

Full Length Research Paper

Neuroprotective effect of Silibinin against middle cerebral artery occlusion induced focal cerebral ischemia and brain injury in Wistar rats

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Silibinin (Silybin) as an active constituent derived from milk thistle (*Silybum marianum*) has been shown to have antioxidant and anti-apoptotic properties. In traditional system of medicine, *Silybum marianum* has been used for treating various kinds of ailments including liver disease and cancer; however, clinical studies are largely heterogeneous and contradictory. The present study was designed to investigate whether Silibinin protects neuronal injury against middle cerebral artery occlusion induced oxidative stress associated damages in focal cerebral ischemia in Wistar rats. Rats weighing 250 to 300 g were pretreated with Silibinin 100 mg/kg and 200 mg/kg body weight, suspended in 0.5% of gum acacia) once daily for seven days. On 8th day, they underwent for middle cerebral artery 2 h suture occlusion by nylon suture. After 120 min of middle cerebral artery occlusion (MCAO) and 22 h of reperfusion, behavioral tests were assessed in terms of neurological deficits. Animals were sacrificed and infarct volume in Triphenyltetrazolium chloride (TTC) stained brain sections was measured. Further, various oxidative biomarkers were estimated in brain homogenates of rats. Pretreatment with Silibinin at doses of 100 and 200 mg/kg significantly improved the neurobehavioural alterations and reduced the infarct volume whereas 200 mg/kg Silibinin significantly increases the Glutathione (GSH) level and the others parameters with respect to control values. These results clearly indicate the neuroprotective effect of Silibinin against middle cerebral artery occlusion associated with oxidative damage induced brain injury due to its antioxidant and anti-apoptotic property.

Key words: Neuroprotective, MCAO, Silibinin, oxidative stress, infarct volume, cerebral ischemia.

INTRODUCTION

Ischemic stroke and neuronal death is the second major cause of death worldwide (Van derWorp and van Gijn, 2007). Cerebral ischemia produces oxygen and glucose deficiency due to reduction and complete blockade of

blood that flows to regions of brain (Zemke et al., 2004). This occlusion, thus damaging physiological activity of brain leads to oxidative stress and further neuronal damage. Ischemic reperfusion injury can causes cellular

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damage by cascade of events such as release of free radicals and cytokines, induction of inflammation, apoptosis, and excitotoxicity that affect structure and function of brain (Kuroda and Siesjo, 1997). It has suggested that oxidative stress, excitotoxicity, inflammation and apoptosis are the major mechanisms that should be involved in the pathogenesis of ischemia/reperfusion injury (Bondy 1995; Lipton 1999). The ischemic mediated neuronal damage might be a result of mitochondrial dysfunctioning of resident cell that disrupts the organized redox balance, and therefore initializing the cascade of damaging events which supports the necrotic and apoptotic cell death pathway (Takemura et al., 1993; Courtois et al., 1998). It has been evidently concluded that oxidative stress is the major mechanism of brain damage in occlusion and reperfusion. That's why it has been concluded that occlusion of middle cerebral artery (MCA) by using nylon suture is a widely suggested experimental model of cerebral ischemia for neuroprotective drug development as it closely resembles stroke injury seen in human patients (Carmichael, 2005; Durukan and Tatlisumak, 2007).

Chain (2001) evidently concluded that oxidative stress is the major mechanism of brain damage in occlusion and reperfusion. In recent years, herbal medicines has been gaining much interest, as these appears to be safe and effective treatment for combating neurodegenerative diseases associated with oxidative damage. It has been reported that the formulations and plant extracts which have antioxidant properties, protect the neurons against ischemic and reperfusion injury (Zhu et al., 2004; Shukla et al., 2006). Among herbal medicines, Silibinin categorized under the group of flavonolignans, that is extracted from milk thistle plant *Silybum marianum*. Silibinin is a potent hepatoprotective used every day in clinical practice for treating liver disorders; it could prevent lipid peroxidation, and also increase antioxidative enzyme levels. It has already been shown at the neuroprotective effect of Silibinin in diabetic mice by Tota et al. (2011).

MATERIALS AND METHODS

Chemicals

1-Chloro-2, 4-dinitrobenzene (CDNB), reduced glutathione (GSH), 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), 2, 3, 5-triphenyltetrazolium chloride (TTC), thiobarbituric acid (TBA), nitroblue tetrazolium (NBT) and trichloroacetic acid (TCA) were purchased from SD fine chemicals, Mumbai, India. All the other chemicals used were of analytical grade.

