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Comparative Evaluation of Non-Steroidal Anti-Inflammatory Drugs on Ovarian Microstructural Anatomy, Serum Antioxidants and Hormones in Wistar Rats

Balakumo E Kia, M.Sc.[1], Innocent A Edagha, Ph.D.[1,2], Idorenyin U Umoh, Ph.D.[1], Edelungudi I Edagha, M.B.B.S.[3], Eno-Obong I Bassey, Ph.D.[1], Alice J Peter, M.Sc.[1], Moses A Ataben, M.Sc.[1]

[1]Department of Human Anatomy, Faculty of Basic Medical Sciences, University of Uyo, Uyo 520001, Nigeria.

[2]Department of Human Anatomy, School of Medicine, Moi University, Eldoret 30100, Kenya.

[3]Department of Family Medicine, Faculty of Clinical Sciences, University of Uyo Teaching Hospital, Uyo 520001, Nigeria.

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Abstract:

Objective: Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for the management of dysmenorrhea. Additionally, self-medication involving combinations of NSAIDs is frequent. We hypothesized that exposure to NSAIDs, either singly or concomitantly, may alter ovarian microstructures, hormones, and antioxidative status.

Material and Methods: Forty young, adult female Wistar rats, weighing between 122-173 g, were divided into eight groups (A-H): five rats per group. Group A served as the control and received distilled water (5 mL/kg body weight). Group B received piroxicam (0.3 mg/kg), Group C received Ibuprofen (6.7 mg/kg), and Group D received diclofenac (1.7 mg/kg). Groups E, F, and G were administered combinations of two NSAIDs: piroxicam+ibuprofen (0.3 mg/kg+6.7 mg/kg), piroxicam+diclofenac (0.3 mg/kg+1.7 mg/kg), and ibuprofen+diclofenac (6.7 mg/kg+1.7 mg/kg), respectively. Group H received a combination of all three NSAIDs: piroxicam, ibuprofen, and diclofenac (0.3 mg/kg+6.7 mg/kg+1.7 mg/kg). All administrations lasted for 3 days, and animals were sacrificed 24 hours later under ketamine anesthesia. Blood and ovaries were harvested for analyses using standard protocols.

Results: Ovarian histology demonstrated stromal hyperplasia, degenerative zona-pellucida, atretic and vacuolated follicles in groups D to H. Progesterone and estradiol concentrations showed increasing trends in groups C, D, F, G, and H compared to A. Superoxide dismutase concentrations significantly (p-value<0.05) increased in drug-administered groups, except in B and H, compared to the normal control (NC). All groups had significantly (p-value<0.05) increased catalase and glutathione peroxidase concentrations compared to the NC group, except group B.

Contact: Innocent A Edagha, Ph.D.
Department of Human Anatomy, Faculty of Basic Medical Sciences,
University of Uyo, Uyo 520001, Nigeria.
E-mail: innocentedagha@uniuyo.edu.ng, innocentedagha@mu.ac.ke

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Conclusion: A 3-day NSAIDs oral administration is associated with gonadotoxicities in the ascending order: diclofenac >ibuprofen >piroxicam, and in the combination groups: piroxicam+ibuprofen+diclofenac >piroxicam+diclofenac; ibuprofen+diclofenac; piroxicam+ibuprofen, respectively, in female Wistar rats.

Keywords: antioxidants, dysmenorrhea, hormones, non-steroidal anti-inflammatory drugs, ovary

Introduction

Dysmenorrhea is a painful menstrual cramp, originating from the uterus and thought to be one of the most prevalent gynecological disorders among women of childbearing age[1]. It is a common occurrence in the population, even though frequently underdiagnosed, because affected women avoid seeking medical attention[2]. It is classified into two broad categories, based on pathophysiology, namely: primary, or secondary dysmenorrhea (SD). Primary dysmenorrhea (PD) is the most common and is associated with spasmodic and painful cramps in the lower abdomen, with onset shortly before or at the beginning of menses in the absence of any pelvic pathology[3]. It starts at adolescence, often within 6 to 24 months after menarche. It manifests as a classical cyclic pattern demonstrating severe discomfort during the first day of menses, lasting until about the third day. This often disturbs or unbalances physical health, quality of life, and productivity. Hence, it elicited the need to seek either temporal remedies or fixes to handle said situations. It is estimated that PD is one of the leading causes of absenteeism from academic work or official duties, translating to an estimated loss of 600 million hours and $2 billion annually in the United States[3].

