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Neuroprotective effect of allopurinol and nimesulide against cerebral ischemic reperfusion injury in diabetic rats

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Abstract. – OBJECTIVES: The main objective of the study was to determine the neuroprotective effect of allopurinol and nimesulide against the cerebral ischemic reperfusion injury in diabetic and nondiabetic rats.

MATERIALS AND METHODS: In this study, Wistar albino rats of either sex weighing 150-250 g were procured from authorized suppliers. Rats were anesthetized by giving thiopentone sodium (45 mg/kg) by i.p. Under anesthesia, midline incision was given. Common carotid arteries were identified and isolated carefully from vago-sympathetic nerve. Rats were made ischemic by occluding bicommon carotid arteries with thread for 30 min, followed by reperfusion for 4 h by removing the occlusion. The drugs allopurinol (15, 30 mg/kg) and nimesulide (20, 40 mg/kg) were administered 10 min before reperfusion. Then after 4 h reperfusion, animals were sacrificed and immediately brain was removed, homogenized, centrifuged and supernatant was collected, various enzyme estimations were done and same procedure was followed in streptozotocin (STZ: 45 mg/kg; i.p.) induced diabetic rats.

RESULTS: Ischemia reperfusion (I/R) group showed significant increase in malondialdehyde (MDA), myeloperoxidase (MPO) and depletion in catalase (CAT) and superoxide dismutase (SOD) levels. Treatment with allopurinol and nimesulide significantly decreased the MDA and MPO levels whereas increased the SOD and CAT levels when compared I/R group in both non-diabetic and diabetic rats.

CONCLUSIONS: These findings suggest the cerebral injury due to over production of free radicals was inhibited by allopurinol and nimesulide that exert a neuroprotective effect probably by radical scavenging and antioxidant activities.

Key Words:

Diabetes mellitus, Reperfusion injury, Cerebral ischemia, Oxidative stress, Allopurinol, Nimesulide.

Introduction

Stroke (cerebral ischemia) is the third most common cause of death and disability in industrialized countries after cardiovascular disease and cancer. Among the various risk factors that appear to play a major role in the severity of stroke are hyperglycemia, hyperlipidemia and hypertension. Hyperglycemia has been documented to be associated with aggravate cerebral damage with increased morbidity and mortality[1,2]. Increased oxidative stress has been implicated in both normo- and hyperglycemic stroke[3]. Furthermore, animal and human studies have linked hyperglycemia in the acute phase of ischemic stroke to worse clinical outcomes regardless of the presence of pre-existing diabetes[3].

Cell injury during periods of ischemia and reperfusion is caused not only by the loss of energy supply caused by deprivation of oxygen and glucose but also by oxidative stress[4]. Moreover, the exacerbation of ischemic injury by reoxygenation (the oxygen paradox) represents an important mechanism of cellular injury and a major clinical problem in treating episodes of cerebral ischemia[4].

Although multiple pathological processes are involved in this injury, experimental data from laboratory animals have provided strong evidence of the role of oxidative stress and inflammation in ischemic brain damage[5,6]. Pathologically, reperfusion-induced inflammation is characterized by deposition of complement, upregulation of adhesion molecules, inflammatory cell infiltration, and cytokine release. Reactive oxygen species (ROS) generation also is implicated in cerebral injury that can react and damage the cellular macromolecules by virtue of the reactivity that leads to cell injury and necrosis. Oxidants

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are also mediators in signaling involving mitochondria, DNA repair enzymes, and transcription factors that may lead to apoptosis after cerebral ischemia[7].

Hyperglycemia is known to intensify ischemic neuronal damage by vascular inflammation, increasing blood-brain barrier permeability, impairing cellular metabolism and promoting tissue acidosis. In fact, diabetes not only increases the stroke risks by two- to three-fold, but also increases mortality and poor recovery following stroke[8]. During ischemia reperfusion, generation of ROS, especially superoxide anions, hydroxyl radicals are increased in hyperglycemic state[9].

Various antioxidant strategies are currently being investigated for determination whether free radicals represent a valuable therapeutic target in cerebral ischemia. These strategies consist of inhibiting free radical production, scavenging free radicals or increasing their degradation. Xanthine oxidase (XO) and COX-II inhibitor prevent the formation of free radicals.

- Allopurinol, a XO inhibitor, reduces cerebral infarct size and edema in permanent ischemia[10].
- Nimesulide, COX-II inhibitor is also neuroprotective after transient and permanent ischemia by scavenging free radicals[11].

