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ORIGINAL ARTICLE

Sub-chronic effects of mefenamic acid alone or in combination with diclofenac on the female reproductive system in albino rats

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Abstract

Objective Although nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used, the long-term effects of prostaglandin inhibition on reproduction are unknown. The current study was designed to assess the effects of sub-chronic low and high mefenamic acid (MA) dosages alone or in combination with diclofenac sodium (DS) on rat reproductive functioning.

Methods Twenty-four female rats were separated into four groups of six animals each. The first group received distilled water as a control, the second MA (10 mg/kg. B.W), the third MA (20 mg/kg. B.W), and the fourth received a combination of diclofenac sodium (1 mg/kg) and mefenamic acid (10 mg/kg) orally for 35 days. Blood was drawn for hormonal, biochemical, and hematological tests. The ovaries were surgically removed and examined.

Results MA alone in low and high doses did not exhibit a significant change in LH, FSH, prolactin, and glutathione, but it did show an increase in progesterone and a decrease in PGE2 and estrogen compared to the control group. In contrast, the combination group demonstrated a significant increase in FSH, LH, and progesterone, as well as a decrease in PGE2, estrogen, and prolactin. In addition, MA alone or combined with DS causes ovarian histopathology abnormalities as compared to the control group. The hematological parameters RBC, HGB, and platelet count were lowered, but there was no change in total WBC.

Conclusion Sub-chronic administration of low or high pharmacological dosages of MA has been shown to have a deleterious effect on the female reproductive system in rats, as evidenced by hormonal, biochemical, and histological alterations. Furthermore, our data demonstrated significant alterations in hematological parameters, particularly with the usage of combination NSAIDs.

Keywords NSAIDs · Mefenamic acid · Sub-chronic · Hematological · Female reproductive toxicity

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used over-the-counter medications in the world, accounting for 5% of all prescriptions [1]. NSAIDs are most commonly used to treat pain and inflammatory conditions such as osteoarthritis, rheumatoid arthritis, postoperative surgical conditions, and menstrual cramps, and are also widely used as analgesics and antipyretics [2, 3]. Despite their broad therapeutic utility, NSAIDs are known to cause a variety of serious side effects, including gastrointestinal toxicity, cardiovascular risks, renal injuries, and hepatotoxicity [4–6].

NSAIDs work by inhibiting the enzyme cyclooxygenase (COX), which includes both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isozymes. From arachidonic acid, this COX enzyme catalyzes the formation of prostaglandins (PGs) and thromboxane [7]. The early 1970s saw the first indications of a possible connection between prostaglandin biosynthesis and ovulation [8]. Ovulation is a complex process that is triggered by a surge in luteinizing hormone (LH). The preovulatory LH surge stimulates COX-II expression and thus increases prostaglandin production

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by granulosa cells [9]. Many periovulatory processes are stimulated by prostaglandins, including the expansion of cumulus granulosa cells [10] and the stimulation of protease activities that degrade extracellular matrix [11]. COX-2 inhibition reduces follicular prostaglandin production, prevents follicle rupture, and inhibits oocyte release, indicating that COX-2 plays an important role in the ovulatory process [12]. Because prostaglandins initiate, regulate, or modulate all of these processes, COX inhibitors may have a significant impact on ovulation and possibly on oocyte release [13].

Indeed, the capacity of NSAIDs to decrease COX-2 activity and hence prostaglandin production is thought to be responsible for their anti-ovulatory effects. NSAIDs can cause reversible infertility in women due to the suppression of cyclooxygenase enzymes [14]. It has been proved the use of NSAIDs in reproductive-age women may lead to "luteinized unruptured ovarian follicles" (LUF) [15]. The correlation between LUF and NSAIDs was originally indicated in rat studies after administering a COX inhibitor. The rat ovaries were much heavier, with big cystic follicles that were identified on histological analysis as luteinized follicles with trapped ova [16].

