CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.2532234

Available online at: http://www.iajps.com Research Article

NEUROPROTECTIVE EFFECT OF SILIBININ AGAINST ISCHEMIA-REPERFUSION INDUCED CEREBRAL INFARCTION IN RATS

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Abstract

Present study was evaluated the neuroprotective effect of silibinin against against ischemia-reperfusion induced cerebral infarction in rats. Silibinin (10 and 20 mg/kg P.o.) was treated for 14 consecutive days followed performed carotid artery occlusion of 30 min then reperfusion period 24 hrs. After 24 hrs of reperfusion, the brains was isolated and subjected to measurement of percentage of cerebral infract volume and malondialdehyde, superoxide dismutase, catalase and inflammatory markers (MPO, TNF-α, IL-10) markers in the brain tissue. The results revealed that the silibinin (20 mg/kg B.Wt.) treated rats brain malondialdehyde levels were significantly decreased and increased superoxide dismutase, catalase and decreased inflammatory mediators like MPO, TNF-α, IL-10 brain tissue in intact group when compared with control group. Silibinin alleviated neurological protection in rats, and the mechanism may be related to augmentation in endogenous antioxidant defense and inhibition of oxidative stress in the rat brain.

Keywords: *Silibinin; Inflammatory mediators, Cerebral ischemic / reperfusion.*

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Please cite this article in press S Praveen Begum et al., ***Neuroprotective Effect Of Silibinin Against Ischemia-Reperfusion Induced Cerebral Infarction In Rats.,*** *Indo Am. J. P. Sci, 2019; 06(01).*

INTRODUCTION:

Oxidative stress and excessive inflammation response are thought to be the key pathological events in ischemia-reperfusion injury. Reactive oxygen species production is one of the most important components of tissue injury after reperfusion of ischemic brain. Over production of reactive oxygen species during reperfusion could cause high rate of oxidative stress and lower anti-oxidative capacity. In response to inflammatory signals from ischemic and reperfused tissue, leukocytes initially accumulate in the vasculature by adhering to the vascular endothelium and plugging capillaries (1,2). Enzymatically active MPO, together with hydrogen peroxide and chloride, produces the powerful oxidant hypochlorous acid, a key contributor to the oxygen-dependent microbicidal activity of phagocytes and radicalized oxygen species (O2•–,ONOO•–) which induces apoptosis and nitrotyrosination of proteins. There is clear indication that the increase in brain MPO activity after cerebral ischemia and reperfusion reflects neutrophil infiltration into the brain. Thus myeloperoxidase (MPO) was used as a marker in our present study to quantify neutrophil accumulation (3,4, 5). In addition, inherent cells such as astrocytes, microglia, or endothelia have been found to be activated by cerebral injuries including ischemic stroke. These cells then become immunologically reactive and interact with each other by producing substances including cytokines and adhesion molecules (6,7,8,9). These molecules appear to be responsible for the accumulation of inflammatory cells in the injured brain, and the resulting immunologic inflammatory cascade produces an environment that may affect the survival of neurons subjected to ischemic injury constitutively express very low levels of MHC antigens, and are thus considered as 'immunologically silent', these cells, once stimulated, can express various cytokines and adhesion molecules. Three major cytokines namely tumor necrosis factor-α and interleukin-10 (IL-10) (10,11, 13, 14). Therefore antioxidant and anti inflammatory agents can protect against the adverse effects of oxidative stress and inflammation produced during ischemia reperfusion injury. Silibinin is a potent antioxidant, anti inflammatory agent and hepatoprotective. There is increasing evidence that supports that potent antioxidants and anti inflammatory agents can provide protection against neurodegenerative changes associated with cerebral ischemia reperfusion injury. Therefore the present study was aimed to evaluate the cerebroprotective action of silibinin against ischemia reperfusion induced cerebral infarction. In the present study, bilateral common carotid arteries occlusion for 30 min and 4 hr reperfusion used for experimental model of global cerebral ischemia. The study carried to estimate percentage of infarction, histological characters, oxidative stress markers (MDA, SOD, CAT) and inflammatory markers (MPO, TNF-α, IL-10) markers in the brain tissue.