Animals

Adult male Wistar rats were obtained from Animal house of Meerut Institute of Engineering and Technology, Meerut India. The rats weighing 250±10 g were used at the start of the experiment. Rats

were housed under standard laboratory conditions, maintained at an ambient temperature of 25±2°C and relative humidity of 45 to 55% with 12 h light: 12 h dark cycle and had free access of food and water. The food was withdrawn for 12 h before the surgical procedure. All the procedures performed were in accordance with Control and Supervision of Experiments on Animals (CPCSEA) guidelines, under Ministry of Animal Welfare Division, Government of India, and New Delhi.

Middle cerebral artery occlusion (MCAO) to induce focal cerebral ischemia

MCAO was carried out according to the procedure described by Koizumi et al. (1986). Rats were anesthetized with Ketamine and Diazepam (70 mg/kg and 5 mg/kg, i.p. respectively) and placed in dorsal recumbency. A longitudinal incision of 1 cm in length was made in the midline of the ventral cervical skin. The right common carotid artery at the level of internal and external carotid artery bifurcation were exposed and carefully isolated. A nylon monofilament (40 mm in length and 0.24 mm in diameter), whose tip was rounded by exposing to flame was inserted from the lumen of the common carotid artery to that of the right ICA to occlude the origin of the right middle cerebral artery (MCA). The right MCA was occluded for 60 min, and thereafter the filament was withdrawn and allowed to be reperfused with blood. 24 h after reperfusion, rats were decapitated. Animals were maintained in a warm condition using a hot blower throughout the surgical procedure.

Post-operative care

After 4 to 5 h of surgery, recovery of anesthesia took place. The animals were kept in a maintained temperature at 25±3°C in individual cages until they gained full consciousness, and then they were kept together in a group of 3 animals per cage. Food with ad lithium water was kept inside the cage for 24 h, so that the animal could easily access it.

Experimental design

Animals were divided into five groups. The first group was sham operated (n=7) (animals were subjected to surgical procedure, but did not occluded MCA except for exposure of ICA and ECA), received 0.5% gum acacia, 10 ml/kg orally; second was MCAO group only (n=12); third was standard group (n= 12) received aspirin before MCAO; fourth was MCAO group pretreated with Silibinin (100 mg/kg in 0.5% gum acacia, orally) (n=12); and fifth was MCAO group pretreated with Silibinin (200 mg/kg in 0.5% gum acacia, orally) (n=12). Vehicle or drugs were fed once daily for 7 consecutive days prior to experimental procedure. After completion of reperfusion period, animals were assessed for neurobehavioural activity, and then sacrificed for biochemical estimations.

Dose selection and drug administration

A dose of 100 and 200 mg/kg of Silibinin was selected on the basis of previous literature of survey (Tota et al., 2011; Lu et al., 2009). These doses have also shown the maximal protection in various types of brain diseases. Rats were pretreated systematically with 100 and 200 mg/kg Silibinin orally suspended in 0.5% gum acacia, once daily for seven consecutive days. On day 8th, MCAO was performed for 120 min followed by 22 h reperfusion (Longa et al., 1989).

Neurological function assessment

After 22 h of reperfusion, neurological test was carried out by an examiner to the experimental groups before the rats were sacrificed. The deficits were scored on a modified scoring system described by Longa et al. (1989), as; 0 = rats moved around in the cage and explored the environment; 1= rats moved in the cage but did not approach to all the sides and hesitated to move; 2= rats barely moved in the cage and showed postural abnormalities curved towards the paretic side; 3= rats unable to move at all with their posture curved towards the paretic side.

Locomotor activity

The spontaneous locomotor activity was recorded by using actophotometer equipped with infrared sensitive photocells. Before locomotor task, animals were placed individually in the actophotometer cage for 2 min for habituation. Thereafter, locomotor activity was recorded for a period of 5 min. The ambulatory activity was expressed in terms of total photo beam counts per 5 min as described by Kulkarni (1999).

Biochemical estimations

The animals were sacrificed under deep ether anesthesia, perfused transcardially with ice-cold saline and the brains were dissected out. A 10% brain homogenate was prepared with ice-cold phosphate buffered saline and was centrifuged at 10,000 rpm at -4°C for 15 min, and the supernatant obtained was used for biochemical estimations.

Estimation of reduced glutathione (GSH)

GSH was measured by the method of Ellman (1959). Equal quantity of brain homogenate was mixed with 10% TCA and centrifuged to separate proteins. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of DTNB and 0.4 ml double-distilled water was added. Mixture was vortexed and the absorbance was recorded at 412 nm. The concentration of GSH was expressed as nmol/mg of protein.