NSAIDs are widely used in the treatment of women with endometriosis, dysmenorrhea, or menorrhagia [17]. As a result, women of childbearing age are typically the recipients of these medications. So the purpose of this study was to assess the effect of sub-chronic administration of different doses of mefenamic acid alone or in combination with diclofenac on the female reproductive system in rats.

Results

Figure 1 shows body weight changes throughout the experiment. Groups 1 and 2 showed less weight gain relative to the control group. However, Group 3 appeared to be different; a significant difference was detected compared with the remaining groups. On the other hand, there were no significant differences between each time point from day 0 to the end of the study on day 35.

The importance of measuring serum PGE2 as a predictive biomarker in this study is crucial in explaining and correlating the association between NSAID usage and female reproductive hormone impact. Figure 2 shows a substantial reduction in serum PGE2 levels in all treated groups compared to the normal control. The oral ingestion of MA alone or combined with DS had a negative impact on serum PGE2 levels.

Figure 3A, B indicates that the combination of MA and DS significantly increased blood LH and FSH levels in group 3. FSH and LH levels in the remaining groups were well matched, and no significant differences were found.

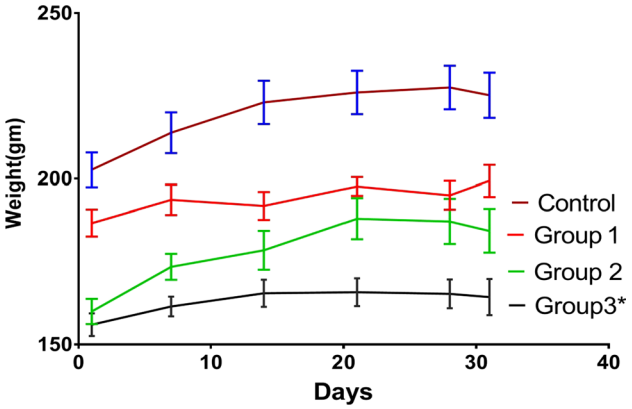

Fig. 1 Rats body weight changes during the course of the study. In group 3, there were no significant changes in body weight over time ($P > 0.05$). * indicates a significant difference from the control $P < 0.05$ The data are presented as a mean ± SER

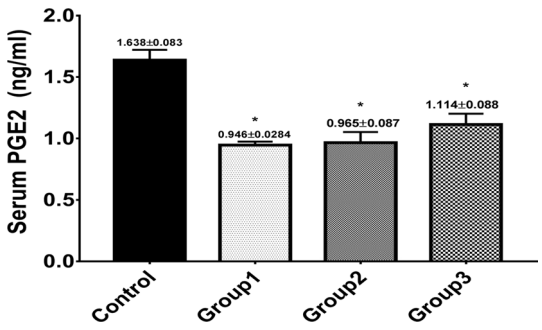

Fig. 2 Effects of sub-chronic usage of low and high pharmacological doses of mefenamic acid alone (Group 1, 2) and in conjunction with diclofenac sodium (Group 3) on serum prostaglandin E2 (PGE2) concentrations in rats ($N = 24$). Data are expressed as Mean ± SER. *represent significant difference $P < 0.05$ between groups

Figure 4A reveals that there has been a sharp decline in serum estrogen concentrations in all treated groups compared to the normal control group. The oral administration of MA and DS had a detrimental effect on serum estradiol levels. Figure 4B shows a marked increase in blood progesterone levels in all treated groups compared to the normal control group.

Figure 5 shows serum prolactin levels to provide a full picture of how NSAIDs affect the female reproductive system. One-way ANOVA and post hoc analysis revealed that no significant differences were found between groups 1, 2, and control groups; they are all well matched. Interestingly, from the graph, it can be seen that Group 3 had a considerable decrease in serum prolactin levels compared to the remaining groups.

