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Effects of diclofenac sodium exposure during pregnancy on COX-2 expression in the ductus epididymis and ductus deferens of the offspring rat

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Abstract

The present study aimed to investigate the effects of diclofenac sodium application during pregnancy on COX-2 expression in the ductus epididymis (DE) and ductus deferens (DD) of the offspring rat. 8 Eight adult Wistar Albino non-pregnant female rats and two breeding male rats were utilized. Rats determined to be pregnant were separated into two categories as sham (S) and diclofenac sodium (DS). It was injected into the S group with 1 ml/kg/day saline for 10 days, and it was injected into the DS group with 6.1 mg/kg/day DS. 7 male offspring were used in each group. DS decreased the serum testosterone levels and incresed the number of mast. DS induced fibrotic thickening. Offspring of S group had moderate COX-2 in the caput epididymis (CPE) and severe COX-2 in the cauda epididymis (CE), while those in the DS group had weak COX-2 in the CPE and moderate COX-2 in the CE. There was no COX-2 expression in the vas deferens and corpus epididymis (COE) of the offspring rats in the S and DS groups. TNF-α expression was not observed in the S and DS groups. It was observed that moderate IL-10 expression in the CPE and CE, and weak IL-10 expression in the vas deferens and COE in the S and DS groups. The findings of our study reveal that DS administration during pregnancy can cause the downregulation of COX-2 expression in the DE and DD of offspring rats and therefore may have adverse effects on the male reproductive system.

Keywords: COX-2, diclofenac sodium, ductus epididymis, rat, vas deferens

1. Introduction

Diclofenac sodium (DS) is extensively utilized in the treatment of various pain and inflammations. DS exerts antipyretic, analgesic, and anti-inflammatory effects by reducing prostaglandins (PGs) through suppression of the cyclooxygenase (COX), particularly COX-2 (1). NSAIDs are frequently prescribed by obstetricians to pregnant women to alleviate pain and pyrexia and treat infection (2). The most critical period of pregnancy is the organogenesis period, which is the riskiest period in terms of teratogenic effects and differentiation in tissues and organs (3). Exposure to teratogens in the early stages of organogenesis may cause damage to the central nervous system, and if exposed in the late stages, urogenital disorders or growth disorders may occur (4). It has been reported that the risk of cryptorchidism increases due to the use of paracetamol or NSAIDs in pregnancy (5).

The enzyme COX is known to have two isoforms. COX-1 is found in many tissues and organs and arranges the production of PGs that it necessary for physiological processes. COX-2 is generally known as an inducible isoform that is responsible for the production of PGs that mediate cell growth and inflammation, regulated by various cytokines and mitogens. COX-2 is structurally expressed in the brain, intestine, thymus, lung, kidney, and reproductive systems (6). Various studies have been conducted showing the importance of COX-2 expression in mammals, including the ductus DE (DE) and ductus deferens (DD) tissues (7).

There are several studies that prenatal application of DS has harmful effects on the various tissues of offspring (8-10). However, as a result of our literature investigation, no experimental research was encountered on the effects of prenatal DS on DE and ductus (vas) deferens of offspring. Based on the studies reporting the adverse effects of prenatal NSAID administration on various organs of offspring, especially reproductive organs, we aimed to reveal the effects of prenatal DS administration on the DE and DD tissues of offspring.

2. Material and Methods

2.1. Animals and experimental protocol

In the study, 8 adult Wistar Albino non-pregnant female rats and 2 breeding male rats with a weight of 200-300 g were utilized. The care and feeding of the animals were carried out in a temperature (21±2 ºC) controlled, 12-hour light and dark environment. Rats were fed standard feed, and water and allowed to mate overnight. The rats determined to be pregnant by the presence of vaginal plaque were randomly divided into 2 groups as sham (S) and diclofenac sodium (DS) groups. S group was injected 1 ml/kg/day saline intramuscular (IM) between the fifth and fifteenth days of pregnancy (total 10

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days), and the DS group was injected IM 6.1 mg/kg/day DS (Dichloron 75mg/3ml IM, Deva, İstanbul). Since the rats reached puberty on average at 8 weeks (11), the offspring rats were expected to reach 8 weeks in the study. Following birth, 7 male offspring rats were randomly selected from each group and fed standard feed, and water (ad libitum) in separate cages until they reached 8 weeks of age. The rats were sacrificed by anesthesia with 50 mg/kg of ketamine and 10 mg/kg of xylazine.

