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(Article begins on next page)



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Effects of prenatal exposure to diclofenac sodium and saline on the optic nerve of 4- and 20-week-old male rats: a stereological and histological study

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Abstract

We investigated the effects of diclofenac sodium (DS) on development of the optic nerve in utero . Pregnant female rats were separated into three groups: control, saline treated and DS treated. Offspring of these animals were divided into 4-week-old and 20-week-old groups. At the end of the 4th and 20th weeks of postnatal life, the animals were sacrificed, and right optic nerves were excised and sectioned for ultrastructural and stereological analyses. We demonstrated that both DS and saline produced structural and morphometric changes in the total axon number and density of axons, but decreased the myelin sheath thickness in male optic nerves. All ultrastructural and morphometric features were well developed in 20-week-old rats. We showed that development of the optic nerve continues during the early postnatal period and that some compensation for exposure to deleterious agents in utero may occur during early postnatal life.

Key words: diclofenac sodium , electron microscopy , male rat , optic nerve , stereology

The first nonsteroidal anti-inflammatory drug (NSAID) was isolated in 1829 when scientists isolated salicylate from willow bark (Vane et al. 1990). The next NSAID, indomethacin, was developed in the 1960s (Green 2001). NSAIDs are among most common drugs (Pascucci 2002) that have anti-inflammatory, analgesic and antipyretic effects (Liu et al. 2005). They have been used widely to treat a variety of diseases and conditions including menorrhagia, dysmenorrhea, migraines, arthritis and many types of pain (Chan et al. 2002, Siu et al. 2000). It has been reported, however, that NSAIDs can cause a premature closure of the fetal ductus arteriosus, which in the fetus leads to pulmonary hypertension, respiratory problems and undesirable effects on kidney function; the latter leads to oligohydramnios and neonatal anuria (Ericson et al. 2001, Ørstensen et al. 2004). The mechanism of NSAID induced teratogenicity, however, is uncertain (Chan et al. 2002, Van den Veyver et al. 1993). The therapeutic effects of NSAIDs are mediated by inhibition of prostaglandin synthesis by cyclo-oxygenase (COX) enzymes (Chan et al. 2002, Van den Veyver et al. 1993). COX-1 and COX-2 are isoforms of the COX enzyme. While COX-1 is constitutively expressed in many tissues, e.g., endothelium, stomach and kidneys (Vane et al. 1998), COX-2 is induced by pro-inflammatory cytokines and endotoxins (Cappon et al. 2003, Mitchell et al. 1993). Two vascular effects of prostaglandin inhibitors are constriction of the fetal ductus arteriosus and reduce renal blood flow. These complications have been described for most nonselective COX inhibitors, and they increasingly are reported for selective COX-2 inhibitors (Ørstensen et al. 2004). Diclofenac sodium [sodium-(*O* -((2, 6-dichloro phenyl)-amino)-phenyl)-acetate]] (DS) is an

NSAID that commonly is used to treat dysmenorrhea and menorrhagia in women of reproductive age (Chan et al. 2002, Dawood 1993). Treatment with DS may be accompanied by adverse effects including serious upper gastrointestinal bleeding and platelet dysfunction (Andreasson et al. 2001, Capone et al. 2007, Liu et al. 2005, Russel 2001). In addition, DS administration during the prenatal period can have adverse effects on both mother and fetus (Siu et al. 2004). Concern for DS induced teratogenicity during organogenesis is exacerbated by limited information regarding the teratogenic effects of the drug (Chan et al. 2001, 2002, Gokcimen et al. 2007, Ragbetli et al. 2007). It is known that COX-2 is expressed selectively in neurons of the central nervous system (CNS) (Andreasson et al. 2001, Breder et al. 1995, Canan et al. 2008, Kaufmann et al. 1996, Yamagata et al. 1993). If DS is administered during pregnancy, especially during the organogenesis period, it may affect the development of the CNS (Canan et al. 2008, Gokcimen et al. 2007, Ragbetli et al. 2007). We studied the effects of prenatal administration DS on the development of the optic nerves of rats using unbiased stereological methods. We also studied the effects of isotonic saline solution to determine possible differences between the DS and control groups. We used the saline injected rats as a sham control group. The saline injected sham control group allowed us to determine whether changes were due to a volumetric increase from injection or to DS itself. We observed that the increase in volume of the abdomen due to saline was the same as for the DS group. We evaluated postnatally axon thickness, myelin thickness, axon cross section area, numerical density of axons, cross section area of optic nerve, and axon number.

