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Sub-Chronic Effect of Different Doses of Diclofenac Sodium on Female Reproductive System in Rats

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely utilized drugs in today's world. These medications are well-known for their anti-inflammatory and analgesic properties. The goal of this study was to suggest and explain the sub- chronic effects of low and high doses of diclofenac on female reproductive system in rats. A total of 24 female rats were divided into 4 groups, six rats in each. The first group was given distilled water as a control for 35 days, the second and third groups were given diclofenac (1 mg/kg) and (5 mg/kg) for 35 days respectively. The fourth group was given a combination of diclofenac and mefenamic acid for 35 days. Hormonal, biochemical, and hematological tests were performed. Low dose diclofenac showed no significant change regarding luteinizing hormone (LH), progesterone, prolactin, and glutathione, but an increase in follicle stimulating hormone (FSH), and decrease in prostaglandin E2 PGE2 and estrogen compared to control group were documented. In contrast, high dose diclofenac alone or combined with mefenamic acid showed significant impact on female reproductive system documented by biochemical and histopathological evaluations. At hematological levels diclofenac decrease red blood cells (RBC), hemoglobin concentration (HGB), and platelet account but no change in the total white blood cells (WBC) were found. Sub-chronic use of diclofenac sodium (DS) alone or in combination with mefenamic acid have a deleterious impact on the female reproductive system, oxidative stress and hematological parameters.

Keywords: NSAIDs, Diclofenac sodium, Female reproductive toxicity.

التأثير دون المزمن لجرعات مختلفة من ديكلوفيناك الصوديوم على الجهاز التناسلي لأناث الجرذان

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الخلاصة

تستخدم مضادات الالتهاب غير الستيرويدية على نطاق واسع في عالم اليوم. تشتهر هذه الأدوية بخصائصها المضادة للالتهابات والمسكنات. كان الهدف من هذه الدراسة هو اقتراح وشرح التأثيرات دون المزمنة للجرعات المنخفضة والعالية من ديكلوفيناك على الجهاز التناسلي للأنثى في الفئران. تم تقسيم إجمالي ٢٤ أنثى من الجرذان إلى ٤ مجموعات ، ستة فئران في كل مجموعة. المجموعة الأولى أعطيت الماء المقطر كعنصر تحكم ، المجموعة الثانية والثالثة أعطيت ديكلوفيناك (١ مجم / كجم) و (٥ مجم / كجم) على التوالي. المجموعة الرابعة أعطيت مزيج من ديكلوفيناك وحمض الميفينامك لمدة ٣٥ يوما. تم إجراء الاختبارات الهرمونية والكيميائية الحيوية والدموية. لم تظهر الجرعات المنخفضة من ديكلوفيناك أي تغيير معنوي فيما يتعلق بـ LH ، البروجسترون ، البرولاكتين ، والجلوتاثيون ، ولكن تم توثيق زيادة في FSH ، وانخفاض في البروستاكلاندين و هرمون الاستروجين مقارنة بمجموعة التحكم. في المقابل ، أظهرت الجرعات العالية من ديكلوفيناك بمفردها أو مع حمض الميفينامك تأثيرًا كبيرًا على الجهاز التناسلي للأنثى موثقًا من خلال التقييمات البيوكيميائية والتشخيص المرضي . في مستويات الدم ، يقلل ديكلوفيناك من عدد كرات الدم الحمراء ، الهيموغلوبين، وحساب الصفائح الدموية ولكن لم يتم العثور على أي تغيير في إجمالي WBC. الاستخدام شبه المزمن لدايكلوفيناك وحده أو بالاشتراك مع حمض الميفينامك له تأثير ضار على الجهاز التناسلي الأنثوي ، والإجهاد التأكسدي والمعايير الدموية.

الكلمات المفتاحية: مضادات الالتهاب غير الستيرويدية ، ديكلوفيناك الصوديوم ، السمية التناسلية للأنثى.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a family of pharmaceuticals authorized by the FDA for the treatment of different diseases as antipyretic, anti-inflammatory, and analgesic [(1)]. They are indicated for muscular discomfort, dysmenorrhea, arthritic diseases, gout, and as opioid-sparing medications in some acute traumatic diseases [(2,4)]. At the mechanistic levels, NSAIDs inhibit the production of prostaglandins by blocking the cyclooxygenase (COX) enzymes. COX enzymes are divided into two types: COX-1 and COX-2. The first one is constitutively responsible for the creation of prostaglandins necessary for organ function, gastric protection, platelet aggregation, and vasoconstriction. The COX-2 isoform is inducible and found in the kidneys and vascular endothelium, and it is only created when the body is inflamed [(5)].

