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The Effect of Diclofenac Sodium on Oxidative Stress and Some Reproductive Hormones in Rat Uterus*

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Diclofenac sodium (DS) is a non-steroidal anti-inflammatory drug (NSAID). DS exerts its effect by binding to cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and preventing the conversion of arachidonic acid into pro-inflammatory prostaglandins by chelation. The aim of this study was to investigate the effect of DS on mRNA expressions of both reproductive tissue antioxidant enzymes and serum reproductive hormones in female rats. A total 28 female albino Wistar rats were randomly divided into 4 groups (Control, I, II and III). The control group received no treatment. Groups I-II-III were injected intramuscularly with a dose of 2.5 mg/kg DS once a day for 3 days. After the 3rd DS application, Group I (2 hours), Group II (48 hours) and Group III (7 days) were sacrificed. Superoxide dismutase1 (SOD1), glutathione-peroxidase (GPx), catalase (CAT) in uterine and ovarian tissues, and serum microsomal prostaglandin E synthase-2 (mPGES-2), estrogen receptor α(ERα)-β(ERβ) and progesterone (PR) analyses were performed by PCR method. CAT decreased in uterus and ovary in all treatment groups (P<0.001). Uterine SOD1 showed increased in groups I-II and decreased in group III (P<0.001). Ovarian SOD1 increased in group I, decreased in groups II-III (P<0.001). ERα, ERβ increased in the treated groups I-II-III (P<0.001). PR increased in groups I-III and decreased in group II (P<0.001). mPGES decreased in group I and increased in groups II-III (P<0.001). In conclusion, it is observed that DS use causes negative changes in mRNA expression of some antioxidant enzymes and hormones in the reproductive system of female rats. It is recommended that the mating of female rats be postponed for seven days following the administration of DS, a common practice in human and animal health

Key Words: *Diclofenac sodium, female, PCR, rat, reproduction*

Diklofenak Sodyumun Rat Uterusundaki Oksidatif Stres ve Bazı Üreme Hormonları Üzerine Etkisinin Araştırılması

Diklofenak sodyum (DS), non-steroit anti-inflamatuar ilaçtır (NSAID). DS, etkisini siklooksijenaz-1 (COX-1), siklooksijenaza (COX-2) bağlanarak ve araşidonik asidin şelasyon yoluyla proinflamatuar prostaglandinlere dönüşümünü önleyerek gösterir. Bu çalışmanın amacı dişi sıçanlarda DS'nin hem üreme dokusu antioksidan enzimlerinin hem de serum üreme hormonlarının mRNA ekspresyonları üzerindeki etkisini araştırmaktı. Toplam 28 adet dişi albino Wistar sıçan rastgele 4 gruba ayrıldı (Kontrol, I-II-III). Kontrol grubuna herhangi bir tedavi uygulanmadı. Grup I-II-III'e 3 gün boyunca günde bir kez 2,5 mg/kg DS dozu intramüsküler olarak enjekte edildi. 3. DS uygulamasından sonra Grup I(2 saat), Grup II(48 saat) ve Grup III(7 gün) sakrifiye edildi. Uterus ve ovaryum dokularında süperoksit dismutaz1 (SOD1), glutatyon-peroksidaz (GPx) ve katalaz (CAT) ile serum mikrozomal prostaglandin E sentaz-2 (mPGES-2), östrojen reseptörü α (ERα), β (ERβ) ve progesteron (PR) analizleri PCR yöntemiyle yapıldı. Tüm tedavi gruplarında uterus ve ovaryumda CAT düzeyleri azaldı (P<0.001). Uterin SOD1 analizinde grup I-II'de artış, grup III'te azalma görüldü (P<0.001). Ovaryum SOD1'i seviyesi grup I'de arttı, grup II-III'de azaldı (P<0.001). ERα, ERβ tedavi edilen gruplar I-II-III'de arttı (P<0.001). PR düzeyleri grup I-III'de arttı, grup II'de azaldı (P<0.001). mPGES grup I'de azaldı, grup II-III'de arttı (P<0.001). Sonuç olarak DS kullanımının dişi sıçanların üreme sistemindeki bazı antioksidan enzim ve hormonların mRNA ekspresyonunda olumsuz değişikliklere neden olduğu gözlendi. Bu çalışmadan elde edilen veriler ışığında hem insan hem de hayvan sağlığında yaygın bir uygulama olan DS uygulamasını takiben dişi sıçanların çiftleştirilmesinin yedi gün ertelenmesi önerilmektedir.

