative damage, TNF-α levels, and infarction rate. However, treatment with carboxyatractyloside, an mPTP opener, 20 min before HSYA, attenuated the protective effect of HSYA.

Conclusions: Our study confirmed that protective effect of HSYA may be conferred through its free radical scavenger action followed by inhibiting the opening of mPTP during reperfusion and HSYA might act as a promising therapeutic agent against cerebral I/R injury.

Keywords: carboxyatractyloside; cerebral ischemic-reperfusion injury; hydroxy safflor yellow A (HSYA); mitochondrial permeability transition pore (mPTP).

Introduction

Restoration of blood flow with effective oxygenation to brain, which is temporarily deprived of vascular supply, often paradoxically results in cerebral ischemia-reperfusion (I/R) injury. Recent studies on several pharmacological agents aimed at various metabolic pathways, apoptotic inhibition, immune system modulation, and promotion of angiogenesis could not successfully translate to clinical application. Moreover, endogenous restoration of blood flow during reperfusion injury leads to production of reactive oxygen species (ROS) which contributes in neuronal I/R injury [1]. Therefore, the interest dwelt in developing neuroprotective strategies to target ROS. Compelling evidence also supports the primary role of ROS in opening of mitochondrial permeability transition pore (mPTP) during I/R injury. In fact, post-ischemic reperfusion conditions, such as overproduction/accumulation of ROS, elevated phosphate concentration, rise in intracellular Ca$^{2+}$, and pH normalization, ideally pave the way to mPTP opening. mPTP is a non-specific pore in the inner mitochondrial membrane penetrating both membranes. Mitochondrial Ca$^{2+}$ triggers the opening of mPTP in the first few minutes of reperfusion phase after I/R injury, which results in mitochondrial swelling, collapse of mitochondrial membrane potential, uncoupling of mitochondrial oxidative phosphorylation, and cytochrome c release, leading to both necrosis and apoptosis depending on duration of opening [2, 3]. It was demonstrated that protection in isolated perfused hearts may be achieved by preventing mPTP opening [4]. Moreover, inhibition of mPTP opening induced both
cardio- and neuroprotective effects in several in vitro and in vivo models [5, 6]. In addition, suppression of mPTP opening induces neuroprotection in vivo against Ca\(^{2+}\) and H\(_2\)O\(_2\) mitochondrial swelling [6]. However, it is still questionable whether modulation of mPTP opening in experimental animal models limits the cerebral I/R injury.

The flowers of safflower plant Carthamus tinctorius L. have been most promising in traditional Chinese medicine for the treatment of various ischemic cardiovascular and cerebrovascular disorders [7]. The extract of C. tinctorius contains several pigments, including hydroxy safflor yellow A (HSYA), safflor yellow B, safflorin A, safflorin C, and other pigments. HSYA, the main chemical component of the safflower yellow pigments, has been demonstrated to prevent dopaminergic neurodegeneration mediated through oxidative stress in rats by decreasing ROS production [8]. In addition, HSYA is also able to reduce myocardial injury in isolated perfused heart of rat through its antioxidative action by regulating nitric oxide and nitric oxide synthase activity [9]. Moreover, HSYA has been shown to reduce brain injury caused by experimental cerebral stroke animal model by attenuating the elevation of malondialdehyde (MDA) and decreasing reduced glutathione (GSH) [10]. Further, recent in vitro data on isolated mitochondria from rat brain suggest that ATP levels and respiratory control ratio were enhanced, besides improving mitochondrial energy metabolism [11]. However, whether HSYA provides cerebroprotection against I/R injury affecting mitochondrial pathway remains poorly understood.

Thus, based on above reports, the present study has been designed to investigate neuroprotective role of HSYA during reperfusion through a mitochondrial pathway, specifically targeting the inhibition of mPTP opening during I/R injury.

