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Effect of melatonin on ischemia reperfusion injury induced by middle cerebral artery occlusion in rats

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Abstract

Free radicals have been implicated in neuronal injury during ischemia reperfusion in stroke. Therefore, in the present study, melatonin, a potent antioxidant, was studied in male Wistar rats subjected to 2 h of transient middle cerebral artery occlusion. Melatonin (10, 20 and 40 mg/kg i.p.) was administered four times in an animal at the time of middle cerebral artery occlusion, 1 h after middle cerebral artery occlusion, at the time of reperfusion and 1 h after reperfusion. Two hours after reperfusion, rats were euthanized for estimation of oxidative stress markers (malondialdehyde and reduced glutathione). The doses of 20 and 40 mg/kg of melatonin significantly attenuated the raised level of malondialdehyde (287 ± 28, 279 ± 52 nmol/g wet tissue, respectively) as compared to the levels (420 ± 61 nmol/g wet tissue) in vehicle-treated middle cerebral artery-occluded rats. There was an insignificant change in levels of reduced glutathione at these doses (95 ± 42, 88.7 ± 36 μg/g wet tissue, respectively) as compared to those in the vehicle-treated middle cerebral artery-occluded rats (108.21 ± 21 μg/g wet tissue). However, there was an insignificant difference between 20 and 40 mg/kg treated rats. Therefore, the dose of 20 mg/kg i.p. was used to evaluate the neuroprotective effect by using diffusion-weighted imaging (30 min after reperfusion), assessing the neurological deficit (24 h after middle cerebral artery occlusion) and estimating oxidative stress markers (72 h after middle cerebral artery occlusion). In the 20 mg/kg melatonin-treated group, percent ischemic lesion volume on diffusion-weighted imaging was significantly attenuated (9.8 ± 3.9) as compared to that in the vehicle-treated group (21.4 ± 4.7). The neurological deficit was significantly improved in the melatonin group (1.8 ± 0.06) as compared to that in the vehicle-treated (2.9 ± 0.38) group. The level of malondialdehyde (321.4 ± 31 nmol/g wet tissue) and reduced glutathione (142.6 ± 13 μg/g wet tissue) in the melatonin-treated group was also significantly decreased as compared to the level of malondialdehyde (623 ± 22 nmol/g wet tissue) and reduced glutathione (226.6 ± 19 μg/wet tissue) in the vehicle-treated group. The present study indicates that melatonin has a neuroprotective action in focal ischemia, which may be attributed to its antioxidant property.

Keywords: Stroke; Antioxidant; Melatonin; Middle cerebral artery occlusion; (Rat)

1. Introduction

Acute ischemic stroke is a leading cause of death and long-term disability in adults. Treatments for limiting neuronal injury after a stroke have proven difficult to develop, primarily because the pathophysiology involved is not yet well understood. (Read et al., 1999). Several mechanisms of neuronal injury in stroke have been proposed, including increased excitotoxicity, calcium overload, protein inhibition and formation of free radicals (Siesjo, 1992). Ample evidence has accumulated showing that the massive generation of free radicals during reperfusion plays a major role in brain injury associated with stroke (Chan, 1996; Mason et al., 2000).

Free radicals are highly reactive species that promote damage to lipids, DNA, carbohydrates and proteins (Chan, 1996; Lewen et al., 2000). Consistent with this, several agents with free radical scavenging properties, viz. α-phenyl-*n-tert* butyl nitrone (Yang et al., 2000), 2,4-diamino pyrrolopyrimidine (U-101033E) (Schmid-Elsaesser et al., 2000), 3-methyl-1-phenyl-pyrazoline-5-one (MCI-186) (Wu et al., 2000), have been investigated in experimental models and a few, e.g. ebselen (Parnham and Seis, 2000) and a nitrone NXY-059 (Lees et al., 2001), are being evaluated clinically.

