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Research report

Melatonin prevents oxidative damage induced by maternal ethanol administration and reduces homocysteine in the cerebellum of rat pups

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HIGHLIGHTS

- Ethanol exposure during pregnancy increased homocysteine levels in pups' cerebellum.
- Increase of homocysteine could induce oxidative stress in pups' cerebellum.
- Ethanol toxicity in part is related to increase of cerebellar homocysteine levels.
- Melatonin had neuroprotective effect against ethanol toxicity in cerebellum.

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ABSTRACT

Chronic alcoholism leads to elevated plasma and brain homocysteine (Hcy) levels, as demonstrated by animal experiments. This study was designed to evaluate the alterations in offspring rat cerebellum following increase of plasma Hcy level induced by maternal exposure to ethanol and to investigate the possible protective role of melatonin administration upon cerebellar ethanol-induced neurotoxicity. The adult female rats were divided randomly into 4 groups, including one control and three experimental groups, after vaginal plagues. Group I received normal saline, group II received ethanol (4 g/kg), group III received ethanol + melatonin (10 mg/kg) and group IV received melatonin on day 6 of gestation until weaning. 21 days after birth, plasma Hcy level, level of lipid peroxidation, the activities of several antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and levels of bcl-2 and bax mRNA expression in cerebellum were determined. Our results demonstrated that ethanol could induce lipid peroxidation, and decrease antioxidants activities and increase plasma total Hcy level. We also observed that ethanol impaired performance on the rotarod and locomotor activities of rats. However, treatment with melatonin significantly attenuated motoric impairment, the lipid peroxidation process and restored the levels of antioxidant activities and significantly reduced plasma total Hcy levels. Moreover, melatonin reduced bax/bcl-2 ratio in the presence of ethanol.

We conclude that these results provide evidence that ethanol neurotoxicity in part is related to increase of plasma Hcy levels and melatonin with reducing of plasma Hcy level has neuroprotective effects against ethanol toxicity in cerebellum.

1. Introduction

Alcohol exposure of the human fetus during pregnancy is responsible for permanent disorders referred to as fetal alcohol syndrome (FAS) [1]. FAS are characterized by physical malformations, behavioral deficits, and mental retardation [2]. Some brain areas, which have been associated with the behavioral deficits, notably the hippocampus, cortex and cerebellum, are very sensitive to alcohol toxicity [3]. The cerebellar cortex of new-born rat is a well-suited model to investigate the effects of neurotoxic or neuroprotective molecules in as much as it exhibits a rather simple structure with four well defined layers and its development are mainly postnatal [4].

From a cellular and molecular perspective, DNA damage and shrinkage or atrophy of several brain regions, particularly the hippocampus and cerebellum, are the major consequences for patients suffering from fetal alcohol spectrum disorder [5–8].

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A variety of mechanisms have been proposed for ethanol neurotoxicity; it is generally accepted that oxidative stress is a major one [9,10]. Ethanol readily crosses the blood–brain barrier and is metabolized in the brain by enzymes, such as catalase, alcohol dehydrogenase, or ethanol-inducible cytochrome P450. This process produces reactive oxygen species (ROS) which includes superoxide free radicals, hydrogen peroxide, and hydroxyl radicals [10]. Disturbance of cellular normal redox state by excessive ROS leads to oxidative stress which causes cellular damage [11]. The CNS is particularly susceptible to oxidative stress due to its high oxygen consumption rate, elevated levels of polyunsaturated fatty acids, and relatively low content of antioxidative enzymes [12].

Previous study in our laboratory demonstrated the role of oxidative stress in alcohol-induced developmental neurotoxicity in cerebellum [13]. The sulfur-containing amino acid homocysteine (Hcy) elicits oxidative neurotoxic effects during alcoholism [14,15] and chronic alcoholism in humans is associated with the development of hyperhomocysteinemia [16–18]. Also, Shirpoor and colleagues have reported that hyperhomocysteinemia-induced oxidative stress plays a crucial role in pathogenesis of fetal alcohol syndrome and ameliorates by vitamin E supplementation [19]. The adverse effects of elevated Hcy levels on the developing brain have been well documented. Hcy has now been implicated in increased oxidative stress, DNA damage, the triggering of apoptosis and excitotoxicity, all important mechanisms in neurodegeneration [20].

Tremendous research efforts has been made to identify potential neuroprotective agents that can ameliorate ethanol-induced developmental CNS damage, so targeting ethanol-induced ROS and oxidative stress would be a logic preventative approach.