Materials and methods

Animals and drugs

Adult male Wistar rats (220–250 g) were procured from the animal house of Birla Institute of Technology and Science (BITS), Pilani, India. The animals were maintained in standard laboratory conditions (temperature 22 °C±2 °C and room humidity 60%±10%) with a 12:12-h light/dark cycle. The animals were fed standard diet and filtered water ad libitum. All the behavioral experiments were carried in between 09:00 and 17:00 h (IST). The experimental procedures on animals were in compliance with the Institutional Animal Ethics Committee of BITS Pilani, India (Protocol No. IAEC/RES/12/07).

HSYA (ChemFaces, Wuhan, Hubei, China; 98% pure, PubChem: 6443665), carboxyatractylloside (CAT; Sigma, MO, USA), nylon filament (Ethicon, Johnson and Johnson), other biochemical reagents and solvents (Central Drug House, New Delhi, India) used in the current study were of analytical grade. Tumor necrosis factor-α (TNF-α) ELISA kit was purchased from Sigma-Aldrich, USA. In the present study, doses of HSYA (8 mg/kg, i.v.) and CAT (1 mg/kg, i.p.) were selected based upon pilot study conducted in our laboratory (data not shown) and from available literature [12, 13]. Solution of drugs and chemicals were always prepared afresh before use.

Surgical protocol

Transient focal cerebral ischemia was induced in male Wistar rats by middle cerebral artery occlusion (MCAO) model. The rats were anesthetized using ketamine (80 mg/kg) and xylazine (10 mg/kg). After retraction of soft tissues over the trachea, right MCAO was achieved by using the intraluminal filament insertion technique (popularly known as suture technique). Briefly, a 3-0 mm poly-L-lysine-coated nylon monofilament was inserted into the internal carotid artery, via the external carotid artery. Once the filament was secured, the incision was sutured back, sterilized with 70% ethanol, and rat was allowed to come out of anesthesia in its home cage. After 60 min of occlusion, rat was briefly reanesthetized to initiate withdrawal of the filament, followed by 24 h of reperfusion. Throughout the surgery, body temperature was monitored at 37 °C using laser Doppler perfusion (LDF) and temperature monitor (mooor VMF-LDF2, UK). The rats were maintained in a well-ventilated room at 25 °C±3 °C until they gained full consciousness and were housed together in a group of two animals per cage. Food and water was kept inside the cages for the 1st week for easy access to animals [14, 15].

Experimental design

Rats were randomized into different groups, each group comprising eight animals.

Group 1: Rats in sham control were subjected to surgical procedure to expose the cerebral artery without any occlusion.

Group 2: Rats in I/R group were subjected to MCAO. Cerebral blood flow (CBF) was monitored by LDF using a flexible probe over the skull. CBF was measured before ischemia, during global ischemia, and during reperfusion. Animals who showed a CBF reduction of at least 70% and animals who were associated with bleeding and died after ischemia induction were excluded from the experimental group.

Group 3: After induction of focal ischemia, rats received HSYA (8 mg/kg i.v.) during onset of reperfusion.

Group 4: After induction of focal ischemia, rats were treated with CAT (1 mg/kg, i.p.), 20 min before HSYA administration.

Behavioral and cognitive parameters

Assessment of neurological scoring: Neurological findings were scored on a 5-point scale. No neurological deficit=0; failure to extend right paw fully=1; circling to parietic side=2; falling to parietic side=3; no spontaneous walking and had depressed levels of consciousness=4 [16, 17].

Assessment of motor coordination: Motor coordination and balance alterations in rodents were assessed by testing the latency to
fall from accelerated rotarod. The rotarod apparatus consisted of horizontal metal rod (3 cm diameter, 70 cm long) which rotates at an adjustable speed. The rod was adjusted at height of 50 cm to discourage jumping of animals from rotating rod. The speed of the rod increases (2 rpm to 15 rpm) with time (300 s), and the amount of time the animal remains on the device was recorded. The rats were given three sessions of habituation trial on stationary rod for 5 min and test was repeated post stroke, and latency to fall off from the rod was determined [18].