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Melatonin, a neurohormone secreted from the pineal gland has potent antioxidant activity (Reiter et al., 1997). Both in vitro and in vivo studies show that melatonin protects cells and tissues against oxidative damage induced by free radical-generating agents (Reiter, 1998). Studies have demonstrated that melatonin scavenges hydroxyl and peroxyl radicals, and in this regard, it is a more potent antioxidant than vitamin E, mannitol and glutathione (Reiter et al., 1997; Hara et al., 1996). Manev et al. (1996) reported that pinealectomized rats were vulnerable to kainate-induced excitotoxicity and photothrombotic brain injury. Further, exogenously administered melatonin ameliorated brain injury after transient forebrain ischemia in rats (Messenge et al., 1998).

Since melatonin readily penetrates the blood–brain barrier and diffuses into neurons and glia equally (Reiter, 1998), it was considered worthwhile to investigate whether melatonin affords protection against ischemic reperfusion injury in rats.

Middle cerebral artery occlusion followed by reperfusion is a model of focal ischemia in rats, which resembles that of human ischemic stroke (Belayev et al.,1996). Reperfusion injury is associated with the generation of free radicals in gerbils, as demonstrated by Yavuz et al. (1997). A recent positive clinical trial with thrombolytic drugs reported that even in patients in whom reperfusion is established, significant necrosis occurs in the ischemic area (Albers, 1999), and it was suggested that pharmacological intervention during reperfusion is a better approach for protecting against neuronal damage. Therefore, in the present study, the effect of acute melatonin treatment was evaluated in the middle cerebral artery occlusion–reperfusion model of acute ischemic stroke in rats.

Since ischemic changes occur rapidly and the neuronal damage needs to be assessed during the early phase, we used diffusion-weighted imaging. Diffusion-weighted imaging is a novel imaging technique based on the principle of magnetic resonance, which has been shown to detect cerebral ischemic lesions in the early phase (i.e. a few minutes after induction of ischemia) (Lo et al., 1997). As no report is available in which diffusion-weighted imaging has been used to evaluate the early changes after melatonin treatment, in the present study, along with diffusion-weighted imaging, neurological deficits and oxidative stress markers were used to illustrate the neuroprotective effect of melatonin in rats.

2. Material and methods

2.1. Animals

Albino male Wistar rats procured from the central animal facility at the All India Institute of Medical Sciences, New Delhi, were group-housed in polypropylene cages ($38 \times 23 \times 10$ cm) with not more than five animals per cage. They were maintained under standard laboratory conditions with a natural dark–light cycle and allowed free access to standard dry rat diet (Golden Feeds, India) and tap water ad libitum. All experimental procedures described in rats were reviewed and approved by the Institutional Animal Ethics Committee.

2.2. Drugs and experimental protocol

Melatonin (courtesy of Dabur India) was freshly dissolved in propylene glycol. It was administered at the doses of 10, 20 and 40 mg/kg i.p. in different groups. In total, four such injections of melatonin were given as follows—at the time of middle cerebral artery occlusion, 1 h after middle cerebral artery occlusion, at the time of reperfusion and the last injection 1 h after reperfusion.

Rats were initially divided into five groups consisting of eight rats each, i.e. sham, vehicle (propylene glycol), vehicle-treated middle cerebral artery occluded group, 10, 20 and 40 mg/kg i.p., melatonin-treated middle cerebral artery-occluded groups. After 2 h of reperfusion, rats were euthanized for estimation of oxidative stress markers (malondialdehyde and reduced glutathione) in whole brain tissue and to select the melatonin dose that would maximally reduce the oxidative stress. Thus, the selected dose was used for detailed evaluation of the neuroprotective effect of melatonin by diffusion-weighted imaging, assessment of neurological function and estimation of oxidative stress markers. Diffusion-weighted imaging was performed 30 min after reperfusion and then the rats were allowed to recover. At 24 h, the neurological deficit was assessed and rats were euthanized at 72 h for estimation of oxidative stress markers in whole brain tissue. Vehicle-treated rats were tested in parallel, using the same experimental protocol.

