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Effects of Melatonin on Oxidative Stress and Spatial Memory Impairment Induced by Acute Ethanol Treatment in Rats

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Summary

Melatonin has recently been suggested as an antioxidant that may protect neurons from oxidative stress. Acute ethanol administration produces both lipid peroxidation as an indicator of oxidative stress in the brain and impairs water-maze performance in spatial learning and memory tasks. The present study investigated the effect of melatonin against ethanol-induced oxidative stress and spatial memory impairment. The Morris water maze was used to evaluate the cognitive functions of rats. Thiobarbituric acid reactive substances (TBARS), which are the indicators of lipid peroxidation, and the activities of antioxidative enzymes (glutathione peroxidase and superoxide dismutase) were measured in the rat hippocampus and prefrontal cortex which form interconnected neural circuits for spatial memory. Acute administration of ethanol significantly increased TBARS levels in the hippocampus. Combined melatonin-ethanol treatment caused a significant increase in glutathione peroxidase activities and a significant decrease of TBARS in the rat hippocampus. In the prefrontal cortex, there was only a significant decrease of TBARS levels in the combined melatonin-ethanol receiving group as compared to the ethanol-treated group. Melatonin did not affect the impairment of spatial memory due to acute ethanol exposure, but melatonin alone had a positive effect on water maze performances. Our study demonstrated that melatonin decreased ethanol-induced lipid peroxidation and increased glutathione peroxidase activity in the rat hippocampus.

Key words

Melatonin • Oxidative stress • Spatial memory • Acute ethanol • Hippocampus

Introduction

Reactive oxygen species (ROS) are involved in several diseases including ischemic injury, aluminum toxicity, Alzheimer's disease, Parkinson's disease and Down's syndrome all of which affect cognitive processes (Poeggeler *et al.* 1993, Reiter *et al.* 1993, Čížová 2004). Therefore several compounds with antioxidative properties are used for the therapy of such neurodegenerative diseases (Poeggeler *et al.* 1993, Patočková *et al.* 2003). Recently, melatonin has been suggested as an antioxidant that may reduce lipid peroxidation, which is an indicator of oxidative stress in the rat brain (Poeggeler *et al.* 1993, Lin and Ho 2000, Pekárková *et al.* 2001, Ortega-Gutierrez *et al.* 2002). Melatonin readily passes all cell membranes, including

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the blood-brain barrier (Reiter *et al*. 1993). It has been shown that melatonin scavenges several free radicals including the peroxyl and hydroxyl radicals. Both these radicals can initiate lipid peroxidation (Poeggeler *et al*. 1993; Reiter *et al*. 2001). Additionally, melatonin also increases the activity of glutathione peroxidase in the brain (Barlow-Walden *et al*. 1995).

Ethanol is able to alter cognitive and behavioral performance in both humans and laboratory animals. One of the principal cognitive effects of ethanol is disruption of learning and memory. Ethanol preferentially impairs hippocampal-dependent learning and memory tasks (Melia *et al*. 1996, Acheson *et al*. 2001). Both ethanol and hippocampal lesions impair water-maze performance on spatial learning and memory tasks (Matthews *et al*. 1999). Therefore, acute ethanol administration is used as a model for spatial memory disorder. However, it was also demonstrated that interactions between the hippocampus and the prefrontal cortex are involved in spatial memory (Stan *et al*. 1997).

Furthermore, acute ethanol administration produces lipid peroxidation, which is an indicator of oxidative stress, in the brain (Renis *et al*. 1996; Somani *et al*. 1996; Mansouri *et al*. 2001). Ethanol can cross cell membranes readily, including the blood-brain barrier. Although ethanol is extensively metabolized in the liver, it has toxic effects in the brain (Mansouri *et al*. 2001). The cytotoxic acetaldehyde produced from oxidation of ethanol can be further oxidized to acetate by acetaldehyde dehydrogenase enzyme, which is present in the brain and is capable of producing ROS (Schlorff *et al*. 1999). It has been demonstrated that ethanol induces synthesis of brain ethanol-inducible cytochrome P450 (CYP2E1). CYP2E1 is present in various brain regions, and it may be an important source of ethanol-induced oxidative stress (Somani *et al*. 1996). It also increases the NADH/NAD ratio, which reduces the ferric ion to ferrous ion, a potent generator of the hydroxyl radical, which can cause lipid peroxidation (Mansouri *et al*. 2001).