**Assessment of spatial working memory:** In Y-maze, three arms (60 cm long, 8 cm wide, 20 cm high) of maze were 120° angle from center, named as A, B and C. The animal was placed at start arm and allowed to move freely in all the arms. An entry into arm was recorded manually when four limbs are within the arm. After testing each rat, the floor was cleaned with 70% ethanol to avoid olfactory clues. Consecutive entry into all the three arms – A, B, and C – is considered to be spontaneous alteration. Over the course of multiple arm entries, rats should show a tendency to enter a less recently visited arm. The spontaneous alteration was calculated as the total number of arm entries minus two, and the percentage of alteration was calculated as (actual alteration/maximum alteration × 100). To estimate spontaneous activity, total number of arm entries was recorded. Data were expressed as percentage of alterations behavior and total number of arm entries [19, 20].

**Biochemical analysis**

**Brain homogenate preparation:** Animals were sacrificed by decapitation, and brains were removed and rinsed with ice-cold sterile saline (0.9%). The whole brain samples were homogenized with 10 times (w/v) of ice-cold 0.1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 10,000 g (4 °C) for 15 min to remove cellular debris, and aliquots of supernatant were separated and used for biochemical estimations [21].

**Estimation of oxidative markers and anti-oxidant enzymes:** Oxidative stress marker, MDA, was quantitatively estimated as described by Wills [21]. Endogenous antioxidant such as GSH and catalase were estimated by Ellman [22] and Sinha [23], respectively. For detailed procedure, see SI materials and methods.

**Protein estimation:** The protein content in whole brain samples for antioxidant and oxidant activity was estimated by biuret method using bovine serum albumin (BSA) (1 mg/mL) as standard [24].

**Estimation of TNF-α**

Rat brain homogenate was used to estimate the level of pro-inflammatory cytokine (TNF-α) using ELISA kit as per manufacturer’s instructions.

**Estimation of infarct volume**

Brains with intact morphology were stained with 2% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC). In brief, brains were sliced into 2 mm thick coronal sections in a brain matrix and stained with 2% (w/v) 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, USA) for 30 min at 37 °C followed by immersion in 4% (w/v) paraformaldehyde in phosphate buffer overnight for color fixation. The non-infarct region turns red, whereas infarct region remained unstained (white). The infarct area was demarcated and analyzed using image J software. The corrected infarct volume was calculated to compensate the effect of brain edema as follows: [contralateral hemisphere area – (ipsilateral hemisphere – measured infarct area)/ contralateral hemisphere area] × 100%. Animals who underwent subarachnoid hemorrhage were excluded from the study [25].

**Statistical analysis**

All data were expressed as mean±SEM. The data obtained from various groups were statistically analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Tukey’s test in SigmaStat software 3.5 (San Jose, CA, USA). A p-value < 0.05 was considered as statistically significant.

**Results**

**Effect of I/R injury and HSYA on neurological scoring**

The effect of I/R injury and HSYA on neurological scoring is shown in Figure 1. The induction of I/R injury resulted in significant (p < 0.001) increase in the neurological deficits when compared to sham control rats. HSYA administration had significantly (p < 0.001) greater functional recovery, whereas CAT treatment significantly reversed (p<0.001) the protective neurological outcome offered by HSYA.

**Figure 1:** Effect of HSYA on neurological scoring. Values are expressed as mean±SEM; n=8. *p<0.001 vs. sham control group, @p<0.001 vs. I/R group, #p<0.001 vs. HSYA. I/R, ischemia-reperfusion group; HSYA, hydroxy safflor yellow A; CAT, carboxyatractyloside.
Effect of I/R injury and HSYA on motor coordination

In this experiment, we examined the impact of HSYA on treatment on skilled motor abilities using rotarod test. After induction of I/R injury, rats spent significantly (p<0.001) less time on rotating rod when compared to sham control (Figure 2). Treatment with HSYA significantly (p<0.001) increased latency time on rotarod, conversely administration of CAT (mPTP opener) attenuated the time spent on rotarod significantly (p<0.001) as compared to HSYA-treated rats.

