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Research Report

The Effect of Intrauterine Acute Ethanol Exposure on Developing Sciatic Nerves and Their Myelination: A Stereological Study

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ABSTRACT

Ethanol (ETH) delays myelination in the sciatic nerve of rat following fetal exposure. It has been suggested that melatonin (MLT), a naturally occurring indole, may be a potential protective agent for ETH toxicity, possibly due to its protective effects against free radical damage in experimental models. However, the precise mechanism of MLT protection in the development of the rat sciatic nerves and their myelination is unclear. In this study, the effect of ETH, MLT and ETH+MLT on the myelin thickness, axon number and axonal area of rat sciatic nerve were investigated. Pregnant rats were injected with either 25% ETH (dosage 2g/kg body weight), 25% ETH plus MLT (dosage 2g/kg and 10 mg/kg, respectively), MLT (dosage 10 mg/kg) or physiological saline (dosage 1 ml/kg) at 7, 15 and 20 days post-conception (first, second and third trimesters, respectively). At postnatal day 14, sciatic nerves of offspring were dissected out and processed for stereological analyses. Our results demonstrated that the myelin thickness was increased by exogenous MLT, and decreased by acute ETH, during first and second trimesters. It was found that MLT was protective to developing myelin thickness following fetal exposure during the first trimester, whereas it was toxic to myelin thickness during the third trimester. The presented study is the first stereological study indicating that exogenous MLT has a toxic effect on the myelin thickness of rat sciatic nerves during the third trimester. Based on our findings, we suggested that exogenous MLT, given during the third trimester as an antioxidant agent, as well as acute ETH, given in the first and second trimesters, may alter the normal development of sciatic nerve myelination.

1. Introduction

Several toxic agents, including various chemicals and drugs, are known to induce certain malformations of the central nervous system (CNS) and/or peripheral nervous system (PNS), and subsequently, cause alterations in cognitive function of exposed offspring (Kodituwakku et al., 2001). Specifically, reactive oxygen species (ROS) are implicated in various toxic injuries, including acute and chronic alcohol toxicity, because increased levels of ROS cause apoptotic cell death (Das and Vasudevan, 2007; Koh et al., 2003). Histologically, a delayed myelination and lamination in the cerebral cortices of rats following fetal ethanol (ETH) exposure has been reported (Das and Vasudevan, 2007; Hofteig and Druse, 1978; Lancaster, 1994; Lancaster et al., 1984) Several researchers have shown that the effect of ETH on whole brain myelin of the rat offspring could be minimized to some extent, although not completely eliminated, by increasing caloric and protein intake of the mothers during gestation (Hofteig and Druse, 1978; Lancaster, 1994; Lancaster et al., 1984)

Melatonin (MLT), N-acetyl-5-methoxytryptamine, is a small lipid and water-soluble indoleamine molecule which can easily cross membrane barriers (Jan et al., 2007). It is a product of tryptophan metabolism in the pineal gland, and is a potent free radical scavenger and antioxidant (Reiter, 1998; Tan et al., 2000; Tunc et al., 2007). It has the ability to protect DNA against oxidative damage and may also have strong anti-inflammatory effects (Genovese et al., 2005). In recent years, MLT has been indicated as having a neuroprotective effect upon a variety of neuropathological events such as ischemia, infarction, and edema formation (Pei et al., 2002; Reiter et al., 2001; Turgut et al., 2005a; Turgut et al., 2005b). Based on these data, it was expected that MLT, given during the prenatal period, may ameliorate the developmental damage to the PNS, and the disruption of nerve myelination, caused by ETH toxicity. There have been only a limited number of studies related to the protective effect of MLT on ETH toxicity in the developing PNS. In this study, we aimed to investigate the postnatal toxic effects of acute ETH and the ameliorative action of exogenous MLT on ETH toxicity following prenatal administration. For this purpose, myelin thickness, axon number and axon area of the sciatic nerve were evaluated, using quantitative stereological methods.