The physiological causes include: the release of prostaglandins (PG) from the endometrium during the onset of menstruation, leading to heightened pain in individuals with elevated PG levels[4]. However, irrespective of the cause of dysmenorrhea, treatment choices are almost the same, because they are all aimed at relieving pain, which often involves the administration of oral analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), contraceptives, or certain non-pharmacological options; such as exercise, heat therapy, acupuncture or dieting[5].

Reactive oxygen species (ROS) play a crucial role in ovarian physiology, by regulating processes like meiosis, ovulation, and corpus luteum function. However, excessive ROS production can overwhelm antioxidant defenses, leading to oxidative stress. This imbalance causes cellular damage, affecting oocyte quality, inducing granulosa cell apoptosis, and accelerating corpus luteum degeneration. Oxidative stress markers in ovarian tissue include: increased malondialdehyde (MDA) levels, decreased superoxide dismutase (SOD) and glutathione (GSH) levels. These changes contribute to various ovarian diseases, including age-related dysfunction, cancer, and polycystic ovary syndrome[6].

The most common and readily available treatment option for dysmenorrhea is a class of drugs belonging to the NSAIDs. All NSAIDs have analgesic, anti-inflammatory, and anti-pyretic properties, and their mechanism of action is via the reduction of prostaglandin production, and relieving associated symptoms[7]. As a result, they are advantageous in the clinical environment, since every patient is affected by at least one of these symptoms. Although NSAIDs may differ in chemical structure, they produce a uniform decrease in PGs synthesis by inhibiting the cyclooxygenase (COX) enzyme responsible for their release[8].

Piroxicam is an NSAID from the oxicam family that inhibits the synthesis of PG in body tissues via COX inhibition[9]. Additionally, piroxicam exhibits analgesic, anti-inflammatory and antipyretic activities[10]. It has a half-life of 14-15 hours, and a peak onset of action at 15-30 minutes for a single dose of 20 mg, and or multiple doses amounting to 20 mg. Its peak plasma concentration time is about 3-5 hours for a single 20 mg dose, and between 3-8 hours for multiple doses of 20 mg daily. It was approved by the food and drug administration in 1982, and is used to treat and manage headaches, migraines, pain, swelling, stiffness and tenderness caused by different types of arthritis as well as other musculoskeletal conditions[10].

Ibuprofen is a propionic acid derivative having the same mechanism of action as piroxicam, having been introduced in 1969[11]. It is particularly effective in dysmenorrhea, due to its inhibition of PG synthesis. Additionally, it is widely utilized in the management of musculoskeletal disorders, such as rheumatoid arthritis and osteoarthritis[9]. Indications for usage include: soft tissue injuries, fractures, vasectomy, tooth extraction, postpartum and postoperative care, effectively suppressing swelling and inflammation[12].

Diclofenac sodium is an analgesic-antipyretic anti-inflammatory drug belonging to the phenylacetic acid derivative class. It inhibits PG synthesis and is somewhat COX-2 selective[13]. Diclofenac is also widely used in conditions, such as rheumatoid and osteoarthritis, bursitis, toothache, dysmenorrhea and post-traumatic/postoperative inflammatory situations, providing rapid pain relief, and reducing wound edema[9].