MATERIALS AND METHODS:

Chemicals Silibinin (Yarrow chem, India), Thiobarbituric acid, 1,1,3,3- tetramethoxypropane and n-butanol were purchased from SD Fine Chemicals. TNF-alpha , IL-10 and IL-6 R & D Systems, India

Animals

In this study, Wistar rats weighing 250-300g were used. The animals were kept in separate cages in a room with 22 ± 2 ºC temperature and 12:12 hours light and dark cycle and free access to enough food and water, proposal was approved by IAEC (315/IAEC/SICRA/PhD/2017).

Drug administration

Silibinin was dissolved in 10% DMSO and injected orally (40 mg/kg, i.p.) once a day for 7 consecutive days (day 0-day 6) subsequent to I/R. The first dose was injected immediately after surgery (15)

Transient bilateral occlusion of common carotid arteries

Rats were anesthetized with an i.p. injection of thiopental sodium (30 mg/kg bd.wt.) and were placed in the supine position. A 2 cm longitudinal incision was made on the ventral neck. Right and left common carotid arteries were then exposed and the arteries were occluded for 30 min followed by reperfusion for 24 hr in control and silibinin groups. At the end of ischemia, the microclips were detached and reperfusion of the arteries was confirmed by visual observation. Finally, the incision was sutured. The rats were kept under a heating pad and lamp for 2 hours until recovery to prevent hypothermia. In the sham group rats subjected to all surgical steps except the occlusion of common carotid arteries (16,17).

Experimental groups

Each group consisted of 6 animals. Experimental protocols was as follows

Group I: Control group: Transient cerebral ischemia was done using the bilateral occlusion of common carotid arteries (BOCCA) method. Vehicle was injected once a day for 14 consecutive days subsequent to cerebral I/C.

Group II: Sham group: Rats underwent surgical

injury except the occlusion of common carotid arteries. Vehicle was injected once a day for 14 consecutive days (day 0 to day 6) subsequent to surgery.

Group III: Silibinin (10 mg/kg/day, P.o) was administered once a day for 14 consecutive days subsequent to cerebral I/C.

Group-IV: Silibinin (20 mg/kg bd.wt. P.o) was administered once a day for 14 consecutive days subsequent to cerebral I/C.

At the end 14 days of experiment, the brains were removed and subjected to measurement of percentage of cerebral infract volume and oxidative stress markers (MDA, SOD, CAT) and inflammatory markers (MPO, TNF-α, IL-10) markers in the brain tissue.

Measurement of percentage cerebral infarct volume

The cerebral infarct volume was determined by using TTC (17).In brief, animals were sacrificed at the end of 4 hr reperfusion and brains were removed rapidly by cervical dislocation and frozen at 4^0C for 5 min. Coronal slices were made in to 2 mm thickness and each slice was immersed in a 1.0% solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) for 30min. The TTC is converted to red formazon pigment by Nicotinamide Adenine Dinucleotide (NAD) and dehydrogenase present in the viable cells. Thus, the viable cells stained deep red and dead cells (infarcted) remain unstained (18). Pale necrotic infarcted tissue was separated and weighed. Percent cerebral infarction was calculated.

RESULTS:

Fig: 1a Effect of Silibinin on percentage infarct size in cerebral ischemia reperfusion injury in rats

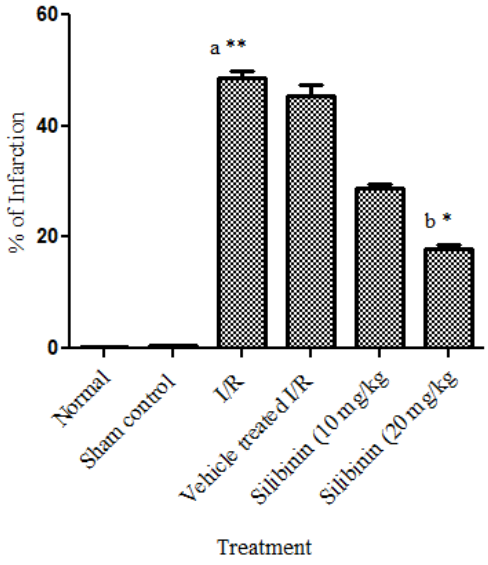


Fig: 1 b Effect of Silibinin infarct size in cerebral ischemia reperfusion injury in rats