2. Material and Methods

2.1. Animals

In this study, male and female Wistar albino rats, weighing between 200-250 g, were obtained from the Surgical Research Center of Adnan Menderes University, Aydın, Turkey. They were mated overnight in separate standard plastic cages, maintained in our laboratory under controlled environmental conditions (an air-conditioned room, room temperature 20 ± 1 ºC and under a 12 h light/dark cycle) and fed ad libitum. Female rats were accepted as pregnant rats when a vaginal plug was found. After mating day, female rats were randomly divided into three main groups as first trimester (between 0 and 7 days), second trimester (between 8 and 15 days) and third trimester (between 16 and 21 days). Each main group divided into four subgroups as ETH, ETH+MLT, MLT and control. Therefore, a total of 12 groups of pregnant rats was included in this study and each group consisted of two pregnant rats (n=24). They were maintained in separate standard plastic cages during pregnancy and during the experiment under the same laboratory conditions as described above. During the first, second and third trimesters, one dose of 2g/kg, 25% of ETH (v:w), one dose of 2g/kg, 25% of ETH (v:w) with 10 mg/kg MLT, one dose of 10 mg/kg MLT and 1 ml/kg physiological saline were administered by means of intraperitoneal (i.p.) injection for ETH, ETH+MLT, MLT and control groups pregnant rats, respectively. The injections were applied in the last days of the trimesters (i.e., in the 7th, 15th, and 21st postconceptional days for first, second and third trimester groups, respectively). After spontaneous delivery, offspring were obtained and the day of birth was designated as PND 0. They were marked by branding on their body and again by coloring with picric acid, and fed for 2 weeks.

Thus, the offspring consisted of the same three main groups as the first, second and third trimester groups, and each trimester was divided into four subgroups as follows: control group (n=5) consisted of offspring from mothers injected with 1 ml/kg serum physiologic saline during their gestation; ETH group (n=5) consisted of offspring from mothers injected with 2g/kg, 25% of ETH (v:w) during their gestation; ETH+MLT group (n=5) consisted of offspring from mothers injected with 2g/kg, 25% of ETH (v:w) plus 10 mg/kg MLT during their gestation; and MLT group (n=5) consisted of offspring from mothers injected with 10 mg/kg MLT during their gestation. Thus, there were 60 offspring (each main group had 20 offspring) in this study.

All experiments and procedures described in the present study were conducted according to institutional guidelines. The Local Ethics Committee on the Use of Live Animals Research of University approved the protocol and appropriate measures were taken to minimize pain or discomfort. Additionally, all efforts were made to minimize suffering and the number of animals used.

2.2. Tissue processing

All offspring were anaesthetized with urethane (1.25g/kg) and perfused through the left cardiac ventricle with 10% neutral-buffered formalin at PND 14. Surgical procedures were performed with the animal in the prone position, using the sciatic nerves of the right limbs. The right limb of the rat's gluteal region was shaved and cleaned with povidone-iodine solution. The sciatic nerves from the sciatic notch to the point of bifurcation were dissected out by means of an oblique gluteal skin incision and a muscle splitting incision. Then, 10-mm nerve segment of the sciatic nerve were removed for analyses. Rats were sacrificed with an overdose of the anesthetic at the end of the experiment.

Dissected sciatic nerves tissues were embedded, the thin sections of these nerves were taken on grids, and they were stained for electron microscopic examination. For this aim, the nerves were stretched to in situ length by being pinned to a card and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) (for 4 to 6 hours, in 4°C). Later, the nerves were rinsed twice in phosphate buffer (pH 7.4) and post fixed in 1% osmium tetroxide (for 2 hours), dehydrated in an ascending alcohol series, and placed in propylene oxide (for 16 minutes). After these procedures, the tissues were embedded in Epon Embedding Kit (Fluka Chemie Gmbt, Switzerland). An

ultramicrotome (Leica RM 2155; Leica, Nubloch, Germany) was used to prepare semithin sections (1-µm thick). Sections were stained with 1% toluidine blue for stereological analysis.

2.3. Stereological analyses

Sciatic nerves sections were evaluated according to stereological principles described earlier (Geuna et al., 2001; Kaplan et al., 2001; Kaplan et al., 2005). A stereologic workstation with a CCD (charge-coupled device) digital camera (JVC, Tokyo, Japan), a personal computer with an image capture card (FlashPoint 3D, Integral Technologies, Indianapolis, Ind, USA), a monitor (Hyundai, Seoul, South Korea), a computer-controlled motorized specimen stage (Prior Scientific, Rockland, Mass, USA), a microcator (Heidenhain, Traunreut, Germany), and a light microscope (Leica DMR; Leica Microsystems, Inc., Wetzlar, Germany) were used for stereological analyses. The CAST-GRID (Computer Assisted Stereological Toolbox; Olympus, Copenhagen, Denmark), a software program, was used to control, measure, and record stereological data and to capture digital images of all sections. This system reproduced microscopic images (obtained through a 100X oil immersion objective, numeric aperture, 1.40) on the computer monitor at a final magnification of 5140X, which allowed accurate recognition and counting of myelin thickness, axon area and axon numbers from nerve fibers. An unbiased counting frame with an area of 59.79 µm2 was used to obtain an estimate of the total axon numbers from nerve cross-sections in an unbiased manner.