Estimation of tissue levels of malondialdehyde (MDA)

The minced brain was homogenized in a buffer containing 30 mM Tris-HCl and 2.5 mM CaCl₂ (pH 7.6). To separate cellular debris the homogenate was centrifuged at 750 gyrations. The supernatant was accurately divided into two portions and centrifuged at 8200 gyrations to obtain mitochondrial fraction. One fraction was utilized for determination of MDA (Yagi, 1982), and the other one was employed for protein estimation (Lowry et al., 1951; Kakkar et al., 1984). The concentration of MDA in brain homogenates was expressed in terms of nM MDA/mg protein.

Estimation of superoxide dismutase (SOD)

SOD activity in cerebral tissues of all the rats were estimated by using the technique of Kakkar et al. (1984), based on inhibition of the formation of nicotinamide adenine dinucleotide, PMS and NBT formazan. Briefly, to 10 µl of homogenate was added 90 µl of 30 mM sodium tetrapyrophosphate buffer (pH 8.3), 30 µl of 0.3 mM NBT, 10 µl of 0.96 mM PMS and 40 µl of double-distilled water. Reaction was initiated by addition of 20 µl 0.72 mM NADH.

Absorbance was measured at 560 nm. A single unit of enzyme was expressed as 50% inhibition of NBT reduction/min/mg protein. Results were expressed as unit of SOD/min/mg protein.

Estimation of catalase (CAT)

CAT was assayed using the method of Aebi (1984). Briefly, the assay mixture of 1.5 ml contained 980 µl of 50 mM sodium phosphate buffer pH 7.0 and 20 µl of homogenate (10 to 15 µg protein). Reaction was started by addition of 500 µl of 30 mM hydrogen peroxide. The decrease in absorbance was then observed for 60 s at every 15 s interval at 240 nm. CAT activity was expressed as U/mg protein.

Estimation of nitrite (NO)

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide, was determined by a colorimetric assay with Greiss reagent according to Green et al. (1982). The absorbance was measured at 540 nm using UV spectrophotometer. The concentration of nitrite in the supernatant was determined from sodium nitrite standard curve.

Estimation of protein

Protein was determined by the method of Lowry et al. (1951) using BSA as a standard. Protein was measured in all brain samples for GSH, MDA, SOD, CAT and nitrite. BSA (1 mg/ml) was used as standard and measured in the range of 0.01 to 0.1 mg/ml.

Measurement of infarct volume

Rats were sacrificed and their brains were quickly removed and sectioned coronally into slices each with a 2 mm thickness. The brain slices were then immersed in 2% triphenyltetrazolium chloride (TTC) for 30 min at 37°C and then fixed with formalin. Infarcted areas were identified as regions lacking brick red staining of normal brain tissues. It was expressed as mm³.

Statistically analysis

The values were expressed as mean ±S.E.M. The statistical analysis was carried out by one way analysis of variance (ANOVA) followed by Tukey's post hoc test. P values <0.05 were considered as significant.

RESULTS

Effect of Silibinin on percent survival of animals after MCAO

After MCAO for 120 min and 22 h reperfusion, approx 50% of animals survived in the vehicle treated group. Pretreatment with Silibinin at 100 and 200 mg/kg increased the survival rate significantly to 66.6 and 83.3% respectively. There was no mortality in sham operated rats treated with vehicle (Table 1).

Table 1. Effect of Silibinin on percent survival after MCAO in rats.

Treatment	No. of animals survived/ used	Survival (%)
Sham operated	7/7	100
MCAO	6/12	50
Standard (Aspirin + MCAO)	11/12	91.66
Silibinin (100 mg/kg) + MCAO	8/12	66.66
Silibinin (200 mg/kg) + MCAO	10/12	83.33

Table 2. Effect of Silibinin on behavioral parameters after MCAO in rats.

Treatment (no of animals used)	Neurological score	Locomotor activity (counts/5 min)
Sham operated (n = 7)	0	130.83±5.73
MCAO (n = 12)	2.83±0.30	27.83±4.28 ^{d, ***}
Standard (Aspirin + MCAO) (n = 12)	0.33±0.21 ^{b, ***}	98.5±5.34 ^{b, ***, e **}
Silibinin (100 mg/kg) + MCAO (n = 12)	1.66±0.2 ^{a, *}	47.33±4.37 ^{c, ***, f ***}
Silibinin (200 mg/kg) + MCAO (n= 12)	1.16±0.30 ^{a, **}	87.16±5.85 ^{a, ***, f ***}

Values are expressed as mean ± S.E.M. Analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post test. ^a = p < 0.05 MCAO vs. Silibinin treated group. ^b = p < 0.05 MCAO vs. standard, ^c = p < 0.05 standard vs. Silibinin treated group, ^d = p < 0.05 sham operated vs. MCAO, ^e = p < 0.05 sham operated vs. standard, ^f = p < 0.05 sham operated vs. Silibinin treated group.