2.3. Middle cerebral artery occlusion to induce focal cerebral ischemia

Rats were anesthetized with chloral hydrate, 400 mg/kg i.p., dissolved in distilled water. Core temperature (rectal) was maintained around 37 °C throughout the surgical procedure using a heating lamp and the thermocontrolled base of the operating table. A midline incision was made and the right common carotid artery, external carotid artery and internal carotid artery were exposed under an operative magnifying glass. A 4.0 monofilament nylon thread (Ethical, Johnson & Johnson) with its tip rounded by rapid heating, by bringing it near a flame, was used to occlude the middle cerebral artery. The filament was advanced from the external carotid artery into the lumen of the internal carotid artery until resistance was felt, which ensured the occlusion of the origin of the middle cerebral artery. The nylon filament was allowed to remain in place for 2 h, after which it was gently retracted so as to allow reperfusion of the ischemic region (Koizumi et al., 1986).

2.4. Assessment of cerebral infarction

2.4.1. Magnetic resonance imaging (MRI)

Magnetic resonance studies were carried out using an animal MRI/MRI scanner (Bruker, BIOSPEC). Experiments were carried out at 4.7 T, using a 69-mm circularly polarized birdcage volume resonator. The ischemic region was identified by acquiring multislice T_2-weighted pilot images using rapid acquisition with a rapid enhancement sequence (TR = 2000 ms, TE = 25 ms, slice thickness = 2 mm and number of slices = 7). After identification of the site of the infarct and selection of the region of interest, diffusion-weighted images were acquired using stimulated echo diffusion-weighted pulse sequence. Diffusion-weighted images were acquired at three different b values with the following acquisition parameters: TR = 2000 ms, TE = 40 ms, TM = 30 ms, gradient duration = 10 ms, and $b = 25$, 50 and 75 mT/m. The area of ischemic tissue damage was calculated from the diffusion-weighted images as the number of pixels with a hyperintensity of 15% relative to the corresponding anatomic structures in the contralateral hemisphere, and expressed as percent hemisphere lesion area. All seven imaged slices were summed to provide the total volume of ischemic tissue injury.

The threshold value of 15% was found to be the lowest cut-off value that did not include pixels in the contralateral nonischemic hemisphere.

2.5. Neurological evaluation

After 24 h of middle cerebral artery occlusion, the animals were subjected to neurological evaluation using a six-point scale (Tatlisumak et al., 1998) Briefly, scoring was as follows, 0 = no deficits, 1 = failure to extend left forepaw fully, 2 = circling to the left, 3 = paresis to the left, 4 = no spontaneous walking, 5 = death.

2.6. Estimation of markers of oxidative stress

To assess the level of oxidative stress, malondialdehyde and reduced glutathione were estimated after 2 and 72 h of middle cerebral artery occlusion. Simultaneous control experiments were also performed. The rats were decapitated under ether anesthesia and the brains were quickly removed, cleaned by rinsing with chilled saline and stored at 70 °C. The biochemical analysis was performed within 48 h.

2.6.1. Measurement of lipid peroxidation

Malondialdehyde (indicator of lipid peroxidation) was measured as described by Okhawa et al. (1979). Briefly, brain tissues were homogenized with 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4). The reagents, acetic acid 1.5 ml (20%), pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulfate (8.1%), were added to 0.1 ml of processed tissue sample. The mixture was then heated at 100 °C for 60 min. The mixture was cooled with tap water and 5 ml of *n*-butanol/pyridine (15:1% v/v) and 1 ml of distilled water was added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was withdrawn and absorbance was measured at 532 nm using a spectrophotometer.

2.6.2. Measurement of reduced glutathione

Reduced glutathione was measured according to the method of Ellman (1959) with a slight modification. Briefly, brain tissues were homogenized with 10 times (w/v) 0.1 M sodium phosphate buffer (pH 7.4). This homogenate was then centrifuged with 5% trichloroacetic acid to remove protein. To 0.1 ml of this homogenate, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5′5 dithiobis (2-nitrobenzoic acid) and 0.4 ml of double-distilled water was added. The mixture was vortexed and the absorbance was read at 412 nm within 15 min.

2.7. Statistical analysis

The data are presented as means ± S.E.M. Analysis of variance (ANOVA) with post hoc comparison (Bonferroni correction) was used for statistical analysis. In diffusion-weighted imaging, the signal intensity of the right cerebral hemisphere was compared to that of the left hemisphere by unpaired Student's *t*-test. $P < 0.05$ represents a level of significance.