Armour et al.[14] reported that most women receive less than satisfactory relief from analgesics, such as paracetamol. This, in turn, possibly leads to dependence on NSAIDs for pain relief during menstruation. However, Omar[8] reported that apart from a rapid onset of action, there is no proven advantage in the use of more than one NSAID at a time. Unfortunately, with the advent of over-the-counter NSAIDs, combination therapy has been thought to maximize the chances of having faster therapeutic success and allow investigation of their impact on ovarian microstructure, antioxidants, and hormones. As a result, it has become common practice for many menstruating women to use such combinations without recourse to the consequences.

Herein, using adult Wistar rats as the animal model, we evaluated the impact of selected, commonly used over-the-counter NSAIDs on ovarian structure and function, including the potential risks associated with their unsupervised use for managing dysmenorrhea. We hypothesized that different NSAIDs would have varying degrees of impact on ovarian structure and function, and that unsupervised use for dysmenorrhea management could potentially lead to adverse effects on ovarian health.

Material and Methods

Drugs and chemicals

Piroxicam, was sourced from Neimeth Pharmaceuticals PLC (Lagos, Nigeria): lot number 30508003. Ibuprofen was obtained from Ranbaxy Nigeria Limited (Ogun, Nigeria): marked with batch number AA76897. Diclofenac, was acquired from Pharmatex Nigeria Limited (Lagos, Nigeria): batch number BJ07537. Hormonal assay kits were procured from Monobind Inc. (Woodland Hills, California).

Ethical consideration

The experimental procedures involving animal care were conducted following the international guidelines for care and use of laboratory animals. Strict adherence to all recommended protocols and regulations was ensured, with ethical clearance obtained from the State Health Research Ethics Committee of the Akwa Ibom State Ministry of Health (Nigeria): Ref No. MH/PRS/99/VOL.V/627. Likewise,

the study was undertaken in accordance with the Basic and Clinical Pharmacology and Toxicology Policy for experimental studies, as reported by Tveden-Nyborg et al[15]. This highlights the ARRIVE guidelines 2.0, as the checklist for the purpose of animal research documentation for in vivo experiments.

Animal care and use

A total of 40 sexually mature, female Wistar rats having weights ranging from 122–173 g were obtained from the Animal House Unit of the Faculty of Basic Medical Sciences, University of Uyo. The animals were housed in plastic cages, with stainless grill tops and maintained at room temperature under hygienic conditions. The animals were maintained on growers pelletized feed (Vital Feeds, Nigeria), with distilled water given *ad libitum.*

Dose conversion and stock preparation

The dosage administered to each animal was determined after calculations taking into consideration the dose of a standard human adult of 60 kg[16]. Recommended doses (mg/kg) of the human doses of the drugs were thus adjusted and calculated using the formula:

$$\text{Dose (mg/kg)} = \frac{\text{Human Dose (mg)}}{\text{60 kg}}$$

The stock was prepared by dissolving the drugs in 100 mL of distilled water, and the stock concentration was determined using the formula:

$$\text{Stock (mg/mL)} = \frac{\text{Drug Concentration (mg)}}{\text{Volume of Water (mL)}}$$

Hence, the dosage administered to each animal in milliliters was therefore calculated using the formula:

$$\text{Dosage (mL)} = \frac{\text{Weight of animal (g)}}{\text{1000 g}} \text{ X } \frac{\text{Dosage (mg/kg)}}{\text{Stock (mg/mL)}}$$

Drug preparation and administration

The drugs were pulverized into a powder using a ceramic mortar and pestle, dissolved in distilled water, and administered based on standard dose regimens for each drug. Oral administration was carried out using a hypodermic syringe attached to an oral cannula. Except for group A, all groups received therapeutic doses of the test drugs. Group B was given piroxicam (0.3 mg/kg), group C was given Ibuprofen (6.7 mg/kg), and group D received Diclofenac (1.7 mg/kg). The double drug combination groups were Group E, Groups F and G, which received therapeutic doses of piroxicam+ibuprofen, piroxicam+diclofenac, and ibuprofen+diclofenac, respectively, while Group H was given all three drugs at therapeutic doses.