Only a few reports have shown the effect of melatonin on brain lipid peroxidation level and/or on antioxidant enzymes activities in ethanol-treated rats (Bondy and Guo 1996, Somani *et al*. 1996, Boveris *et al*. 1997). It is not clear whether acute ethanol administration has an effect on the antioxidant enzymes activities in the hippocampus and the prefrontal cortex. The aims of this study were 1) to investigate the effect of ethanol on superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, and on thiobarbituric acid reactive substances (TBARS) as an indicator of lipid peroxidation, in the prefrontal cortical and hippocampal areas of the rat brains, that form interconnected neural circuits for spatial memory, and 2) to determine the effects of melatonin on ethanol-induced impairment of spatial learning and memory as well as on antioxidant enzymes activities and TBARS levels.

Methods

Animals and treatment

We used 250-300 g male Wistar rats in our study. These animals were kept under standard laboratory conditions with a 12-h light/dark cycle and ad libitum food and water throughout the experiments which were approved by local ethical committee. National Institutes of Health Guidelines for the Care and Use of Laboratory Animals were used for all the experiments in this study.

The animals were randomly divided into four animal groups, with seven rats in each. We performed all experiments between 09:00 and 12:00 h. One group received ethanol (Ethanol group), the second group was treated with melatonin (Melatonin group), the third group received melatonin before the ethanol injections (Melatonin plus ethanol group), and control rats received equivalent volumes of saline and a vehicle (saline containing 0.1 % ethanol) at the respective time-points (Control group). Ethanol and melatonin groups also received an injection of saline or the vehicle at the respective time-points. Ethanol was diluted to 15 % in saline and administrated intraperitoneally (i.p) in a dose of 2.5 g/kg 30 min before the first training session on each day during the spatial memory tasks (Acheson *et al*. 2001). Previously, it has been shown that 2.5 g/kg ethanol results in peak blood ethanol levels in rats 30 min after the intraperitoneal injection (Givens and Breese 1990).

Melatonin (Sigma) was dissolved in ethanol and further diluted in saline; it was administered in a dose of 10 mg/kg i.p. The reason for the application of melatonin 15 min before the ethanol injections was the rapidity of melatonin metabolism (Vakkuri *et al*. 1985).

Morris water maze testing

For the spatial memory, performance in the Morris water maze was evaluated. The experiments were carried out in a circular, galvanized steel maze (2 m in diameter and 75 cm in depth), which was filled with 50 cm deep water kept at 22 °C and rendered opaque by the addition of a non-toxic, water soluble dye. The maze

was located in a large quiet test room, surrounded by many visual cues external to the maze (e.g. the experimenter, ceiling lights, rack, pictures, etc.), which were visible from within the pool and could be used by the rats for spatial orientation. Locations of the cues were unchanged throughout the period of testing. A video camera fixed to the ceiling over the center of the maze was used for recording and monitoring movements of the animals. There were four equally divided quadrants in the pool. In one of the quadrants, a platform (1.0 cm below water surface, 10 cm in diameter) was submerged centrally and fixed in position which was kept constant throughout the acquisition trials. The rats performed five trials *per* day for four consecutive days (20 trials). In the swimming trials, each individual rat was released gently into the water at a randomly chosen quadrant except the one that contained the hidden platform for facing an extra maze cue. The rat swam and learned how to find the hidden platform within 60 s. The escape latency which is the time elapsed before the rat reached the platform in each trial, is a measure of acquisition of spatial navigation. The mean latency of finding the invisible platform was measured for individual animals on each day (mean daily escape latency). After reaching, the rat was allowed to stay on the platform for 15 s and was then taken back into the cage. The rats were placed on the platform by hand for 15 s if they could not escape to the platform within 60 s by themselves, and their escape latency was accepted as 60 s. During the inter-trial intervals, animals were kept in a dry home cage for 60 s.

For assessing the spatial memory, the platform was kept away from the maze for 24 h in the final trial. The probe trial lasted 60 s, each rat was placed into the water as in the training trials and the time in seconds spent in the quadrant formerly occupied by the platform (correct quadrant) were recorded. We applied all injections before the probe trial.

Preparation of tissue extract, enzymes and lipid peroxidation assay

Previous studies have shown that the optimum time for elevation of plasma lipid peroxidation levels is about 1-1.5 h following ethanol exposure (Schlorff *et al*. 1999). The rats were sacrificed by cervical dislocation under ether anesthesia 30 min after the probe trial (60 min after ethanol administration) and the hippocampus and the prefrontal cortex were separated on an ice-cold surface. Tissue homogenates were prepared as described by Carrillo *et al*. (1991). An aliquot of the homogenate and supernatant was stored at –80°C until the determination of TBARS levels, SOD and GPx enzyme activities.