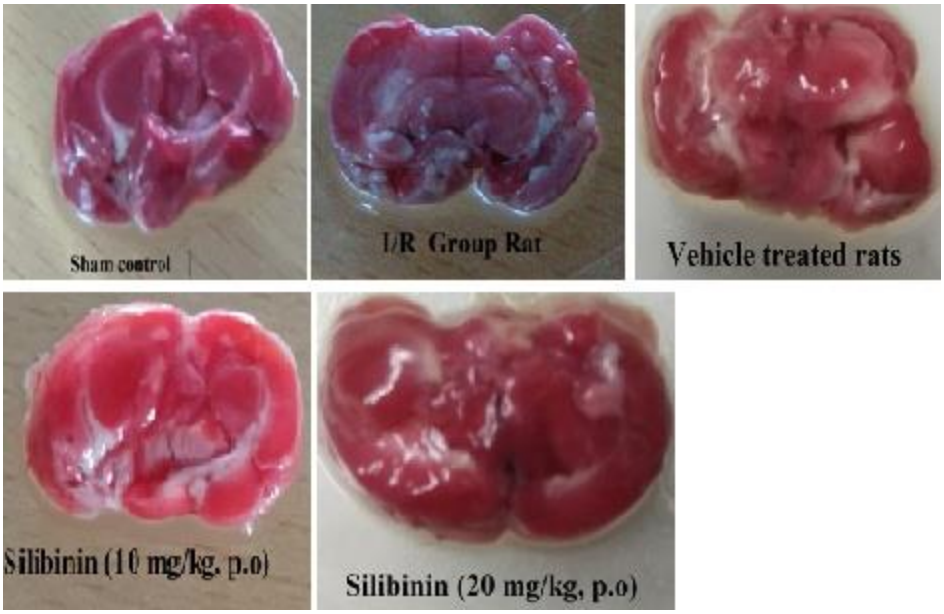


Fig: 2 Effect of Silibinin on MDA (nmol/g Wet Tissue) levels in infarcted tissue in cerebral ischemia reperfusion injury in rats

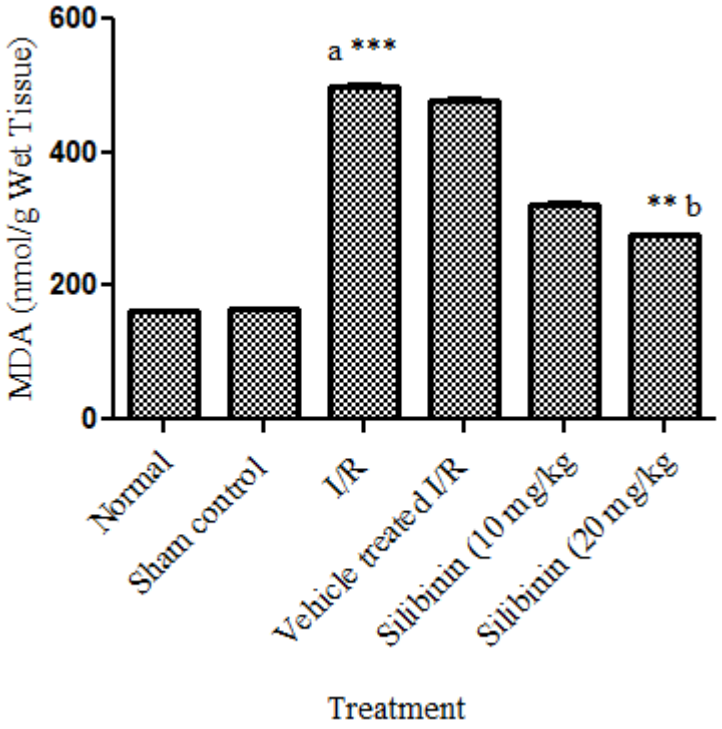


Fig: 3 Effect of silibinin on SOD (U/mg protein) levels in infarcted tissue in cerebral ischemia reperfusion injury in rats