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Prostaglandins are made from the polyunsaturated phospholipid (arachidonic acid) by a series of multistep enzymatic processes, such enzymes are targeted by a variety of medications, the most common of which being (NSAIDs). It appears that chronic use of NSAIDs might have a negative impact on bodily tissues including renal dysfunction, blood pressure, hepatic damage, platelet inhibition, gastrointestinal, cardiovascular and reproductive system as recently studied organs[6,7].

Diclofenac sodium (DS) is one of the most commonly prescribed analgesic. It is a phenylacetic acid derivative that has been used as human medicine for many years to treat rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and primary nocturnal enuresis [8]. In the short term, it might potentially be used to treat acute musculoskeletal injuries and dysmenorrhea [9]. The availability of DS as an over-the-counter medication may lead to its misuse, resulting in gastrointestinal, liver, renal, and neurological consequences[10–12]. Furthermore, it can be seen that the hematological and biochemical indicators of rats may be significantly affected by DS. As a consequence, it can be inferred that DS, at various dosages and over different periods, may have negative impacts on critical animal tissues, resulting in hematological disorders, hepatic and renal impairments [13]. It was found that DS produce histological changes in the male rat's testicles, reduce sperms count, motility, viability, and significantly reduce testosterone level[10].

Little data are available regarding the effect of NSAIDs on the female reproductive system (FRS), however, ovulation, whether primary or secondary may be affected. Normally, prostaglandins play an essential role in the ovulation process. When luteinizing hormone (LH) surges and progesterone levels increase before the onset of ovulation, prostaglandins help to facilitate ovulation by digesting the collagen surrounding the ovarian follicle and also by stimulating smooth muscle contraction. However, the exact mechanism by which prostaglandins accomplish these steps is still unclear and required further research to declare it(14).NSAIDs can cause reversible infertility in women due to the suppression of cyclooxygenase enzymes and prevent formations of prostaglandin. Since NSAIDs are widely used and abused in women during their reproductive years, it has been proven that these women will have "luteinized unruptured ovarian follicles"[15]. Fortunately, natural ovulation was reported to be retained after these agents were stopped[16]. Cölçimen N et al. investigated the morphological and prenatal follicle number after low dose (1mg/kg) DS administration, no significant differences were observed in pregnant rats compared to the remaining groups[17]. Due to the limited knowledge are available on the effect of NSAIDs on the female reproductive system, the present work was designed to evaluate the possible effect of subchronic administration of different doses of DS alone or in combination with mefenamic acid on the female reproductive system in rats.

Materials and methods

Materials

Diclofenac sodium obtained from AL-Fayhaa pharmaceutical company in AL-Basrah city, Iraq, Mefenamic acid (Ponstan Forte) tablet as 500 mg active ingredient was purchased from (Pfizer manufacturing Deutschland GmbH, Betriebsstatte Freiburg, Germany). All hormonal analysis kits were purchased from Abbott Co. (USA), while PGE2 and GSH ELISA kits, were obtained from Shanghai YL Biotech Co. (China).

Animals

This study employed twenty-four mature female albino rats weighing between 150 and 250 g. They purchased from the Animal Holding Unit of the University of Basrah, The College of Veterinary Medicine, Iraq. The rats were maintained in plastic cages with a temperature of 25–30 °C and a 12-hour light/12-hour dark photoperiodicity. They were fed a standard pelletized diet and had free access to water daily, and were weighed before the beginning of the work as the starting point, then every 7days, and at the end of the experiment. After two weeks of acclimatization to the new laboratory environment, the rats were enrolled in the current study. The study followed the National Institute of Health Guidelines for the Use of Laboratory Animals, and ethical approval was received in October/2021 from the Pharmacy College/University of Basrah/Ethics Committee 3/5/293.

Experimental design

Twenty-four female rats were chosen randomly and divided into control and three (3) treated groups, with six animals in each group. The control group received only distilled water (as a vehicle of diclofenac sodium) orally administered daily to be compared and exclude vehicle, stress, and environmental effects. Group 1 rats received 1mg/kg (9) of DS gavaged daily. Group2 rats treated with a high dose of DS (5mg/kg) (18) were gavaged in a daily manner. Group3 rats received a combination of both DS (5mg/kg) and mefenamic (20mg/kg) via the oral route. All control and treated groups administered their scheduled doses for a period of 35 days as sub-chronic study model. The rats were weighed every 7 days, and the doses changed accordingly. After the last day of medication treatment, 5 mL of blood from each rat was withdrawn via posterior vena cave with a needle syringe while they were anesthetized with chloroform. Then the blood was distributed into two tubes one of them for hematological parameters evaluation, while the remaining were allowed to clot

and centrifuged at 10000rpm for 20 minutes, and the resulting serum was extracted, frozen, to be used later for biochemical and hormonal analysis. The ovaries of the rats were excised and fixed in formalin for histopathological investigation.