However, no investigative reports exist till date pertaining to neuroprotective activity of xanthine oxidase inhibitor and COX-II inhibitor on cerebral ischemia reperfusion injury in diabetic conditions. Hence, we decided to study the neuroprotective activity of allopurinol and nimesulide in cerebral ischemia reperfusion injury in both diabetic and nondiabetic rats.

Materials and Methods

Chemicals

The drugs, allopurinol and nimesulide were obtained as gift sample from Indoco Pharma Remedics, Ahmedabad, Gujarat – India and Micro Labs Ltd, Karnataka, Bangalore – India respectively. All other chemicals and reagents used are of analytical grade and procured from approved vendors.

Preparation of Drug Solutions

To solubilise allopurinol and nimesulide, dimethyl sulphoxide (DMSO) and polyvinylpyrrolidone (PVP) were used respectively and different concentrations of drugs, allopurinol (15 mg/kg, 30 mg/kg) and nimesulide (20 mg/kg, 40 mg/kg) were prepared.

Animals

After obtaining permission from IAEC, Wistar albino rats (150-250 gm) were obtained from Raghavendra Enterprises, Bangalore, Karnataka. All the experiments were performed in accordance with the guideline established by the above Committee. They were acclimatized for one week prior to experiment. Rats were caged in fully ventilated room. Animals were maintained in 12:12 h light and dark cycle and were housed at temperature of 25 ± 2°C. They had free access to a standard chow diet and water *ad libitum*.

Induction of Diabetes Mellitus

Wistar albino rats (150-250 gm) of either sex were induced by i.p injection of a single dose of streptozotocin (STZ: 45 mg/kg) after 18 h fasting. STZ was dissolved in cold citrate buffer (pH 4.5) immediately before use and solution were made fresh daily. The rats were allowed access to sucrose (5%) in drinking water for next 24 h to prevent hypoglycemia. STZ has been shown to induce a diabetic state[12] in 72 h. Rats with blood glucose level more than 250 mg/dl were considered to be diabetic and included in the study.

Induction of Cerebral Ischemia

Wistar albino rats of either sex weighing 150-250 g were procured from authorized suppliers. They were anesthetized by giving thiopentone sodium (45 mg/kg) i.p.

Occlusion: Surgical technique for the induction of cerebral ischemia was adopted from the earlier published method[13]. Under anesthesia, midline incision was given. Common carotid arteries were identified and isolated carefully from vago-sympathetic nerve. Ischemia was induced by occluding the bicommon carotid arteries (BCCA) with a thread for 30 min and the *reperfusion* was allowed for 4 h by removing the thread. Then, the drugs allopurinol and nimesulide were administered 10 min before the reperfusion.

Preparation of Post-Mitochondrial Supernatant (PMS)

Following decapitation, the brain was removed and washed in ice cold 0.9% saline, and was kept on ice and subsequently blotted on filter paper, then weighed and homogenized in cold phos-

phate buffer (0.1 M, pH 7.4) using a homogenizer. The homogenate was centrifuged at 10,000×g for 20 min at 4ºC and post-mitochondrial supernatant was kept on ice until assayed.

Superoxide Dismutase (SOD)

The SOD level was estimated by the method described by Kakkar et al[14] Aliquot of supernatant 0.1 ml was added to 1.2 ml of 0.052 M sodium pyrophosphate buffer (pH 8.3) followed by addition of 0.1 ml of 186 µM phenazonium methosulphate, 0.3 ml of 300 µM nitroblue tetrazolium, 0.2 ml of 780 µM NADH. Reaction mixture was incubated for 90 sec at 30°C, and the reaction was stopped by the addition of 0.1 ml of glacial acetic acid. Reaction mixture was stirred vigorously and shaken with 4.0 ml of *n*-butanol and centrifuged at 4000 rpm for 10 min. the absorbance of organic layer was measured at 560 nm using UV-Vis spectrophotometer, Shimadzu (Asia Pacific: PTE Ltd, Singapore Science Park, Singapore – 118227). A control was prepared using 0.1 ml of distilled water devoid of 0.1 ml of homogenate. One unit of the enzyme activity is defined, as enzyme concentration required inhibiting the absorbance of chromogen production by 50% in control sample under the assay conditions.

Catalase (CAT)

The CAT level was measured by the method of Aebi[15]. Supernatant 0.1 ml was added to cuvette containing 1.9 ml of 50 mM phosphate buffer. To this mixture, 1.0 ml of freshly prepared 30 mM H_2O_2 was added and changes in absorbance for 3 min at 240 nm at an interval of 30 sec was measured. A control was prepared using 0.1 ml of distilled water devoid of 0.1 ml of homogenate. One unit of the enzyme activity is defined as enzyme concentration required inhibiting the change in the absorbance by 50% in one min in the control sample.

