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Solasodine protects rat brain against ischemia/reperfusion injury through its antioxidant activity

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ABSTRACT

Ischemic stroke is the second leading cause of death worldwide. The major limitation of stroke management is the lack of clinically effective therapy. Antioxidants have been demonstrated as potent neuroprotective agents by enhancing the defense mechanism(s), whereas reducing the oxidative stress in the ischemic stroke models. In the present study, we evaluated neuroprotective potential of solasodine, an antioxidant glycoalkaloid of Solanum species, against global model of ischemia in rats. Ischemia/reperfusion (I/R)-injury produced marked elevation in lipid peroxidation (LPO) and nitric oxide (NO), whereas superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) levels were decreased in experimental animals. Prior administration of solasodine (100 and 200 mg/kg, p.o.) significantly heightened SOD, CAT, GSH and total thiols, whereas reduced LPO and NO levels in the brain. Interestingly, brain coronal sectioning and histopathology studies revealed a marked reversal of I/R-provoked neuronal damage in the solasodine treatment groups. Taken together, our study, for the first time, demonstrates neuroprotective potential of solasodine against global ischemia-induced cerebral injury in experimental rats. We propose that the neuroprotection offered by solasodine could be attributed, at least in part, to its anti-oxidant property.

1. Introduction

Cerebrovascular diseases (CVD) are a group of brain dysfunctions which includes disorders such as ischemic stroke, hemorrhagic stroke and cerebrovascular anomalies. It has been estimated that approximately 6 million annual deaths are due to stroke alone, making it the second leading cause of mortality worldwide (Anonymous, 2004). Ischemic stroke may result as a consequence of events such as cardiac arrest, coronary artery bypass surgery and/or carotid artery occlusion that lead to reduction in blood supply and failure of energy metabolism in the brain (Wang et al., 2002).

The mechanism of ischemia/reperfusion (I/R) injury-induced neurodegeneration is complex. It is well-established that I/R generates reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2), which leads to oxidative damage of membrane lipids, proteins and nucleic acids. Moreover, increased ROS formation has been shown to inactivate protective mechanisms such as superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH), whereas augment lipid peroxidation (LPO) and inducible nitric oxide synthase activity (Candelario-Jalil et al., 2001; Choi et al., 2009). This has lead to evaluate beneficial role of antioxidants, the free radical scavengers, on neuroprotection in stroke models. The major limitation of stroke management is the lack of clinically

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effective therapy except recombinant t-plasminogen activator (rt-PA). Therefore, developing safe and effective compounds, preferably from the plant origin, could be beneficial in stroke therapy (Yu et al., 2008).

Solasodine, a glycoalkaloid, is a chief constituent of solanum species (Nakamura et al., 1996). It has been shown that solasodine possesses CNS activities such as antipyretic (Kulcsar-Gergely, 1976), anticonvulsant (Chauhan et al., 2011) and memory enhancing effects in Alzheimer's disease (Desai et al., 2011). Furthermore, Lecanu et al. (2011) explored the *in vitro* and *in vivo* neurogenesis effects of solasodine and reported that solasodine possesses neurogenesis properties. Importantly, Emmanuel et al. (2006) demonstrated anti-inflammatory, whereas Koduru et al. (2007) showed potent antioxidant activities of solasodine. It has been documented that isolated plant constituents having antioxidant and anti-inflammatory properties showed potent neuroprotective actions (Margaill et al., 2005). For instance, antioxidants such as quercetin (Dajas et al., 2003), curcumin (Thiyagarajan and Sharma, 2004) and glabridine (Yu et al., 2008) demonstrated potent neuroprotective effects in different experimental models. In the light of above background, we studied neuroprotective potential of solasodine against global model of ischemia in experimental animals.

2. Materials and methods

2.1. Chemicals and reagents

Solasodine, trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), glutathione, 5-5-dithiobis (2-nitrobenzoic acid) (DTNB) and (±)-epinephrine were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. 2,3,5-triphenyltetrazolium chloride (TTC) was purchased from Hi-Media, Mumbai, India. All other chemicals were of high purity analytical grades.