Hormonal assay

Serum levels of LH, FSH, progesterone, estradiol, and prolactin were measured using prepared kits of Chemiluminescent Microparticle Immunoassay (CMIA) for the quantitative determination of these hormones following the manufacturer's procedure while utilizing a diagnostic automated laboratory analyzer (Abbott Architect i4000, USA).

Biochemical assay

Serum glutathione and PGE2 were determined using an ELISA (enzyme-linked immune sorbent assay) based on the Biotin double antibody sandwich technology according to the manufacturer's instructions.

Hematological parameters

The anticoagulated blood was used to calculate the hematological parameters. red blood cells (RBC) count, hemoglobin (Hb) concentration, packed cell volume (PCV), total white blood cells (WBC) count, monocytes count, lymphocytes count, granulocytes count, platelets count, mean corpuscular volume (MCV), mean hemoglobin concentration (MCHC), and mean corpuscular hemoglobin (MCH) were all determined using automated hematology analyzer Nano 3 (GenoLab-TEK, Windsor, Canada).

Histopathological examination

Ovaries of each rat were sliced into tiny pieces, cleaned in normal saline, and stored in 10% formaldehyde. A sample of tissue was dried and buried in paraffin before being sliced into 3–4 mm slices and mounted on a thin glass slide. Staining was done with hematoxylin and eosin (H&E), and then a comprehensive histopathological investigation was performed under supervision of professional pathologist.

Statistical analysis

One-way ANOVA analysis was used among groups. Then Turkey's post-hoc analysis test was utilized for further assessment of data to obtain clear *P* values between groups. Data were expressed as Mean± SEM with $p<0.05$ significance. GraphPad Prism software (Version 6.0).

Results

Administration of DS for 35 days in Group 1 and Group2 decreased body weight gain compared to the control group as shown in figure (1). Group3 rats received co-treatment of DS and MA as a combination to evaluate the possible sub-chronic unwanted effect of these compounds. No changes in body weights were documented when correlating statistically between changes in body weights and periods of time (35 days) P=0.0687. Compared to the control group, changes in body weight in Group3 were different and no weight gain was documented, nevertheless, there is a significant increase in body weight in the remaining groups.

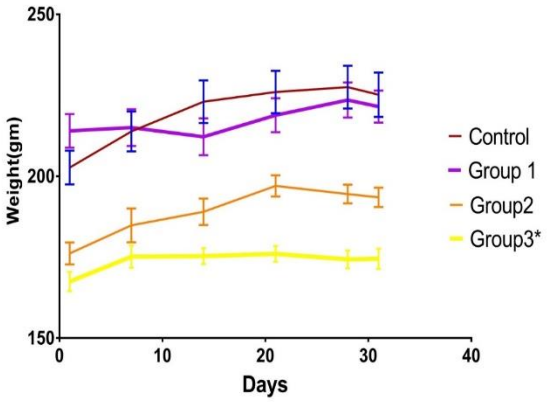

Figure 1. Changes in rats body weights with respect to days of the experiments. No significant changes in body weights during time were documented in in group 3 P>0.05. * Represents significant difference in comparison to control P<0.05. Results are presented as Mean ± SEM

In this study, measurement of serum PGE2as prognostic biomarker is important point to explain and correlate the relationship between NSAIDs use and female reproductive hormone effect. Figure (2) clearly proves this idea where a significant reduction in serum PGE2 level in group1 (treated with low dose DS), group2 (treated with a high dose of DS) P=0.0051 and group3 (treated with combination) P=0.0006 compared to the control group.

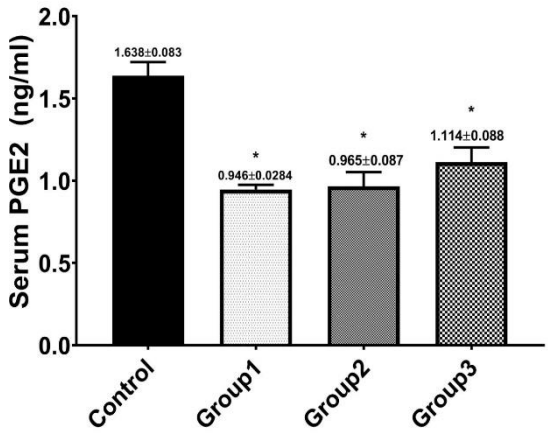

Figure 2. Impact of sub-chronic use of low and high pharmacological doses of Diclofenac sodium alone (Group1, 2) and in combination with mefenamic acid (Group3) on serum concentration of prostaglandin E2 (PGE2) in rats (N=24). Values are expressed as Mean± SEM. *represent significant difference P<0.05 among groups.