Melatonin, which is secreted by the pineal gland, is a powerful scavenger of oxygen free radicals, hydroxyl radicals and peroxyl radicals [21–23] and it is a highly effective antioxidant. Melatonin is a scavenger of both oxygen- and nitrogen-based reactive molecules, including peroxynitrite anion (ONOO-) and its decomposition products, including hydroxyl radical ($OH^{\bullet}$), nitrogen dioxide (NO_2), and carbonate radical ($CO_3^{\bullet -}$) [24]. Besides its ability to direct scavenge radicals and radical products, melatonin also augments the activities of antioxidative enzymes, including GPx, SOD and glutathione reductase [21,24–26]. Melatonin (10 mg/kg) administration to pregnant rats has been reported to increase antioxidant enzyme activities such as SOD and GPx and to protect against oxidative mitochondrial damage in the fetal rat brain [27,28]. On the other hand, Murawska-Cialowicz reported that plasma homocysteine level significantly decreased in melatonin administration together with a methionine rich diet in rats [29]. Also, Baydas and colleagues have indicated that there is a link between melatonin and homocysteine. They reported that homocysteine levels are increased due to lack of melatonin in pinealectomized rats [30].

Thus, this study was designed to evaluate the alterations in offspring rat cerebellum following increase of plasma Hcy level induced by maternal exposure to ethanol and to investigate the possible protective role of melatonin administration upon cerebellar ethanol-induced neurotoxicity, using behavioral, histological, and biochemical and molecular analyzes.

2. Materials and methods

2.1. Drugs and chemicals

Absolute ethanol, melatonin, 2-Thiobarbituric acid (TBA), 1.1.3.3 tetramethoxypropan, nitro blue tetrazolium (NBT), trichloro acetic acid (TCA), were all purchased from Sigma–Aldrich chemicals.

2.2. Experimental animals and treatment

The experimental protocol was approved by the Research and Ethics Committee of Damghan University. Adult female and male Wistar rats were obtained from the breeding colony of the Pasture Institute of Iran. They were housed in a temperature and light controlled room under a 12/12 h light/dark cycle with food and water provided ad libitum. After one-week acclimatization in the laboratory conditions, female rats were housed overnight with males and checked on the following morning for the presence of copulation plugs. The day at which a vaginal plug found was used to define the beginning of gestation (day 0). Pregnant females were individually housed in plastic cages. Pregnant rats were randomly divided into four different treatment groups of 5 rats: control, ethanol, ethanol + melatonin and melatonin groups.

Control group were given vehicle only (normal sterile saline). Rats in ethanol group received 4 g/kg of ethanol solution in saline (40% v/v) by oral gavages once per day [19]. In the ethanol + melatonin group, rats received melatonin (10 mg/kg) through oral gavage at 16:00 h in addition to ethanol exposure. Melatonin group received melatonin (10 mg/kg) through oral gavage at 16:00 h once per day.

All groups were treated from the 6th day of gestation until weaning (Postnatal days 21). Melatonin dosage was selected on the basis of earlier report which has demonstrated its neuroprotective effects in rats [31–33]. Melatonin was dissolved in ethanol and further diluted in saline. The final concentration of alcohol was <2%. At birth, eight pups were left with each dam. Whenever possible, only male rats were kept within the litters and females were kept only if necessary to maintain equal litter sizes. Pups were not directly exposed to ethanol. On postnatal day 21, 5–8 pups (per group) were used to evaluate plasma Hcy levels, malondialdehyde (MDA) levels, SOD, catalase (CAT), GPx activities and determination of bax and bcl-2 mRNA expression and the other 10 pups (per group) were used for behavioral and histological evaluation on Postnatal days (PD) 31 and 33, respectively. The design of experiment is shown in Fig. 1.

2.3. Hcy concentrations

EDTA plasma samples were used for the determination of Hcy. Total plasma Hcy levels were determined with enzyme immunoassay kit (Axis-Shield AS, Oslo, Norway).

2.4. Behavioral study

2.4.1. Open field test

Pups were individually placed at the center of a clean open field apparatus (40 cm × 40 cm × 15 cm, divided into nine squares). Prior to the evaluation, animals were habituated to the box for 1 min within the box. The observed parameter, number of squares crossed (locomotor activity), rearing (number of times the rat stood completely erect on its hind legs) and occurrences of grooming (number of times the rat scratched its face with its forepaws) were recorded for 5 min by two blind observers.

2.4.2. Accelerating rotarod assay: a motor performance test

Rotarod test, which measures balance, coordination, and motor control, was used to evaluate motor performance. The rotarod apparatus (Hugo Basil, Biological Research Apparatus, Italy) consists of a suspended rod able to run at constant or at accelerating speed. Each pup was placed on a rod covered with rubber to evaluate rotarod performance. The accelerating rotarod set to accelerate gradually from 4 to 40 rpm for each trial. The starting speed was 4 rpm, and the total time of each trial was 300 s. On a given trial, four pups were placed on the cylinder, one pup in each compartment.