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Evaluation of neuroprotection by melatonin against adverse effects of prenatal exposure to a nonsteroidal anti-inflammatory drug during peripheral nerve development

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ABSTRACT

The potential ability of melatonin to protect against impairment of the fetal peripheral nerve system due to maternal consumption of diclofenac sodium (DS) was investigated. Eighty-four pregnant rats were divided into seven groups: control (CONT), saline administered (PS), DS administered (DS), DS with low-dose melatonin administered (DS + MLT10), DS with high-dose melatonin administered (DS + MLT50), low-dose melatonin administered (MLT10), and high-dose melatonin administered (MLT50). After the pregnancy, six male newborn rats from each group were sacrificed at 4 and 20 weeks of age. Their right sciatic nerves were harvested, and nerve fibers were evaluated using stereological techniques. Mean numbers of myelinated axons, axon cross-section areas and the mean thickness of the myelin sheet were estimated. Four-week-old prenatally DS-exposed rats had significantly fewer axons, a smaller myelinated axonal area, and a thinner myelin sheath compared to CONT group ($p < 0.05$). Although melatonin at both doses significantly increased axon numbers, only a high dose of melatonin increased the diameter of those axons ($p < 0.05$). At 20-weeks of age, myelinated axon number in the DS group was not only significantly lower than all other groups ($p < 0.05$) but also the cross-sectional area of these axons was smaller than all other groups ($p < 0.05$). There were no differences between the groups regarding the mean thickness of the myelin sheet. The current study indicates that prenatal exposure to DS decreases the number and the diameter of sciatic nerve axons and that melatonin prophylaxis can prevent these effects.

1. Introduction

Female patients in the age spectrum to become pregnant are frequently prescribed nonsteroid anti-inflammatory drugs (NSAIDs) by their physicians for the treatment of primary dysmenorrhea, intrauterine contraceptive device-induced dysmenorrhea, and menorrhagia (Schoenfeld et al., 1992). NSAIDs can also be used during pregnancy for the effective prevention of preterm labor, preterm uterine contractions, polyhydramnios, and preeclampsia (Gokcimen et al., 2001). However, there is a lack of knowledge on the use of NSAIDs during pregnancy, causing difficulty in the prescription of these drugs for patients. Diclofenac sodium (DS) is the NSAID of choice since the 1980s due to its high specificity for the arachidonic acid-degrading cyclooxygenase-2 (COX-2) enzyme rather than its isoform COX-1. Prenatal DS exposure results in severe effects ranging from stillbirth to miscarriage and to development defects (Cook et al., 2003). The severity of the manifestations of prenatal exposure to DS depends on several factors, such as the time of exposure, total DS consumption by the mother, the genotypic background, and other factors that may exert an influence on the developing fetus. Information about the effects of prenatal exposure to DS is still quite limited, and results have not always been consistent (Cook et al., 2003; Ragbetli et al., 2007).

Several previous studies confirmed neurotoxic effects of prenatal exposure to DS on the central nervous system (CNS), namely the pyramidal and granular cells of the hippocampus and the Purkinje cells of the cerebellum (Gokcimen et al., 2007; Ragbetli et al., 2007). Canan et al. later demonstrated that 15-day maternal administra-

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tion of DS during pregnancy has negative effects on the developing peripheral nervous system as shown by relevant changes in the morphology of nerve fibers from the sciatic nerve (Canan et al., 2008b). To explain their results, they stated that DS suppresses cell proliferation in the spinal cord and the dorsal root ganglia by affecting cell cycle regulators. Previous studies showed that DS constrains the differentiation of neuronal stem cells into neurons and that the drug suppresses cell proliferation via the induction of apoptosis (Andreasson et al., 2001; Kudo et al., 2003). Aygün et al. has demonstrated the possible neuroprotective effects of MLT on DS toxicity (Aygün et al., 2012).

Melatonin (*N*-acetyl-5-methoxytryptamine) is a molecule which is secreted from pineal gland (Aygün et al., 2012). The main task of this hormone is adjusting the rhythm of body and protecting the biological clock of the body (Claustrat et al., 2005 Singh and Jadhav, 2014). Melatonin is also a powerful antioxidant which reduces lipid peroxide concentrations in the cells (Nakano et al., 2012; Vargas et al., 2011; Wang et al., 2013).

The related studies show that Mel has a neuroprotective effect through the antioxidant activity (Odaci and Kaplan, 2009; Kaplan et al., 2011). The prenatal exposure of NSAIDs, especially diclofenac sodium, has been reported that have negative effects on nervous system (Ekici et al., 2012 Zengin et al., 2013). At this point; Reiter et al. reported that coadministration of melatonin with other drugs which have possible side effects reduces the toxic efficacy of these drugs (Reiter et al., 2002).

This study investigated the effect of chronic maternal exposure of rats to DS on the development of the sciatic nerve in offspring and the potential ability of melatonin to reduce the side effects of maternal DS exposure during the development of the peripheral nervous system.