Serum concentrations of FSH were significantly elevated in all treated groups as shown

in figure 3A in comparison to the control group. Rats ingroup1 showed the highest significant value compared to other treated and control groups. Administration of pharmacological doses of DS in combination with mefenamic acid significantly increase serum LH value in group3 as shown in figure 3B. Groups 1 and 2 are well-matched with the control group and no significant differences were documented.

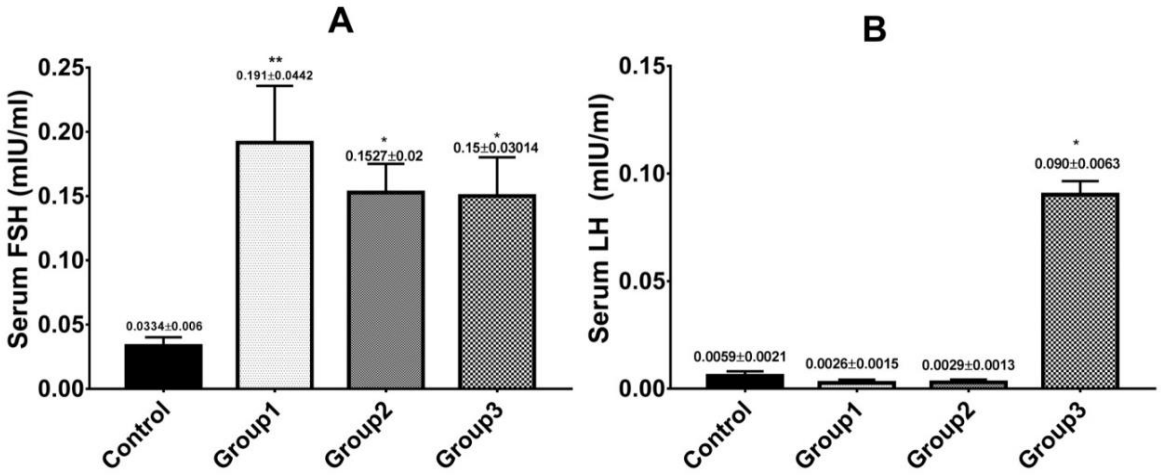

Figure 3. Impact of sub-chronic use of low and high pharmacological doses of Diclofenac sodium alone (Group1, 2) and in combination with mefenamic acid (Group3) on A: serum concentration of (FSH), B: serum concentration of (LH) in rats (N=24). Values are expressed as Mean± SEM. *represent significant difference P<0.05 among groups. ** represent significant difference P<0.01 among groups.

Serum concentrations of estrogen were significantly reduced in all treated groups as shown in figure 4A compared to normal control group. Oral administration of DS is associated negatively with serum estradiol concentrations. In contrast, Administration of a high pharmacological dose of DS in (Group2) and in combination with mefenamic acid (Group3) significantly increases serum progesterone value compared to the normal control group and Group1 as seen in figure 4B. Groups 1 associated with a non-significant (P>0.05) compared to control.

