

Investigation of the effects of diclofenac sodium in rat ovary on the number of preantral follicles by stereological methods in prenatal period

Neşe Çölçimen^{1*}, Murat Çetin Rağbetli¹, Mikail Kara², Okan Arıhan³, Veysel Akyol¹

¹Departments of Histology and Embryology, Van Yuzuncu Yil University, Van, Turkey

²Departments of Histology and Embryology, Mustafa Kemal University, Hatay, Turkey

³Departments of Physiology, Van Yuzuncu Yil University, Van, Turkey

ABSTRACT

For healthy future generations pregnancy is a period in which maximum amount of attention should be given. In this period, choice, duration and dosage of medication as well as the changing physiology of the mother should be taken into account. Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of drugs that have analgesic, anti-pyretic and anti-inflammatory effects. This group of drugs can be used in the treatment of the ongoing rheumatic diseases or which occur during the pregnancy period and the complications that occur due to pregnancy. We aimed to show how it affects the number of ovarium preantral follicles of newborn fetus on rats by using Diclofenac Sodium which belongs to this drug family group.

Diclofenac Sodium (1 mg / kg, IM) was injected for 15 days from the 5th day of pregnancy to pregnant rats in Diclofenac Sodium group and saline (1 ml / kg, IM) was injected to the pregnant rats of the sham group at the same time period. In the 4th week after birth, ovarian tissue preparates of rat pups were stained with hematoxylin-eosin and ovarian total tissue volumes and preantral follicle counts were evaluated under light microscope.

There was no statistically significant difference between groups for ovarium total tissue volumes and preantral follicle numbers ($p>0.05$). Lowest value of follicular intensity was observed in Diclofenac sodium group among study groups however no statistically important difference was found ($p>0.05$).

Results obtained in our study may be related with administration of drug in not a high dosage (1mg/kg). Further studies may be performed with higher doses.

Key Words: Diclofenac sodium, ovary, preantral follicle number, rat, stereology

Introduction

Pregnancy is a very sensitive period for selection and use of drugs since developing fetus may get harm from this administration although those drugs are used for their desired beneficial effects. Drugs and chemical substances may pass placental barrier and pose untoward effects on fetus and they may cause permanent morphological and functional alterations (1). Nonsteroidal Anti-inflammatory drugs (NSAID) are used widely since they have analgesic, anti-pyretic, anti-inflammatory, anti-thrombotic, anti-cancer and chondroprotective effects (2). In Turkey antibiotics, pain killers and anti-rheumatic drugs are among the mostly used drug groups (3). Among them NSAIDs are also very commonly used in the World. This drug group has a ratio of 25% among all used drugs in Turkey (4).

Diclofenac, fenoprofen, ibuprofen, indometacine, meklofenamat, flurbiprofen, naproxen, piroxicam and sulindac which are NSAIDs are classified as category B/C in first and second trimester and as D in third trimester by the American Food and Drug Administration (FDA) (1,5). They are used for treatment of rheumatism as well as preterm birth and treatments of complications such as polyhydramnios (6). Since NSAIDs don't cause addiction they are used safely in long term treatment of many rheumatic diseases and generally known as anti-rheumatic drugs (6). They are preferred in musculo-skeletal and joint diseases due to their anti-inflammatory properties (7). In addition Diclofenac Sodium containing drugs are frequently prescribed by Gynecologists and Obstetrics for avoiding preterm birth, symptomatic polyhydramnios and pre-eclampsy and treatment of primary dysmenorrhea,

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*Corresponding Author: Neşe ÇÖLÇİMEN, Van Yuzuncu Yil University, Faculty of Medicine, Departments of Histology and Embryology, Van, Turkey, Tel: 0 (432) 225 17 03/ 25185, E-mail: colcimennese@hotmail.com

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dysmenorrhea due to intra uterine devices and menorrhagia (6).

In literature, effects of Diclofenac Sodium on over tissue are not elucidated in detail. Although there are studies conducted on effects of Diclofenac Sodium on pregnant and fetus, no study was found on the effect on number of preantral follicle number of ovarium of four weeks old female fetus stereologically. Guven et al. (8) studied over volume and graaf follicle however they did not focus on preantral follicles. In order to contribute for the clarification of the subject we aimed to study effect of Diclofenac Sodium on the fetal reproductive system when used during pregnancy. We focused on overs of fetus and assessed effect of NSAID on morphological volume and preantral follicle number via stereological methods.