Anahtar Kelimeler: *Diklofenak sodyum, dişi, PCR, rat, üreme*

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Introduction

NSAIDs, a group of non-opioid (non-narcotic) drugs, are among the most widely used medicines to treat symptoms of inflammation such as pain, fever, swelling (edema) and redness. In the US alone, more than 172 million COX inhibitors were prescribed in 2004 (1, 2).

The activity of NSAIDs is characterized by inhibiting the biotransformation of arachidonic acid (AA), a membrane-bound phospholipid, into prostaglandins, prostacyclins (PGI2) and thromboxane A2 (TXA2) via cyclooxygenase (COX) enzymes (2). The use of drugs in the NSAID group has been reported to inhibit COX-1 and COX-2 activity. COX-1 is constitutively present in most tissues, while COX-2 is mainly expressed at sites of inflammation (3, 4). Diclofenac sodium (DS) is a non-steroidal

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anti-inflammatory drug used to reduce inflammation and relieve pain. Diclofenac is a non-narcotic painkiller with a strong analgesic effect. DS is frequently used in both animal and human health for inflammatory and degenerative forms of rheumatism, rheumatoid arthritis, ankylosing spondylitis, osteoarthritis and spondylarthritis, painful syndromes of the vertebral column, extra-articular rheumatism, post-traumatic and postoperative pain, inflammation and swelling, dysmonera. Damage to the liver, kidney, heart and brain tissues caused by DS use is known (5, 6).

DS has been reported to adversely affect ovarian cortex volume, graff follicle volume, corpus luteum size, oocyte diameter, granulosa layer and thecal layers. It has also been reported to cause a decrease in the volume of the lumen, epithelial layer and lamina propria in the uterine cornua (7). Studies examining the effect of the use of DS on the uterus are limited to histopathology. The objective of this study was to examine the effects of DS on the mRNA expression of antioxidant enzymes (CAT, SOD1, GPX) in reproductive tissues and the levels of serum reproductive hormones (ERα, Erβ, mPGES, PG) in female rats.

Materials and Methods

Research and Publication Ethics: This study was conducted at Van Yüzüncü Yıl University Experimental Medicine Application and Research Center in accordance with the decision of Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee dated 30/11/2023 and numbered 2023/13-37.

Animals: In this study, 28 3-month-old healthy adult female Wistar Albino rats with an average weight of 150-200 g were used. Experimental animals were housed in standard cages at the Experimental Medicine Application and Research Center in an environment with 12 hours of light, 12 hours of darkness and a temperature range of 20-22ºC. Animals were fed with standard pellet feed and tap water. 28 adult female rats were randomly divided into 4 groups (Table 1).

Table 1. Experiment treatment groups

Group	n	Treatment
Control	7	No application was made.
Group I (2. hour)	7	Diclofenac Sodium (Diklovet; Vetas, Türkiye) was administered at a dose of 2.5 mg/kg for 3 days and the animals were sacrificed at the 2nd hour following the 3rd application.
Group II (48. hour)	7	Diclofenac Sodium (Diklovet; Vetas, Türkiye) was administered at a dose of 2.5 mg/kg for 3 days and the animals were sacrificed at the 48th hour following the 3rd application.
Group III (7. day)	7	Diclofenac Sodium (Diklovet; Vetas, Türkiye) was administered at a dose of 2.5 mg/kg for 3 days and the animals were sacrificed at the 7th day following the 3rd application.

The duration of DS use and sacrification times of rats in our study were designed according to the package insert of the drug used. Diclovet (Vetas, Türkiye) is recommended to be used at a dose of 2.5 mg/kg for a maximum of three days in animal health. It is also stated that the drug reaches the maximum level in serum concentration in the second hour after administration and remains in circulation for 48 hours (8).