A counting frame was placed onto sections in a systematic, uniform, random manner, and appropriately sampled nerve fibers were counted. Meander sampling of sectioned nerve profiles was performed in 40 X 40-µm, successive, systematic, random steps. This ensured that all locations within a nerve cross-section were equally represented and all axon profiles were sampled with an equal probability, regardless of shape, size, orientation, and location. Data were entered into a Microsoft Excel (Microsoft, Seattle, Wash.) file for subsequent processing in spreadsheets.

Estimation of the mean myelin sheet thickness and axon area of the sciatic nerves was performed on the sampled sections. Another stereological workstation that was composed of a CCD digital camera, image capture card (Flash Point 3D, Integral Technologies, Indianapolis, Ind.), personal computer, and computer-controlled motorized specimen stage (Prior Scientific, Cambridge, United Kingdom), a microcator (Heidenhein Traunreut, Germany) and a light microscope (Leica, Wetzlar, Germany) were used for stereological analyses of myelin thickness and axon cross sectional area. A software program (CAST-GRID®-Computer Assisted Stereological Toolbox-Olympus, Copenhagen, Denmark) was used to control, measure and record stereological data and to capture digital images of the sections. This system reproduced microscopic images on the computer monitor at a final magnification that allowed accurate recognition and quantifying the myelinated nerve fibers. A two-dimensional isotropic uniform random nucleator (Geuna et al., 2001; Larsen, 1998) was used for estimation of cross sectional axon area and the thickness of myelin sheet using an oil objective (100x, NA 1.35) at a final magnification of 5111. Meander sampling of each sectioned nerve profiles was done over successive, systemic-random steps of 55x55, step size. A two-dimensional nucleator at isotropic uniform random positions was used for estimation of axonal areas and the thickness of myelin sheet using an oil objective (100x, NA 1.35).

2.4. Statistical analyses

For axon numbers, parametric test assumptions were not available. Because distributions of the variables were not normal and variances were not homogeneous, we used the Friedman test for dependent group comparisons and a Kruskal-Wallis one-way analysis of variance by ranks test for independent group comparisons. Results have been expressed as number of observation (n), Mean ± SD and median (M).For myelin thickness and axon area, a Shapiro-Wilk test was used to test the normality of the distribution of the variables. The distributions were not normal and, according to Levene's test, group variances were not homogeneous. Therefore, the groups were compared by the Kruskall-Wallis one way analysis of variance by ranks test and then multiple comparisons between pairs of groups were carried out according to the Dunn test. Results have been expressed as number of observations (n), mean ± standard deviation, median and min-max values. A p value less than 0.05 was considered significant. All statistical analyses were performed using the SPSS 13.0 for Windows (SSPS Inc, Chicago, IL, USA).

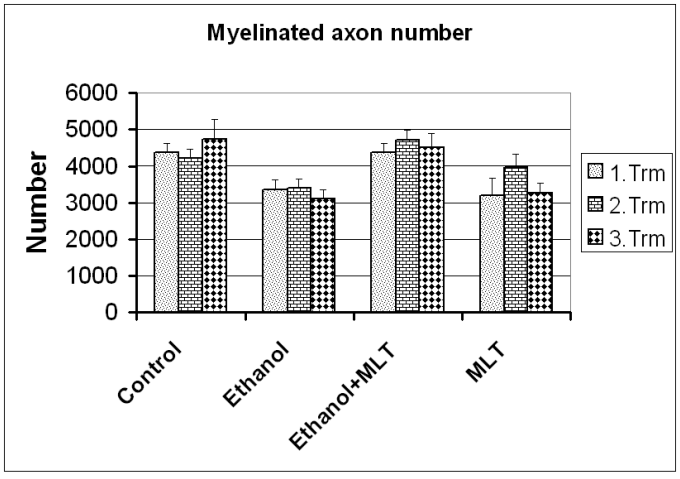

Fig. 1- A comparison of axon numbers of each group during the first, second, and third trimesters of sciatic nerve is seen. There was no significant difference among the trimester of the same group for control, ETH, ETH plus MLT (ETH+MLT) and MLT groups, respectively, (Mean SEM). CONT, control; ETH, ethanol; ETH+MLT, ethanol+melatonin; MLT, melatonin groups.