Estimation of MDA Level

MDA level was measured by the method developed by Ohkawa et al[16]. 0.2 ml of brain homogenate was treated with 0.2 ml sodium dodecyl sulphate (8.1%), 20% of 1.5 ml of acetic acid (pH 3.5), 1.5 ml thio-barbituric acid (0.8%). The mixture was made up to 5 ml with distilled water and then heated at 95°C in oil bath for 60 min. The mixture was cooled and 5 ml of n-butanol and pyridine mixture (15:1 v/v) was added. The mixture was shaken vigorously. After centrifugation of the mixture at 4000 rpm for 10 min, the organic layer was taken and the absorbance was measured at 532 nm. The concentration of MDA formed is expressed as nmol/g wet tissue.

Estimation of MPO Level

MPO level was estimated by the method described by Mullane et al[17] 40 µl supernatant was added to 960 µl of phosphate buffer containing O-dianisidine dihydrochloride (0.167 mg/ml) and hydrogen peroxide (0.0005%) solution and shaken vigorously. The change in the absorbance was measured at 460 nm for 3 min at an interval of 60 sec. One unit of enzyme activity was defined as the amount of MPO that causes a change in absorbance measured at 460 nm for 3 min. 1 unit is the amount of enzyme that consumes 1 µmol H_2O_2/min. MPO activity data are presented as units/ml.

Estimation of Protein

Protein was estimated by the method of Lowry et al[18]. The levels of protein were expressed as milligram of protein per g tissue.

Statistical Analysis

The results were expressed as (Mean ± SEM). MDA, SOD, CAT and MPO were determined by factorial One-way ANOVA. Individual groups were compared using Dunnet's test. Differences with $p < 0.05$ were considered statistically significant. Statistical analysis was performed using Prism software (Version 5).

Results

Estimation of Biochemical Parameters

SOD and CAT activities decreased in the ischemic reperfused group compared to sham group in both non diabetic and diabetic group (Table I). Administration of allopurinol (15, 30 mg/kg) and nimesulide (20, 40 mg/kg) in non-diabetic and diabetic condition increased SOD level and CAT levels (Table I).

In the present study, increase in the tissue MDA activity was observed in ischemic reperfused group when compared with sham group in both non diabetic and diabetic group (Table I). Administration of allopurinol (15, 30 mg/kg) and nimesulide (20, 40 mg/kg) to cerebral ischemic reperfused group reduced MDA level significantly in both non-diabetic and diabetic condition (Table I).

Table I. Effect of allopurinol and nimesulide on biochemical parameters of oxidative stress and inflammation in brain in 30 min bilateral common carotid artery occlusion followed by 4 h reperfusion.

Groups	CAT (μ moles of H_2O_2 metabolized/mg protein/min)	SOD (units/mg protein)	MDA (nmol/g wet tissue)	MPO (units/ml)
Non diabetic				
Sham	8.692 ± 0.26***	23.25 ± 0.41***	132.63 ± 5.12***	0.0086 ± 0.0002***
I/R	4.171 ± 0.26	12.12 ± 0.28	272.02 ± 2.99	0.0231 ± 0.0001
DMSO	4.197 ± 0.25	12.10 ± 0.15	262.76 ± 13.34	0.0213 ± 0.0003
PVP	4.240 ± 0.15	12.32 ± 0.28	267.26 ± 8.72	0.0213 ± 0.0002
APR-15 mg/kg	5.746 ± 0.22***	19.48 ± 0.17***	164.90 ± 4.69***	0.0176 ± 0.0001***
APR-30 mg/kg	6.379 ± 0.14***	21.15 ± 0.22***	147.89 ± 4.39***	0.0163 ± 0.0002***
NMS-20 mg/kg	6.126 ± 0.16***	16.34 ± 0.25***	175.42 ± 10.16***	0.0174 ± 0.0002***
NMS-40 mg/kg	6.541 ± 0.14***	18.64 ± 0.17***	159.45 ± 7.69***	0.0163 ± 0.0002***
Diabetic				
Sham	7.81 ± 0.11***	22.08 ± 0.29***	134.38 ± 6.84***	0.0098 ± 0.0004***
I/R	3.46 ± 0.11	11.80 ± 0.17	274.02 ± 7.98	0.0234 ± 0.0006
DMSO	3.50 ± 0.07	11.97 ± 0.16	269.31 ± 4.02	0.0208 ± 0.0003***
PVP	3.63 ± 0.26	11.58 ± 0.12	271.52 ± 4.54	0.0217 ± 0.0003**
APR-15 mg/kg	4.33 ± 0.12ns	16.88 ± 0.19***	167.03 ± 5.08***	0.0171 ± 0.0003***
APR-30 mg/kg	5.44 ± 0.10***	19.67 ± 0.11***	152.52 ± 5.23***	0.0158 ± 0.0001***
NMS-20 mg/kg	5.66 ± 0.23***	15.55 ± 0.11***	182.42 ± 5.44***	0.0191 ± 0.0001***
NMS-40 mg/kg	6.09 ± 0.06***	16.45 ± 0.13***	166.66 ± 7.11***	0.0163 ± 0.0003***