2. Materials and methods

This study was performed in accordance with the guidelines of Selcuk University Experimental Medical Research Center Animal Care Committee, and the ethics committee approved all the experimental procedures. Primiparous Wistar female rats weighing 250–280 g were used. The animals were allowed free access to food and water. They were housed at constant room temperature (20–22 °C) and exposed to a light cycle of 12 h/day (08:00–20:00) for 1 week before the experiment. Four females were placed with single male rats in the late afternoon. Vaginal smears were taken the following morning at 09:00. The presence of a copulatory plugin the vagina was considered to be an indication of pregnancy (Gestational day 1; GD1). After the vaginal plugs were discovered the males were removed from the cage and the females were housed as single for the remainder of the pregnancy period. Pregnant animals were housed in standard recyclable rat cages which had dimensions of 43 cm (length) × 34 cm (width) × 20 cm (height). Cages had sawdust bedding in an air-conditioned room at 20 °C under a 12/12 h light/dark cycle. They had free access to a commercial rat diet Ayvet (Konya, Turkey) and water ad libitum.

2.1. Group design

Eighty-four pregnant rats were randomly divided into seven groups with 12 rats in each group:

Control Group (CONT): Control group. No drug was administered.

Physiological Serum Group (PS): Beginning from the fifth day after mating for 10 days during pregnancy, physiological serum (1 mL/kg dose) was intraperitoneally injected in the control group over the same period.

Diclofenac Sodium Group (DS): Beginning from the fifth day after mating for 10 days during pregnancy, DS (Voltaren, 75 mg/3 mL ampoules, Novartis, Mefar Ilac Sanayi A.S., Istanbul, Turkey) in a dose of 1 mg/kg daily was intraperitoneally injected between 16:00 and 17:00 h.

Diclofenac Sodium + Melatonin 10 mg Group (DS + MLT10): The same as Group 2 but melatonin (Sigma–Aldrich, Milano, Italy) in a dose of 10 mg/kg was also injected intraperitoneally between 16:00 and 17:00 h.

Diclofenac Sodium + Melatonin 50 mg Group (DS + MLT50): The same as Group 2 but melatonin (Sigma–Aldrich) was injected intraperitoneally in a dose of 50 mg/kg between 16:00 and 17:00 h.

Melatonin 10 mg Group (MLT10): Only melatonin (Sigma–Aldrich) was injected intraperitoneally in a dose of 10 mg/kg between 16:00 and 17:00 h.

Melatonin 50 mg Group (MLT50): Only melatonin (Sigma–Aldrich) was injected intraperitoneally in a dose of 50 mg/kg between 16:00 and 17:00 h.

After spontaneous delivery, 12 male offspring were collected from 12 different female rats for each 7 group (total 84) and were fed for either four or 20 weeks. At the end of the fourth and 20th weeks, the animals were weighted and then anaesthetized with ketamine (Ketalar-Eczacibasi, Istanbul, Turkey) 50 mg/kg intraperitoneally and perfused intracardially with neutral formalin. The entire sciatic nerves were then removed en bloc from all the rats. The nerves were stretched to in situlength by pinning onto a card and then fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4–6 h at 4 °C. Once they were fixed, they were rinsed twice in a phosphate buffer (pH 7.4). The specimens were then postfixed in 1% osmium tetroxide for 2 h, dehydrated in an ascending alcohol series, and put into propylene oxide for 16 min. These procedures were completed by embedding the tissues overnight in an araldite CY212 + 2-dodecenysuccinic acid anhydride + benzyl dimethylamine and dibutyl phthalate mixture. Semi-thin sections of 1 μm thickness were cut using an ultramicrotome (Super Nova Reichert-Yung, Austria), and the sections were stained with 1% toluidine blue.

2.2. Stereological analysis

A stereology workstation was used; it consisted of a modified light microscope, a motorized specimen stage for automatic sampling (Prior, Rockland, MA, USA), an electronic microcator (Heidenhain, Traunreut, Germany), a CCD color video camera (JVC, Tokyo, Japan), a PC with a frame grabber board (type Flash Point 3D, Integral Technologies, Indianapolis, IN, USA), and a 17″ television screen monitor. The analysis was carried out at a final magnification of ×5090. A software program (Stereo Investigator-Micro Bright Field) was used to control, measure, and record the stereological data and to capture digital images of the sections.

The stereological analyses of the sciatic nerves were conducted according to principles described previously (Canan et al., 2008a). The area of the unbiased counting frame was 30 × 30 μm (900 μm^2). The myelinated axons were counted if they appeared inside the unbiased counting frame and if the inclusion lines of the frame located on each sampling area intersected them. In this study, the upper and right borders of the unbiased counting frame were the inclusion lines, and the lower and left borders, including their extensions, were accepted as the exclusion lines. Any profile of a myelinated nerve fiber section hitting the exclusion lines was excluded from counting, and any profile hitting the inclusion lines and located inside the frame was counted. This ensured that all locations within a nerve cross-section were equally represented and that all axonal profiles were sampled with an equal probability, regardless of shape, size, orientation, and location. A two-dimensional isotropic uniform random nucleator was used to