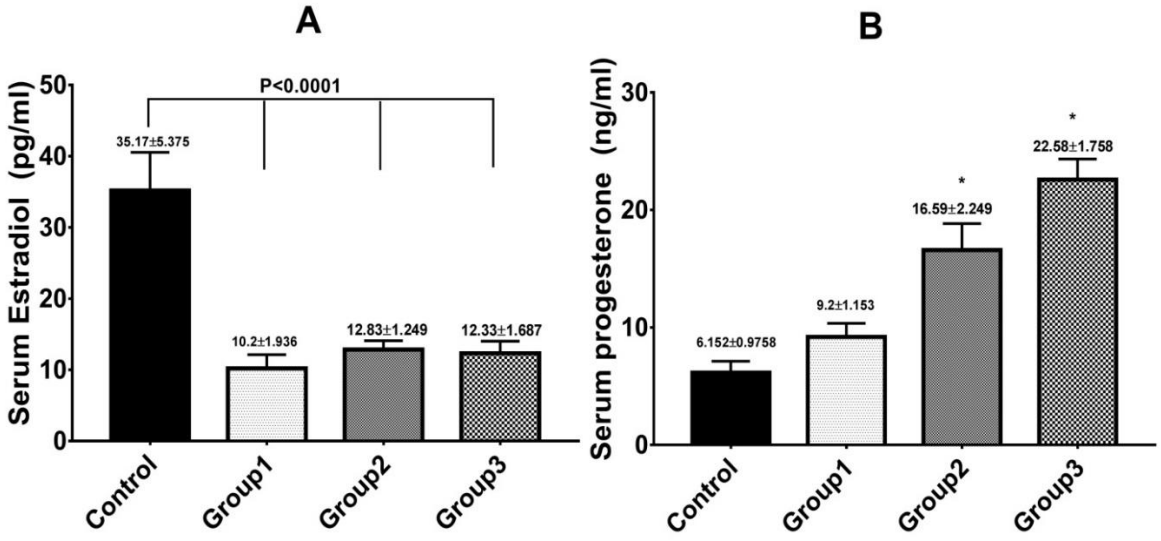

Figure 4. Impact of sub-chronic use of low and high pharmacological doses of Diclofenac sodium alone (Group1, 2) and in combination with mefenamic acid (Group3) on A: serum concentration of Estradiol, B: serum concentration of Progesterone in rats (N=24). Values are expressed as Mean± SER. *represent significant difference P<0.05 among groups.

Serum prolactin levels were evaluated in this work to obtain a complete explanation about the effect of NSAIDs on the female reproductive system. There are no significant differences were observed between Groups 1, 2, and control groups, all of them are well-matched as seen in figure 5. A significant reduction in serum prolactin level was detected in Group3 compared to the remaining group.

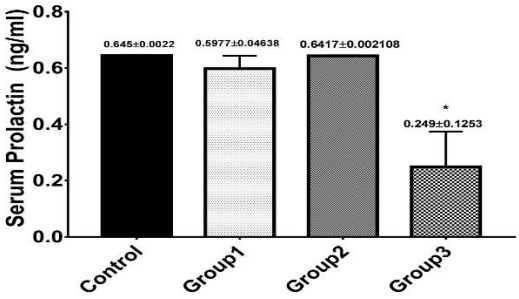

Figure 5. Impact of sub-chronic use of low and high pharmacological doses of Diclofenac sodium alone (Group1, 2) and in combination with mefenamic acid (Group3) on serum concentration of Prolactin in rats (N=24). Values are expressed as Mean± SER. *represent significant difference P<0.05 among groups.

Figure 6 summarizes serum concentrations of Glutathione in control and all studied groups. Serum Glutathione concentration significantly decreased in Group 2(rats exposed to a high pharmacological dose of DS) compared to the control group. No significant differences were documented between Groups1, 3, and control groups, all of them have comparable glutathione concentrations.

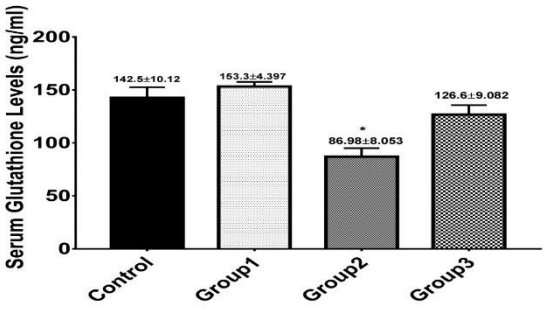

Figure 6. Impact of sub-chronic use of low and high pharmacological doses of Diclofenac sodium alone (Group1, 2) and in combination with mefenamic acid (Group3) on serum concentration of Glutathione levels in rats(N=24). Values are expressed as Mean± SEM. *represent significant difference P<0.05 among groups.

The microscopic finding of ovaries of control rats showed normal primordial follicles, primary and secondary follicles, Graafian's follicles, and normal parenchymal cells (figure 7A). In contrast to the treatment groups showing numerous follicles with thick and dark dyed granulosa and theca layers, Graafian follicles with pyknotic oocyte, multi follicles ovary, corpus luteum (CL), and asterisk fibrotic (figure 7B,C and D).