Determination of SOD activity

SOD activity was determined using a RANSOD kit (Randox Labs, Grumlin, UK) (Delmas-Beauvieux *et al*. 1995). To generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol-5) phenyl tetrazolium chloride (INT) to form a red formazan dye. Concentration substrates were used at levels of 0.075 μmol for xanthine and 0.037 μmol for INT. SOD inhibits this reaction by converting the superoxide radical to oxygen. A SOD unit inhibits the rate of reduction of INT by 50 % in a complex system with xanthine and xanthine oxidase. Due to small linearity range of the test, the sample must be diluted so that the percentage of inhibition falls between 30 % and 60 %. Using the standard, provided in the kit, a standard curve was prepared, and the value for the supernatant was determined from this curve. SOD activity in the supernatant was measured at 505 nm on a Shimadzu UV-1201V spectrometer and the results were expressed in SOD U/mg protein.

Determination of GPx activity

GPx was determined using a Randox test combination (RANSEL). GPx catalyses the oxidation of glutathione (at a concentration of 5 μmol/l) using cumene hydroperoxyde according to the method of Paglia and Valentine (1967). In the presence of glutathione reductase (at a concentration $\geq$ 0.75 mU) and 0.35 μmol of NADPH, the oxidized glutathione is immediately converted to a reduced form with concomitant oxidation of NADPH to NAD^+. The decrease in absorbance is measured at 340 nm and 37 °C. The assay was performed on the supernatant. The necessary enzyme activity to convert one μmol of NADPH to NADP in one minute was defined as the GPx unit and the results were expressed as GPx U/mg protein.

Determination of TBARS levels

TBARS level was estimated according to the method of Rehncrona *et al*. (1980). 0.5 ml of homogenates was extracted with 0.5 ml of trichloroacetic acid (20 % w/v). After centrifugation, 1 ml of thiobarbituric acid (0.67 % wt/vol) added to 0.9 ml of supernatant and the samples were heated in boiling water for 10 min. After cooling, the absorbance was recorded at 532 nm. Using 1,1,3,3-tetraethoxypropane, a standard

curve was prepared and the value of the homogenate was determined from this curve. The results were expressed in nmol/mg protein.

Determination of protein concentration

Protein contents of supernatant and homogenate were determined using a U/CSF protein kit (Roche Diagnostics, Germany). The sample was preincubated in an alkaline solution containing EDTA, which denaturates the protein and eliminates interference from magnesium ions. Benzethonium chloride is then added, producing a turbidity that was read at 600 nm.

Statistics

For each parameter of the water maze task, one-way ANOVA measures were used repeatedly, followed by Bonferroni post-hoc analysis. To compare the data on SOD or GPx enzyme activities and TBARS levels, one-way ANOVA and post-hoc Bonferroni tests were used. The results of the experiments were expressed as means ± SEM.

Table 1. The effects of melatonin and/or ethanol treatment on activities of GPx and SOD, and TBARS levels in the rat hippocampus.

	SOD U/mg protein	**GPx U/mg protein**	**TBARS nmol/mg protein**
Control	3.16±0.18	0.014±0.01	0.73±0.02
Ethanol	3.04±0.16	0.013±0.01	1.10±0.03 [a]
Melatonin plus ethanol	3.50±0.09	0.027±0.01 [a,d]	0.69±0.03 [c]
Melatonin	3.61±0.16	0.028±0.01 [b,d]	0.59±0.03 [b,c]

[a] p< 0.001 compared with control group, [b] p< 0.05 compared with control group, [c] p< 0.001 compared with ethanol group and [d] p< 0.05 compared with ethanol group.

Table 2: The effects of melatonin-and/or ethanol treatment on activities of GPx and SOD, and TBARS levels in the rat prefrontal cortex.

	SOD U/mg protein	**GPx U/mg protein**	**TBARS nmol/mg protein**
Control	3.03 ± 0.09	0.012 ± 0.01	0.44 ± 0.02
Ethanol	2.88 ± 0.28	0.012 ± 0.01	0.49 ± 0.02
Melatonin plus ethanol	3.55 ± 0.15	0.013 ± 0.02	0.41 ± 0.02 [d]
Melatonin	3.18 ± 0.2	0.015 ± 0.01	0.44 ± 0.01

[d] p< 0.05 compared with ethanol group.

Table 3A: The effects of melatonin treatment on spatial learning in the Morris water maze.