Values are expressed in mean (n=6) ± SEM, ***$p < 0.05$ Vs I/R group, **$p < 0.05$ Vs I/R group ns $p < 0.05$ Vs I/R group. APR = Allopurinol; NMS = Nimesulide; I/R = Ischemic reperfusion.

The level of enzyme MPO was found to be increased in ischemic reperfused group compared to sham group in both non diabetic and diabetic group (Table I). Administration of allopurinol (15, 30 mg/kg) and nimesulide (20, 40 mg/kg) to cerebral ischemic reperfused group reduced MPO level significantly in both non-diabetic and diabetic condition (Table I).

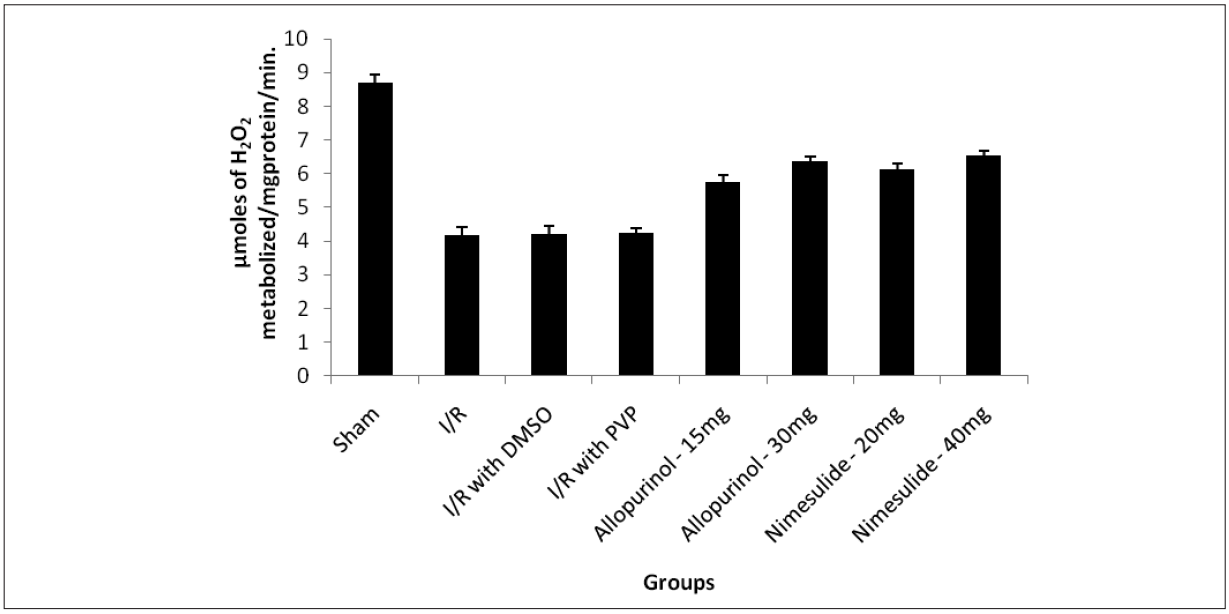

Figure 1. Histogram representing effect on CAT levels after treatment protocol in nondiabetic group.