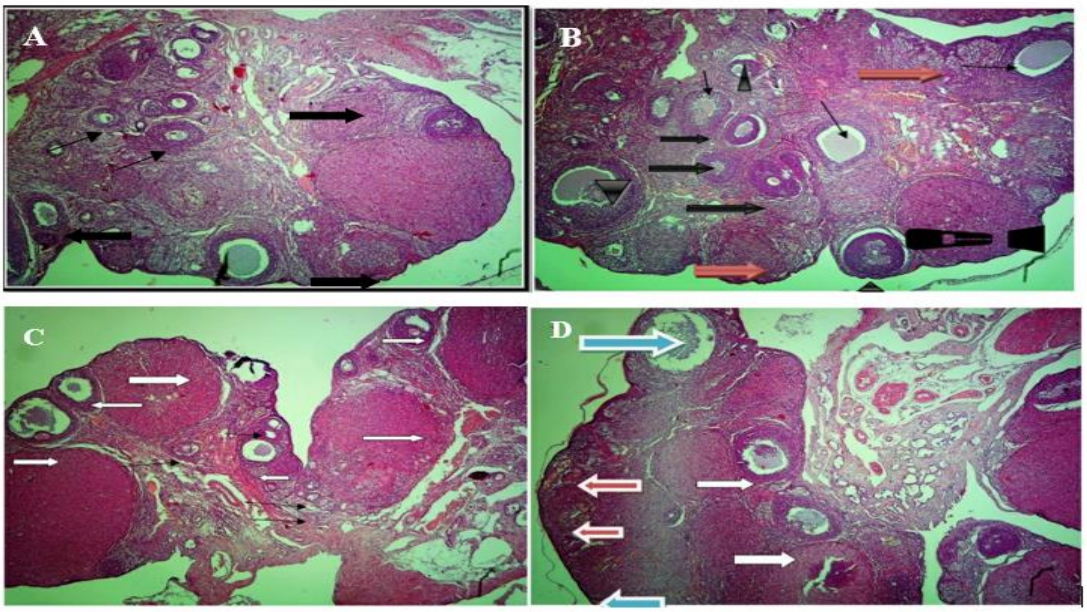

Figure 7. Light micrographs of the ovary section stained with H&E X20, A) representative image showed normal follicle growth, primary and secondary (thick arrowhead), oocyte with healthy nucleus (thin arrow), graafian and corpus luteum with healthy appearance (thick arrow). B) representative image showed numerous follicles with thick and dark dyed granulosa and theca and layers(black arrow), Graafian follicles with pyknotic oocyte(thick arrowhead), multi follicles ovary (thin arrow), corpus luteum (Cl); asterisk, fibrotic(red arrow).C) representative image showed numerous follicles(thin arrow), thick arrow, graafian follicles, oocyte, nucleus with healthy appearance, and Graafian follicles with pyknotic nucleus(whit arrow).D) representative image showed (white arrow) graafian follicles with healthy appearance, thick and dark dyed granulosa and theca and layers(blue arrow), corpus luteum with asterisk, fibrotic(red arrow).

Blood was collected form rats and characteristic complete blood count of all groups are summarized in Table (1). The concentrations of WBC were comparable in all treated and control groups. The finding of differential cell count showed that the percent of lymphocyte, and MID% were significantly reduced in Group3 and Group1 respectively compared to control. No significant differences were documented in granulocyte concentration in all treated and control groups. The red blood cell count and hemoglobin values were negatively associated with NSAIDs administration in all treated groups compared to control. Data on MCV, MCH, and MCHC give us an idea about the amount and average concentration of hemoglobin in RBC. Significant reduction in their values was detected in Groups 1 and 2 and 3 in comparison to that in control group. Results of the present work indicate a significant reduction in platelet count in all Groups that received DS alone or in combination with mefenamic acid compared to the control group.

Table 1. Impact of sub-chronic use of low and high pharmacological doses of Diclofenac sodium alone (Group1, 2) and in combination with mefenamic acid (Group3) on complete blood count in rats (N=24). Values are expressed as Mean± SEM. *represent significant difference P<0.05 compared to control.

parameter	Control Mean± SER	Group1 Mean± SER	Group2 Mean± SER	Group3 Mean± SER	P-Value
WBC (10^3/Ul)	10.8± 1.45	11.29 ± 2.35	13.8 ± 1.52	12.25 ± 1.46	P>0.05
Lym%	78.3±2.19	71.95± 4.65	72.65±3.53	60.37± 3.81*	P<0.05
Gran%	13.46 ±2	12.86 ± 2.57	16.5 ±3.01	17.23± 2.158	P>0.05
Mid%	8.23 ±0.53	5.18 ± 2.15*	10.83 ± 1.02	7.4± 0.86	P<0.05
RBC(10^6/Ul)	6.93± 0.15	5.16 ± 0.17*	5.43± 0.22*	6.22 ± 0.09*	P<0.05
HGB(g/dL)	13.22±0.2	11.08 ± 0.24*	11.08± 0.57*	11.5± 0.15*	P<0.05
HCT %	38.76 ± 1.1	34.06 ± 0.71	34.4± 1.96	36.26 ± 0.27	P>0.05
MCV(fL)	65.75±1.75	59.72± 0.41*	59.25± 1.6*	59.52±0.53*	P<0.05
MCH(pg)	20.81 ±0.97	21.55± 0.54	19.81 ± 0.4	18.25±0.2*	P<0.05
MCHC(g/dL)	33.3±0.31	32.5± 0.1	33.18 ± 0.07	33.55±0.1	P>0.05
RDW-CV %	13.18±0.27	15.01± 0.66*	14.46 ± 0.44	13.85± 0.32	P>0.05
RDW-SD(fL)	34.38±1.17	39.81± 1.46*	34.38 ±1.08	32.15± 1	P>0.05
PLT(10^3/uL)	567.7±20.28	421.5±23.42*	466.7±19.96*	399.3 ± 20.23*	P<0.05
MPV(fL)	8.75±0.522	7.06 ± 0.25*	7.08 ± 0.13*	6.96 ±0.16 *	P<0.05
PDW(fL)	8.75±0.52	8.08 ± 0.46	8.35± 0.31	8.13 ± 0.27	P>0.05
PCT%	0.3618±0.007	0.29 ± 0.01	0.46 ± 0.05	0.519±0.05 *	P<0.05
P-LCR%	11.41±1.6	8.9± 1.45	8.5± 0.6	8.18 ±0.99	P>0.05
P-LCC10^9/L)	62.5±8.58*	34.67±3.49	59± 7.96	58.83 ± 7.687	P<0.05