An overview of serum glutathione concentrations in control and all studied groups is summarized in Fig. 6. There

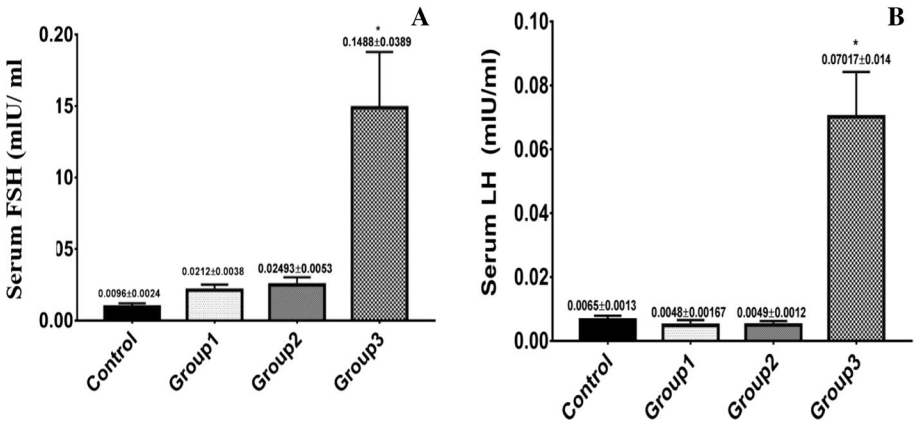

Fig. 3 A: serum concentration of Follicle stimulating hormone (FSH), B: serum concentration of Luteinizing Hormone (LH) in rats ($N=24$) after sub-chronic administration of low and high pharmacological doses of mefenamic acid alone (Group 1, 2) and in combination with diclofenac sodium (Group 3). The mean ± SER is used to express the values. *Represents a significant difference between groups $P<0.05$

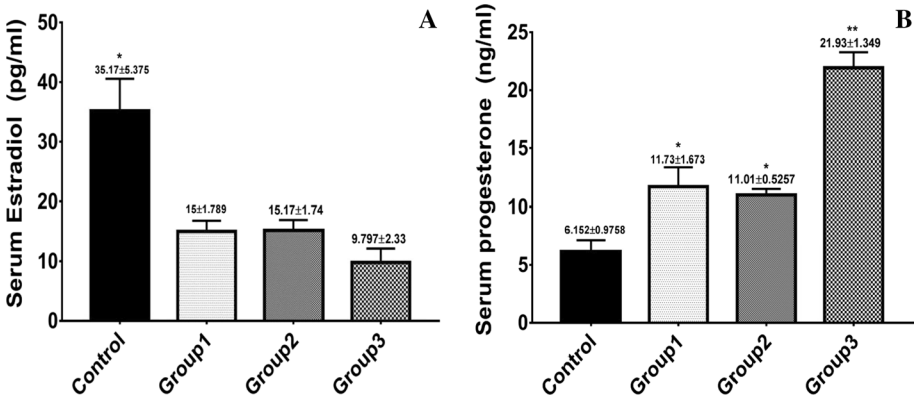

Fig. 4 A: serum concentration of Estradiol, B: serum concentration of Progesterone in rats ($N=24$) after sub-chronic administration of low and high therapeutic doses of mefenamic acid alone (Group 1, 2) and in combination with diclofenac sodium (Group 3). The values are given as Mean ± SER, with * representing a significant difference between groups ($P<0.05$)

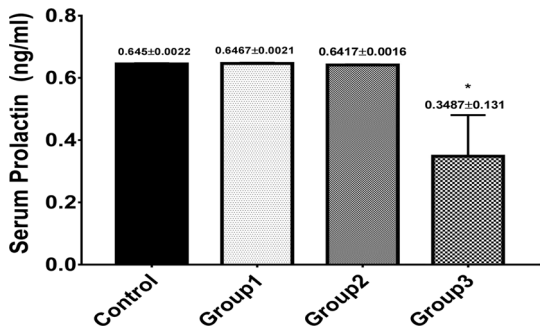

Fig. 5 Effects of sub-chronic use of low and high pharmacological doses of mefenamic acid alone (Groups 1 and 2) and in combination with diclofenac sodium (Group 3) on serum prolactin concentrations in rats ($N=24$). The values are given as Mean ± SER, with * indicating a significant difference between groups ($P<0.05$)