Materials and methods

Animals

We obtained from the Surgical Research Center of Yüzüncü Yıl University (Van, Turkey) 18 male and 18 female albino rats weighing between 150 and 200 g. Rats were mated overnight in separate standard plastic cages, and females were considered pregnant when a vaginal plug was observed. Pregnant rats were housed in standard plastic cages on sawdust bedding at 20 ° C, and with a 12 h light-dark cycle, and they were fed ad libitum . Pregnant rats were divided into three groups of six: control, saline injected, and drug treated. Beginning from day 5 (GD5) after mating through day 15 (GD15) of pregnancy, 1 mg/kg DS (Voltaren, 75 mg/3 ml ampoule; Novartis, Mefar İlaç Sanayi A.Ş., İstanbul, Turkey), was injected intraperitoneally (i. p.) daily into the drug-treated group (DS group). During the same period, 1 ml/kg isotonic saline solution, dose, was injected i. p. into the saline group. Rats in the control group were not injected. After spontaneous delivery, male offspring were obtained (two male pups from each mother). We used only males to avoid possible anatomical and physiological variation between the genders. The 36 male offspring were assigned to groups: 12 offspring for the control group, 12 offspring for the saline group and 12 offspring for the DS group, and these groups were fed for 4 or 20 weeks. At the end of 4th week, half (6) of the animals of each group was sacrificed. Also at the end of 20th week, the remaining animals (6) of each group were sacrificed. Eventually, there were 6 male animals in each of the six groups. At the end of the 4th or 20th weeks, animals were anesthetized with an injection i. p. of 1.25 g/kg urethane and perfused intracardially with neutral formalin. After perfusion fixation, the right optic nerves were removed.

Tissue processing

The optic nerves were stretched to their length in situ by pinning them onto a card, then fixing them with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 – 6 h at 4 ° C. After fixation, 0.5 cm tissue

samples were cut from each stretched optical nerve. The tissue samples were rinsed twice in pH 7.4 phosphate buffer and postfixed in 1% osmium tetroxide for 2 h. Blocks were dehydrated through an ascending alcohol series and placed in propylene oxide for 16 min. The tissues then were embedded for 48 h using an Epon embedding kit (Fluka Chemie GmbH, Buchs Switzerland). Semithin sections and thin sections were cut at 1 μ m and 90 nm, respectively, using an ultramicrotome (Super Nova Reichert-Jung, Vienna, Austria). Semithin and thin sections were stained with 1% toluidine blue and uranyl acetate-lead citrate for stereological and histological analyses. Tissue ultrastructure was examined using a transmission electron microscope (JEM-1010, JEOL, Tokyo, Japan) with a Mega-View III digital camera and Soft-Imaging System (SIS, Munster, Germany).

Stereological analyses

Optic nerve sections were analyzed using stereological principles (Geuna et al. 2001, Larsen 1998, Kaplan et al. 2010). The total cross sectional area of the optic nerve was obtained using a stereological workstation that comprised a digital camera (Nikon COOLPIX5400, Tokyo, Japan), a manual dial indicator controlled specimen stage (Kaplan et al. 2001, 2005) and a light microscope (Nikon Microphot-FX, Tokyo, Japan). Other parameters including cross sectional areas of axons, numerical density of axons, total number of axons, and thickness of the myelin sheaths were analyzed using electron microscopy images and Image J software (National Institutes of Health (NIH), <http://rsbweb.nih.gov/ij>). These images were obtained using the rules of systematic random sampling. The area of the unbiased counting frame was 38.248 μ m² (Gundersen et al. 1986).

The sampled areas were selected in a systematic, uniformly random manner using grid bars (Fig. 1). This procedure ensured that all locations within a nerve cross section were represented equally and that all axon profiles were sampled with equal probability regardless of shape, size, orientation or location (Canan et al. 2008, Geuna et al. 2000, 2001, Gundersen et al. 1986, 1999, Kaplan et al. 2010). The cross sectional areas of the axons and thickness of the myelin sheaths were estimated using Image J software.

Statistical analyses

Myelin thickness, axon cross sectional area, numerical density, total cross sectional area of the optic nerve, and numbers of axons for control, saline and DS groups were compared. Homogeneity of variables was controlled by Levene’s test. Variances were not homogeneous, so data were analyzed by Kruskal-Wallis one-way analysis of variance by the rank test. Multiple comparisons between pairs of groups were carried out according to the Dunn test. Results are expressed as number of observations (n), mean SEM median. Values of p

0.05 were considered significant. Statistical analysis was performed using SPSS 13.0 for Windows.