3.2. Myelin thickness

Myelin sheet thickness in the rat sciatic nerves of the first, second and third trimesters of the control group, and also among three trimesters of ETH group, were not significantly different. A significant difference in the myelin sheet thickness was observed between the first and second trimester of ETH+MLT group ($p<0.01$), but in the third trimester sheet thickness was not different from the earlier trimesters in this group. A significant difference in the myelin sheet thickness was observed between the second and third trimester of MLT

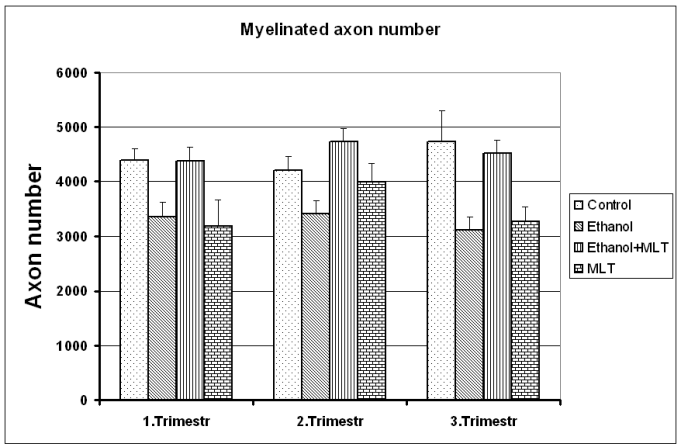

Fig. 2- A comparison of axon numbers in the sciatic nerve of each group during the first, second, and third trimesters. There is no significant difference between groups of control, ETH, ETH+MLT and MLT during the first, second and third trimesters (Mean ± SEM). CONT, control; ETH, ethanol; ETH+MLT, ethanol+melatonin; MLT, melatonin groups.

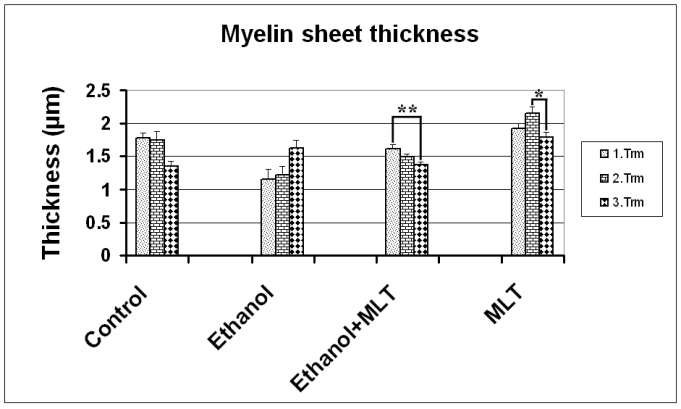

Fig. 3- A comparison of myelin sheet thickness for each group is seen. A significant difference in the myelin sheet thickness was observed between the first and second trimester of ETH+MLT group, and also between the second and third trimester of MLT group (Mean ± SEM). CONT, control; ETH, ethanol; ETH+MLT, ethanol+melatonin; MLT, melatonin groups, * p<0.05; ** p<0.01.

group (p<0.05), but myelin sheet thickness in the first trimester of MLT was not different from the second and third trimesters **(Fig.3).**

Myelin sheet thickness in the first trimester of the ETH group was significantly thinner than that in the control, ETH+MLT and MLT groups (p<0.01). Myelin thickness of MLT group in the first trimester was significantly thicker than that in the ETH+MLT group (p<0.05). Although a toxic effect of ETH on myelin sheet thickness, when compared to the control group was observed but a protective effect of MLT after exposure of ETH was not found in ETH+MT group in the second trimester. On the other hand, an increased myelin sheet thickness at the second trimester was seen in the MLT group, when compared to that of other groups (p<0.001) (Fig.4).

3.3. Axon cross section area

The cross sectional area of the axons was not different among first, second and third trimesters of the control, ETH+MLT, or MLT groups. However, a significant difference was found between the first and second trimesters of the ETH group (p<0.01), but there was no difference between second and third trimesters for ETH (Fig. 5).