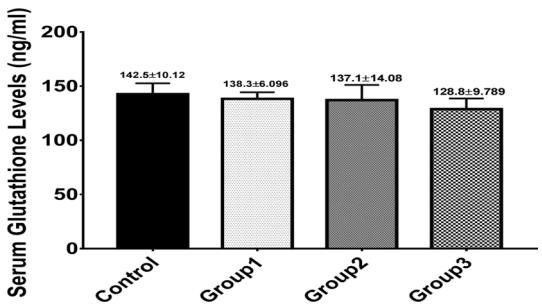

Fig. 6 Effects of sub-chronic administration of low and high pharmacological doses of mefenamic acid alone (Groups 1, 2) and in conjunction with diclofenac sodium (Group 3) on blood Glutathione levels in rats ($N=24$). The values are given as Mean ± SER, with * denoting a significant difference between groups ($P<0.05$)

was no significant reduction in serum glutathione levels among groups 1, 2, or 3, versus control.

On histopathological section, the ovaries of control rats showed normal follicle growth, primary and secondary oocyte with healthy nucleus, graafian and corpus luteum with healthy appearance as shown in (Fig. 7A). On the other hand, the ovarian section of female rats in the treated groups exhibited thickened multicystic follicles, unovulated graafian's follicles, congestive area of in the parenchymal tissue of ovary, and vaculation lining epithelial of follicles with lyses of oocyte (Fig. 7B–D).

In comparison with control rats, oral treatment of mefenamic acid (MA) alone or in combination with DS resulted in substantial reductions in RBCs, HGB, HCT, MCV, and MCH (Table 1). WBC concentrations were