Materials and Methods

Study was initiated with 9 female albino rats obtained from Experimental Medicine Research Center (26.03.2009 No:04). Rats used in the study were weighing 150 to 200 grams and none was copulated prior to the study. An ethical permission was obtained from Animal Studies Ethical Commission. 3 female and 1 male rats which are mixed for copulation were then separated into different cages. Female rats were divided into 3 groups as Diclofenac Sodium (3 individuals), control (3 individuals) and sham (3 individuals). Following copulation, observation of vaginal plaques was accepted as the "0 day" of pregnancy. Standard pellet and water were given to rats *ad libitum* under normal light and dark cycle with constant temperature (21 ± 2 °C). Rats separated for

Diclofenac Sodium group were administered with daily 1mg/kg Diclofenac Sodium (Diclofenac Sodium, 75mg/3 ml ampulla) intramuscular (I.M) for 15 days following the 5th day of their pregnancy. Same amount of saline was injected to pregnant rats in sham group. No intervention was administered to control group. Each rat was caged individually prior to labor. Postnatal offspring were separated into different cages as males and females. Diclofenac Sodium, control and sham groups were formed with 6 individuals per group which are all aged 4 weeks. Infant female rats were anesthetized with ketamine (50 mg/kg) and perfused with 10% neutral formaldehyde via intracardiac route. In the perfused animals, lower abdominal incision was administered and position of right over was localized within the abdominal cavity. Then over was extracted with uterus. Obtained overs were detached from their tuba uterina and paraffin blocks were prepared after completing routine histological procedures. Slices of 5µm thickness obtained from paraffin blocks were deparaffinized and were exposed to Hematoxylin-eosin staining. Follicles were classified according to their progress stages in accordance with the relevant literature on the subject. Prenatal follicles were evaluated in our study (Fig. 1). For the volume measurement of stereological analysis a modified method of Cavalieri principle was used (9). Following total tissue volume ratios were measured with dotted area ruler which is provided in Shetereom 1.5 version software program (10,11). AxioVision 3,1 (Zeiss axioplan 2 imaging Germany, Göttingen) program was used for imaging. Ovarium total tissue volumes, densities of preantral follicle numbers were compared with Kruskal-Wallis test.

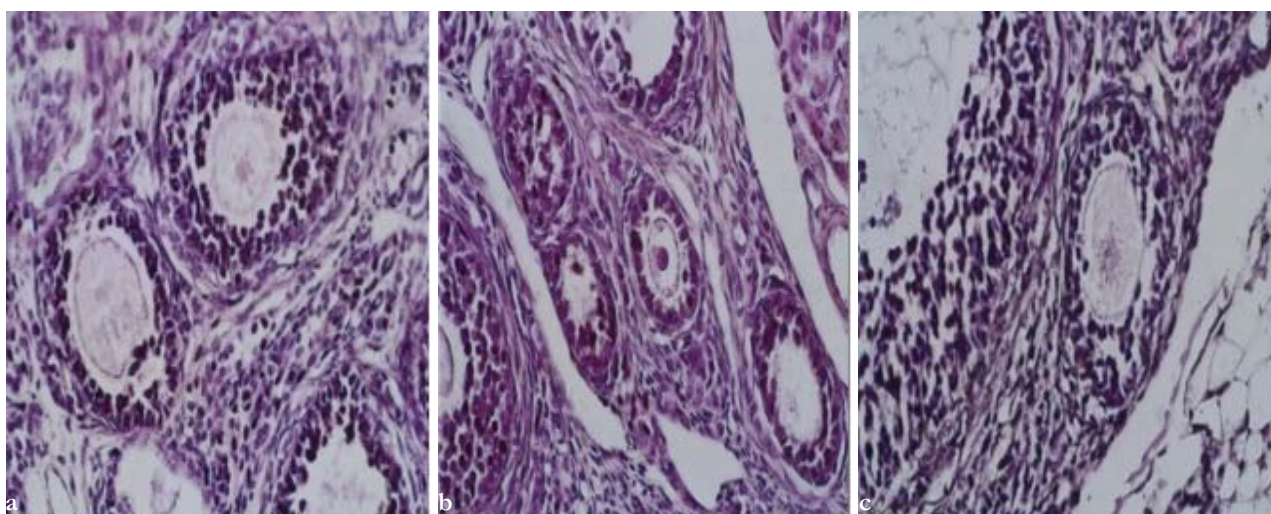


Fig. 1-a: View of follicles in an ovarian section from the control group **b:** View of follicles in an ovarian section from the Diclofenac Sodium group. **c:** View of follicles in an ovarian section from the sham group.