The axonal cross sectional areas in the control, ETH, ETH+MLT and MLT were not significantly different from each other in the first, second and third trimesters (Fig. 6).

The morphology of nerve fibers in the sciatic nerves from each group is shown in Figure 7. The sciatic nerve fibers in the control group were seen to have normal structure (Fig. 7, CONT 1-3). In the ETH group, most of myelin sheet of nerve fibers was degenerated as a result of the toxic effect of ETH (Fig. 7, ETH 1-3). A neuroprotective effect of MLT was not seen at the level that was anticipated as only a partial preservation of myelin sheet was observed (Fig. 7, ETH+MLT 1-3). An increased myelin sheet thickness was seen in comparison to the other groups in response to MLT alone (Fig. 7, MLT 1-3).

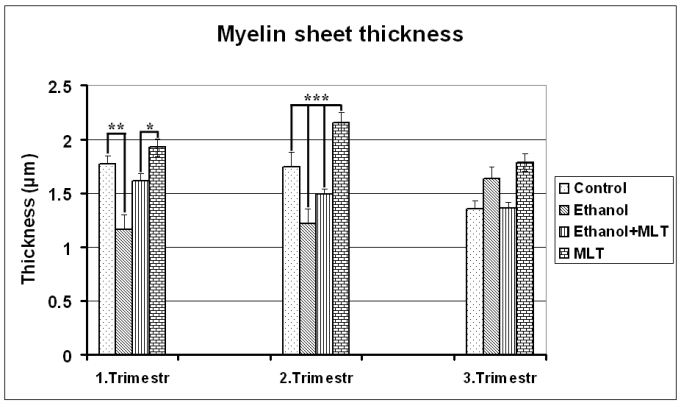

Fig. 4- A comparison of myelin sheet thickness for each trimester is seen. Myelin sheet thickness in the first trimester of ETH group was significantly thinner than the control, ETH+MLT and MLT groups, and myelin thickness in MLT group in the first trimester was significantly thicker than ETH+MLT group. MLT group has an increased myelin sheet thickness at this second trimester in comparison of other groups (Mean ± SEM). CONT, control; ETH, ethanol; ETH+MLT, ethanol+melatonin; MLT, melatonin groups, * p<0.05); **p<0.01; ***p<0.001.

4. Discussion

The present study clearly demonstrated that one dose of ETH had a toxic effect on the cross sectional area of axons and on myelin sheet thickness in developing rat sciatic nerve, but there was no effect of ETH on total axon number. Many previous studies have revealed that fetal exposure to ETH can damage various developing regions of the CNS, such as the cerebral cortex, corpus callosum, basal ganglia, and cerebellum in humans (Riley et al., 2004; Roebuck et al., 1998; Sowell et al., 2008). MLT has been widely tested to determine its efficacy in protecting against free radical damage in experimental models, including trauma, ischemia and reperfusion injury (Maldonado et al., 2007; Ozdemir et al., 2005; Turgut et al., 2005a; Turgut et al.,

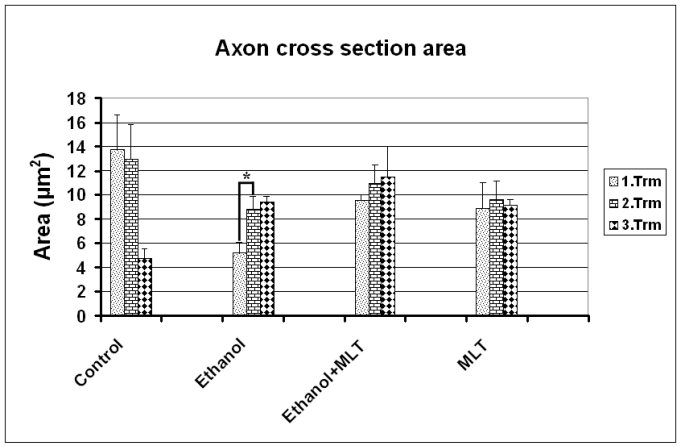

Fig. 5- A comparison of cross section area of axons in the sciatic nerve for each group is seen. A significant difference is only found between the first and second trimesters of ETH group. There is no significant difference among the different trimesters of each group (Mean ± SEM). CONT, control; ETH, ethanol; ETH+MLT, ethanol+melatonin; MLT, melatonin groups * p<0.01.