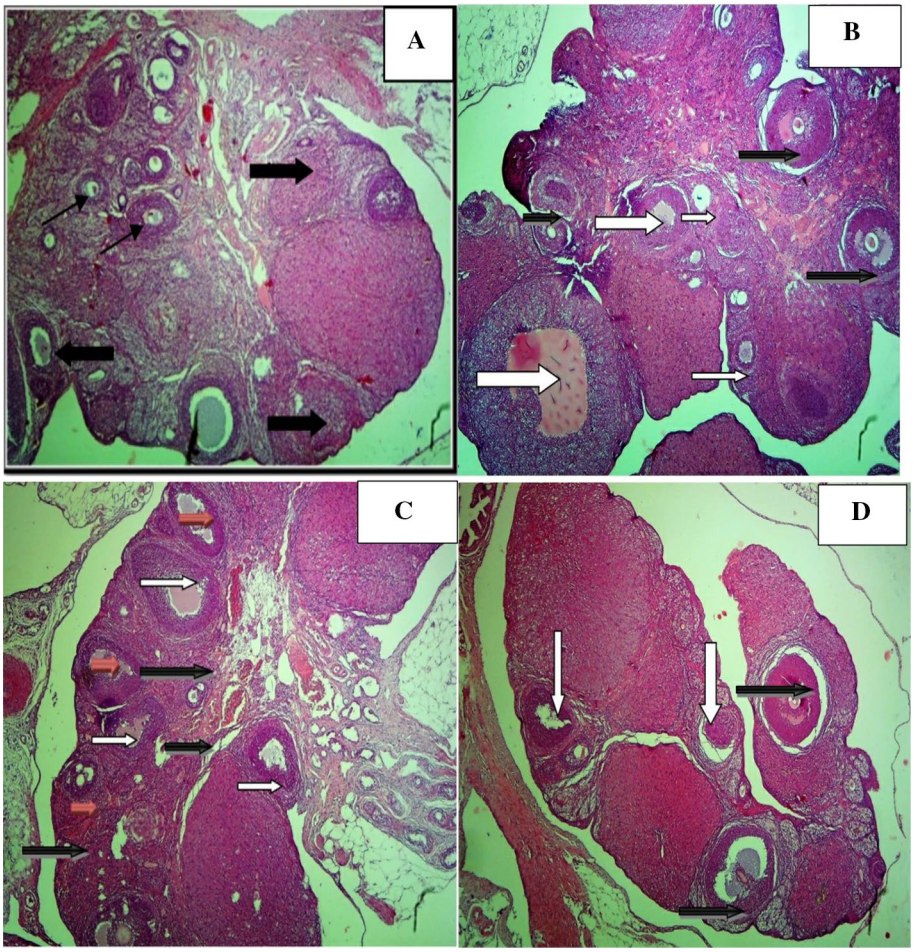

Fig. 7 H & E X20 stained light micrographs of the ovarian sections A) section of ovary in control group showed normal follicle growth, primary and secondaryoocyte with healthy nucleus (thin arrow), graafian and corpus luteum with healthy appearance (thick arrow). B) section of ovary in group 1 showed multi cystic follicles with thickness (white arrow), an ovulatory graafian's follicles (black arrow). C) section of ovary in group 2 showed congestive area of in the parenchymal tissue of ovary (black arrow), vaculation lining epithelial of follicles with lyses of oocyte (white arrow), and an ovulatory graafian's follicles (red arrow). D) section of ovary in group showed an ovulatory graafian's follicles (black arrow), vaculation lining epithelial of follicles with lyses of oocyte (white arrow)

Table 1 Complete blood count in rats ($N=24$) after sub-chronic administration of low and high pharmacological doses of mefenamic acid alone (Group 1, 2) and in conjunction with diclofenac sodium (Group 3)

Parameter	Control Mean ± SER	Group1 Mean ± SER	Group2 Mean ± SER	Group3 Mean ± SER	*P*-Value
WBC (10^3/Ul)	10.8 ± 1.45	13.57 ± 0.93	11.1 ± 1.76	9.42 ± 0.94	$P > 0.05$
Lym%	78.3 ± 2.19	71.86 ± 4.96	76.73 ± 1.18	69.96 ± 2.4	$P > 0.05$
Gran%	13.46 ± 2	16.48 ± 3.44	14.71 ± 1.27	22.7 ± 2.91	$P > 0.05$
Mid%	8.23 ± 0.53	11.17 ± 0.7526*	8.55 ± 0.42	7.33 ± 0.3621	$P < 0.05$
RBC (10^6/Ul)	6.93 ± 0.15	5.8 ± 0.396*	5.8 ± 0.23*	5.742 ± 0.14*	$P < 0.05$
HGB (g/dL)	13.22 ± 0.204	11.8 ± 0.58*	11.48 ± 0.31*	11.67 ± 0.13*	$P < 0.05$
HCT%	38.76 ± 1.1	34.1 ± 1.59*	33.98 ± 0.92*	36.11 ± 0.52	$P < 0.05$
MCV (fL)	65.75 ± 1.757	59.72 ± 0.41*	59.25 ± 1.64*	59.52 ± 1.5*	$P < 0.05$
MCH (pg)	20.81 ± 0.97	18.38 ± 0.72*	17.18 ± 0.39*	18.3 ± 0.26*	$P < 0.05$
MCHC (g/dL)	33.3 ± 0.31	32.93 ± 0.28	32.96 ± 0.21	33.23 ± 0.22	$P > 0.05$
RDW-CV%	13.85 ± 0.27	13.16 ± 0.36	13.13 ± 0.32	13.53 ± 0.32	$P > 0.05$
RDW-SD (fL)	34.38 ± 1.17	32.51 ± 1.25	33.78 ± 1.2	33.1 ± 1.05	$P > 0.05$
PLT (10^3/uL)	567.7 ± 20.28	374.2 ± 44.83*	506.2 ± 26.09	410.5 ± 37.76*	$P < 0.05$
MPV (fL)	8.75 ± 0.52	7.4 ± 0.19*	7.167 ± 0.22*	8.15 ± 0.2405	$P < 0.05$
PDW (fL)	8.75 ± 0.52	7.4 ± 0.19*	7.16 ± 0.22 *	8.15 ± 0.23	$P < 0.05$
PCT%	0.41 ± 0.019	0.28 ± 0.05	0.46 ± 0.01	0.36 ± 0.05	$P > 0.05$
P-LCR%	11.41 ± 1.6	7.08 ± 0.37*	6.13 ± 0.46	9.93 ± 0.85	$P < 0.05$
P-LCC (10^9/L)	62.5 ± 8.58	30.16 ± 5.67	43.16 ± 3.24	48 ± 4.5	$P < 0.05$