3. Results

3.1. Mortality

No mortality was observed in the sham-operated group ($n = 8$). In the vehicle-treated middle cerebral artery-occluded group, out of 10 rats, seven survived, one died during ischemia and two died immediately after reperfusion. In melatonin-treated group, out of 10 rats, two died during induction of ischemia.

3.2. Effect of graded doses of melatonin on levels of malondialdehyde and reduced glutathione after 2 h of reperfusion

The level of malondialdehyde was significantly higher in vehicle-treated middle cerebral artery-occluded rats (420 ± 61 nmol/g wet brain tissue) after 2 h of reperfusion than in sham-operated rats (194 ± 29 nmol/g wet tissue). In the rats treated with 20 and 40 mg/kg i.p. melatonin, the elevated levels of malondialdehyde seen after middle cerebral artery occlusion were significantly attenuated, the mean malondialdehyde values being 287 ± 28, 279 ± 52 nmol/g wet brain tissue, respectively. In the 10 mg/kg

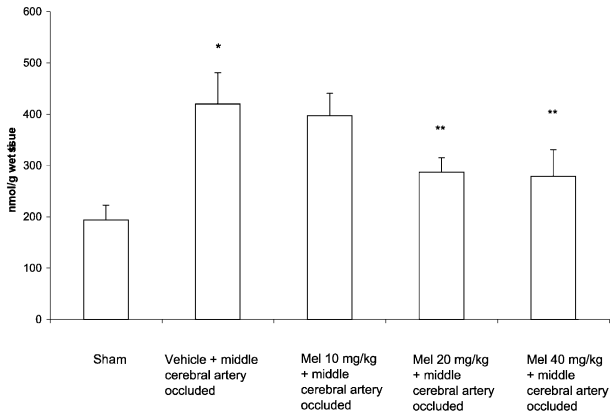

Fig. 1. Effect of different doses of melatonin (10, 20 and 40 mg/kg i.p.) on the levels of malondialdehyde at 2 h after reperfusion in middle cerebral artery-occluded rats. Values are expressed as nmol/g wet tissue (mean ± S.E.M.); $^{*}P < 0.05$ versus sham.

melatonin-treated group, the change in levels (397 ± 44 nmol/g wet tissue) of malondialdehyde was insignificant compared to that in vehicle-treated rats (Fig. 1).

The change in levels of reduced glutathione in vehicle-treated middle cerebral artery-occluded rats after 2 h of reperfusion was insignificant (108.1 ± 21 μg/g wet brain tissue) compared to than in the sham-operated rats (94.2 ± 32 μg/g wet brain tissue) ($P < 0.05$). Similarly, there was no change in the levels of reduced glutathione in all the melatonin-treated middle cerebral artery-occluded groups, the mean values being 98.9 ± 26, 95 ± 42, and 88.7 ± 36 μg/g wet brain tissue, respectively.

3.3. Effect of melatonin (20 mg/kg i.p.) treatment on diffusion-weighted imaging

Focal ischemia was evident in all rats. The ischemic lesion was manifested as increased signal intensity on the diffusion-weighted imaging scans with a high *b* value. The lesions were distributed within the middle cerebral artery vascular territory and included the basal ganglia and the overlying cortex. (Fig. 2A,B). The ischemic lesion area appeared smaller in the melatonin-treated rats than in the vehicle-treated middle cerebral artery-occluded rats (Fig. 2C,D).

The signal intensity of the right hemisphere was compared to that of the contralateral hemisphere in the region of interest. In the ischemic right peripheral parietal cortex as well as the caudate of putamen, there was a significantly (228.7 ± 13.2, 227.5 ± 22.8 arbitrary units) higher intensity than in the contralateral hemisphere (186.2 ± 41.2, 182.6 ± 27.4 arbitrary units) in same slices of brain. In the melatonin-treated group, the signal intensity was not significantly different compared to that of the contralateral side in same slice of brain. The mean signal intensity in the cortex as well as the caudate putamen in the right cerebral hemisphere was 186.3 ± 31.7 and 184.9 ± 28.3 arbitrary units, respectively, and 178.3 ± 11.2, 181.7 ± 29.6 arbitrary units in the contralateral hemisphere.