Experimental design

Group treatment and dosage

A=normal control (NC) – distilled water (5 mL/kg), B=piroxicam (0.3 mg/kg), C=ibuprofen (6.7 mg/kg), D=diclofenac (1.7 mg/kg), E=piroxicam (0.3 mg/kg)+ibuprofen (6.7 mg/kg), F=piroxicam (0.3 mg/kg)+diclofenac (1.7 mg/kg), G=ibuprofen (6.7 mg/kg)+diclofenac (1.7 mg/kg), H=piroxicam (0.3 mg/kg)+ibuprofen (6.7 mg/kg)+diclofenac (1.7 mg/kg).

All the drugs and NC groups were administered once daily for 3 days by oral route via oro-gavage needle. A 3-day administration period is often sufficient to observe the immediate effects of the drug on pain and inflammation without introducing confounding factors associated with long-term use; additionally, NSAIDs generally reach steady-state plasma concentrations within 2-3 days of regular dosing. At the beginning of the study, the estrous stage of the rats was determined using vaginal cytology. To mimic the human menstrual cycle, only rats that were

in their Metestrus stage of their estrus cycle were used for the study. This was to ensure that all the animals were in estrus at the termination of the experiment. The standard regimens of drugs for the treatment of dysmenorrhea in humans are as follows: piroxicam 20 mg, ibuprofen 400 mg/8 hours, and diclofenac 100 mg/day[17].

Termination of experiment

The animals were sacrificed by injection of 0.1 mL/100 g of ketamine intraperitoneally on day 4 of the experiment. Their ovaries were also removed and examined grossly for the presence of any anatomical alterations.

Hormonal assay procedures

The concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), progesterone (P4), and estradiol (E2) were determined using similar enzyme-linked immunosorbent assay procedures. The general protocol for each hormone assay involved the following steps: arrangement of coated wells in a holder; thereafter, dispensing of standards, specimens, and controls into appropriate wells, and then the addition of specific enzyme reagents and/or biotin reagents. They were then incubated at room temperature (duration varied by hormone), after which washing and rinsing of wells was undertaken, and then the addition of substrate solution and further incubation. An addition of stop solution to halt the reaction was performed, and the mixture was read for absorbance at 450 nm (with a reference wavelength of 620-630 nm) using a microplate reader. Specific details for each hormone assay:

FSH: 60-minute incubation; method described by Odell et al[18].

LH: 60-minute incubation; method described by Kosasa[19].

P4: 60-minute incubation; method outlined by Abraham[20].

E2: 30-minute and 90-minute incubations for initial antibody-antigen binding and competitive binding with enzyme reagent; method outlined by Abraham[20].

In our study, the main differences between assays were in the volumes of reagents used, specific incubation times, and the addition of biotin reagents for P4 and E2 assays. All procedures aimed to ensure thorough mixing, complete color changes, and timely absorbance readings to obtain accurate hormone concentration measurements.

Antioxidant enzymes assay

Method for homogenizing a sample

The ovaries were rinsed in an ice-cold 1.15% KCl solution, patted dry, and weighed. Subsequently, they were homogenized using 0.1M phosphate buffer (pH 7.2). The resulting homogenate underwent centrifugation at a speed of 2500 rpm for 15 minutes. After removal from the centrifuge, the supernatant was decanted and stored at -20 °C until needed for analysis.

Determination of SOD activity

SOD activity was assessed by its capacity to impede the auto-oxidation of epinephrine, determined through the increase in absorbance at 480 nm, following the method outlined by Sun and Zigma[21]. The reaction mixture (3 mL) consisted of 2.95 mL of 0.05 M sodium carbonate buffer at pH 10.2, 0.02 mL of ovary homogenate, and 0.03 mL of epinephrine in 0.005N HCL to initiate the reaction. The reference cuvette included: 2.95 mL buffer, 0.03 mL of substrate (epinephrine), and 0.02 mL of water. Enzyme activity was calculated by measuring the absorbance change at 480 nm over a 5-minute period ($\sum$= 4020M^{-1} cm^{-1}).