2.2. Histological analysis

DE and vas deferens (VD) tissues of the offsprings were removed, they were fixed in a 4% buffered paraformaldehyde. After fixation, tissues were followed by a histological tissue processing procedure. Sections of 4 µm thickness were deparaffinized and passed through xylene and ethyl alcohol series. For the evaluation of connective tissue, the Masson-trichrome staining procedure was applied. Sections were evaluated semiquantitatively under the light microscope (12).

2.3. Immunohistochemical analysis

An immunohistochemical staining procedure was applied to 4µm thick sections from the tissues. Immunohistochemical analysis was performed according to previously reported study protocols. The slides were incubated in COX-2 (Santa Cruz, sc-514489, USA, dilution: 1/100), TNF-α (Santa Cruz, sc-52746, USA, dilution: 1/100), and IL-10 (Santa Cruz, sc-365858, USA, dilution: 1/100) primary antibodies. For immunohistochemical evaluations, 10 randomly selected areas were examined and the severity of immunopositivity was evaluated semiquantitatively. According to the intensity of the immunopositive staining; negative (–), weak (+), moderate (++), and strong (+++) (13).

2.4. Stereological analysis

Consecutive sections of 4 µm thickness were taken by systematic random sampling from paraffin blocks. After the first sections were chosen randomly, the other sections were systematically taken every 25 steps and an average of 10 sections were obtained from each tissue. The number of mast cells was counted by applying the physical dissector counting method in tissues stained with toluidine blue staining. The numerical density formula used for mast cell count in tissues;

$NV = \Sigma Q^{-} / \Sigma V_{dis}$

NV: Numerical density

ΣQ^{-}: Total mast cells seen in all sections of animals

ΣV_{dis}: Total volume of sections (14).

2.5. Biochemical analysis

For biochemical analysis, intracardiac blood samples taken from rats were centrifuged at 3500 rpm for 10 minutes in a centrifuge device (Electro-mag M-615 P-Turkey). Testosterone levels in serum samples were measured in an autoanalyzer (Roche HITACHI, C702, 1263-13, Germany).

2.6. 2.6. Statistical analysis

One Way ANOVA test was applied to compare the groups statistically by applying a one-way analysis of variance to the experimental groups. The mean and standard deviation were given as descriptive statistics of the groups. The $p<0.05$ value was considered significant in the comparison of all groups.

3. Results

3.1. Histological analysis

In the sections stained by the Masson-trichrome staining method, it was determined that the connective tissue arrangement was disturbed and there was fibrotic thickening in the DS group (Fig. 1).

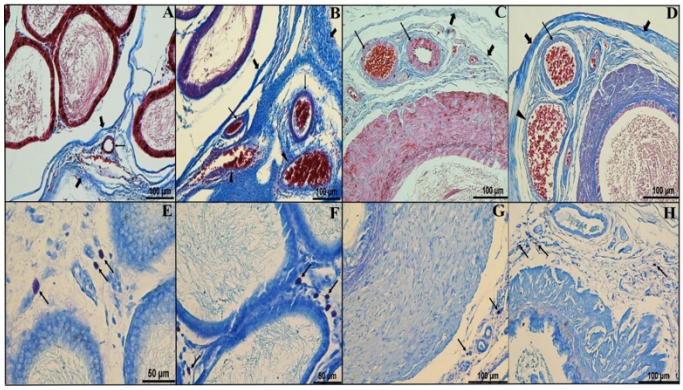

Fig. 1. Images of Masson trichrome (**A,B,C,D**) and Toluidine blue (**E,F,G,H**) stainings of tissues. Micrograph of the CPE in S and DS groups in **A-B,** respectively. Connective tissue (thick arrows), artery (thin arrows), vein (arrowheads) are shown in the interstitial space. Micrograph of the distal VD in S and DS groups in **C-D,** respectively. Connective tissue (thick arrows), artery (thin arrows), vein (arrowhead) are shown. Micrograph of the CPE in S and DS groups in **E-F,** respectively. Mast cells (arrows). Micrograph of the proximal VD in S and DS groups in **G-H,** respectively. Mast cells (arrows)

3.2. Stereological results

The number of mast cells increased in the caput epididymis (CPE), COE, CE, proximal and distal parts of the VD of rats in the DS group compared to S group rats ($p<0.05$) (Fig. 2).