2.2. Animals

Male Wistar albino rats (150–250 g; eight weeks old) were used for study. The animals were housed five rats per cage under well-controlled conditions of temperature (25 ± 1 °C), humidity (55 ± 5%) and light-dark cycle (lights on 07:00–19:00 h). Animals had free access to standard rat chaw (Amrut, Pranav Agro Ind. Ltd., Vadodara, India) and water. All the experimental protocols were approved (IAEC/DPS/SU/1210) by Institutional Animal Ethics Committee. The experimental procedures were performed as per the guidelines laid by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, Ministry of Environment and Forest, Government of India, New Delhi. Animals were allowed to acclimatize for one week prior to the experiments.

2.3. Determination of LD_{50}

The toxicity profile of solasodine was performed on Wistar albino rats (150–250 g; eight weeks old) and the median lethal dose (LD_{50}) was determined as per the method of Litchfield and Wilcoxon (1949). Briefly, solasodine suspended in tween 80 (1% v/v) in saline at a dose of 125, 250, 500, 1000, 1500 and 2000 mg/kg was orally administered to different groups ($n=5$). The animals were examined every 30 min up to a period of 3 h and then, occasionally, for further 4 h; and finally at 24 h.

2.4. Experimental design

Fig. 1 depicts schematic representation of experimental design and treatment timeline. Briefly, the animals were divided into six groups ($n=8$) and received different drug treatments or vehicle (Tween 80, 1% v/v) for 10 days prior to the experiment. The treatment groups were as follows: Sham control group (Group I), Ischemia group (Group II), Ischemia/reperfusion group (Group III: negative control), Quercetin group (Group IV: standard group; 25 mg/kg) and Solasodine groups (Group V and VI; 100 and 200 mg/kg, respectively). Solasodine and quercetin were suspended in tween 80 (1% v/v) and orally administered once a day for 10 days.

2.5. Induction of ischemia

Global cerebral ischemia was induced according to the method of Farbiszewski et al. (1995). Briefly, Groups II to VI were subjected to Bilateral Common Carotid Artery (BCCA) occlusion under general anesthesia induced by intraperitoneal (i.p.) administration of mixture of xylazine (10 mg/kg) and ketamine (45 mg/kg). Animals were placed on the back and ventral neck incision (2 cm) was made; the left and right common carotid arteries were separated carefully from vagus nerve and were occluded simultaneously for 30 min with atraumatic clamps. This was followed by reperfusion of blood for 60 min in Group III–VI, whereas Group II animals (Ischemia only group) were decapitated immediately after ischemia. The change of color from pale white to reddish confirmed the reperfusion of blood flow into BCCA. Surgical procedures were performed between 08:00 and 13:00 h under controlled conditions of temperature (37 ± 0.5 °C) and artificial ventilation (95% O_2 and 5% CO_2). Sham-operated non-ischemic animals underwent the same surgical procedures except BCCA occlusion.

2.6. Preparation of tissue homogenate

Immediately after reperfusion, animals were decapitated and brains were removed. Subsequently, the brains were washed in cooled saline (0.9%), kept on ice and blotted on fimmed paper to remove excess saline. Immediately, brains were weighed and

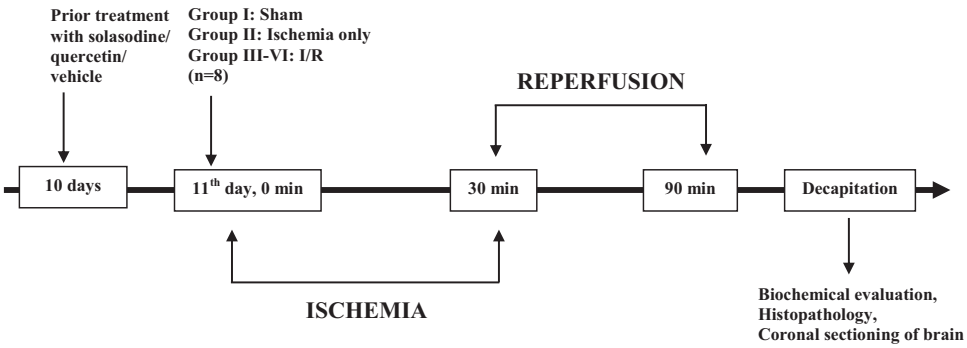

Fig. 1. Experimental protocol time line showing surgical procedure, drug treatment, biochemical assays, coronal sectioning and histological evaluation of rat brain.