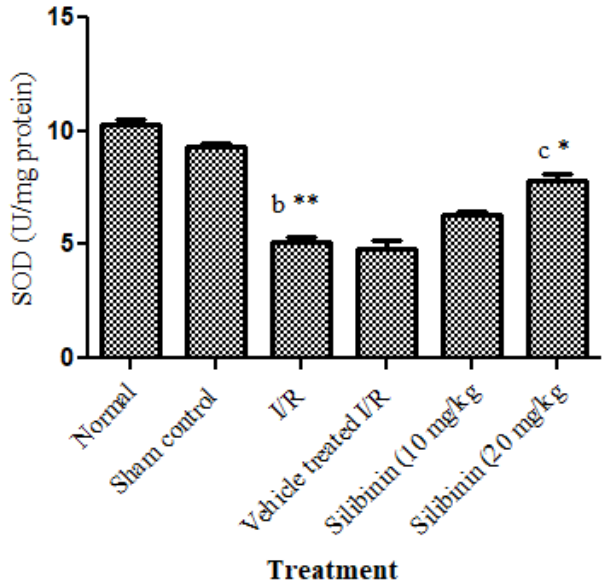


Fig: 4 Effect of silibinin on catalase (µmoles/min/ mg Protein) levels in infarcted tissue in cerebral ischemia reperfusion injury in rats

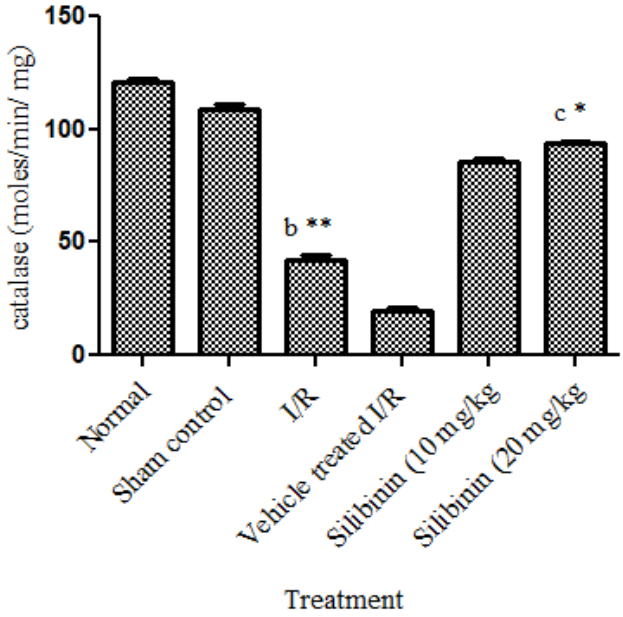


Fig: 5 Effect of silibinin on Myeloperoxidase (U/g Tissue) levels in cerebral ischemia reperfusion injury in rats

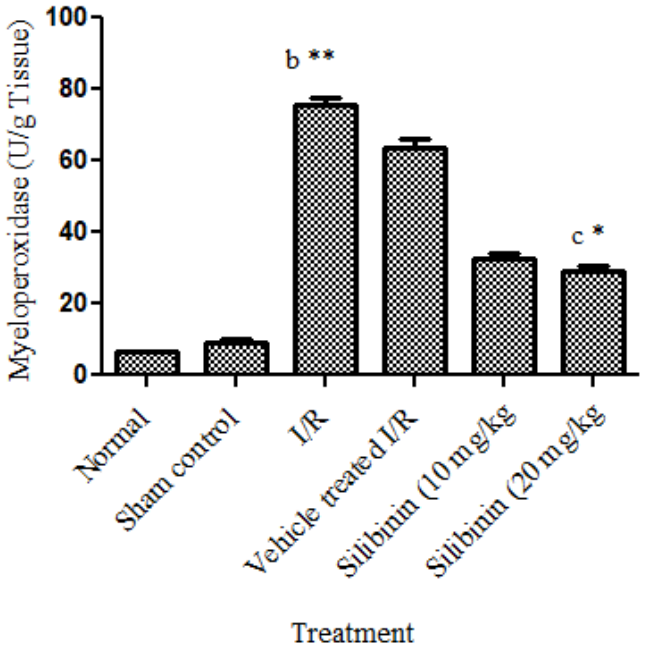


Fig: 6 Effect of silibinin on TNF-α (pg/mg Of Tissue) levels in cerebral ischemia reperfusion injury in rats