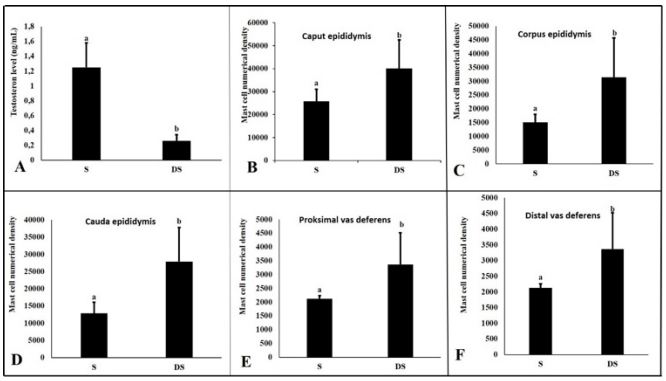

Fig. 2. Mean of serum testosteron levels among S and DS groups are shown in **A**. Statistically, "b" shows a significant decrease compared to "a" in this diagram. Means of mast cell numerical density of the CPE, COE, CE and proximal and distal VD are shown in **B, C, D, E and F,** respectively. Statistically, "b" shows a significant increase compared to "a" in B, C, D, E and F diagrams. $p < 0.05$

3.3. Immunohistochemical results

The score of expressions of COX-2 (Fig. 3), TNF-α (Fig. 3) and IL-10 (Fig. 4) in the VD and DE of offspring rats belonging to the S and DS groups are given in Table 1. TNF-α and IL-10 expressions were similar in both groups. While COX-2 expression was weak in the caput epididymis of the DS group

and moderately expressed in the S group, it was moderately expressed in the cauda epididymis of the DS group and severely expressed in the S group. Expression was not observed in other parts.

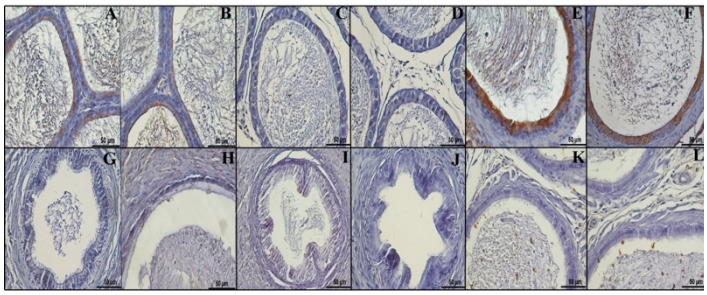

Fig. 3. Immunohistochemical images of COX-2 (**A,B,C,D,E,F,G,H,I,J**) and TNF- α (**K,L**) expressions of tissues. **A.** Micrograph of the CPE in S group, moderate expression of COX-2. **B.** Micrograph of the CPE in DS group, weak expression of COX-2. **C-D.** Micrographs of the COE in S and DS group, respectively, COX-2 expressions are negative. **E.** Micrograph of the CE in S group, strong expression of COX-2. **F.** Micrograph of the CE in DS group, moderate expression of COX-2. **G-H.** Micrographs of the proximal VD S and DS group, respectively, COX-2 expressions are negative. **I-J.** Micrographs of the distal VD, S and DS group, respectively, COX-2 expressions are negative. **K-L.** Micrographs of the CE, S and DS group, respectively, TNF-α expressions are negative

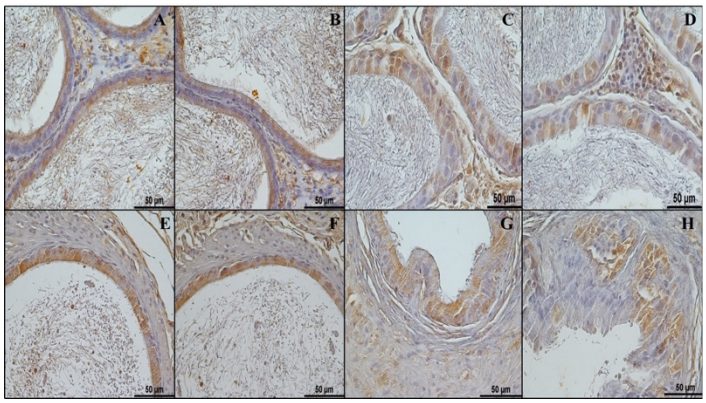

Fig. 4. Immunohistochemical images of IL-10 expressions of tissues**. A-B.** Micrographs of the CPE in S and DS groups, respectively, moderate expressions of IL-10 were observed. **C-D.** Micrographs of the COE in S and DS groups, respectively, weak expressions of IL-10 were observed. **E-F.** Micrographs of the CE in S and DS groups, respectively, moderate expressions of IL-10 were observed. **G-H.** Micrographs of the distal VD in S and DS groups, respectively, weak expressions of IL-10 were observed

3.1. Serum testosterone levels

The testosterone levels of the offspring rats in the DS group significantly increased compared to the serum testosterone levels of the offspring in the S group ($p<0.05$) (Fig. 2).