homogenized as 10% (w/v) in cold phosphate buffer saline (0.05 M, pH 7.4) under standardized conditions. The homogenates were centrifuged at 10,000 × *g* for 10 min at 4 °C (MPW-350 R, Korea) and supernatant thus obtained was kept in deep freeze (−20 °C) until biochemical estimation (UV 1800, Shimadzu, Japan).

2.7. Biochemical estimation

2.7.1. Lipid peroxidation

The protocol described by Braughler et al. (1987) was followed to measure thiobarbituric acid reactive substances (TBARS) content as an index of alterations in LPO in the brain homogenates. Briefly, the brain homogenate (500 μl) was incubated with TCA (15%), TBA (0.375%) and HCl (5 N) at 95 °C in the water bath for 15 min. The mixture was cooled, centrifuged and absorbance of the supernatant was measured at 512 nm against appropriate blank. The amount of LPO was determined using the formula $e = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as TBARS nmol/mg of protein.

2.7.2. Superoxide dismutase

The method is based on the ability of SOD to inhibit the autooxidation of epinephrine to adrenochrome at alkaline pH (Misra and Fridovich, 1972). Briefly, the supernatant (25 μl) was added to a mixture of epinephrine (0.1 mM) in carbonate buffer (pH 10.2) in a total volume of 1 ml and the formation of adrenochrome was measured at 295 nm. The SOD activity was calculated using the standard plot and represented as U/mg of protein.

2.7.3. Catalase

The method described by Claiborne (1985) was followed to assess CAT activity. Briefly, the assay mixture comprised of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1 ml hydrogen peroxide (0.019 M) and 0.05 ml brain homogenate (10% w/v) in a total volume of 3 ml and the decrease in absorbance was recorded at 240 nm. CAT activity was expressed in terms of nM H_2O_2 consumed/min/mg of protein.

2.7.4. Total thiols

The assay of total thiols determination is based on the principle of formation of relatively stable yellow color by sulfhydryl groups with DTNB (Sedlak and Lindsay, 1968). Briefly, brain homogenate (0.2 ml) was mixed with phosphate buffer (0.8 ml; pH 8), DTNB (40 μl; 10 mM) and methanol (3.16 ml). This mixture was incubated for 10 min and the absorbance was measured at 412 nm against appropriate blanks. The total thiols content was derived by using formula $e = 13.6 \times 10^{31}\ cm^{-1}\ M^{-1}$.

2.7.5. Glutathione

GSH was determined as per the method of Sedlak and Lindsay (1968). Briefly, brain homogenate (10% w/v) was prepared in EDTA (20 mM; pH 4.7). The EDTA-brain homogenate (100 μl) or pure GSH was added to 0.2 M tris–EDTA buffer (1 ml, pH 8.2) and 20 mM EDTA (0.9 ml; pH 4.7), followed by addition of 20 μl of Ellman's reagent (10 mM DTNB in methanol). After 30 min of incubation, the test samples were centrifuged and the absorbance of the supernatants was measured at 412 nm.

2.7.6. Nitric oxide metabolites

The method described by Miranda et al. (2001) was followed. This assay determines nitrites (NO_2) based on the fact that a chromophore with an absorbance at 550 nm is formed by reaction of nitrite with the Griess reagent (the mixture of 0.1% naphthylethylenediamine and 1% sulfanilamide). Briefly, the mixture of brain homogenates (500 μl) and Griess reagent (500 μl) was incubated for 20 min and the absorbance was measured at 550 nm. $NaNO_2$ was used as a standard to calculate NO_2 concentrations, which was expressed as μM/mg of protein.