Effect of Silibinin on neurological deficit

The spontaneous motor activity showed no neurological deficits in sham operated rats, while in MCAO group followed by reperfusion for 22 h caused marked change in the behavior of the animals. In spontaneous motor activity test, MCAO animals spend most of the time in the center of the cage with posture curved toward the paretic side. The animals in the vehicle treated group subjected to ischemic reperfusion injury exhibited severe neurological deficit (score: 2.83±0.30), and showed circling towards the contralateral side and had a reduced mobility when compared to the sham operated animals. The rats in the Silibinin 100 and 200 mg/kg treated animals subjected to MCAO showed significant (p < 0.05) improvement in the behavior when compared to the ischemic rats (score: 1.66±0.21 and 1.16±0.30 respectively) (Table 2).

Effect of Silibinin on locomotor activity

In comparison to the sham operated rats, the locomotor activity of the MCAO group rats were significantly decreased (p < 0.05). A dose dependent increase in locomotor activity was observed in Silibinin pretreated groups. Pretreatment with Silibinin 100 mg/kg in ischemic rats showed an increase in locomotor counts, whereas Silibinin 200 mg/kg pretreated ischemic rats exhibited a significant increase (p < 0.05) in the locomotor counts when compared to vehicle treated rats (Table 2).

Effect of Silibinin on tissue Glutathione (GSH) in MCAO rats

The brain glutathione levels were estimated in all the groups. Level of reduced glutathione in MCAO rats were significantly reduced as compared to sham operated rats whereas in all pretreated groups glutathione were significantly increased when compared with MCAO group (Table 3).

Effect of Silibinin on tissue Lipid Peroxidation (LPO) or MDA in MCAO rats

The MDA level measured after 24 h of middle cerebral artery occlusion were found to be significantly increased in the MCAO rats than in normal rats. High dose of Silibinin produced significantly reduction in MDA levels when compared to that of MCAO group (Table 3).

Effect of Silibinin on tissue superoxide dismutase (SOD) in MCAO rats

The level of SOD after 22 h of reperfusion in MCAO occluded group were significantly reduced as compared to the normal rats. In both Silibinin pretreated group, levels of SOD were significantly increased as compared to the MCAO group (Table 3).

Table 3. Effect of Silibinin on brain oxidative and nitrative stress parameters after MCAO in rats.

Treatment (no of animals used)	LPO (nmoles of MDA/mg protein)	CAT (U/mg protein)	SOD (U/min/mg protein)	GSH (nmoles/mg protein)	Nitrite (nmoles/mg protein)
Sham control (n = 7)	2.817±0.13	16.95±1.35	12.06±0.33	3.00±0.080	62.41±0.22
MCAO (n = 12)	4.656±0.38 ^{a,*}	8.51±0.50 ^{a,***}	6.02±0.40 ^{a,***}	1.00±0.069 ^{a,***}	111.71±0.38 ^{a,***}
Standard + MCAO (n = 12)	2.419±0.50 ^{b,*}	13.26±0.20 ^{a,**,b,***}	9.69±0.30 ^{a,***,b,***}	2.35±0.20 ^{a,*;b,***}	55.72±0.12 ^{a,***,b,***}
Silibinin (100 mg/kg) + MCAO (n = 12)	3.966±0.85	9.77±0.32 ^{a,***,c,**}	7.29±0.12 ^{a,***,b,*;c,***}	1.37±0.05 ^{a,***,c,***}	64.23±0.21 ^{a,***,b,***,c,***}
Silibinin (200 mg/kg) + MCAO (n = 12)	2.638±0.10 ^{b,*}	12.46±0.22 ^{a,***,b,**}	9.09±0.20 ^{a,***,b,***}	2.12±0.17 ^{a,***,b,***}	53.46±0.18 ^{a,***,b,***,c,***}

Values are the means SEM. ^ap < 0.05 as compared to sham operated; ^bp < 0.05 as compared to MCAO group; and ^cp < 0.05 as compared to pretreated group with standard + MCAO (repeated measures one-way ANOVA followed by Tukey's test for multiple comparisons).

Effect of Silibinin on tissue catalase (CAT) in MCAO rats

The levels of catalase were reduced in MCAO occluded group as compared to normal rats. All Silibinin pretreated rats showed elevation in the levels as compared to the MCAO group (Table 3).