Table 1. Effect of DS application on COX-2, TNF-α and IL-10 expressions expression in the vas deferens and epididymis

	COX-2		TNF-α		IL-10	
	DS	S	DS	S	DS	S
Caput epididymis	+	++	-	-	++	++
Corpus epididymis	-	-	-	-	+	+
Cauda epididymis	++	+++	-	-	++	++
Proximal vas deferens	-	-	-	-	+	+
Distal vas deferens	-	-	-	-	+	+

Staining intensities: (–): negative; (+): weak; (++): moderate; (+++): severe

4. Discussion

COX-2 first converts AA to PGG_2 and then to PGH via peroxidase activity. In the next step, eicosanoids such as PGD_2, PGE_2, PGI_2, and $PGF_{2\alpha}$ are synthesized by PG synthases (15). Additionally, as intracellular mediators for GnRH release in the hypothalamic-pituitary axis, PGs act important roles in both male and female gonads (16). While it provides decidualization, implantation, uterine contraction and ovulation in women; In men, it affects sperm motility, contraction of smooth muscles around the seminiferous tubules, and the development of ventral prostate and seminal vesicle. Female mice lacking COX-2 have been reported to be infertile due to reproductive function disorders associated with fertilization, ovulation, decidualization and implantation (6). It has been shown that COX-2 is expressed in the male reproductive tract and its expression was modulated by androgens (6,7).

McKanna et al. reported that COX-2 was expressed only in the distal VD of rats, and there was no COX-2 expression in the proximal VD, DE and testis (17). Wong et al. revealed the presence of COX expression in the epithelium of the CE of rats (18) Similarly, Lazarus et al. reported that COX-2 is expressed in the epithelium of the VD and distal CE of mice (19). In the previous study to explore the effect of COX-2 in the maturation of the reproductive tract of male rats, Stanfield and Khan reported that COX-2 is expressed in the VD and DE of immature male rats during sexual maturation, and COX-2 expression increases during adolescence and sexual maturation. In the same study, it was reported that PGs derived from COX-2 may act a role in the maturation of the DE and VD by increasing androgen production and regulating smooth muscle contraction in these tissues (6).

PGs are found in many parts of the male generative system and are mainly under the control of androgens. Known to be located in the epididymal fluid, PGD_2 contributes to the fertility capacity of sperm by facilitating lipid and protein exchange between sperm and epididymal fluid during epididymal maturation (20). Previous studies have demonstrated that PGD_2 stimulates Leydig cells to produce testosterone (21). In our study, moderate COX-2 expression

was observed in DE epithelial cells of the CPE of postnatal eight-week-old offspring rats in the S group, while severe COX-2 expression was observed in both muscle layer and epithelial cells of the CE. It was determined that the distribution of COX-2 in the CPE and CE of the offspring animals in the DS group decreased compared to the S group. Our findings support the findings reported in previous studies of COX-2 expression in the CPE and COX-2 expression in the CE (6,7,18,19). Although there are studies showing weak COX-2 distribution in the COE (6), our findings are in accordance with studies showing that there is no COX-2 expression in these regions of the DE (17,19).

It has been reported that the frequent use of therapeutic drugs to treat diseases causes an increased incidence of male infertility (22) NSAIDs reduce the transcript levels of two important proteins which provide cholesterol transport to the inner mitochondrial membrane in steroid synthesis. These proteins are steroidogenic acute regulatory protein (StAR) and peripheral type benzodiazepine receptor (PBR) (23) It has been reported that ibuprofen administration induces a simultaneous decrease in testosterone levels and expression of steroidogenic enzymes (24). Balin et al. suggested that male offspring of rats exposed to ibuprofen during pregnancy had a reduction in the nuclear volume of Leydig cells, percentage of normal sperm morphology and testosterone levels, and ibuprofen adversely affected the reproductive parameters of male rats by altering the programming of the hypothalamus (25). In another study, it was reported that testosterone production was suppressed by indomethacin, paracetamol and acetylsalicylic acid (26). It also was shown that NSAIDs cause down-regulation of gene transcription in the hypothalamus-pituitary-gonad axis and decrease plasma testosterone levels (27). A recent study showed that testosterone levels decreased in rats treated with DS (28). According to the biochemical findings of our study, serum testosterone levels in DS group rats were considerably lower compared to S group rats. This finding of our study shows that prenatal DS administration leads to a reduction in serum testosterone levels in offspring rats. It is considered that a decrease in the testosterone levels is due to inhibition of StAR protein production in Leydig cells of offspring as a result of prenatal DS application (23,29).