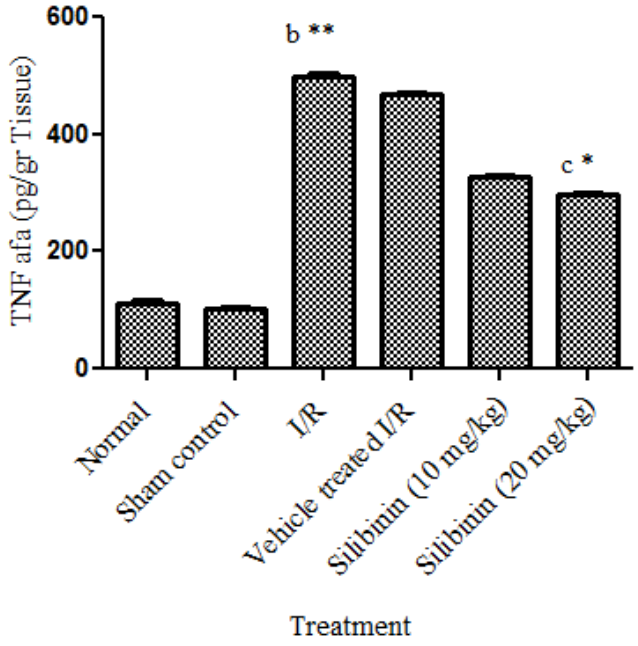
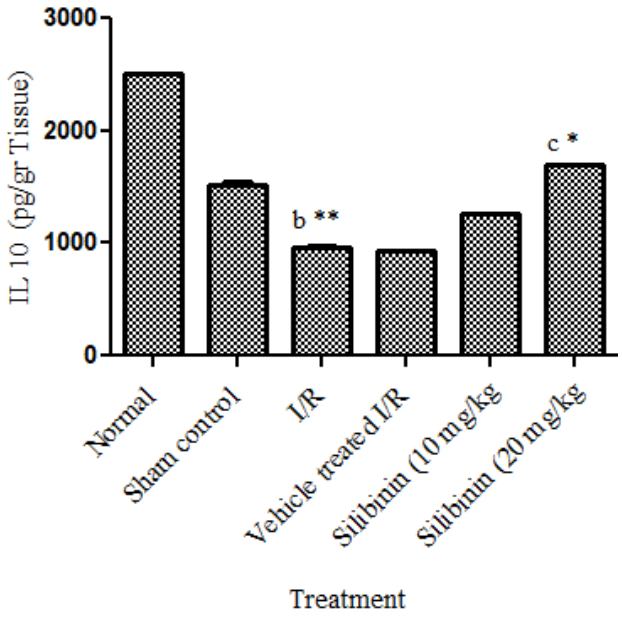


Fig: 7 Effect of silibinin on IL-10 (pg/mg of Tissue) levels in cerebral ischemia reperfusion injury in rat



The I/R groups rats were exhibited significant increase % of cerebral infarction from 0.2 ± 0.01 to 48.7 ± 1.2 ($P < 0.01^{a**}$), similararly size was significantly decreased 48.7 ± 1.2 to 17.9 ± 0.7 ($P < 0.01^{b*}$) in silibinin (20 mg/kg bd. wt.) treated rats. The MDA levels were significantly increased 162 ± 0.98 to 499±2.8 ($P < 0.001^{a***}$) in I/R group rats compared to normal rats, similarly MDA was significantly ($P < 0.01^{b**}$) decreased from 499±2.8 to 276±1.9 in silibinin (20 mg/kg, p. o) treated rats compared to I/R group rats. The SOD levels were significantly ($P < 0.01^{b**}$) decreased from 10.3±0.21 to 5.1±0.21 in I/R group rats compared to normal rats, SOD levels were significantly 5.1±0.21 to 7.8±0.32 ($P < 0.05^{c*}$) increased in silibinin (20 mg/kg bd.wt.) treated rats. Catalase levels were significantly ($P < 0.01^{b**}$) decreased from 121.76±1.54 to 42.43±1.9 in I/R group rats compared to normal rats, similararly Catalase levels were significantly increased 42.43±1.9 to 93.54±1.3 ($P< 0.05^{c*}$) in silibinin treated rats. Myeloperoxidase levels were significantly ($P< 0.01^{b**}$) incecreased from 6.5±0.21to 75.4±2.1 in I/R group rats compared to normal rats, similarly myeloperoxidase levels were significantly ($P < 0.01^{b**}$) decreased from 75.4±2.1 to 29.6±1.2 in silibinin (20 mg/kg bd.wt.) treated rats. TNF alfa levels were significantly ($P < 0.05$ c^{*}) incecreased from 110.78±5.4 to 498.95±4.8 in I/R group rats compared to normal rats, similararly TNF alfa levels were significantly decreased from 498.95±4.8 to 298.43±2.7 ($P < 0.05$ ^{c*}) increased in silibinin (20 mg/kg bd.wt.) treated rats. IL 10 levels are significantly decreased from 2500.67±12.87 to 965.65±6.9 ($P < 0.01$ ^{b**}) in I/R group rats compared to normal rats, Similararly IL 10 levels are significantly ($P <0.05^{c*}$) decreased from 965.65±6.9 to 1698.87±3.5 in silibinin treated rats.