The results are given as Mean ± SER, with * indicating a significant difference from the control ($P < 0.05$)

similar in both the treatment and control groups. The results of the differential cell count revealed that the MID percent in Group 1 was much higher than that observed in the control group. In both treatment and control groups, no significant variations in granulocyte and lymphocyte concentrations were found. The current study's findings show that groups 1 and 3 had significantly lower platelet counts than those in the control group.

Discussion

NSAIDs are frequently utilized in several areas of pain management, such as following operational procedures and for the relief of dysmenorrhea and menorrhagia for dual effects as analgesics and reducing menstrual blood loss [18]. Abuse of NSAIDs can adversely inhibit normal reproductive processes, causing reversible infertility [14].

In this study, food intake and the rate of body weight gain were both lowered in the low- and high-dosage MA-treated groups compared to the control group. Our findings are supported by Van Lieshout et al. and Alabi et al. They showed that NSAIDs produced a decrease in food intake, which was accompanied by a decrease in body weight gain [19, 20]. However, Chouhan et al. found that sub-chronic treatment of mice with diclofenac did not result in weight loss [21]. Actually, high doses of NSAIDs lead to gastric irritation and subsequent weight loss [22].

Prostaglandin plays a key role in female fertilization; it is required for sexual maturation, ovulation, implantation, and pregnancy [11]. This factor is the cornerstone of our findings regarding the female reproductive system. A substantial reduction in serum PGE2 levels in all treated groups came in line with Shabani et al. and Amiri Farahani et al. who showed that MA improves the symptoms of primary dysmenorrhea and reduces menstrual bleeding by reducing PGE2 levels [23, 24]. In addition, Abdel- Halem et al. discovered that DS was a potent inhibitor of PGE2 biosynthesis [25].

One of the most integrative and functional endpoints for reproduction assessment has been recommended to be the measurement of pituitary and sex steroid hormones. The most striking observation to emerge from our results was a significant reduction in serum estrogen levels and a subsequent increase in serum LH and FSH values. The plasma amount of estrogen regulates FSH and LH release via a feedback mechanism. A dual positive feedback of estrogen on pituitary hormones explains such a finding [26]. Ji et al. found greater abundances of FSH receptors and LH receptor transcripts in the gonads of female fish exposed to NSAIDs due to increased pituitary FSH and LH production [27]. However, Chijioku-Agbonifo et al. showed that piroxicam has a deleterious influence on female fertility by reducing gonadotropin levels [28].

NSAIDs impeded the completion of the ovulatory process by inhibiting PG production in preovulatory follicles, resulting in follicular rupture failure and cyst development. The failure of ovulation of a specific number of graafian follicles that had been transformed into luteinized unruptured follicles resulted in significant histological alterations [15]. Our results are well matched with those observed in earlier studies. Furthermore, our findings were consistent with the fact that chronic NSAID use has been linked to ovulation suppression by delaying or preventing prostaglandin-mediated ovarian follicle rupture, resulting in reversible infertility in women of reproductive age [29].