2.7.7. Total protein

The total protein contents of brain homogenates (10% w/v) were determined as per the method of Lowry et al. (1951).

2.8. Coronal sectioning of rat brain

Immediately after ischemia and/or reperfusion, the animals were decapitated and brains were removed. Subsequently, four coronal brain sections (2 mm thick) were made using steel brain matrix (Alto, Stoelting Co., Illinois, USA) and incubated in phosphate-buffered saline (pH 7.4) containing 2% TTC (staining reagent) at 37 °C for 10 min on the dorsal and ventral side of a section and then kept in neutral-buffered formalin overnight. The images of the TTC-stained sections were obtained using a high resolution scanner (Scanjet 6100C/T, Hewlett-Packard, Palo Alto, USA).

2.9. Histopathology

Immediately after ischemia and/or reperfusion, animals were killed by decapitation and brains were dissected out. The brains were fixed with 10% formalin overnight, embedded in paraffin wax, and cut into sections (5 μm thick). The brain sections were stained with Hematoxylin–Eosin (H&E) using standard methods. The normal morphology and the presence and nature of ischemic damage were verified by a neuropathologist who was unaware of the experimental design or results of the TTC assay.

2.10. Statistical analysis

All the data are presented as mean ± S.E.M. The significance of difference in means between control and treated animals for different parameters was determined by One-way Analysis of Variance (ANOVA) followed by multiple comparisons Dunnett's test using GraphPad Prism (Version 5.0). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Determination of LD_{50}

The LD_{50} of solasodine was found as 2000 mg/kg, *per os* (p.o.) in rats. Two doses corresponding to 1/20th and 1/10th of LD_{50} value, i.e. 100 and 200 mg/kg, p.o., respectively were selected for the present study.

3.2. Effect of solasodine on lipid peroxidation

Table 1 depicts effect of different treatments on LPO in brain homogenate. The ischemia and I/R (negative control) groups showed a significant ($P < 0.001$) elevation in the TBARS levels, a marker of LPO, as compared to sham-operated animals. Interestingly, prior treatment of solasodine (100 and 200 mg/kg) significantly reduced LPO levels ($P < 0.001$) when compared to negative control group.

3.3. Effect of solasodine on antioxidant enzymes

The effect of solasodine on the antioxidant enzyme levels was studied by estimation of SOD and CAT levels in brain homogenate. Animals subjected to ischemia and I/R showed a significant

reduction in SOD ($P<0.001$) and CAT ($P<0.01$) levels as compared to sham group. As compared to I/R group, solasodine (100 and 200 mg/kg, p.o.) treatment significantly increased these enzyme levels in a dose-dependent manner ($P<0.01$ and $P<0.001$, respectively) (Table 2). The effect could be attributed to the antioxidant potential of solasodine.

3.4. Effect of solasodine on non-enzymatic antioxidants

Table 2 shows effect of different treatments on GSH and total thiols levels, the two major non-enzymatic antioxidants, in brain homogenates. A marked decrease in GSH ($P<0.001$) and total thiols ($P<0.001$) levels was observed in ischemia and I/R groups as compared to sham animals. These levels were significantly augmented to normal by solasodine (100 and 200 mg/kg, p.o.) treatment in dose-dependent manner.

3.5. Effect of solasodine on nitric oxide metabolites

As evident from Table 2, a marked increase in nitric oxide (NO) contents in brain homogenates of ischemia ($P<0.01$) and I/R groups ($P<0.001$) was observed as compared to sham group. Solasodine (100 and 200 mg/kg, p.o.) administration ameliorated NO levels in a dose-dependent manner ($P<0.01$ and $P<0.001$, respectively) when compared to I/R group.

Table 1
Effect of solasodine on oxidative stress markers (LPO and NO) in rat brain.