	Day 1	**Day 2**	**Day 3**	**Day 4**
Control	44.69±4.83	34.51±2.32	32.66±3.94	27.26±2.78
Ethanol	58.14±1.86	59.28±0.71 a	56.83±0.21 a	58.74±0.94 a
Melatonin plus ethanol	58.74±0.81b	59.5±0.01 a	59.63±0.37 a	59.8±0.01 a
Melatonin	52.40±3.4	33.46±3.67 c	20.56±1.49 b,c	17.29±2.07 b,c

Ethanol-treated group had longer escape latencies at the second, third and fourth days of training trials than control group. ([a] P<0.001). Melatonin-treated group had shorter escape latencies at the third and fourth days of training trials compared with control group ([b] P<0.05) and melatonin plus ethanol group ([c] P<0.001).

Table 3B: The effects of melatonin treatment on the time spent in the correct quadrant in the probe trial at the fifth day.

	Time in the correct quadrant (s)
Control	23.26 ± 0.96
Ethanol	9.09 ± 1.35 [a]
Melatonin plus ethanol	2.21 ± 0.70 [a,d]
Melatonin	31.17 ± 1.17 [b,c]

The time spent in the target quadrant was significantly less in ethanol-treated group than the control group ([a] $P<0.001$). Melatonin-treated group had spent longer time in the target quadrant in the probe trial than the control group ([b] $P<0.01$) and ethanol group ([c] $P<0.001$). Melatonin-Ethanol treatment had spent very shorter time in the correct quadrant in the probe trial than the control ([a]$p<0.001$) and ethanol group ([d]$p<0.05$).

Results

Table 1 presents the GPx and SOD activities and TBARS levels in the rat hippocampus. Acute administration of ethanol significantly increased TBARS levels in the hippocampus compared to control rats ($p<0.001$). Combined melatonin-ethanol treatment caused a significant decrease of TBARS levels in the hippocampus as compared to the ethanol group ($p<0.001$). Melatonin administration alone also decreased TBARS levels in the hippocampus when compared to the control group ($p<0.05$). Melatonin plus ethanol-treated rats showed a significant increase in hippocampus GPx activities as compared to the ethanol group ($p<0.05$) and control group ($p<0.001$). There was also a significant increase in the hippocampal GPx activity in the melatonin-treated group as compared to control group ($p<0.05$). SOD activity in the hippocampus was not changed by ethanol or melatonin treatment (Table 1).

As shown in Table 2, there was only a significant decrease in TBARS levels in the combined melatonin-ethanol treated group as compared to the ethanol group in the rat prefrontal cortex ($p<0.05$).

Table 3A presents the mean daily escape latency data. Ethanol impaired the acquisition of spatial memory. Melatonin administration prior to ethanol did not improve ethanol-induced spatial learning impairment. However, there were no significant differences in escape latency throughout the training period between ethanol and ethanol plus melatonin treated rats. The mean latency to find the platform declined progressively during the training period on four consecutive days in control and rats treated with melatonin alone. However, the melatonin administered alone significantly shortened the mean latency on the third and fourth days of training trials compared to the control group ($p<0.05$) (Table 3A).

The time spent in the correct quadrant was used for evaluating spatial memory in probe trials (Table 3B). Ethanol-treated animals spent significantly less time than the control group ($p<0.001$). Melatonin plus ethanol treatment did not change this deficit as compared with the ethanol-treated group. However, the melatonin-treated animals spent more time in the correct quadrant compared to the control group ($p<0.01$) (Table 3B).

Discussion

Our results indicate that melatonin decreases ethanol-induced lipid peroxidation and increases GPx activities in the rat hippocampus. Melatonin had a potent protective effect against ethanol-induced lipid peroxidation due to increased GPx activities in the rat hippocampus. Melatonin had a positive effect on spatial learning and probe trial performance when used alone; on the other hand, melatonin-ethanol co-administration did not reverse ethanol-induced memory impairment in the Morris water maze.

Acute ethanol administration leads to oxidative stress in the brain (Renis *et al*. 1996, Somani *et al*. 1996, Mansouri *et al*. 2001). Results from previous studies concerning the effect of acute ethanol administration on antioxidant enzyme activities in rat brain were conflicting. Acute ethanol administration did not change antioxidant enzyme activities in the rat brain (Boveris *et al*. 1997). Ethanol increased SOD activity in the cerebral cortex and increased GPx activities in the hypothalamus and striatum (Somani *et al*. 1996). Dietary administration of ethanol to rats for 2 weeks decreased SOD activities in several brain regions except the hippocampus and prefrontal cortex (Bondy and Guo 1996). These discrepancies result from variations in experimental species, doses and protocols of ethanol administration. We have shown that ethanol caused an increase of TBARS levels and was not able to change SOD and GPx activities in the hippocampus when compared to control rats. In the prefrontal cortex, ethanol did not change antioxidant enzymes and TBARS levels. The hippocampus is a brain area particularly vulnerable to ethanol-induced oxidative stress (Renis *et al*. 1996), so that ethanol may cause more severe change in the hippocampus than in the prefrontal cortex. Only a few reports have shown an effect of melatonin on brain lipid peroxidation levels and/or antioxidant enzymes activities in ethanol-treated rats. Melatonin prevents lipid