Determination of catalase (CAT) activity

CAT activity was determined following the method of Sinha[22], wherein the decrease in absorbance at 620 nm, attributed to the decomposition of H_2O_2, was measured using a UV recording spectrophotometer at 60-second

intervals over 5 minutes. The assay was conducted colorimetrically at 620 nm and expressed as µmoles of H_2O_2 consumed per minute per milligram of protein at 25 °C. The reaction mixture (1.5 mL) comprised of 1.0 mL of 0.01M phosphate buffer at pH 7.0, 0.1 mL of ovary homogenate, and 0.4 mL of 2M H_2O_2. To halt the reaction, 2.0 mL of dichromate-acetic acid reagent (mixture of 5% potassium dichromate and glacial acetic acid in a 1:3 ratio) was added. The extinction coefficient used was: $\sum=40M^{-1}$ cm^{-1}.

Lipid peroxidation assay

MDA, an indicator of lipid peroxidation, was determined following the protocol outlined by Buege and Aust[23]. To 1.0 mL of the supernatant, 2 mL of TCA-TBA-HCl reagent (comprising thiobarbituric acid 0.37%, 0.24N HCl, and 15% TCA in a 1:1:1 ratio) was added. The resulting tricarboxylic acid-thiobarbituric acid-hydrochloric acid mixture was boiled at 100 °C for 15 minutes, then allowed to cool. After centrifugation at 3000 rpm for 10 minutes to remove flocculent materials, the supernatant was collected, and the absorbance was measured at 532 nm against a blank. MDA levels were calculated using the molar extinction coefficient for the MDA-TBA complex, set at 1.56×105 M-1CM-1.

Assay of glutathione peroxidase (GPx)

Tris-buffer, EDTA, sodium azide, and enzyme preparation (ovary homogenate), each at 0.2 mL, were combined and thoroughly mixed. Subsequently, 0.2 mL of GSH and 0.1 mL of H_2O_2 were added to the mixture. The contents were well-mixed and incubated at 37 °C for 10 minutes. To stop the reaction, 0.5 mL of 10% TCA was added. After centrifugation, the remaining content was determined following the GSH procedure, and the activities were expressed as µg of GSH consumed per minute per milligram of protein, in accordance with the method described by Rotruck et al[24].

Histopathological assessments

After the extraction of the ovaries, they were fixed whole in 10 % buffered formalin. Dehydration was performed by passing the tissues through ascending grades of alcohol (70%, 95%, and 100%), respectively. The tissues were then cleared in xylene and further embedded with paraffin wax. The blocks were then sectioned using a microtome and stained using standard procedures for ovary histopathological analysis. The tissues were deparaffinized by putting sections in xylene and then rehydrated by passing the slides through descending grades of alcohol (100%, 95%, and 70%), then rinsed in distilled water for 5 minutes. The tissues were stained in hematoxylin for 3–5 minutes, then washed under running tap water for another 5 minutes. Sections were then differentiated in 1% acid alcohol for 5 minutes, and washed again under running tap water. Slides were then stained with eosin for 10 minutes and rinsed under running tap water for 1–5 minutes. Tissues were thereafter dehydrated in ascending grades of alcohol and cleared in xylene. They were then mounted in a mounting medium[25]. Photomicrographs of the ovaries were evaluated by three independent histopathologists, blind to details of the study, to avoid bias. The processed slides were examined under a light microscope (OlympusCX31), attached to an AmScope® digital camera (MU1000), China.

Statistical analysis

The study data was analyzed using the GrapPad Prism version 10.2.0 software, with the "One-Way" analysis of variance (ANOVA) and a Tukey post hoc test. Data was presented as mean±standard error of mean. Values were statistically significant at p-value<0.05.

Results

Effect of NSAIDs on body weight

There was an increase in weight across all the test groups. However, the weight gained across the groups was not statistically significant, as shown in Table 1.