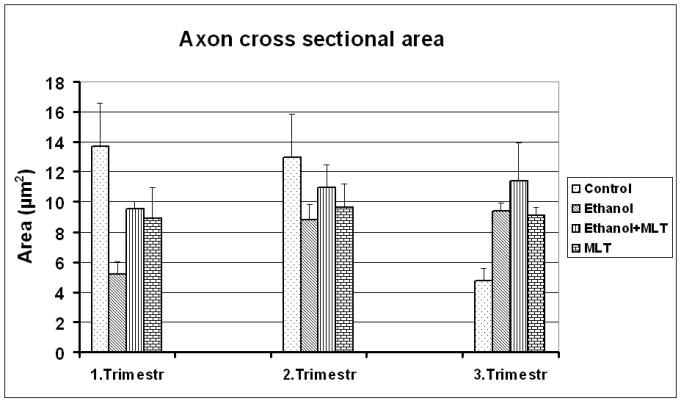

Fig. 6- Comparison of cross section area of axons in the each group of sciatic nerve for each trimester is seen. The cross section area of axon in the control, ETH, ETH+MLT and MLT was not significantly different from each other in the first, second and third trimesters (Mean ± SEM). CONT, control; ETH, ethanol; ETH+MLT, ethanol+melatonin; MLT, melatonin groups.

2005b). Numerous studies have revealed that MLT can be highly effective in reducing molecular damage, cellular death and tissue loss (Maldonado et al., 2007). Since the development of the CNS is profoundly disturbed by exposure to ETH during the prenatal period (Heaton et al., 2003), it has been reported that its most harmful effects are caused by ROS, especially by some oxygen and other free radicals (Agar et al., 2003; Baydas and Tuzcu, 2005; Kozan et al., 2007; Montoliu et al., 1994; Nordmann et al., 1990; Nordmann et al., 1992). Recently, it has been suggested that apoptosis is involved, although the precise mechanism of ETH toxicity on the developing CNS is not elucidated so far (Antonio and Druse, 2008). In this experiment, our hypothesis was that MLT can act as a protective agent against ETH damaging in the developing PNS during the prenatal period, due to the fact that it is a potent free radical scavenger and antioxidant (Reiter, 1998; Reiter et al., 1994; Tan et al., 2000; Tunc et al., 2007).

Neurotoxicity of ETH in the developing CNS and/or PNS is widely accepted, because the development of the nervous system is profoundly disturbed by exposure to ETH during prenatal period (Druse and Hofteig, 1977; Heaton et al., 2003). However, a neuroprotective effect of MLT on the developing sciatic nerve and its myelination in the early postnatal period has not been well documented using quantitative stereological methods. The results presented here for myelin sheets showed that exogenous MLT has a neuroprotective effect against ethanol toxicity effects on myelin thickness. This effect may occur by scavenging of free radicals and stabilizing glial activity against the damaging effects of ETH, as previously suggested (Baydas and Tuzcu, 2005). In the present study, not only did MLT treatment during development of nerve cells have no toxic effects on myelin thickness during the three trimester periods, it also had a positive effect on myelin sheet thickness. Interperitoneal treatment of the mother rat with MLT was probably effective during gestation of the offspring, since maternal circulation and