The percent ischemic lesion volume in vehicle-treated middle cerebral artery-occluded rats after 30 min of reperfusion was 21.4 ± 4.7 by diffusion-weighted imaging. In the melatonin-treated rats, the percent ischemic lesion

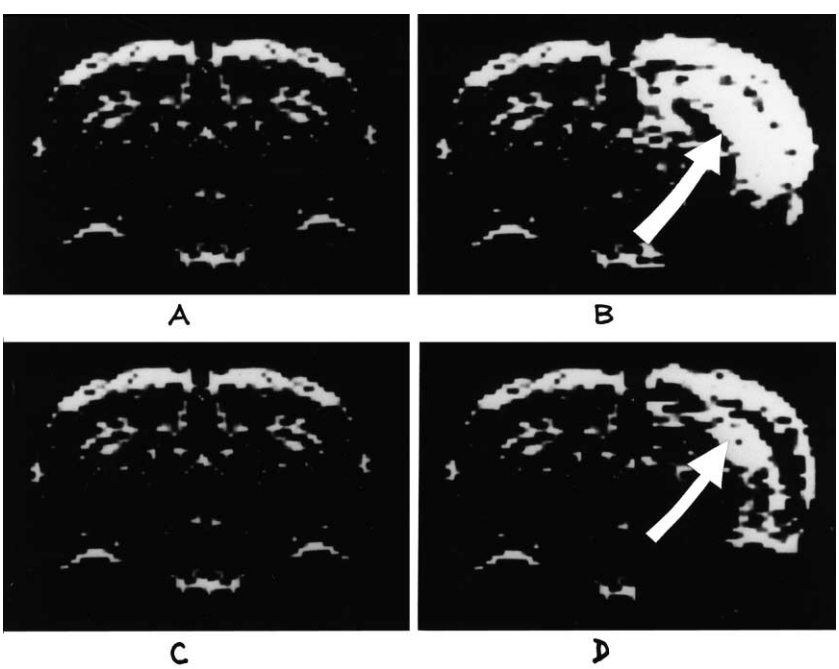

Fig. 2. Diffusion-weighted imaging scans of vehicle-treated middle cerebral artery-occluded rats A (normal), B (30 min after reperfusion) and melatonin-treated rats C (Normal) and D (30 min after reperfusion). Focal ischemia was evident as regions of increased signal intensity in the right cerebral cortex. Melatonin treatment appeared to ameliorate the lesions.

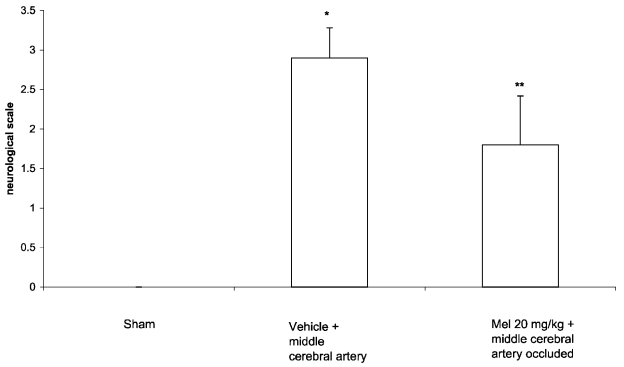

Fig. 3. Effect of melatonin (20 mg/kg i.p.) on neurological function at 24 h after middle cerebral artery occlusion in rats.

volume (9.8 ± 3.9) was significantly less than that in the vehicle-treated rats ($P < 0.05$).

3.4. Neurological evaluation:

The neurological deficit was evaluated after 24 h of middle cerebral artery occlusion. The mean score of middle cerebral artery-occluded rats was 2.9 ± 0.4, indicating that the neurological scores at 24 h after middle cerebral artery occlusion were significantly ($P < 0.05$) better in the melatonin-treated group (1.8 ± 0.06) than in the vehicle-treated group. (Fig. 3).