Table 1. Descriptive statistics and comparative results

Variable	Group	N	Median	Mean	St. Dev.	Min.	Max.	p
Ovarium Volume	Diclofenac Sodium	6	0.039	0.038	0.004	0.033	0.043	0.514
	Control	6	0.036	0.040	0.09	0.033	0.057	
	Sham	6	0.036	0.036	0.02	0.032	0.039	
Preantral Follicle Numerical Intensity	Diclofenac Sodium	6	3080900	3833720	2255548	2359400	8331360	0.133
	Control	6	6145640	5969260	2278184	2730200	8404760	
	Sham	6	3216600	3555873	1811412	1141720	6619200	

Results are presented as mean / median \pm standard deviation ($P < 0.05$).

Table 2. Volumetric point numbers of Diclofenac Sodium groups and Total volume of ovary

Rat	Volumetric point numbers	Total volume of ovary (cm ³)
Diclofenac Sodium 1	1556	0.0406
Diclofenac Sodium 2	2433	0.0330
Diclofenac Sodium 3	1371	0.0396
Diclofenac Sodium 4	3063	0.0341
Diclofenac Sodium 5	1255	0.0425
Diclofenac Sodium 6	1429	0.0380
Mean	1851.16	0.03796

Results

Coefficient of Variation values of groups (CV), were 0.13 in Diclofenac Sodium group, 0.19 in sham group and 0.21 in control group ($p < 0.01$). Therefore number of rats was considered as sufficient. Coefficient of error (CE) in groups were determined as 0.0379 in Diclofenac Sodium group, 0.0360 in sham group, 0.0344 in control group. Therefore it is accepted as $P < 0.10$.

When total volume of ovarium of Diclofenac Sodium group was compared with control group, no change in ovarium total volume was detected and values were not statistically significant ($P > 0.05$) (Table 2,3,4). When ovarium total preantral follicle numbers of Diclofenac Sodium group and control group were compared, no statistically significant change was detected ($P > 0.05$) (Table 5) (Table 1).

Discussion

NSAIDs show their anti-inflammatory, anti-pyretic and analgesic properties via inhibiting Cyclooxygenase-1 and 2 which are key enzymes for prostanoid formation (12,13). Inhibition of such enzymes does not only attenuate prostanoid level but also affect Renin-Angiotensin-Aldosterone system and may have an impact on kidney and many pathophysiological processes by affecting renal vascular tonicity. NSAIDs are among the mostly used drugs in the World and Turkey (4).

Table 3. Volumetric point numbers of Sham groups and Total volume of ovary

Rat	Volumetric point numbers	Total volume of ovary (cm ³)
Sham 1	1241	0.0385
Sham 2	2340	0.0354
Sham 3	2298	0.0321
Sham 4	2758	0.0367
Sham 5	1719	0.0382
Sham 6	1655	0.0353
Mean	2001.83	0.0360

Table 4. Volumetric point numbers of Control groups and Total volume of ovary

Rat	Volumetric point numbers	Total volume of ovary (cm ³)
Control 1	1216	0.0430
Control 2	1324	0.0382
Control 3	935	0.0570
Control 4	2293	0.0342
Control 5	2385	0.0342
Control 6	2309	0.0334
Mean	1743.66	0.0344

Table 5. Number of ovary total primordial follicles of the groups

Rat	Number of ovary total primordial cells (N)	Number of ovary primordial cells (Q-)
Diclofenac Sodium 1	3688.74	211
Diclofenac Sodium 2	3547.36	258
Diclofenac Sodium 3	5081.99	295
Diclofenac Sodium 4	3956.31	264
Diclofenac Sodium 5	4634.71	249
Diclofenac Sodium 6	6012.35	285
Control 1	2918.14	322
Control 2	5536.86	248
Control 3	4308.11	273
Control 4	4541.65	244
Control 5	5644.85	315
Control 6	9174.92	274