Results

Histopathological results

The axons of the control group were well developed and myelinated. The boundaries of the myelin sheaths and axoplasm were normal. The myelinated axons showed electron homogeneity and appeared intact. Electron microscopic (EM) examination showed myelin degeneration of the optic nerves of the offspring of the saline group. The myelin degeneration also was conspicuous in the rats of the DS group at 4 and 20 weeks of age (Fig. 2B, C, E, F). The axons of these groups were characterized by disorganized cytoskeletal elements and electron dense material apposed to the axolemma (Fig. 2B, C, F). Axon profiles were shrunken and showed irregular boundaries. Also evident were interrupted myelin sheaths and axon membranes (Fig. 2B, thin arrow) and the myelin bundles of these axons were loose (Fig. 2B, C, arrowhead).

“ Onion bulb ” structures were seen in the areas with irregular myelin bundles (Fig. 2C). The periaxonal compartment was poorly defined (Fig. 2C). In both 4- and 20-week-old animals of the saline and DS groups, the optic nerves showed normal histological structure. The myelin sheaths of 20-week-old animals in the DS group were electron dense and their boundaries were normal like the 4-week-old ones. Axonal cytoplasm of the 20-week-old saline and DS groups were homogeneous, unlike the 4-week-old saline and DS groups (Fig. 2 E, F).

Stereological results

Our stereological findings are summarized in Fig. 3

Total number Axons

The number of axons in the DS group was greater than in the control and saline groups for 4-week-old rats ($p < 0.05$) (Fig. 3A). The control group of 20-week-old rats had more myelinated axons than the other groups at the same age ($p < 0.05$) (Fig. 3A). No significant differences were found between the saline and DS groups (Fig. 3A). Twenty-week-old rats in the control, saline and DS groups had more nerve fibers than the 4-week-old rats (Fig. 3A).

Cross sectional area of axons

There were no significant differences in the cross sectional areas of axons among the 4-week-old control, saline and DS group males (Fig. 3B). The axonal cross sectional area for the 20-week-old DS group was greater than that of the other groups ($p < 0.05$) (Fig. 3B). Comparison of 4- and 20-week-old groups showed a significant difference between axonal cross sectional areas for the saline and DS groups. Saline and DS groups of 20-week-old rats had greater axonal cross sectional areas than the 4-week-old groups (Fig. 3B).

Myelin thickness

Among 4-week-old male rats, both saline and DS groups had thinner myelin sheaths than the controls ($p < 0.05$). By contrast, 20-week-old male rats showed no significant difference in myelin sheath thickness among the groups studied (Fig. 3C). Control 4-week-old rats had thicker myelin sheaths than the 4- and 20-week-old saline and DS groups ($p < 0.05$) (Fig. 3C).

Cross sectional area of optic nerve

Comparison of 4-week-old male rats in the control, saline and DS groups showed no significant differences in the cross sectional areas of optic nerve among groups (Fig. 3D). The cross sectional areas of optic nerves in the 20-week-old DS group was greater than the other groups ($p < 0.05$) (Fig. 3D). The optic nerves of 20-week-old rats had larger cross sectional areas than the 4-week-old groups for control, saline and DS groups (Fig. 3D).

Numerical density of axons Four-week-old control rats exhibited lower axonal density than 4-week-old saline and DS groups ($p < 0.05$) (Fig. 3E). Among 20-week-old males, the saline group showed greater numerical density than the other groups ($p < 0.05$) (Fig. 3E), but no significant differences were found between the control and DS groups (Fig. 3E). The 20-week-old control group showed greater numerical density of axons than 4-week-old controls, but the 20-week-old DS group showed lower numerical density of axons than the 4-week-old DS group (Fig. 3E). There was no significant difference between the 4-week-old and 20-week-old saline groups.