Groups	LPO (nmol/mg of protein)	NO (μmol/mg of protein)
Sham	16.58 ± 1.820	0.06 ± 0.006
Ischemia	53.49 ± 2.620^{c}	0.11 ± 0.004^{b}
Ischemia/Reperfusion	85.05 ± 6.630^{c}	0.17 ± 0.011^{c}
Quercetine (25 mg/kg)+I/R	17.30 ± 1.650^{z}	0.08 ± 0.009^{z}
Solasodine (100 mg/kg)+I/R	25.59 ± 1.130^{z}	0.12 ± 0.012^{y}
Solasodine (200 mg/kg)+I/R	12.14 ± 1.060^{z}	0.09 ± 0.012^{z}

Values are presented as mean ± S.E.M., $n=8$, One-way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test, $^{b}P<0.01$ and $^{c}P<0.001$ vs. sham-operated animals and $^{y}P<0.01$ and $^{z}P<0.001$ vs. I/R group. LPO—Lipid peroxidation, NO—Nitric oxide, I/R-Ischemia/Reperfusion.

Table 2
Effect of solasodine on endogenous antioxidant levels (SOD, CAT, GSH, and Total thiols) in brain homogenates.

Groups	SOD (U/mg of protein)	CAT (U/mg of protein)	GSH (μmol/mg of protein)	Total thiols (μmol/mg of protein)
Sham	99.88 ± 3.874	0.03 ± 0.003	4.29 ± 0.490	12.55 ± 1.050
Ischemia	53.01 ± 2.372^{c}	0.02 ± 0.002^{a}	2.69 ± 0.100^{b}	7.67 ± 0.800^{b}
Ischemia/Reperfusion	49.32 ± 2.257^{c}	0.01 ± 0.002^{b}	2.37 ± 0.100^{c}	5.81 ± 0.350^{c}
Quercetine (25 mg/kg)+I/R	108.60 ± 9.771^{z}	0.04 ± 0.004^{z}	4.13 ± 0.340^{z}	12.22 ± 1.410^{z}
Solasodine (100 mg/kg)+I/R	78.30 ± 2.826^{y}	0.03 ± 0.004^{y}	3.49 ± 0.280^{y}	11.50 ± 1.290^{y}
Solasodine (200 mg/kg)+I/R	91.99 ± 4.524^{z}	0.04 ± 0.004^{z}	5.25 ± 0.080^{z}	13.09 ± 0.640^{z}

Values are presented as mean ± S.E.M., $n=8$, One-way Analysis of Variance (ANOVA) followed by multiple comparison Dunnett's test, $^{a}P<0.05$, $^{b}P<0.01$ and $^{c}P<0.001$ vs. sham group and $^{y}P<0.01$ and $^{z}P<0.001$ vs. I/R group. SOD—Superoxide dismutase, CAT—Catalse, GSH—Glutathione, I/R—Ischemia/Reperfusion.