Effect of I/R injury and HSYA on spatial working memory

When compared to sham control, I/R injured rats showed significantly (p<0.001) reduced alteration behavior. Moreover, HSYA group significantly (p<0.001) displayed high alteration behavior (Figure 3A) as compared to I/R group, whereas CAT treatment significantly (p<0.01) resulted in low alterations. However, there was no significant difference among the groups in number of entries indicating the similar extent of spontaneous activity (Figure 3B).

Effect of I/R injury and HSYA on MDA, GSH, and catalase levels

I/R injury significantly increased (p<0.001) lipid peroxidation along with reduction of antioxidant enzyme levels, that is, GSH and catalase as compared to sham control. Treatment with HSYA during onset of reperfusion attenuated the rise in MDA (p<0.001, Figure 4A) and prevented the fall of antioxidant enzymes when compared to I/R-treated rats. However, administration of CAT, abolished the antioxidative effects of HSYA as evident by increased MDA level and decreased GSH (Figure 4B) and catalase (Figure 4C) levels.

Effect of I/R injury and HSYA on TNF-α levels

Compared to sham control, TNF-α levels were significantly high in I/R injury group. Pharmacological intervention of HSYA resulted in significant (p<0.001) attenuation of TNF-α levels as compared to I/R group (Figure 5). In contrast, administration of CAT, significantly (p<0.001) raised the levels of inflammatory marker (TNF-α) compared to HSYA-treated rats.

Effect of I/R injury and HSYA on infarct volume

An increased infarction volume was significantly (p<0.001) visible after induction of I/R injury as compared to sham control. HSYA treatment significantly (p<0.001)
reduced the volume of infarction when compared to I/R-injured rats, whereas CAT treatment increased the infarction rate significantly (p<0.01) than HSYA-treated rats (Figure 6).

**Discussion**

According to recent research, HSYA has shown promising neuroprotective effect against brain I/R injury as a newly identified chemical [26, 27]. Moreover, it has been demonstrated that HSYA showed cardioprotective and cerebroprotective actions through its antioxidant property [7, 8]. However, the molecular mechanism underlying this protection was not fully elucidated. In the present study, HSYA-treated rats showed less neurological deficits, reduced oxidative damage, and less infarction, conversely CAT, an mPTP opener, abolished the protective effects of HSYA. Thus, we demonstrated for the 1st time, a connecting link between neuroprotective effects of HSYA and mitochondrial pathways.

MCAO is a well-documented experimental stroke model to induce I/R injury and to study its pathophysiology, as it produces persistent neurological and cognitive
deficits [28]. Moreover, recent reports suggest that MCAO mimics the human stroke and produces selective neuronal damage [29]. Therefore, in the present study, cerebral I/R injury was induced by MCAO model.

Cerebral I/R injury in the present study resulted in severe behavioral and cognitive abnormalities, in accordance with previous studies [30, 31]. Several studies also supported the protective role of HSYA to stabilize the behavioral abnormalities induced by I/R injury [7, 8].

Neurological scoring is a valuable indicator of I/R injury in rodents. NSS was rated for equilibrium, righting reflex, motility, and circling behavior [16]. In our study, data showed loss of sensorimotor abilities in I/R-injured rats as evidenced by high neurological scoring. Moreover, HSYA treatment significantly improved the neurological outcome, whereas CAT administration abolished the protective effects of HSYA (Figure 1). Similar pattern of behavioral deficits in motor function were observed in I/R injured rats as evidenced by rotarod task. HSYA treatment markedly suppressed I/R-induced motor abnormalities, by contrast CAT abolished the HSYA protection (Figure 2).