Those drugs are used increasingly since they don't cause addiction during long term use which is essential for the treatment of the rheumatic diseases (14). Acetylsalicylic acid is the prototype of NSAIDs. In a study conducted in USA in 1991, it was found that 6000 tons of acetylsalicylic acid was used per year (15). In our study we used non selective Diclofenac Sodium which is belonging to NSAID group. In the last 20 years Diclofenac Sodium are sold in different names (Voltaren, miyadren, dikloron, etc.) in Turkey (16). Diclofenac Sodium which has a common use has been shown to deteriorate many organ and tissue structures and cause side effects in many systems. Among the most well-known side effects are as follows; ulceration, bleeding, stomatitis in stomach; acute renal failure, hypertension, interstitial nephritis in kidney; erythema multiforme, bullous eruptions, eruptions, drug eruptions, pustular psoriasis in skin; hepatitis, Reye syndrome in liver; aseptic meningitis, head ache, dizziness, nausea in central nervous system; aplastic anemia, thrombocytopenia, neutropenia, hemolytic anemia in blood; pulmonary edema, pulmonary alveolitis, asthma and anaphylactic reactions in lungs (7,14).

Treatment of active rheumatic diseases during pregnancy is vital for both mother's health and for proper development of fetus. Present knowledge about use of anti-rheumatic drugs during pregnancy is limited since conducting prospective clinical studies during that period are not ethical. Therefore it must be considered well before using NSAIDs in order to avoid preterm birth or treatment of rheumatic disease (6). It was reported that NSAID use during pregnancy cause side effects in both mother and the fetus. It was observed that administration of Diclofenac Sodium during pregnancy prolongs pregnancy

period and decreased number of litters. Kökçü et al. (17) compared the pregnancy period and distribution of number of litters in Diclofenac Sodium administered and non-administered groups and reported pronounced differences. In experimental studies normal pregnancy period of rats prolong from 21-23 days to 28-29 days due to NSAIDs. Same situation was also observed in pregnant using NSAIDs (18,19). We observed no alteration in period of pregnancy or birth in our study. Chan et al. (20) administered different doses of Diclofenac Sodium to pregnant rats and found teratogenic effects due to high dosage. Carp et al. (21), administered 3 mg/kg Diclofenac Sodium to rats and observed almost complete prevention of blastocyte implantation of rat embryos. Contraction in Ductus Arteriosus (DA), hydrops fetalis, oligohydroamnios, ileal perforation, cystic lesions in brain and parenchymal degeneration especially in liver were observed in postnatal period due to NSAID use in pregnancy (6). Needs and Brooks (22), administered 4 mg/kg Diclofenac Sodium to rat embryos and observed rib defects in fetus. These studies reveal that different effects may occur on fetus due to different doses of Diclofenac Sodium administered in different time periods of pregnancy. On the other hand Russell (23) reported no teratogenic effect of 10 mg/kg Diclofenac Sodium administration on rabbits. In our study we did not observe a teratogenic effect during investigation of delivered offspring. Üstün et al. (24) investigated overs of Diclofenac Sodium (0.2 mg/kg) administered rats histopathologically and observed significant decrease in secondary follicle number compared to control and no significant difference in alteration of tertiary follicle number. In our study we did not find a change in preantral follicle number. Güven et al.

(8) investigated ovarium (medulla, cortex, Graaf follicle and corpus luteum volumes as well as granulosa, teka cells and antrum volumes) and volumes of uterine horns stereologically on female rats which were exposed to Diclofenac Sodium during their prenatal life period. No significant change in volumes of tissues was detected and this finding is parallel with total volume finding in our study.

Recent observations of rare congenital anomalies in Turkey (such as Jarko-Levin Syndrome, Spondylocostal Dysostosis) also support common use of NSAIDs in Turkey. Therefore it is advised to be cautious during prescription of NSAIDs such as Diclofenac Sodium in pregnancy by the physicians (7).

In our study effect of prenatal administration of Diclofenac Sodium (1mg/kg) on postnatal 4 weeks of aged rat ovarium preantral follicle number and on total tissue volume by stereological methods were studied and no statistically difference was observed between groups (Diclofenac Sodium, sham and control). Results obtained in our study may be related with dosage of administered drug (1mg/kg) which is not very high compared to higher doses in literature. Further studies may be performed with higher doses and focus on density of follicle granulosa cell mitochondria via stereological methods using electron microscopy.

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