DISCUSSION:

The brain is highly susceptible to cerebral ischemia-reperfusion injury, it produces cerebral damage (Cheung 2003). Cerebral infarction is considered one of the important components of cerebral ischemia, it can be atherothrombotic or embolic (19). Cerebral infarctions fluctuate in their severity with one third of the cases resulting in death. Cerebral ischemia-reperfusion injury produces cerebral infarction via a complex cascade of patho biological events that progresses over a short period of time (20,21). The cerebral infarction has been associated with ROS, which react with cellular macromolecules such as proteins, lipids and nucleic acids leading to damage of the neuron (22, 23). At surrounding area of ischemic event (penumbral region) spontaneous peri-infarct depolarization (PID) waves are involved (24). PID may be triggered by Ca+ or K+ during energy depletion. The core ischemic cells undergo anoxic depolarization and they are destined to die due to lack of energy but those which are present in the surrounding area can be depolarized and repolarized again (25). As the number of depolarizations increases, infarct size grows larger (26). Delayed PIDs after reperfusion contribute to a complex secondary pathology involving delayed edema, hypoperfusion and intracranial hypertension. The manifestation of PIDs in electroencephalography

recordings may enable continuous real-time monitoring of infarct progression (27).

Ischemia and reperfusion cause brain injury via multiple pathways. Previous studies demonstrate that reactive oxygen species are elevated during cerebral ischemia and reperfusion, which plays a major role in the pathophysiology of ischemic stroke or cerebral I/R related injury. MDA is end product of lipid peroxidation, the results clearly indicates the cytotoxic effect of free radical by peroxidation on brain tissue, because it contains large amount of phospholipids that are rich in polyunsaturated fatty acids leading to neuronal death. Silibinin treatment significantly reduced the elevated tissue MDA levels contributing to partial protection. This may be because of involvement of multiple pathways in global cerebral ischemia-reperfusion injury. The beneficial effects of these Silibinin are attributed to their antioxidant and anti inflammatory properties. The evidence from our study clearly indicates that besides the peripheral organs, these Silibinin may also help to prevent tissue damage from oxidative stress in the brain (28). Increased nitric oxide concentrations associated with ischemia and reperfusion may have dual effects on lipid peroxidation. Nitric oxide reacts with free radicals, thereby producing the highly damaging peroxynitrite. Nitric oxide injury takes place for the most part through the peroxynitrite route because peroxynitrite can directly oxidize low density lipoproteins, resulting in irreversible damage to the cell membrane. When these Silibinin are used free radicals are scavenged and therefore can no longer react with nitric oxide, resulting in less damage. In contrast, nitric oxide itself may directly inhibit lipid peroxidation by intercepting alkoxyl and peroxyl radical intermediates thereby terminating chain propagation reactions (29). Present study demonstrated that SOD and CAT levels were significantly reduced in ischemia-reperfusion (I/R) control group when compared to sham control. To prevent oxidative damage, mammalian cells have developed a complex antioxidant defense system that include enzymatic activities (SOD, CAT, glutathione peroxidase) and free radical scavengers such as glutathione, vitamin C and E. SOD catalyzes the dismutation of superoxide radicals forming hydrogen peroxide. GPx and CAT are the unique enzymes scavenging hydroperoxides and therefore act in concert with SOD. Decrease in the antioxidant enzyme activities during ischemia and reperfusion is due to the attack of sulphydril (-SH) groups of enzymes by oxygen free radicals and interaction of enzymes with peroxidation products, which can affect the active site of the enzyme. Another reason for reduction of enzyme activities can be attributed to the reduction in pH, i.e. acidosis. Ischemia renders the cells to undergo anaerobic metabolism there by producing lactic acid and acidosis. Enzymes that are pH-sensitive will therefore be