According to previous reports, induction of I/R injury in the present study also resulted in hippocampal damage leading to cognitive dysfunction [32]. Moreover, Y-maze is well established to explore the spatial working memory in rodents. According to recent reports, HSYA improved the cognitive parameters in Aβ_{1-42}-induced mice with Alzheimer disease (AD) [33]. In the present study, HSYA showed neuroprotective effect based on improved short-term memory, which might be because of its powerful antioxidant property. However, CAT administration abolished the protective effect of HSYA by loss of spatial working memory (Figure 3A and B).

Accumulating evidence suggests that oxidative stress appears to be a critical factor in the pathogenesis of cerebral I/R injury. It is a well-known fact that cerebral ischemia significantly increases ROS concentration and causes damage to various cellular components including lipids, proteins, and DNA. Moreover, oxidative stress due to I/R injury also results in opening of mPTP through a cascade of pathway involving mitochondrial calcium, pH neutralization, and inhibition of oxidative phosphorylation [13, 34, 35]. In the present study, the concentration of oxidative stress markers, such as MDA, which are proportional to oxidative stress and lipid peroxidation were high, whereas antioxidants, such as GSH and catalase, were low in brain homogenates of I/R injury rats. In accordance with previous studies, HSYA prevented the ascent of MDA (Figure 4A) and diminution of GSH (Figure 4B) and catalase (Figure 4C) level indicating antioxidative effect. However, a significant rise of oxidative stress was observed in CAT+HSYA-treated rat brains, indicating the loss of protective role of HSYA. It seems that I/R injury resulted due to severe oxidative stress that prime the opening of mPTP during reperfusion which was attenuated by HSYA treatment. This indicates that the neuroprotection offered by HSYA may be due to inhibition of mPTP opening. Our results were in agreement with earlier reports where HSYA attenuated the oxidative stress and protected the spinal cord against I/R injury in rabbits [36].

A growing body of evidences suggests inflammatory reaction as a major pathophysiological mechanism associated with I/R injury. Among the factors, TNF-α is considered to be strong immunological factor in the pathogenesis of cerebral I/R injury. It has been reported that few hours after MCAO, TNF-α triggers the macrophage, microglia, and astrocyte proliferation leading to demyelination and reactive gliosis [37, 38]. Moreover, increased TNF-α levels after I/R injury reflected the neurological severity and functional abnormality in stroke patients [39]. In the present study, a significant elevation of TNF-α level was observed in I/R-treated rats. Neuroinflammation was significantly controlled by HSYA treatment indicated by reduced cytokine levels, according to previous reports [36]. Attenuation of inflammatory marker, TNF-α levels, was prevented in CAT+HSYA group which might be due to opening of mPTP (Figure 5).

The results were further supported by histochemical estimation of infarction volume using TTC stain. In our study, the brain damage was significantly higher after induction of I/R injury as indicated by large infarct volume, whereas HSYA treatment resulted in significant reduction in infarct volume. This report was in agreement with previous reports indicating that HSYA reduced the infarction rate after cerebral I/R injury in vivo [40]. Compared to HSYA-treated rats, administration of CAT significantly attenuated the protective effect of HSYA by increasing the infarct volume, indicating the neuroprotective role of HSYA possibly through inhibition of mPTP opening (Figure 6).

Taken together the above-mentioned data, the symptomatic improvement in behavior and cognitive deficits are related to HSYA neuroprotective action. Noteworthy, HSYA prevents I/R injury during reperfusion phase through inhibition of mPTP opening as evidenced by biochemical and oxidative stress parameters. Thus, the present study strongly supported HSYA as a possible therapeutic strategy against reperfusion injury and its development as a promising candidate for cerebral I/R injury.

Acknowledgments: The authors are thankful to DST, New Delhi, India and BITS, Pilani, Rajasthan, India for their financial support for this study.
Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: None declared.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References