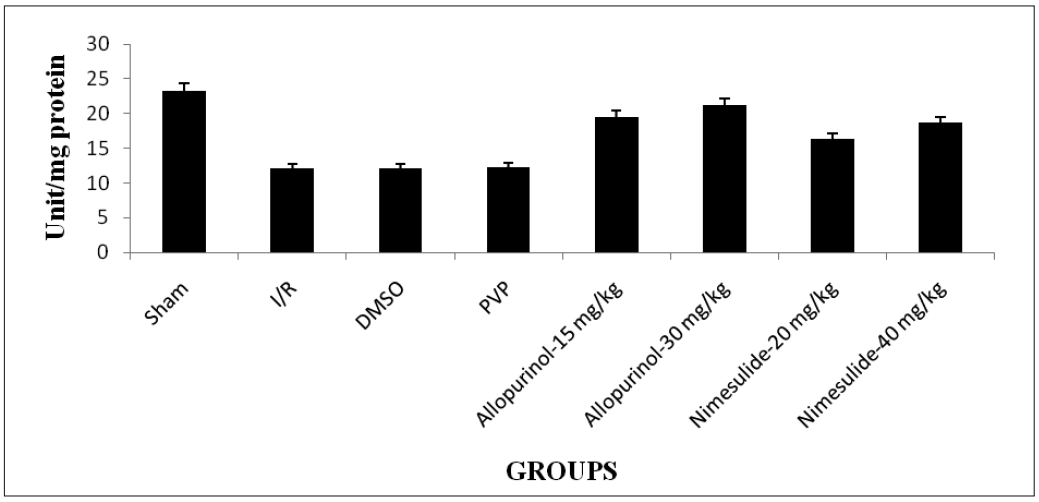

Figure 2. Histogram representing effect on cerebral SOD levels after treatment protocol in nondiabetic group.

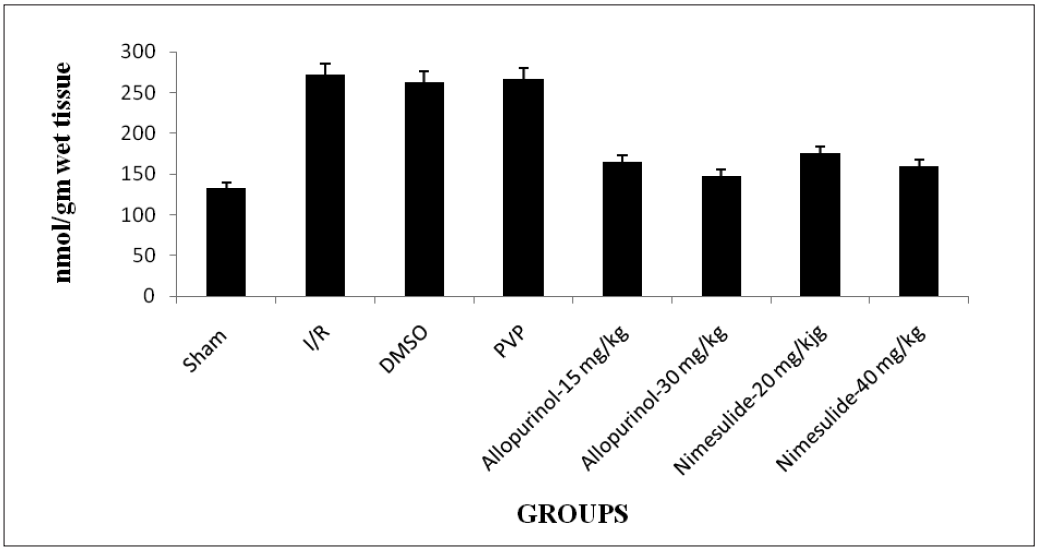

Figure 3. Histogram representing effect on cerebral MDA levels after treatment protocol in nondiabetic group.