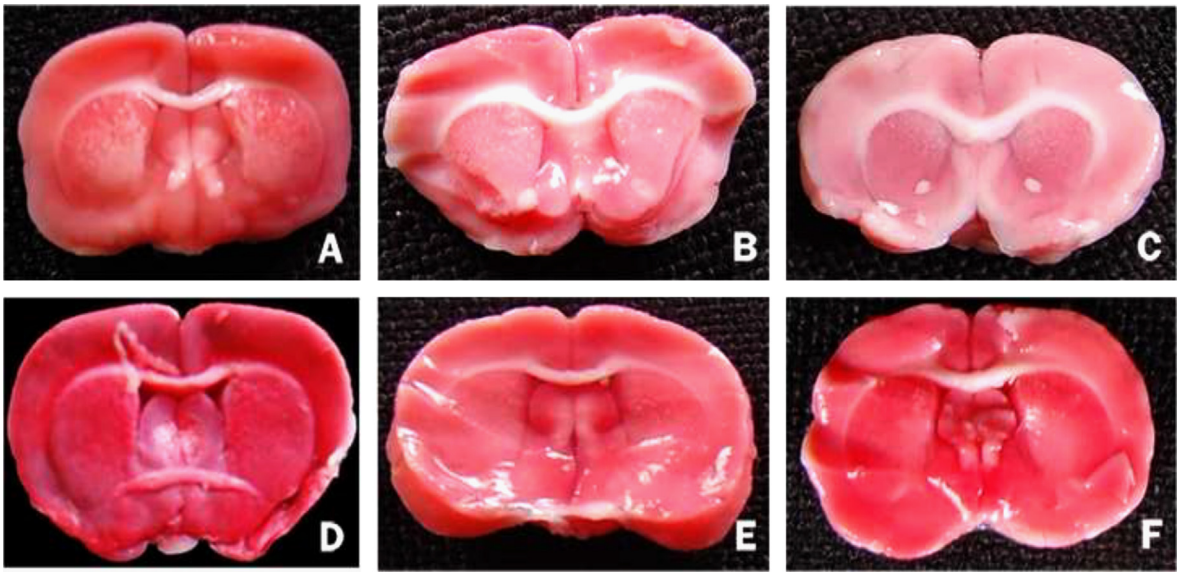

Fig. 2. Representative coronal sections stained with 2,3,5-triphenyltetrazolium chloride (TTC) from each studied group. A: Normal; B: Ischemia group; C: I/R group; D: Quercetin 25 mg/kg+I/R; E and F: 100 mg/kg+I/R and 200 mg/kg+I/R of solasodine, respectively. Ischemia-reperfusion group showed marked cerebral infarction, mainly in the caudal and rostral side of hippocampus (C), whereas infarction was markedly reduced in the rat brains treated with 100 mg/kg (E) and 200 mg/kg (F) of solasodine and also in quercetin-treated animals (D).

3.6. Coronal sectioning of rat brain

As evident from Fig. 2, sham group did not exhibit ischemic tissue (Fig. 2A), whereas a progressive increase in the cerebral infarction (white colored tissue) was observed in ischemia and I/R groups (Fig. 2B and C). Further, quercetin (Fig. 2D) and solasodine-treated rats showed a predominant red colored tissues indicating neuroprotection (Fig. 2E and F).

3.7. Histopathological studies

Fig. 3 shows effect of different treatments on histopathology of brain. Fig. 3A demonstrates different hippocampal regions of rat brain. As compared to the sham group (Fig. 3B), ischemia (Fig. 3C) and I/R (Fig. 3D) groups showed progressively damaged, shrunken and clustered pyramidal neurons in the hippocampal CA1 region.