3.5. Oxidative stress markers

3.5.1. Effect of melatonin (20 mg / kg i.p.) on brain malondialdehyde levels after 72 h of occlusion

The levels of malondialdehyde after 72 h of middle cerebral artery occlusion were found to be significantly higher in the vehicle-treated rats (623 ± 22 nmol/g wet tissue) than in the sham-operated rats (195 ± 26 nmol/g wet tissue) ($P < 0.05$). In the melatonin-treated group, the levels of malondialdehyde were significantly lower (321.4 ± 31 nmol/g wet tissue) ($P < 0.05$) than those in the vehicle-treated group (Fig. 4).

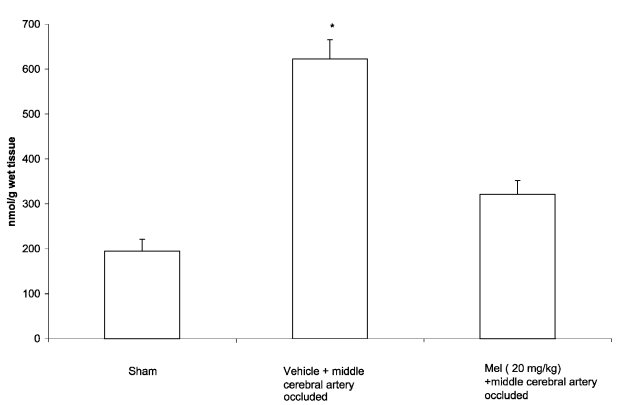

Fig. 4. Effect of melatonin (20 mg/kg i.p.) on the levels of malondialdehyde at 72 h after middle cerebral artery occlusion in rats. Values are expressed as nmol/g wet tissue (mean ± S.E.M.); $^{*}P < 0.05$ sham versus vehicle-treated middle cerebral artery-occluded; $^{**}P < 0.05$ vehicle-treated rats versus melatonin-treated rats.

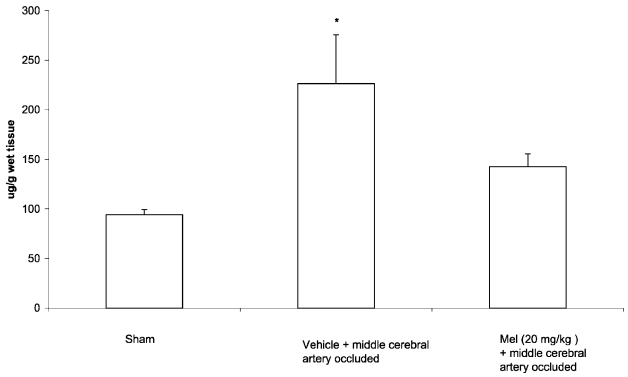

Fig. 5. Effect of melatonin (20 mg/kg i.p.) on the levels of reduced glutathione at 72 h after middle cerebral artery occlusion in rats. Values are expressed as μg/g wet tissue (mean ± S.E.M.); $^{*}P < 0.05$ sham-operated rats versus vehicle-treated middle cerebral artery-occluded rats; $^{**}P < 0.05$ vehicle-treated rats versus melatonin-treated rats.

3.5.2. Effect of melatonin (20 mg / kg i.p.) on brain reduced glutathione levels after 72 h of occlusion

Similar to the malondialdehyde protocol, the brain levels of reduced glutathione were estimated 72 h after middle cerebral artery occlusion in vehicle- and melatonin-treated rats. Levels of reduced glutathione in vehicle-treated middle cerebral artery-occluded rats were significantly higher ($P < 0.05$) than those in the sham-operated rats, the values being 226.6 ± 19 μg/g wet tissue and 94.2 ± 4.9 μg/g wet tissue, respectively. These elevated levels of reduced glutathione were significantly lower ($P < 0.05$) in the melatonin-treated group (142.6 ± 13 μg/g wet tissue) than in the vehicle-treated group (Fig. 5).

4. Discussion

Stroke causes brain injury in millions of people worldwide each year (Read et al., 1999). Despite the enormity of the problem, there is no approved therapy that can reduce infarct size or neurological disability. Recently, both animal as well as human studies have provided evidence that the oxidative damage to membrane lipids and proteins is increased during ischemia and reperfusion (Mason et al., 2000). Exploration of oxidative stress is important to evolve neuroprotective strategies so as to enhance neuronal survival after cerebral ischemia.