Mechanistically, at the molecular level, inhibition of PGE2 stimulates the synthesis of estrogen by stimulating the activity of aromatase, the enzyme that transforms androgens into estrogens [30]. These findings are consistent with Hudson et al. suggesting that NSAID use is linked to decreased levels of circulating estradiol, which might be one mechanism by which NSAIDs protect women from breast cancer [31].

In contrast to earlier findings, other studies found that NSAIDs has estrogenic potential and is also considered a leading cause of endocrine disturbances [32].

Contrary to estrogen, progesterone levels were the total opposite. Serum progesterone levels were considerably higher in all treated groups. These findings match those of Tomioka et al. who found that the use of NSAIDs causes an increase in blood progesterone levels in individuals with juvenile idiopathic arthritis [15]. These findings, on the other hand, contradict previous research in humans and dogs that found MA causes a drop in serum progesterone levels and prevents ovulation by blocking the production of the yellow corpus luteum, which results in a drop in blood progesterone levels [33, 34].

Serum prolactin levels were evaluated to understand how NSAIDs affect the female reproductive system. Serum prolactin levels were not significantly different among groups, with the exception of group 3, which exhibited a substantial drop in serum prolactin levels compared to the control group. It is difficult to explain this result. However, PGS may promote prolactin release in the hypothalamus, so inhibiting production in the central nervous system (CNS) by NSAIDs inhibits prolactin secretion [25]. This came in tune with prior research that found diclofenac sodium induces a quick and prolonged drop in prolactin plasma levels in healthy people [35]. In another study, Koizumi et al. found that MA has no effect on serum prolactin secretion [36].

Another possible explanation for our finding is oxidative stress, which is caused by an imbalance between reactive oxygen species (ROS) and protective antioxidants and affects men and women throughout their reproductive

lives. GSH is a significant endogenous antioxidant generated by cells that participates directly in the neutralization of free radicals and reactive oxygen species. It is also involved in fertilization and early embryo development [37]. In the present work, there are no significant variations in glutathione concentrations between the control and all study groups. However, Ahmad et al. found the glutathione (GSH) content of albino rats was significantly reduced after treatment with mefenamic acid [38], and Ahmed et al. showed that the diclofenac-treated group induced a significant reduction in GSH concentration [39].

On hematological parameters, the total WBC count in all treated groups remained unchanged compared to the control. This came in agreement with Aziz et al. who found that Diclofenac has no effect on the total number of WBCs [40]. However, Asia et al. found NSAIDs cause an increase in the polymorphonuclear leukocytes [41]. The considerable fall in RBC, Hb, and HCT values in all treatment groups might indicate drug-induced toxicity, which is described by excessive destruction of red blood cells, resulting in anemia. It could also be caused by gastrointestinal bleeding, causing erythrocyte loss. The results of this study agree with those of Abdel-Rahman et al., who found that oral administration of DS at 11.6 mg/kg resulted in a significant drop in RBCs, HGB, and HCT [42]. Similarly, in other articles, significant dose-dependent decreases in RBC, Hb, PCV, MCV, and MCHC are caused by DS [43]. Platelet count, on the other hand, was significantly lower in groups 1 and 3 when compared to the control group. The lower platelet count in this study might be due to MA inhibitory influence on platelet formation via inhibiting the production of thromboxane A2 and prostacyclin. This is in accordance with previous studies that showed that NSAIDs caused a reduction in platelet count [44].

When two or more NSAIDs are used concurrently, there is an increased risk of side effects. Even though there is no medical reason to take multiple systemic NSAIDs at the same time, the growing use of over-the-counter nonsteroidal anti-inflammatory drugs could cause a serious problem with public health [45].