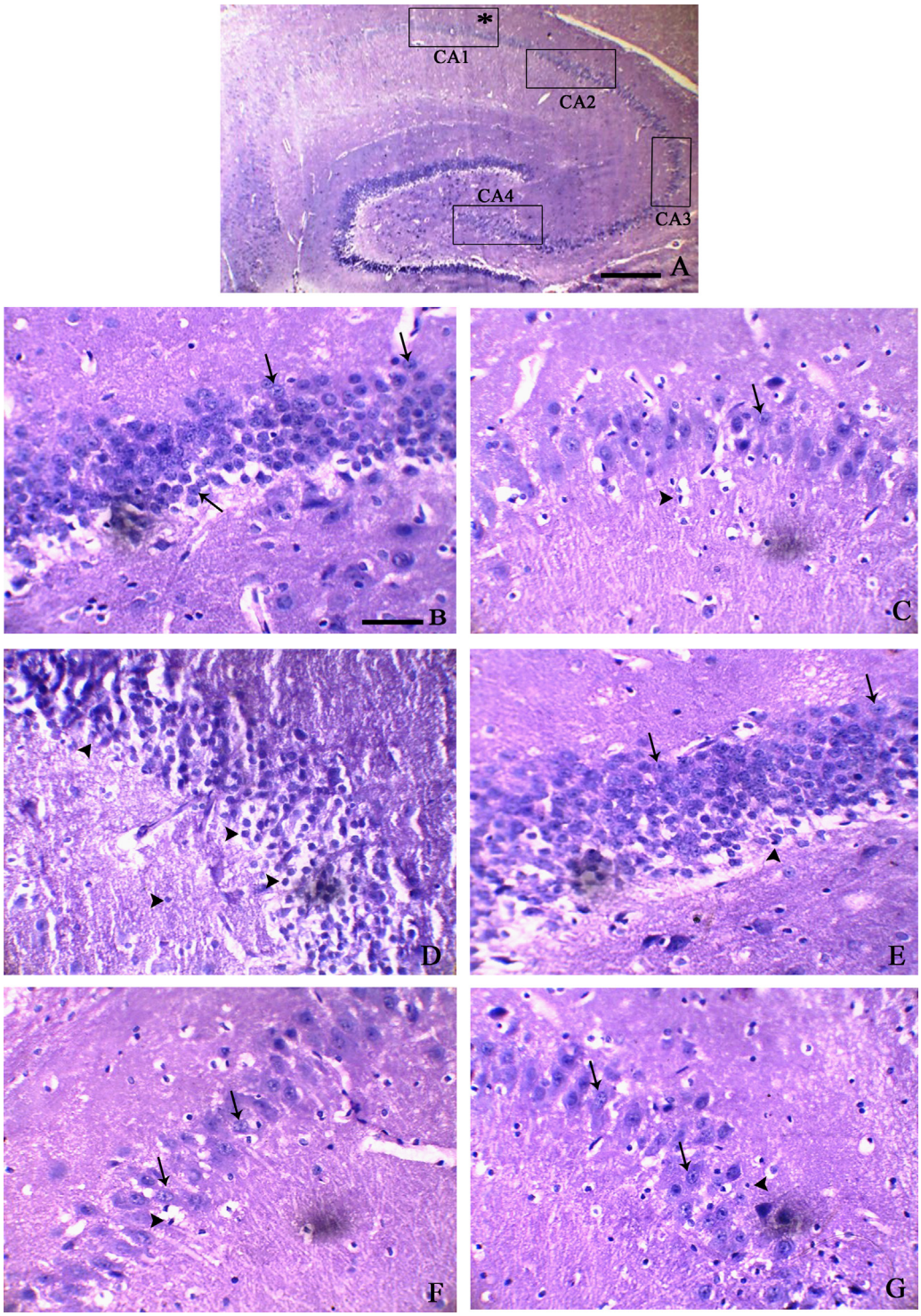

Fig. 3. Representative photomicrographs of Hematoxylin and Eosin (H&E) stained hippocampus region in rat brain from each studied group. A: Hippocampal region (4 ×); B: sham group; C: ischemia group; D: I/R; E: quercetin 25 mg/kg+I/R; F and G: 100 mg/kg+I/R and 200 mg/kg+I/R of solasodine, respectively. Sham group (B) showed normal cells with round bodies and clear nuclei and nucleoli. Ischemia (C) and ischemia/reperfusion (D) groups showed neuronal damage in the hippocampal region as indicated by pyramidal cells without round bodies and prominent nucleoli. Solasodine (F and G) and quercetin (E) pre-treatments significantly reduced neuronal damage in the hippocampal region; The asterisk indicates the middle CA1 regions for higher magnification. Arrow head indicates damaged neurons and arrow represents intact pyramidal cells in CA1 hippocampal region. Magnification: 40 ×. Bar=500 μm (A) and 50 μm (B,C,D,E,F and G).

This neuronal damage was markedly reduced by quercetin and solasodine treatments (Fig. 3E and F,G, respectively).

4. Discussion

Lack of safe and effective therapy for stroke has raised the attention to test possible beneficial effects of antioxidants, especially from herbal origin. Natural product always seems to be a viable approach to develop safe and effective treatment for different ailments (Yu et al., 2008). Solasodine, a glycoalkaloid, possess diverse pharmacological activities such as antioxidant, antipyretic and CNS action such as anticonvulsant effects. However, neuroprotective potential of solasodine is largely unexplored. The present study evaluated cerebroprotective effect of solasodine (100 and 200 mg/kg, p.o.) on global model of ischemia in experimental animals. The selected doses were devoid of any toxic signs or behavioral abnormalities in experimental animals.