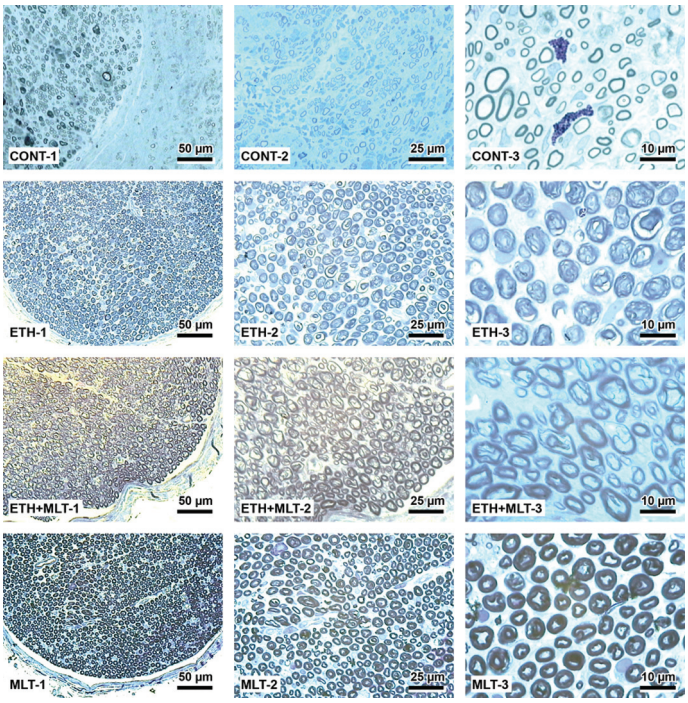

Fig. 7- Effects of ethanol and melatonin on rat sciatic nerve structure. Four treatment conditions (control, ETH, ETH+MLT, and MLT) were used, and figures show photographs taken at low, medium and high magnification. The structure of sciatic nerve fibers in the control group shows normal structure (CONT 1-3), but as a result of the toxic effect of ETH most of myelin sheet of nerve fibers are degenerated (ETH 1-3). Partial preservation of myelin sheet was observed with added MLT (ETH+MLT 1-3). An increased myelin sheet thickness is seen in comparison to other groups (MLT 1-3). CONT, control; ETH, ethanol; ETH+MLT, ethanol+melatonin; MLT, melatonin groups.

the fetal pineal gland are two potential sources of MLT, and MLT is readily transferred from maternal to fetal circulation (Okatani et al., 1998).

Although exogenous supply of MLT to the mother rat increased MLT in fetal circulation, MLT treatment was not shown to have any deleterious effects on myelin thickness in the

developing rats during this period. However, some researchers have suggested that exposure to excess MLT can have a toxic effect on peripheral nerve development, even though the underlying mechanism behind this idea is unclear. It has been proposed that excess MLT may affect developing Schwann cells, resulting in an undeveloped myelination process of the rat sciatic nerves in postnatal life. Schwann cell formation is preceded by the generation of two other cell types, the Schwann cell precursors (SCPs), which are the glial cells of embryonic day (ED) of 14-15 rat nerves, and immature Schwann cells (ISCs), which are generated from the SCPs from ED 15 to ED 17 (Jessen et al., 1994; Jessen and Mirsky, 2005; Riethmacher et al., 1997). The nerves consist of irregular axon-Schwann cell bundles at ED 18 in rats. Around ED 18 in rats, ISCs begin to surround large groups of axons, although myelination starts some 3 days later, at birth (Jessen and Mirsky, 2005; Ziskind-Conhaim, 1988). While these events take place, premature myelination seems to be prevented by the activity of a number of signaling systems that function as 'myelination brakes' (Jessen and Mirsky, 2005). In this study, exogenous MLT was administered at ED 21 for the third trimester group. Therefore, it may induce the activity of signaling systems that function as 'myelination brakes' and they may progress during the early postnatal days, causing decreases of developing myelin thickness of rat sciatic nerves (Jessen and Mirsky, 2005).

Importantly, it has been reported that one critical window of ETH vulnerability coincides with the period of gastrulation (Mooney and Miller, 2007). In the present study, the result that one dose of ETH had no toxic effects on developing myelin thickness during the third trimester was not expected. Recently, it has been suggested that antioxidant/free radical scavenging properties of various antioxidants, e.g. MLT, have a putative role in their anti-apoptotic effects (Antonio and Druse, 2008). Reportedly, MLT prevents H_2O_2 activation of the pro-apoptotic enzyme, caspase-3 (Juknat et al., 2005). Furthermore, it has been reported that exogenous antioxidants exert both neuroprotective and anti-teratogenic effects against the toxic effects of ETH (Heaton et al., 2004). Nevertheless, it is speculated that high doses of some antioxidants exert pro-oxidant effects (Watjen et al., 2005). In the present study, the toxic effect of exogenous MLT on the myelin thickness of rat sciatic nerves during third trimester may be explained by its pro-oxidant effect augmenting apoptosis. Additional studies are needed to establish the neuroprotective effects of MLT against ETH-associated apoptosis in the PNS of rat.

In conclusion, based on results on myelin sheet thickness of rat sciatic nerves, it is suggested that intrauterine exposure to exogenous MLT seems to have a toxic effect during the third trimester, whereas acute ETH is toxic during the first and second trimester. Both give rise to defects in sciatic nerve myelination. To our knowledge, this is the first report indicating that exogenous MLT has a toxic effect on sciatic nerve myelination, and that it may alter postnatal development of myelin thickness. Additional developmental studies are required to elucidate the precise mechanism for the observed effects of exogenous MLT, both toxic and neuroprotective, in the development of the sciatic nerve of the rat.

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