Discussion

Reactive oxygen species (ROS) frequently are formed during drug use, because many drugs are oxidized to form radicals (Halliwell et al. 1985, Orhan et al. 2001). DS causes morphologic or metabolic alterations in some organs or cells by forming free radicals that cause oxidative stress (Gómez-Lechón 2003, Orhan et al. 2001). DS causes apoptosis in hepatocytes by an oxidative stress-induced opening of the mitochondrial permeability transition pore (Gómez-Lechón et al. 2003). The molecular mechanism of DS-induced apoptosis involves the generation of ROS as an initial step (Inoue et al. 2004, Milusheva et al. 2008). It is known that oxidative stress damages proteins, lipids and nucleic acids, which result in cell membrane and synaptic disorganization, and neural cell signaling dysfunction as a consequence of apoptotic and/or necrotic events (Cui et al. 2004). Because the myelin sheaths consist mainly of protein and lipids (Inuzuka et al. 1988, Trapp et al. 1982), tissue integrity, including myelin and the axon, may be disrupted and embryonic development of nerves may be inhibited by ROS caused by DS. Because DS administered during gestation can affect the development of the CNS and CNS-related structures (Carp et al. 1988, Gokcimen et al. 2007, Ragbetli et al. 2007), it has been suggested that the number of nerve fibers and the cross sectional area of axons formed during development of the sciatic nerve are affected by DS administration during the embryonic period (Canan et al. 2008). It has been suggested that some NSAIDs can overcome growth restrictions caused by some types of CNS inhibitors (Fu et al. 2007). We postulated that DS administered from embryonic day 5 to 20 would affect the morphology features of nerve fibers in the rat optic nerve. We found no significant difference in cross sectional areas of the axons and total cross section area of the optic nerve between the control and experimental groups in 4-week-old rats. Significant differences were found, however, in the numerical density of the axons and total number of axons in 4-week-old rats in the saline and DS groups compared to the age matched control groups. On the other hand, in both the saline and DS groups, myelin sheath thickness decreased compared to the 4-week-old control group. Earlier, we investigated the effects of prenatal exposure to DS on the number of Purkinje cells in female rats (Odaci et al. 2010) and found that DS increased the total Purkinje cell number in female rat cerebellum compared to controls. The results of the experiment reported here suggest that prenatal administration of either saline or DS produces morphological and histopathological changes in the developing optic nerve of rats. These changes include an increased number of axons and a decrease in myelin sheath thickness in 4-week-old rats. In 20-week-old rats, the experimental groups showed fewer axons than the control group, but the numerical density of axons was greater than in the control group. The axonal cross sectional area of the DS group was greater than the other groups. These results were expected, because during exposure to any stressor, such as prenatal exposure to a toxic agent or increase in abdominal volume of the mother, the body responds with stress. Different substances including cortisol, adrenaline, corticotropin-releasing hormone and ACTH are released into the circulation. It is well known that each individual would respond differently to stress stimuli because of genetics, experience and social environment (Mulder et al. 2002). Animals exposed to prenatal stress show impaired motor development, impaired adaptive behavior, impaired sexual behavior and impaired learning-memory function (Huizink et al. 2000, Clarke et al. 1994, Weinstock 1997, Schneider et al. 1999). Increased cortisol is known to produce reduced myelination of the optic nerve in young rats (Bohn et al. 1982). In our study, increased cortisol levels owing to maternal stress due to injection of DS or saline may have caused transient changes in myelination and axon numbers in the optic nerves of the offspring. All morphometric parameters of 20-week-old rats were higher than the 4-week-old animals. Because development of the optic nerve begins during late prenatal and early postnatal life (Kuwabara 1975), we administered saline and DS through the 20th day. Saline and DS application may increase the generation of new neurons and axons during the 4th week of postnatal life, which eventually would be reduced by apoptosis (Knabe et al. 2009, Zacharaki et al.

2000). It has been reported that DS has a neurotoxic effect on CNS and CNS related structures when administered during the prenatal period (Canan et al. 2008, Gokcimen et al. 2007, Kudo et al. 2003, Ragbetli et al. 2007). We found no reports in the literature concerning the effects of DS and saline on the development of the optic nerve. Our results indicate that both DS and saline cause an increase in the total number of axons and the numerical density of axons, but that these agents decrease the myelin sheath thickness in the male rat optic nerve. It may be that both saline and DS can stimulate optic nerve fibers during the developmental period. It is known that exercise, stress, ischemia and drug administration during the perinatal period can increase the number of neurons in the CNS (Gao et al. 2010, Kannangara et al. 2011, Schmitz et al. 2000). Schmitz et al. (2000) also reported that the number of granular cells in the cerebellum increased during the postnatal period following prenatal application of low-dose X-irradiation. Chen et al. (2003, 2006) found that neither prenatal nor early postnatal nicotine exposure reduced the number of pyramidal, granular or Purkinje cells in the CNS. Contrary to expectations, nicotine exposure and X-irradiation caused an increase in the number of neurons. A similar result was obtained after exposure to DS, i.e., an increased the number of Purkinje cells in the female rat offspring after the prenatal administration of DS (Odaci et al. 2010). We have shown that saline and DS administered in utero affect the development of myelination of rat optic nerves and increase the number of axons. Further investigations of different developmental periods and different doses of DS are needed to establish the effect of DS administration on the development of optic nerve during gestation. Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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