Discussion

(FRS) maybe affected by different toxicants that may in turn induce direct or indirect adverse outcomes. Certain chemicals digested and bio-transformed locally into toxic free radicals, whereas others cause toxic damage through hormonal regulation [19].DS is one of the most commonly used NSAIDs among women of reproductive age for the management of a range of women's illnesses, including dysmenorrhea and menorrhagia[20]. Chronic use and abuse of NSAIDs may lead to an unexpected effect on FRS, from this the current work was intended to explain how the sub-chronic use of DS affected the female reproductive system in rats.

An experiment in this study has shown administration of DS resulted in a reduced body weight gain in group1 (received low dose of DS) and group 2 (received a high dose of DS) as compared to the control group. Decrease in food intake, gastric discomfort associated with NSAIDs causes stomach and intestinal lesions as the most prominent adverse effects are the principal reasons for our results. Our findings are seen to be consistent with different studies, Elliott SN et al showed that use of DS in dose (5 mg/kg), bodyweight growth was significantly reduced and Alabi QK et al found the

treatment of the rats with DS resulted in a reduction in body weight. [18,21]. On other hand, other articles demonstrated a positive correlation between NSAIDs use and an increase in body weight. this may be explained by edema and impairment of renal function occurring in DS abuse [22].

In the current study, low and high doses of DS (Group1 and 2) alone or when combined with mefenamic acid (Group3) diminished serum PGE2 concentration significantly compared to the control group. Mechanistically, This reduction can be explained due to inhibition of PGE2 synthesis both centrally and peripherally by competitive reversible inhibition of COX enzymes [23]. These findings align with Nakatsugi S et al. who found that NSAIDs have the ability to inhibit the PGE2 production through COX-2 from exogenous and endogenous arachidonic acid [24]. Furthermore, Abdel- Halem MS et al found that DS was a powerful inhibitor of PGE2 biosynthesis in the brain [25].

Measurement of serum prostaglandins in this experiment is important to correlate and further interpret our finding regarding the impact of NSAIDs abuse on FRS.

Oral administration of DS alone and in combination with MA resulted in a significant elevation in serum FSH levels in comparison to the control group, the highest significant value seen in group 1. On the other hand, significant elevation in serum LH level in group 3, compared to the remaining groups. Measurement of serum menotropins levels in relation to other female hormones are interesting because the impact of negative and positive feedback mechanisms of all these hormones cascade is considered the key to discussing our results. Unfortunately few articles have been carried out on this field, however, these hormonal changes are similar to that found by. Ji K et al found a significant increase in transcription of FSHr and LHr, genes after exposure to NSAIDs [26]. In contrast, some studies showed treatment with NSAIDs causes a decrease in both serum LH and FSH levels [7], or non-significant changes in the serum levels of FSH and LH [27,28].The cystic appearance of the treatment animals' ovaries compared to the normal control group confirm these results. These histopathological changes was due to the failure of ovulation of a certain number of graafian follicles that had been converted into luteinized unruptured follicles [29]. Inhibition of PG synthesis in the preovulatory follicles by NSAIDs hampered the completion of the ovulatory process, resulting in follicular rupture failure and thus cyst formation[30].Our findings matched those of Tomioka RB et al, who reported an increase in LUF syndrome in Juvenile idiopathic arthritis (JIA) patients on NSAIDs [15]. Also Jesam C et al who showed Non-steroidal anti-inflammatory medicines (meloxicam or rofecoxib) are known to have comparable effects on ovulation when used systemically over several days. These medications caused luteinized un-ruptured follicles (LUF), or dysfunctional, delayed ovulation [31].