Melatonin, the chief secretory product of the pineal gland, possesses free radical scavenging and antioxidant properties. It has been shown to decrease oxidative damage to DNA, proteins and lipids under various pathological conditions (Ikeda and Long, 1990). Recently, melatonin administration has been reported to attenuate kainic acid-induced oxidative damage and also to reduce the brain infarct volume in rats subjected to forebrain ischemia.

Therefore, in the present study, the effect of melatonin treatment was assessed on brain injury induced by middle cerebral artery occlusion followed by reperfusion in rats.

Middle cerebral artery occlusion significantly increased the level of malondialdehyde at 2 and 72 h after reperfusion as compared to level in the sham-operated rats. However, at 72 h, the values were comparatively higher. An increased level of malondialdehyde in the post-ischemic period suggests an increased load of free radicals during reperfusion, which could contribute to, if not be solely responsible for, neuronal injury. Earlier studies have also reported an increased level of malondialdehyde after reperfusion in a model of bilateral carotid occlusion in gerbils (Yavuz et al., 1997). The values of malondialdehyde were higher at 72 h than at 2 h, indicating a progressive generation of free radicals, at least beyond the 2-h period, which is detrimental to the survival of neurons. This finding suggests the possibility of therapeutic intervention by free radical scavengers even in a later stage in stroke.

There was an insignificant change in the level of reduced glutathione at 2 h while there was a significant increase in the level of reduced glutathione at 72 h as compared to levels in the sham-operated rats. Glutathione is an endogenous antioxidant found in all animal cells, and reacts with free radicals and can provide protection from singlet oxygen, hydroxyl radical and superoxide radical damage. The insignificant change at 2 h in levels of reduced glutathione, even though there was increase in malondialdehyde levels, may be attributed to sufficient endogenous capacity of the animal to maintain reduced glutathione levels. However, at 72 h, the sharp increase in reduced glutathione levels could be due to compensatory mechanisms to combat the oxidative stress. Such a compensatory increase in reduced glutathione levels has also reported by Shi et al. (1994).

In rats treated with 20 and 40 mg/kg melatonin, the middle cerebral artery occlusion caused a significantly smaller increase in brain malondialdehyde levels than it did in vehicle-treated rats The lower dose of melatonin, 10 mg/kg i.p., was ineffective as there was an insignificant difference between the melatonin and the vehicle-treated middle cerebral artery-occluded rats. Also, there was an insignificant difference between the effect of melatonin on oxidative stress in the 20 and 40 mg/kg dose-treated groups. Therefore, a dose of 20 mg/kg of melatonin was selected for further studies to evaluate its neuroprotective effect by diffusion-weighted imaging, assessment of neurological deficits and estimation of oxidative stress markers in the same rats.

Diffusion-weighted imaging is sensitive to early changes in ischemia and is superior to routine T1 and T2-weighted imaging, which is unable to detect infarcts during the initial hours after the onset of stroke (Back et al., 1994). Diffusion-weighted imaging reveals early ischemic lesions as regions of increased signal intensity, i.e. decreased water diffusitivity (Lo et al., 1997), and the hyperintense regions demonstrated by diffusion-weighted imaging eventually become infarcted without therapeutic intervention (Li et al., 2000). Evidence has shown that the ischemic hyperintensity is potentially reversible when reperfusion is performed quickly (30 min) after ischemia; however, reperfusion does not reduce the extent of the initial hyperintensity when it is performed 2 h after focal ischemia. It can partially reduce initial diffusion-weighted imaging lesions after 45 to 60 min of transient ischemia (Li et al., 2000; Hasegawa et al., 1994). Persistent areas of diffusion-weighted hyperintensity seen after reperfusion after 2 h of middle cerebral artery occlusion suggest that irreversible changes are caused in this time period, which are not reversed by reperfusion. Moreover, several studies have reported that there is a significant correlation between 2,3,5-triphenyltetrazolium postmortem infarct volume and the diffusion-weighted imaging-derived ischemic lesion volume after reperfusion, which suggests that early in vivo estimation by diffusion-weighted imaging could be used to evaluate the therapeutic efficacy of neuroprotective agents (Minematsu et al., 1992; Tatlisumak et al., 1998).