Involvement of free radicals in the development of I/R-induced cerebral injury is well investigated. Excessive production of ROS during reperfusion produces detrimental effects on cellular macromolecules such as nucleic acids, proteins, and lipids in ischemic tissues (Reynolds et al., 2007). We may recall that $O_2^{\bullet -}$ and $OH^{\bullet}$ are potent inducers of LPO, a self-propagating chain-reaction which leads to oxidative degradation of lipids in cell membrane (Mylonas and Kouretas, 1999). Malondialdehyde (MDA), an end product of LPO that reacts with TBA, is estimated as TBARS from the brain homogenates. In this study, ischemia and reperfusion groups showed significantly increased TBARS levels in the rat brain. Interestingly, prior administration of solasodine significantly attenuated TBARS levels.

Antioxidant enzymes such as SOD, glutathione peroxidase (GPx), CAT and non-enzymatic antioxidants such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), GSH, and carotenoids are believed to play key role in the defense mechanisms against free-radical induced oxidative stress (Ahmad et al., 2006; Zhang et al., 2007). Amongst these, SOD plays an important role in detoxification of ROS by metabolizing $O_2^{\bullet -}$ to H_2O_2, which is subsequently neutralized to H_2O by CAT to end the cycle of superoxide (Michiels et al., 1994). GSH acts directly by detoxifying ROS and indirectly as a substrate for various peroxidases thus, protecting the cells against oxidative damage. It is well-documented that oxidative stress markedly reduces these endogenous antioxidants during I/R insult in experimental animals (Ahmad et al., 2006; Buch et al., 2012). In our study, ischemia and reperfusion significantly decreased, whereas solasodine administration increased GSH, SOD, CAT, and total thiols levels in rat brains. The results suggest neuroprotective potential of solasodine through modulation of these endogenous antioxidants.

Nitric oxide (NO), a potent ROS responsible for ischemic damage, produces toxic effects on neurons. The detrimental effects of NO is attributed to peroxynitrite ($ONOO^{\bullet -}$), which is a reaction product of NO with $O_2^{\bullet -}$ (Sekhon et al., 2003). In the present study, we found that ischemia and I/R augmented, whereas solasodine pre-treatment attenuated NO levels in the rat brain. Further, the effects of various treatments on I/R-induced cerebro-vasculature damage were assessed by histopathological analysis and coronal sectioning of brain. It is well-known that TTC imparts deep red color to live neurons, while areas of necrosis lack dehydrogenase activity therefore, does not receive stain and remain white and/or pale white in color (Fishbein et al., 1981). In our study, brain coronal sectioning revealed a marked cerebral damage (white colored tissue) in the ischemia and I/R groups, whereas prior treatment of solasodine reduced cerebral infarction (red colored tissue). It has been shown that ischemia and I/R provoke a robust inflammatory response in the rat brain that leads to neuronal damage in hippocampal CA1 region (Yang et al., 2002). In consistent to this, we observed progressively damaged, shrunken and clustered pyramidal neurons in the hippocampal CA1 region neutrophil infiltration and marked cerebral damage in ischemia and I/R groups. Interestingly, solasodine pre-treatment markedly reduced neuronal damage, indicating anti-inflammatory potential of solasodine which could be beneficial to the neuroprotection against I/R injury.

In conclusion, the present study, for the first time, shows neuroprotective potential of solasodine in experimental animals. Solasodine markedly reduced cerebral infarction and oxidative stress, and enhanced the defense mechanisms in the global model of ischemia in rats. We propose that antioxidant property, at least in part, could account for neuroprotective action of solasodine. Although prior treatment of solasodine ameliorated/normalised oxidative damage induced by I/R injury, an assessment of early-treatment efficacy and window of opportunity need to be studied in future to identify clinical relevance of solasodine in stoke treatment.

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