easily affected. Thus significant alteration in the antioxidant enzyme activities during cerebral ischemia and reperfusion may be responsible for more neurodegeneration than ischemia (30). Silibinin treatment in our present investigation increased the endogenous antioxidant enzymes SOD and CAT indicating enhanced biochemical defenses to scavenge the overproduced free radicals. The present study explored the cerebral damage in terms of infarction observed in ischemia-reperfusion injured brain tissue as evidenced by the increased percentage of infarct volume in I/R group rats when compared to Sham control. Infarction volume in the brain is an important determinant in assessing the consequences of cerebral ischemia which leads to neurological impairment. Myeloperoxidase activity was significantly lower in animals treated with Silibinin as compared with the nontreated group. These results suggest the anti-inflammatory role of Silibinin by inhibiting neutrophil adherence to the endothelium during cerebral I/R period. Transient ischemia of the cerebral vasculature followed by reperfusion leads to a secondary cascade of pathophysiologic events, characterized by a complex inflammatory response. Inflammation in stroke has been traditionally identified on histopathology as neutrophil infiltration, which correlates positively with ischemic damage (31, 32, 33, 35). Myeloperoxidase (MPO) is the most abundant component in azurophilic granules in neutrophils and has often been used as a histopathological marker for neutrophils. It is also expressed in the myeloid line, especially in monocytes and macrophages/microglia. MPO interacts with hydrogen peroxide to generate highly reactive species including hypochlorite (OC1–) and radicalized oxygen species (O2•–, ONOO•–). MPO-mediated radicalization of molecules induces apoptosis and nitrotyrosination of proteins. Thus, MPO is a key component of inflammation and has been shown to play a major role in animal models of stroke in the post hypoxic inflammatory response. MPO genotypes are associated with increased brain infarct size and poorer functional outcome. Therefore, inflammation is a complex cascade of events involving different types of cells and molecules, MPO could be used as an excellent biomarker for inflammation to access the extent of neutrophil infiltration during cerebral I/R injury (36, 37, 38,39,40). Silibinin treatment significantly reduced the inflammation characterized by decrease in myeloperoxidase activity in animal subjected to cerebral I/R injury. The evidence relating to

neuroinflammatory modulation by Silibinin supports the following mechanisms (41, 42, 43) The inhibitory role on the release of cytokines, such as TNF-α from activated glia, inhibitory action against iNOS induction and subsequent NO• production in response to glial activation, ability to inhibit the activation of NADPH oxidase and subsequent ROS generation in activated glia, capacity to down regulate the activity of pro-inflammatory transcription factors such as NF-KB. The potential to modulate signaling pathways such as MAPK cascade. Similarly, Silibinin treatment significantly reduced the inflammation characterized by decrease in tumor necrosis factor-α (TNF-α) in animal subjected to cerebral I/R injury. In the present study, significant increase in cerebral infarction in I/R group rats and significant decrease in cerebral infarction in Silibinin treated rats was observed. These results were in accordance with earlier reports. Therefore, the results in the present study propose that Silibinin has significant neuroprotective action as evidenced by significant decrease in cerebral infarct volume, TNF alfa, and Interleukin 10 activity was considerably increased as compared with un treated rats.

CONCLUSION:

Silibinin treated group rats showed tremendous recovery from abnormalities in ischemic reperfusion insult. Therefore we suggest that silibinin has potent cerebroprotective action against cerebral ischemia reperfusion injury. The cerebroprotective actions of silibinin are partially attributed to their anti inflammatory effects against I/R injury in rats as evidenced by significant reduction in pro-inflammatory markers MPO, TNF-α and significant increase in anti-inflammatory marker IL-10.

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