Effect of Silibinin on tissue nitrite (NO) level in MCAO rats

The NO level measured after 24 h of middle cerebral artery occlusion were found to be significantly increased in the MCAO rats than in normal rats. High dose of Silibinin produced significantly reduction in NO levels when compared to that of MCAO group (Table 3).

Effect of Silibinin on infarct volume in MCAO rats

TTC dye staining of brain slices of all Silibinin pretreated animals showed significant improvement in the infarct volume, aspirin, and Silibinin 200 mg/kg pretreated animals showed a highly significant reduction in infarct volume as compared to MCAO group (Figure 1).

DISCUSSION

In the present study critically evaluated the most abundant naturally occurring flavonoid Silibinin isolated from *S. marianum* as a potential new prophylactic anti-oxidative and anti-apoptotic target in cerebral stroke. MCAO is a classical and well-characterized experimental model of cerebral ischemia. The cytotoxic response occurs within minutes after the onset of cerebral ischemia, which then encompasses proinflammatory response, apoptosis, oxidative stress and neurological damage. Characteristically, ischemic/reperfusion induced brain injury is associated with biochemical, behavioral and histopathological alterations which is seen to be well ameliorated with the pretreatment of Silibinin. Herein, the study observed that Silibinin prevents cerebral ischemia/reperfusion injury by ameliorating oxidative damage. The development of pathogenesis of cerebral ischemia, which is associated with an increased production of free radicals, specifically hydroxyl radical, superoxide, higher lipid peroxidation and lower enzymatic antioxidant defenses (Halliwell 1992; Dringen et al., 2000). The above damages can be prevented by detoxification of free radicals. Beneficial effects of various antioxidants and free radical scavengers

in ischemic investigate the pathology of cerebral ischemia. However, the success behind these models lies only when they can reproduce the end results. Silibinin at dose of 200 mg/kg BW treatment (p.o.) showed significant reduction in brain infarct volume in MCAO induced focal cerebral ischemia model of stroke in rats; and improves brain impairment due to its antioxidant effect. Silibinin at dose of 100 mg/kg also decreases oxidative stress induced lipid peroxidation reaction and increased glutathione peroxidase with increased activity of SOD.

Conclusion

Conclusively, the present study demonstrates that Silibinin possess Neuroprotective effect against ischemia-reperfusion induced brain oxidative damage in rats.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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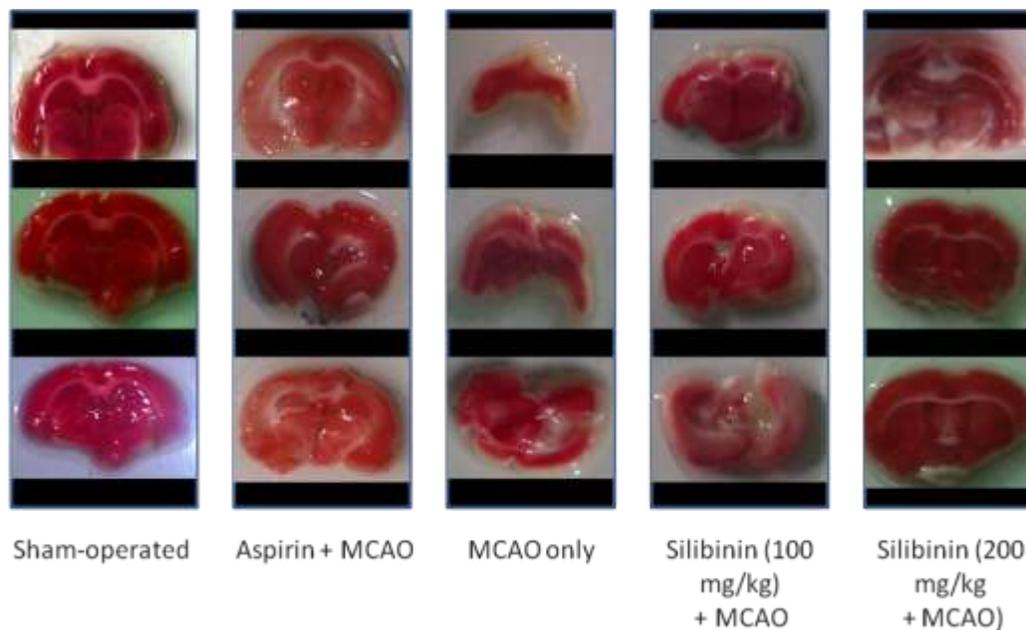


Figure 1. Effect of Silibinin on ischemic rat brains. The infarct volume is significantly reduced in Silibinin (200 mg/kg) group and aspirin group when compared with MCAO only.

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