Evaluation of serum estrogen along with serum progesterone levels in this study are important to fully summarized the effect of DS on complete blood hormones of FRS. Serum estrogen levels were significantly reduced in all treated groups compared to the control group. Estrogen is a hormone that plays a major role in female reproduction. It is responsible for the ovulatory follicle's development, activating the pre-ovulatory surge of gonadotropins in the middle of the menstrual period, changing the condition of cervical mucus to make it easier for sperm to travel, and preparing the uterine endometrial lining for implantation [32]. PGE2 increases estrogen production by increasing the activity of aromatase, the enzyme that converts androgens to estrogens [33]. These results are in agreement with Wangwa EK et al found that NSAIDs (Piroxicam) has been shown to have detrimental effects on female fertility by lowering levels of estrogen, progesterone, and gonadotropin, also Hudson AG et al found that serum estradiol levels in NSAID users are considerably lower than in non-users [7,34]. Regarding progesterone levels assay, we found the opposite results compared to estrogens values. Serum progesterone levels were significantly elevated in both groups 2 and 3 compared to the control group while the group1 showed a non-significant effect because of limited articles talking about this project. However, drug molecules that inhibit PG synthesis could adversely affect progesterone value and consequently female hormones. These findings are similar to that of Amiridis GS et al who reported that meloxicam was used at various periods after insemination to reduce PGF2a release, raise luteal progesterone levels, and prevent early embryonic death [35]. On other hand, these results disagree with previous studies that found NSAIDs cause a decrease in serum progesterone levels like Dzięcioł M et al showed single dose administration of meloxicam cause a significant drop in progesterone levels, also Amina S et al showed that usage of mefenamic acid cause the progesterone levels to drop with dose equal to 0,5 mg/Kg 1 mg/Kg, 1,5 mg/Kg, and 2 mg/Kg[36,37].

To complete the view about the effect of NSAIDs on FRS, serum prolactin levels were measured. A non-significant change in the serum prolactin levels in treated groups compared to the control group were obtained except in group 3 which showed a significant reduction in serum prolactin level compared to the control group. One reason for that was inhibition of prostaglandins (PGs) biosynthesis in the central nervous system (CNS), PGS may stimulate prolactin release in the hypothalamus [25]. The current result comes in tune with previous studies that showed DS causes a rapid and sustained decrease in prolactin plasma levels in healthy volunteers [38]. In contrast, our finding was

challenged by Adeyemi WJ et al demonstrated DS treatment cause an increase in the level of prolactin in male rats [39].

Glutathione is the main abundant thiol anti-oxidant in the body. It's a natural antioxidant found in both male and female gametes, in varying quantities. It has been proven that it is involved in the fertilization process and early embryo development. It protects eggs from oxidative stress during folliculogenesis, and as a result, egg quality is highly dependent on it [40]. Excessive reactive species generation, such as reactive oxygen species (ROS), has been linked to diclofenac metabolism in vivo, resulting in oxidative stress, genomic damage, and cell death via apoptosis [41]. In this study, there is a significant decrease in serum glutathione concentration in group 2 treated with high dose DS compared to the control group. These findings are consistent with the results that reflect high doses DS administration to rats lower glutathione concentrations significantly compared to control groups [10,42].

Finally, the considerable changes in hematological markers in diclofenac sodium-treated rats might indicate toxicity. The overall WBC count did not change significantly in any of the treatment groups during this study. This is in agreement with previous studies [43]. Although Abd-El Megid S et al showed that NSAIDs causes a significant decrease in WBC count [44]. The considerable drop in white blood cell counts found in the treatment groups might be attributed to the participants' reduced feed consumption, as noted during the experiment. This is in line with previous research, which found that reduced feed intake had a significant influence on the hematopoietic system, resulting in lower numbers of white blood cells, platelets, and reticulocytes [45].The significant decrease in RBC and HGB levels in all treatment groups when compared to the control group might indicate drug-induced toxicity, which is characterized by excessive red blood cell destruction and anemia [46]. It might also be attributed to erythrocyte loss from gastrointestinal hemorrhage. When the body loses a significant amount of blood. The results of this investigation are consistent with those of Orinya OA et al [13], and Abdel-Rahman ON et al [47]. Significant reductions in MCV, MCH, and MCHC values were detected in all treated groups compared to the control group. Similar outcomes were observed in previous studies [48]. Significant reduction in platelet count in all treated groups compared to control group, it's reasonable that the lower platelet count in this study might be due to DS's inhibitory influence on platelet synthesis, lowering platelet aggregating strength. This is in agreement with earlier studies [13,49].

In conclusion, according to the present finding, we can assume that sub-chronic use of DS alone or in combination with MA has a deleterious impact on the female reproductive system through disruption of hormonal concentrations and regulations, shifting the balance toward oxidative stress by reduction of glutathione concentrations, and ovarian histopathological changes. Furthermore, considerable reduction in leukocytes, RBC, hemoglobin, and platelet counts were documented at hematological levels

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