Materials and procedures

Materials

Mefenamic acid (Ponstan Forte) 500 mg tablet was purchased from (Pfizer Manufacturing Deutschland GmbH, Betriebsstatte Freiburg, Germany). Diclofenac sodium was obtained from Al-Fayhaa company for pharmaceutical industries in Al-Basrah, Iraq.

Experimental animals

Twenty-four sexually mature female albino rats weighing 150–250 g were recruited from the college of science, Kufa University. The animals were housed in plastic cages with standard bedding. Every day, all of the animals were evaluated for general behavior. The amount of food and drink consumed was measured twice a day. Body weight was measured once a week. The room temperature was kept between 18 and 24 degrees Celsius, with a light/dark cycle of 12/12 h. The rats were fed a conventional rodent diet and drank tap water (the water was changed twice per week). All potentially uncomfortable situations have been avoided. All trials began after two weeks of acclimating to laboratory settings.

Experimental work

Twenty-four rats were divided into 4 groups (control and three treated groups) of 6 rats each. The female rats were given the treatment by oral gavage for 35 days. The first group represents the normal control group that received only D.W. Group 1 received mefenamic acid (10 mg/k). Group 2 received mefenamic acid in a higher dose (20 mg/kg). Group 3 received a combination of 10 mg/kg mefenamic acid and 1 mg/kg diclofenac sodium.

One day later, the animals inhaled chloroform for anesthesia by putting them in a sealed container with a cotton ball loaded with an anesthetic agent. The rat's chest was opened, and 5 mL of blood was drawn directly from the vena cava. The blood was then separated into two tubes, one for measuring hematological parameters and the other for centrifugation at 10,000 rpm for 20 min to provide serum for biochemical and hormonal analysis. The ovaries were promptly dissected and kept in 10% formalin before being embedded in paraffin for histopathological inspection.

Hormonal assay

The quantitative assessment of serum levels of LH, FSH, progesterone, estradiol, and prolactin was assessed using prepared Chemiluminescent Microparticle Immunoassay (CMIA) kits and a diagnostic automated laboratory analyzer (Abbott Architect i4000, USA).

Biochemical assay

An ELISA (enzyme-linked immunosorbent assay) based on the Biotin double antibody sandwich method was used to

quantify serum glutathione and PGE2. They were carried out following the manufacturer's recommendations.

Hematological assay

Blood was collected in anticoagulated capillary tubes, and a full haemogram was performed to count total white blood cells (WBC), monocytes, lymphocytes, granulocytes, red blood cells (RBC) count, hemoglobin (Hb) concentration, packed cell volume (PCV), platelet count, mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) was determined using an automated hematological analyzer, Nano 3 (GenoLab-TEK, Windsor, Canada).

Histological examination

Each rat's ovaries were fixed in a 10% formalin solution. After dehydration, the samples were embedded in paraffin. Using a microtome, 4–5 mm tissue slices were cut and put on a thin glass slide. Hematoxylin and eosin (H&E) staining was used, followed by a complete histological examination.

Statistical analysis

The results are shown as mean ± SEM. A one-way analysis of variance with Turkey post hoc was performed to compare the treatment and control groups. At $P < 0.05$, values are considered substantially different. GraphPad Prism was used to conduct the analysis (Version 8).

Conclusions

Sub-chronic administration of low or high pharmacological doses of MA has a negative impact on the female reproductive system in rats, as evidenced by hormonal, biochemical, and histopathological changes. In addition, our findings revealed considerable changes in the hemodynamic parameters, especially with the abuse of combination NSAIDs.

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Declarations

Conflict of interest Bedoor A. Salim, Muhsin S. G. AL-Moziel, and Ausama Ayob Jaccob declare that they have no conflict of interest.

Ethical approval Animal experiments were performed in accordance with the guidelines established by the National Institutes of Health (NIH). Permission for these experiments was obtained by the Animal Ethics Committee of the College of Pharmacy at the University of Basra in October 2021, 3/5/293.

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