Anaesthesia Protocol and Sacrification: Xylazine hydrochloride (Xylazinbio, Bioveta, Czech Republic) at a dose of 3 mg/kg and Ketamine hydrochloride (Ketasol, Richter Pharma, Interhas, Türkiye) at a dose of 90 mg/kg were used for anaesthesia. Rats were sacrificed by exsanguination method under anaesthesia (9).

Oxidative Stress

***Collection of tissue samples for RNA isolation and preparation for analysis*:** Ovarian and uterus tissues were collected under sterile conditions and stored at –80 °C (ILDAM, DF–210, Turkey) until the study day. On the study day, the tissues were allowed to thaw at room temperature, and approximately 30 mg of tissue was taken into sterile tubes. The tissues were then homogenized by adding 0.2 mL of sterile phosphate buffer. The homogenized tissues were centrifuged (Hettich, Rotofix 32, Germany), and the liquid portion of the tube was discarded. The pellet was used for total Ribonucleic acid (RNA) isolation (10, 11).

RNA extraction and analysis – cDNA extraction: Total mRNA was extracted from the obtained pellets using the Trizol Reagent–chloroform method. The amount and purity of the extracted mRNA were measured using a spectrometer (Biochrom, Anthos Zenyth 200RT, UK). A nanodrop spectrophotometer device (BioDrop, UK) was used for the quantitative evaluation of total RNA. To obtain complementary DNA (cDNA), reverse transcription was performed using the Wizscript kit (Wizbio WizScript cDNA Synthesis Kit, Korea) according to the protocol, with the Rotor–Gene Q Software–Run device. The expression levels of oxidative/antioxidant genes (CAT, GPx, SOD1, mPGES, ER-a, ER-b, PR) were analyzed. Real Time–qPCR Using the obtained cDNAs, the mRNA transcription levels of the target genes (CAT, GPx, SOD1, mPGES, ER-a, ER-b, PR) were determined in Table 2 (10, 11).

Optimized primer conditions were determined for each gene. An example of RT–qPCR reaction conditions is provided in Table 3. The RT–qPCR reactions were performed using the ROTOR–GENE Q system (Qiagen, Germany). To determine gene expression patterns related to oxidative stress, the transcription levels of CAT, GPx, SOD1, mPGES, ER-a, ER-b, PR were measured. ACTB (Actin Beta) was used as a control gene in the expression analysis. SYBR Green master mix (ENZO Life Science, cat: ENZ–NUC104–0200) was used for amplification detection. The Ct (cycle threshold) values were determined at the beginning of the logarithmic phase of the amplifications for each sample. The gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method, and the fold changes in expression were compared to the control group (10, 11).

Statistical Analysis: Data were analyzed using SPSS Windows 20.0 statistical software package. Mean values and standard deviations were calculated for each of the evaluated indicators. Kolmogorov-Smirnov test was performed and it was determined that the groups showed normal distribution. One-way ANOVA was used to evaluate the differences among groups, followed by Duncan test for multiple comparisons. Results with P<0.05 were defined as significant.

Results

As a result of PCR analysis of uterine tissue, CAT value was found to be significantly decreased at 2 hours, 48 hours and 7 days following DS administration compared to the control group (P<0.001). As a result of PCR analysis performed from ovarian tissue, CAT value was significantly decreased at 2 hours, 48 hours and 7 days following DS administration compared to the control group (P<0.001). As a result of PCR analysis from uterine tissue, it was determined that SOD1 value increased statistically at the 2nd and 48th hour and decreased at the 7th day following DS administration compared to the control group (P<0.001). As a result of PCR analysis from ovarian tissue, it was determined that SOD1 value statistically increased at the 2nd hour and decreased at the 48th hour and 7th day following DS administration compared to the control group (P<0.001). GPX value was negative in all groups. The analysis was repeated 3 times to avoid errors (Tablo 4).