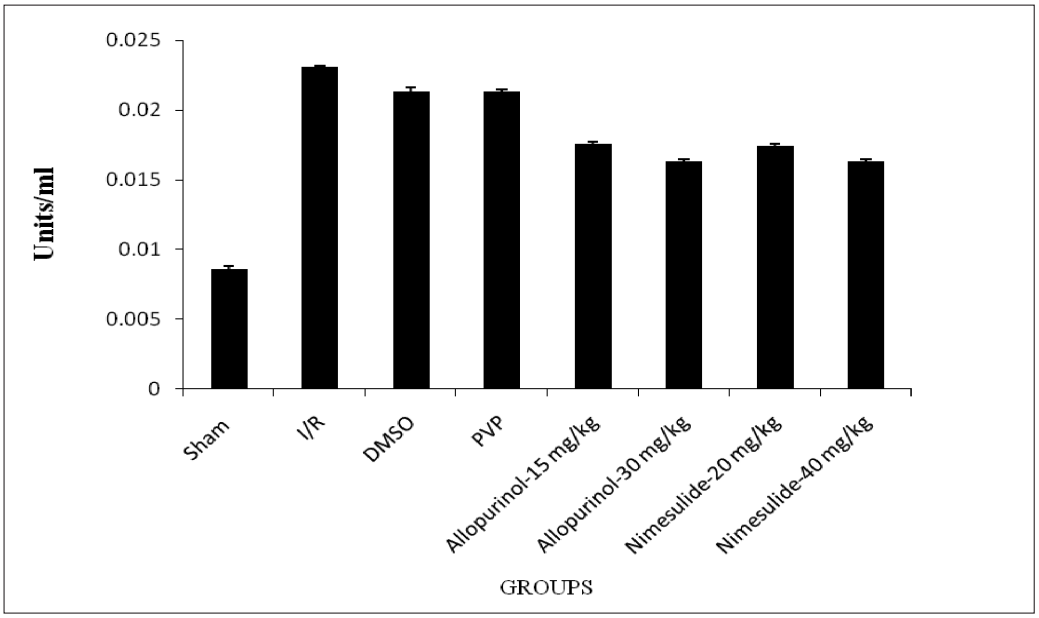

Figure 4. Histogram representing effect on cerebral MPO levels after treatment protocol in nondiabetic group.

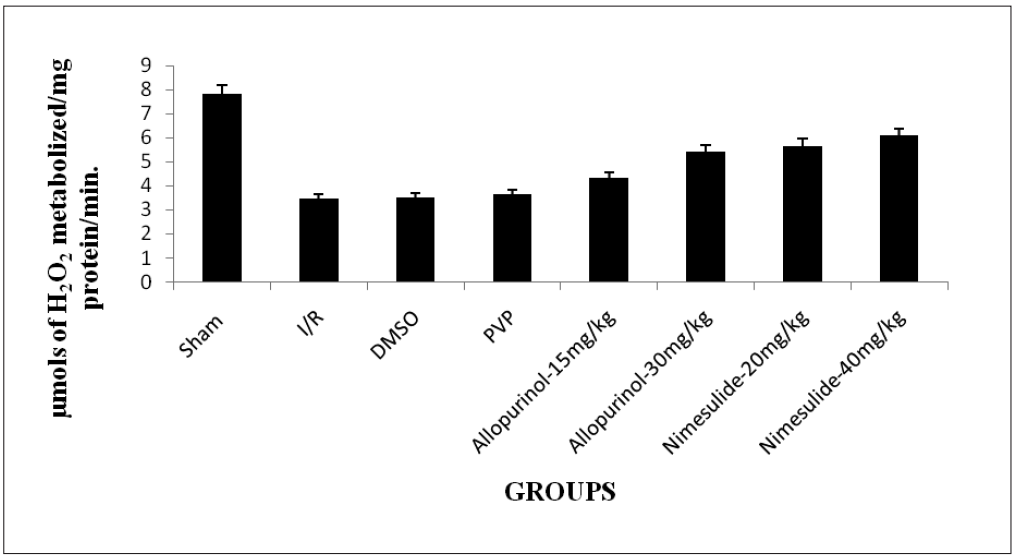

Figure 5. Histogram representing effect on cerebral CAT levels after treatment protocol in diabetic group.

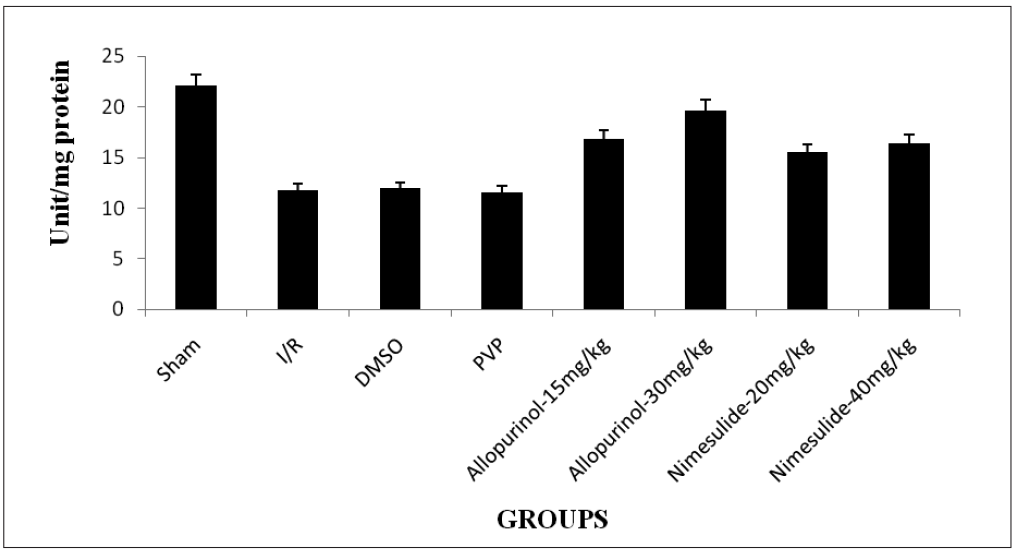

Figure 6. Histogram representing effect on cerebral SOD levels after treatment protocol in diabetic group.