peroxidation resulting from chronic ethanol exposure in whole brain (El Sokkary *et al*. 1999) and also prevents forebrain lipid peroxidation in chronically ethanol-treated rats (Raghavendra and Kulkarni 2001). We have shown that melatonin-ethanol co-administration increased GPx activities and decreased TBARS levels in the hippocampus compared to ethanol-treated rats. To our knowledge, this is the first report indicating that melatonin decreases ethanol-induced lipid peroxidation and increases GPx activities in the rat hippocampus. Melatonin administration alone increased GPx activity in the hippocampus compared to control rats. Melatonin application before ethanol decreased TBARS levels without changing antioxidant enzymes in the prefrontal cortex, compared to the ethanol group. The concentration of melatonin is higher in brain ventricles than in the peripheral plasma following its exogenous administration (Reiter *et al*. 2001). Because of its proximity to the ventricles, the hippocampus is one of the most peculiar brain structures, which may be susceptible to the action of melatonin (El Sherif *et al*. 2002). This may explain the reason why melatonin is more effective on the hippocampus than on the prefrontal cortex after acute ethanol exposure in our study.

In this study, acute dose ethanol (2.5 g/kg) impaired spatial learning in the water maze, and the results were in conformity with the other study, which had demonstrated the acquisition of spatial learning impairment after the same dose of ethanol exposure (Acheson *et al*. 2001). Although we did not test the motor performance of animals in our study, previous studies reported no significant differences in motor performance between rats receiving 2.5 g/kg ethanol or saline-treatment (Little *et al*. 1996, Acheson *et al*. 2001). It has only been suggested that melatonin improved chronic ethanol-induced amnesia (Raghavendra and Kulkarni 2001). Melatonin administration prior to ethanol had no effect on ethanol-induced spatial learning impairment; meanwhile, while the group treated with melatonin only displayed significantly shorter escape latencies, compared to the control rats. During the probe trial, the ethanol-treated and melatonin plus ethanol-treated rats had no spatial bias towards the previous position of the platform. This indicates that these rats never learned the location of the hidden platform during the acquisition of spatial memory and thus had no spatial memory of the target location to recall in the probe trial. However, the only melatonin-treated group showed a clear spatial bias towards the training quadrant during the probe trial as compared to control rats. These results suggest that the melatonin treatment *per se* had a positive effect on water-maze performance. This is consistent with the other study, which has suggested that melatonin facilitated short-term memory (Argyriou *et al*. 1998). One of recent studies suggested that melatonin could be involved in structural remodeling of synaptic connections during memory and learning processes (Baydas *et al*. 2002). Another recent publication has also suggested that melatonin may influence memory formation in the hippocampus (El Sherif *et al*. 2003). In our study, rats which had been treated with melatonin plus ethanol had the worst performance in the water-maze tasks. We observed a tendency to sleep and sedation only in rats which had been treated with melatonin plus ethanol. Melatonin has sedative and analgesic effects in rats receiving high dose (Golombek *et al*. 1996, Pekárková *et al*. 2001). A single melatonin injection (25 mg/kg) increased significantly GABA turnover in the hypothalamus, while a dose of 100 mg/kg was needed to affect GABA turnover in the cerebral cortex (Golombek *et al*. 1996). Ethanol also enhanced activities of the GABAergic system (Wan *et al*. 1996). Melatonin may potentiate ethanol sedation in the co-administrated group (melatonin plus ethanol group) so that this group shows the worst water-maze performance.

In conclusion, this study demonstrated that melatonin decreases ethanol-induced lipid peroxidation and increases glutathione peroxidase activity in the rat hippocampus. ROS seem to play an important role in ethanol-induced neurotoxicity and melatonin protects neurons from acute exposure to ethanol-induced oxidative stress in the rat hippocampus. However, in the present study, melatonin had no effect on spatial memory impairment due to acute ethanol exposure; on the other hand, melatonin had a positive effect on water maze performances when used alone. The results of our study indicate that acute ethanol-induced memory deficit appears to be without any relation to oxidative stress.

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