Table 2. Primary sequence sequence of target genes

The Name of the Gene	Primary sequence sequencing	
	F. 5'-3'	R: 5'-3'
β-actin	F-5'-TTGCTGACAGGATGCAGAAG-3'	R-5'-GTACTTGCGCTCAGGAGGAG-3'
Catalase	F-5'-GGACGCTCAGCTTTTCATTC -3'	R-5'-TTGTCCAGAAGAGCCTGGAT -3
Glutathione Peroxidase	F-5'-GATGTGAACGGGGAGAAAGA-3'	R-5'-TTCATGGGTTCCCAAAAGAG-3'
Superoxide Dismutase 1	F- 5'-CAGAAGGCAAGCGGTGAAC-3'	R- 5'-CAGCCTTGTGTATTGTCCCCATA-3'
Prostaglandin E	F-5′-GACCCTGTACCAGTACAAGAC-3′	R- 5′-GAGGAGTCATTGAGCTGTTGC-3′
Estrogen Receptor-a	F-5-AATTCTGACAATCGACGCCAG-3	R-5-GTGCTTCAACATTCTCCCTCCTC-3
Estrogen Receptor-b	F-5-AACAAGGGCATGGAACATCTGCT-3	R-5-TCCGCCTCAGGCCTGGCCATCA-3
Progesterone	F-5-CATGTCAGTGGACAGATGCT-3	R-5-ACTTCAGACATCATTTCCGG-3

Table 3. RT-qPCR Reaction conditions

Reaction content	For one sample	Reaction Cycle
Tampon (2X)	10 µL	95°C 2' denaturation
Primer and control Primer (Beta aktin)	Forward : 0.5 µL	
	Reverse : 0.5 µL	95°C 5"
dH_2O	8.4 µL	*58°C -60°C
cDNA	0.6 µL	40 cycle
Total	20 µL	

* The binding temperature varied according to the primers. Melting Curve Ramp: 50-99 (1 degree increment) 90ºC 5 s.

Table 4. Uterus and ovary PCR results

	CAT		SOD1		GPx	
	Uterus	Ovary	Uterus	Ovary	Uterus	Ovary
Control	1.03±0.34[a]	1.07±0.55[a]	1.05±0.47[a]	1.06±0.5[a]		
Group I (2. hour)	0.48±0.2[b]	0[b]	22.94±12.44[b]	2.90±0.64[b]		
Group II (48. hour)	0.57±0.12[b]	0[b]	63.87±19.67[b]	0.50±0.14[b]		
Group III (7. day)	0.51±0.06[b]	0[b]	0.24±0.08[b]	0.45±0.11[b]	GPX value was negative in all groups.	

The difference between groups with different signs (a, b) in the same column is significant (P<0.001).

Table 5. Serum PCR results

	mPGES	ER-a	ER-b	PR
Control	1.00±0.12[a]	1.08±0.56[a]	1.04±0.41[a]	1.02±0.25[a]
Group I (2. hour)	0.45±0.08[b]	2.00±0.1[b]	4.36±1.58[b]	1.66±0.21[b]
Group II (48. hour)	1.94±0.43[b]	12.35±1.26[b]	1.40±0.39[b]	0.66±0.15[b]
Group III (7. day)	1.22±0.05[b]	7.59±1.56[b]	3.26±0.59[b]	1.26±0.13[b]

The difference between groups with different signs (a, b) in the same column is significant (P<0.001).

As a result of PCR analysis of serum samples, it was determined that there was a statistically significant increase in ERα value at 2 hours, 48 hours and 7 days following DS administration compared to the control group (P<0.001). There was a statistically significant increase in ERβ value at 2 hours, 48 hours and 7 days following DS administration compared to the control group (P<0.001). PR value was statistically increased at 2 hours and 7 days and decreased at 48 hours following DS administration compared to the control group (P<0.001). mPGES value was statistically decreased at 2 hours and increased at 48 hours and 7 days following DS administration compared to the control group (P<0.001) (Tablo 5).

Discussion

Diclofenac sodium from the NSAID group, which is frequently preferred in both human and veterinary medicine, was used. Diclofenac sodium has been put on the market under different names by many companies in Türkiye. Dikloron (tablet, ampoule), Voltaren (ampoule, eye drop), Miyadren (ampoule, tablet), Diclomec (gel, ampoule) are some examples (12).

In studies using diclofenac sodium, it has been reported to increase oxidative stress and cause histopathological changes in kidney, liver (13), heart (14) and testis (15) tissues. In addition, it has been reported to have teratogenic effects in the spinal cortex (16), increase sperm DNA damage (17), cause deterioration in hematological values (18) and stomach ulcers (19).