Niu et al. demonstrated that DS may exert toxic impacts via mitochondrial damage, oxidative stress and ER stress (30). As reported in a previous study, administration of DS has been shown to initiate the production of free radicals and oxidative stress, which may cause dysfunction of GnRH secretion in the hypothalamus by crossing the blood-brain barrier (28). Altindağ and Rağbetli reported that Sertoli and Leydig cells diminished in the testicular tissues of the offspring of pregnant rats administered DS (10).

The mechanism behind the regulation of epithelial functions in the DE remains a mystery, and information on its effects on sperm cells is limited. However, the processes that sperm undergo in the DE are of great importance and any negativity that may occur during these processes can be an important cause of male infertility (31,32). Available data on the physiological role of COX-2 in the DE indicates that it may be involved in DE maturation and pubertal development, as well as in sperm maturation, motility, and transport (6). Our findings show that DS administration during pregnancy can reduce COX-2 expression in the DE. It is considered that this decrease in COX-2 expression in the DE tissues of the offspring in the DS group may have negative effects on epididymal cells and sperm cells. In addition, our hormonal findings show that a diminish in serum testosterone levels supports the data showing that COX-2 expression is under the influence of androgens (6,7).

Mast cells have been demonstrated to be found in the testis, DE and excretory ducts in the male reproductive system. Mast cells are considered into 2 types; MMC and CTMC. Both CTMC and MMC can be found in the DE and testis (33). In the present study, it was observed that there was a significant increase in the number of mast cells in the DE and VD of the offspring of rats treated with prenatal DS compared to the S group, and mast cells were especially abundant in the CPE. An increase in the number of mast cells in the VD and DE in the DS group is considered to be associated with hypogonadism due to the decreased serum testosterone level (25,27,29). An increased number of mast cells in the male generative tract has been associated with infertility Significant levels of tryptase have been reported in the semen of infertile patients (33). According to the Masson-trichrome staining findings of our study, an increase in connective tissue and fibrotic thickening was observed in the DE and VD of the DS group compared to the S group in the prenatal period. We believe that the increase in the number of mast cells in the VD and DE may cause fibrosis in the connective tissues, resulting in negative effects on the functions of these tissues.

TNF-α, COX-2, IL-6 and iNOS are cell signaling proteins involved in systemic inflammation and the formation of the acute phase reaction. In a study in which a seminal vesiculitis model was created in rats, it was shown that TNF-α and COX-2 expression were importantly raised in the experimental group compared to the control group (34). In our study, TNF-α expression was not encountered in the DE and VD of both the S group and the DS group. These findings show that COX-2 is physiologically expressed in the DE without inflammation. High levels of IL-10 were encountered in the semen of fertile men, while IL-10 levels were found to be considerably reduced in the seminal plasma of infertile men (35). In our study, moderate IL-10 expression was observed in the CPE and CE of both the S group and DS group in postnatal rats, while weak IL-10 expression was observed in the COE and proximal and distal parts of the VD. Our findings show that prenatal DS administration does not cause a change in IL-10 expression in the reproductive tract of postnatal offspring. As a result, the findings of our study reveal that DS application during

pregnancy can cause the downregulation of COX-2 expression in the DE and DD of offspring rats and therefore may have adverse effects on the male generative system. In addition, the findings of the current study showed that DS applied to pregnant rats caused a diminish in serum testosterone levels of offspring rats, a rise in the number of mast cells in the VD and DE and fibrotic thickening in the connective tissues of both VD and DE. The results of our study reveal that exposure to DS during pregnancy may have noxious impacts on the reproductive system of offspring rats.

Conflict of interest

The authors declared no conflict of interest.

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None to declare.

Authors' contributions

Concept: O.K., M.Ç.R., Design: O.K., M.Ç.R, Data Collection or Processing: O.K., M.Ç.R., Analysis or Interpretation: F.A., M.Ç.R., R.A., Literature Search: O.K., F.A., M.Ç.R., R.A., Writing: O.K., F.A., M.Ç.R., R.A.

Ethical Statement

Approval was obtained from Van Yüzüncü Yıl University Animal Experiments Ethics Local Committee, the study started. The ethics committee decision date is 25/02/2021 and the number of ethical committee decisions is 2021/02-24.

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