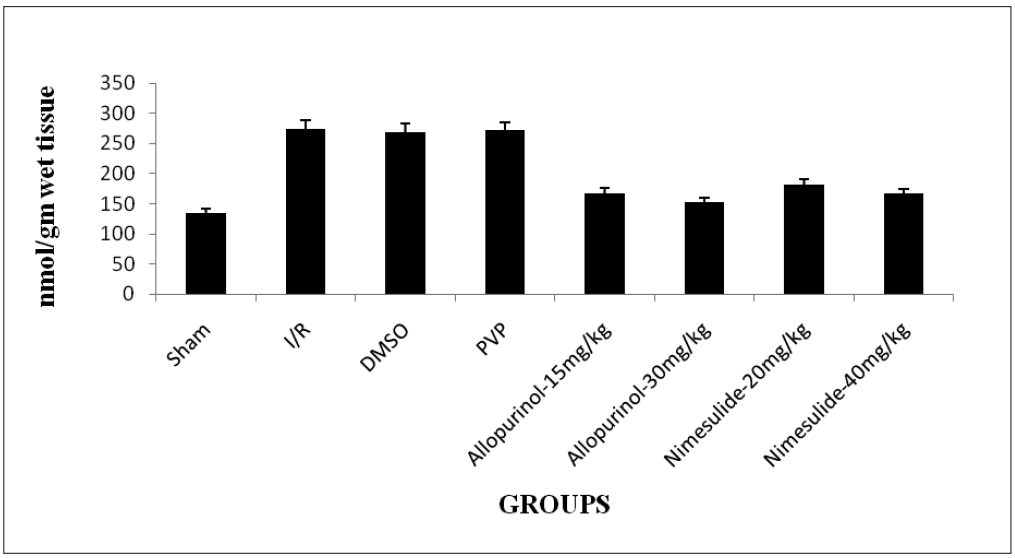

Figure 7. Histogram representing effect on cerebral MDA levels after treatment protocol in diabetic group.

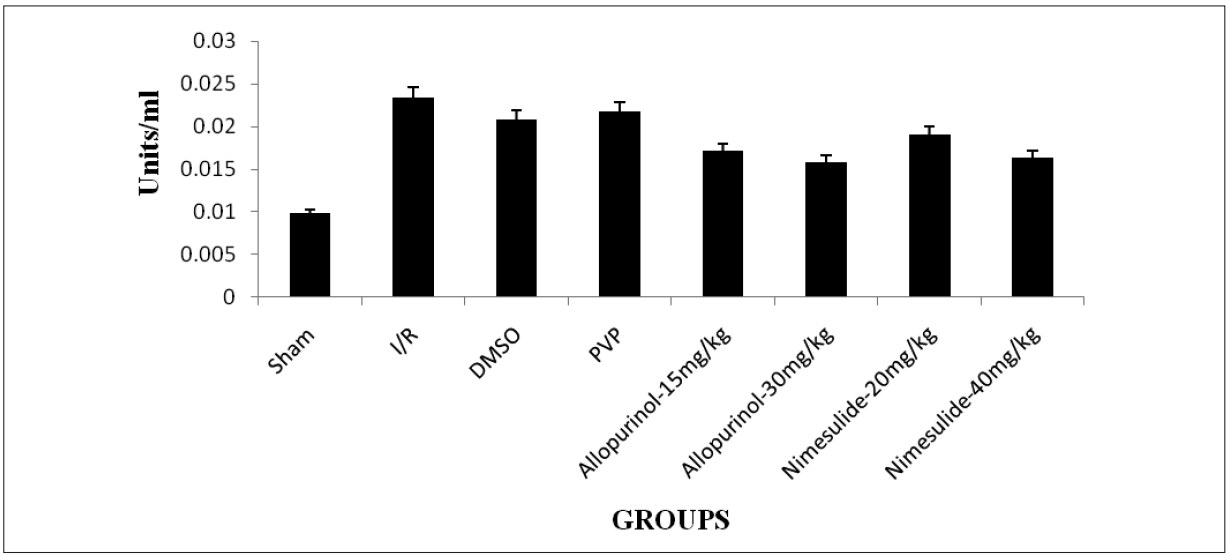

Figure 8. Histogram representing effect on cerebral MPO levels after treatment protocol in diabetic group.