In this study, SOD1, one of the oxidative stress factors, was found to be statistically higher in the uterine tissue at the 2nd hour and 48th hour following DS administration compared to the control group (P<0.001). SOD1 level in ovarian tissue was found to be statistically higher at the 2nd hour following DS administration compared to the other groups (P<0.001). The results obtained are consistent with the drug's package insert. DS reaches its peak in the blood within two hours and begins to lose its effect after 48 hours (8).

CAT levels were statistically decreased in uterine tissue samples taken at the 2nd hour, 48th hour and 7th day after DS administration compared to the control group (P<0.001). In ovarian tissue, it was determined that CAT activity was present only in the control group and there was no activity in the other groups (P<0.001). The CAT enzyme protects against oxidative stress by converting 6 million H_20_2 molecules into H_20 and O_2 per minute. DS use reduces CAT enzyme activity in the uterus and stops it in the ovary for seven days, causing an increase in oxidative stress in these tissues (20).

Studies have reported that oxidative stress causes mitochondrial dysfunction, oocyte senescence, triggering apoptosis, degeneration of cumulus cells, poor embryo quality, anomalies, decreased fertilization rates, ovulation disorders, problems in meiosis, shortened telomere length and shortened embryo life span (21, 22).

It was found that mPGES levels decreased in serum samples at the 2nd hour and increased at the 48th hour and 7th day compared to the control group (P<0.001). As with all NSAIDs, DS exerts its effect by inhibiting PGE synthesis. As stated in the package insert of the drug, the drug reaches its maximum level in the blood in the 2nd hour after administration and loses its effect in the 48th hour. It was determined that the PGE results obtained as a result of the analysis were in parallel with the drug's package insert information. It is known that low PGE level causes relaxation of uterine smooth muscles, while high PGE level negatively affects ovulation, fertilization and embryonal development (23). In addition, NSAIDs have the side effect of triggering premature birth (24). Since PGE levels are important during reproduction and pregnancy periods, NSAID use is dangerous.

ERα levels were found to be higher at the 2nd hour, 48th hour and 7th day compared to the control group. ERβ levels were found to be higher at the 2nd, 48th hour and 7th day compared to the control group (P<0.001). Mechanistically, at the molecular level, inhibition of PGE stimulates estrogen synthesis by stimulating the activity of aromatase, the enzyme that converts androgens to estrogens in 2nd hour (25). The increase in estrogen levels may be explained by the inhibition of PGE synthesis due to DS use. The increase in ERα and ERβ levels at 48th and 7th day is not compatible with the PGE level at the same time. However, as shown in Table 5, ERα increases at 48th hour and ERβ increases at 2nd hour compared to the control group. This may be explained by the fact that ERα and ERβ are present at different levels in all cells and tissues and differ in their distribution and release mechanisms (26). Therefore, the increase in ERα and ERβ levels is parallel with the pharmacological properties of the drug and the PGE level is related. Estrogen levels are of high importance during reproductive periods. Estrogen levels may cause inhibition of implantation during the mating period. Since high estrogen levels during implantation periods will decrease the fertility rate, estrogen levels should be low during this period (27-30).

In this study, it was found that progesterone levels increased in the 2nd hour and 7th day after DS administration compared to the control group (P<0.001). The increase in PR levels at the 2nd hour has similar results with the study conducted by Salim et al (31). It is known that the relationship between PR and PGE is inversely proportional in hormonal mechanism (32). It is expected that the pharmacological properties of the drug used are compatible with the PGE level and the PR level shows an inverse ratio compared to PGE in the 2nd hour and 48th hour. PR level is expected to be at basal level in the hormonal mechanism of estrous cycle during ovulation and mating period. In this period, high PR levels constitute an obstacle for ovulation and fertilization (33-35).

In conclusion, it is observed that DS use causes negative changes in mRNA expression of some

antioxidant enzymes and hormones in the reproductive system of female rats. In light of the data obtained from this study, it is recommended that the mating of female rats be postponed for seven days following the administration of DS, a common practice in both human and animal health.

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