In the present study, diffusion-weighted images revealed hyperintensity in the lateral caudoputamen and parts of the lower frontoparietal cortex. During middle cerebral artery occlusion, the signal intensity in the right cerebral hemisphere was significantly enhanced. This may be attributed to the failure of energy metabolism, which leads to an influx of Na^+ and osmotically driven water into cells. The shift of water into cell results in restricted diffusion of water protons, leading to hyperintensity on diffusion-weighted imaging confined to the region of the middle cerebral artery (Lo et al., 1997). In melatonin-treated rats, the signal intensity in the right hemisphere did not change as compared to that in the contralateral hemisphere of the same slice. Moreover, the ischemic lesion volume also decreased significantly as compared to that in the vehicle-treated rats. These results might account for the beneficial effect of melatonin in the acute phase of ischemia. The finding is in accordance with the observation of Muller et al. (1996), who reported that U743899, a free radical scavenger structurally similar to tirilazad mesylate, attenuated the enhanced signal intensity and decreased ischemic lesion volume induced by middle cerebral artery occlusion.

Significant impairment in neurological function was observed after middle cerebral artery occlusion, as evidenced by the increased neurological score in the rats. This corroborated clinically the neuronal damage seen in the territory of the middle cerebral artery occlusion, i.e. caudate putamen and cortex areas that control motor function. Melatonin-treated middle cerebral artery-occluded rats showed an improvement in neurological function as compared to the vehicle-treated middle cerebral artery-occluded rats.

The finding suggests that the improvement in neurological function could be due to a decreased volume of the

ischemic lesion in the territory area. This finding is in accordance with a previous study, which reported that exogenous administration of melatonin improved neurological deficits and decreased the volume of infarction in rats with focal ischemia (Kilic et al., 1999).

The level of malondialdehyde was significantly attenuated by melatonin treatment. Cuzzocrea et al. (2000) also reported that melatonin attenuated the increased level of malondialdehyde induced by ischemia reperfusion and thereby showed a neuroprotective effect against transient ischemia in gerbils. Though the level of reduced glutathione was significantly lower in the same rats as compared to the vehicle-treated rats, the level was still higher than in the sham-operated rats, indicating that melatonin has a significant antioxidant property which can be attributed to the higher reduced glutathione levels in melatonin-treated animals as compared to the sham-operated rats. This result is consistent with previous reports that demonstrated that melatonin increased the level of reduced glutathione in the body by its action on rate-limiting enzymes. Apart from its direct free radical-scavenging activity, melatonin also has the capability of increasing mRNA levels or the activity of endogenous antioxidant enzymes (Reiter, 2000). Furthermore, the pro-oxidative enzyme nitric oxide synthase is also inhibited by melatonin (Reiter, 1998). This property affords antioxidant protection by reducing levels of NO, which are highly toxic, during ischemia reperfusion and by lowering the generation of $ONOO^{\cdot}$. These indirect antioxidant actions of melatonin may also be important in providing protection against neuronal injury. In summary, melatonin treatment of middle cerebral artery-occluded rats caused a decrease in oxidative stress, which was associated with a decreased volume of the ischemic lesion on diffusion-weighted imaging, as well as neurological recovery. The antioxidant property of melatonin may be the mechanism involved in neuronal protection.

In recent clinical trials with thrombolytic therapy, e.g. recombinant tissue plasminogen activator, although reperfusion could be established, significant pan necrosis occurred due to sudden oxidative stress. The present study is of clinical significance since melatonin was shown to have a neuroprotective effect against reperfusion injury. Thus, the protection of neurons by melatonin seen after reperfusion indicates the beneficial effect of pharmacological intervention with melatonin. Therefore, neuroprotective treatment with melatonin in combination with thrombolytics may provide a beneficial outcome in the treatment of acute ischemic stroke.

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