Discussion

The present study demonstrated cerebral injury after 4 h of reperfusion following 30 min of cerebral ischemia by BCCA (bilateral common carotid artery occlusion) in STZ induced diabetic rats causes more damage than in non-diabetic rats. Oxidative stress is believed to be implicated in the pathogenesis of postischemic cerebral injury. Several experimental models of cerebral ischemic reperfusion injury showed significant neuroprotection when treated with antioxidants[19].

Most of the researchers proved that oxidative stress and inflammatory responses are involved in the pathogenesis of ischemic reperfusion injury[20,21]. ROS have been indicated as one of the earliest and most important components of tissue injury after reperfusion of ischemic organ. The extent of brain injury appears to depend on the experimental pattern of ischemia/reperfusion: free radicals production is continuous during ischemia, while during reperfusion it is primarily confined to the early stage, when fresh oxygen is supplied to the ischemic region[19]. The brain is very susceptible to the damage caused by oxidative stress, due to the high rate of oxidative metabolic activity, high polyunsaturated fatty acid contents, relatively low antioxidant capacity and inadequate neuronal cell repair activity[20]. Oxygen radicals, the products of biochemical and physiological reactions, are known to damage cellular lipids, proteins, and nucleic acids and to initiate cell signaling pathways after cerebral ischemia[22].

The brain is extremely dependent on oxygen supply[23]. Peroxidation of lipids can disrupt the organization of the membrane, causing changes in fluidity and permeability. Damage to mitochondria induced by lipid peroxidation can lead to further ROS generation[24]. The reaction of these radicals with double bonds of fatty acids in lipids produces peroxides that give rise to α, ß-unsaturated aldehydes including malondialdehyde (MDA), 4-hydroxynonenal (HNE) and acrolein. These aldehydes covalently bind to proteins through reaction with thiol groups and alter their function[25].

Hyperglycemia is also found to promote lipid peroxidation of low density lipoprotein (LDL) by a superoxide-dependent pathway resulting in the generation of free radicals[26].

We observed increase in the tissue MDA activity in ischemic reperfused brain when compared with the sham groups and the results were in agreement with previous studies[27]. Treatment with different doses of allopurinol (15 mg/kg, 30 mg/kg; i.p.) and nimesulide (20 mg/kg, 40 mg/kg; i.p.), reduced the levels of MDA in both diabetic and non-diabetic groups in comparison with ischemic reperfusion group.

Superoxide dismutase (SOD) and catalase constitute a mutually supportive team of defense against ROS. SOD, immediately dismutates super-

oxide to generate hydrogen peroxide, a more stable and less toxic compound. Hydrogen peroxide is a lipid-soluble molecule which diffuses easily across cell membranes and exerts remote effects like peroxidation of the membrane cell lipids, induction of DNA damage, oxidation of cellular enzymes. Defense mechanisms against the deleterious effects of hydrogen peroxide include the activities of catalase (CAT) and myeloperoxidases[28]. Catalase prevents hydroxyl radical generation by dismutating hydrogen peroxide. Therefore, the measurement of these endogenous antioxidants enzymes i.e. SOD and CAT has been performed to estimate the amount of oxidative stress. In the present study, SOD and CAT activity were decreased in the ischemic reperfused group in comparison with the sham group and the results were in agreement with previous studies[29].

The reduced SOD and CAT activity were increased by administration of allopurinol (15 mg/kg, 30 mg/kg; i.p.) and nimesulide (20 mg/kg, 40 mg/kg; i.p.) in both non-diabetic and diabetic groups.

Inflammation can extend ischemic brain injury and adversely affect outcome in experimental animal models[30]. Myeloperoxidase (MPO), a key inflammatory enzyme secreted by activated neutrophils and macrophages/microglia, can generate highly ROS to cause additional damage in cerebral ischemia[30]. In our present report the activity of enzyme MPO was increased in ischemic reperfused group compared to sham group. Administration of allopurinol (15 mg/kg, 30 mg/kg; i.p.) and nimesulide (20 mg/kg, 40 mg/kg; i.p.) to cerebral ischemic reperfused group reduced the MPO level significantly in both diabetic and non-diabetic conditions suggesting the possible role of anti-oxidant and anti-inflammatory mechanism in the neuroprotective actions of nimesulide and allopurinol.

Conclusions

The present research showed that allopurinol and nimesulide have neuroprotective activity against cerebral ischemic reperfusion injury in diabetic condition. Further detailed studies are to be developed to understand molecular and cellular mechanisms involved in healing processes which could provide new directions in the treatment of ischemic reperfusion induced cerebral injury in STZ induced diabetic rats.

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