Table 1 Effect of non-steroidal anti-inflammatory drugs on body weight

Group	Initial body weight (g)	Final body weight (g)	Change in weight (g)	Change in weight (%)
A	154.75±6.57	168.25±6.47	13.50±0.65	8.02
B	141.75±6.43	157.75±7.52	16.00±3.49	10.14
C	142.00±7.95	159.50±9.54	17.50±2.10	10.97
D	150.00±4.69	164.00±1.47	14.00±3.81	8.53
E	136.75±5.41	146.50±5.69	9.75±1.49	6.66
F	143.75±5.66	157.75±6.39	14.00±0.91	8.87
G	149.75±10.77	162.00±12.28	12.25±2.72	7.56
H	139.50±10.99	149.75±11.60	10.25±3.47	6.84

Data are presented in Mean±Standard Error of Mean, A=normal control (NC) – distilled water (5 mL/kg), B=piroxicam (0.3 mg/kg), C=ibuprofen (6.7 mg/kg), D=diclofenac (1.7 mg/kg), E=piroxicam (0.3 mg/kg)+ibuprofen (6.7 mg/kg), F=piroxicam (0.3 mg/kg)+diclofenac (1.7 mg/kg), G=ibuprofen (6.7 mg/kg)+diclofenac (1.7 mg/kg), H=piroxicam (0.3 mg/kg)+ibuprofen (6.7 mg/kg)+diclofenac (1.7 mg/kg).

Effect of NSAIDs on antioxidant and oxidative stress biomarkers

SOD concentrations were significantly increased (p-value<0.05) in the test groups compared to group A. The Piroxicam group alone (B) and the groups administered with the three NSAIDs showed a significant (p-value<0.05) decrease compared to group A. The SOD concentrations in groups administered with piroxicam and ibuprofen (group E), piroxicam and diclofenac group (F), and ibuprofen and diclofenac group (G) were significantly (p-value<0.05) increased compared to piroxicam group (B) and the three-cocktail group (H); as shown in Figure 1A.

CAT concentrations exhibited a significant increase (p-value<0.05) in groups administered within the ibuprofen group (C), the diclofenac group (D), the piroxicam and the ibuprofen group (E), the piroxicam and diclofenac group (F) combined as well as the ibuprofen and diclofenac group (G) compared to group A. The piroxicam group (B) had significantly reduced CAT concentrations compared to the other test groups. Also, the group administered with three drugs (H) had significantly reduced CAT concentrations compared to the diclofenac group alone (D) and the piroxicam and diclofenac group (F): as presented in Figure 1B.

GPx showed a significant increase (p-value<0.05) in the various test groups compared to group A. The piroxicam group alone (B) had significantly (p-value<0.05) decreased GPx concentrations compared to group A. All other test groups had significantly increased GPx concentrations when compared to group B; as shown in Figure 1C.

There was no significant difference (p-value>0.05) in MDA concentrations between the various test groups in comparison to the NC group (A). Among the various test groups, no statistically significant difference was observed either; as shown in Figure 1D.

Effect of NSAIDs on female reproductive hormones

The FSH concentration showed a significant difference (p-value<0.05) in group D when compared with the NC group (A). Likewise, FSH concentration was decreased compared to the NC and group B. No significant differences were seen in the other test groups compared to the NC or among various test groups; as indicated in Figure 2 (A-D).

E2 concentrations were increased in groups C, D, F and H, with higher concentrations in group C and the

least concentrations being in group F. P4 concentrations were increased in groups C, D, F, G, and H compared to groups A, B, and E, with the least concentrations being in the group E. The LH concentrations were decreased in all treatment groups, with the lowest concentrations being in group D.

There was no significant difference (p-value>0.05) in E2, P4 and LH concentrations, respectively, when NSAIDs-administered groups were compared to the NC: shown in Figure2 (C-D).

Effect of NSAIDs on ovarian microanatomy

In the NC group (A), the ovarian section showed normal developing follicles at various maturation stages. The cortical region of the ovary was in focus, and primordial follicles, primary follicles, secondary follicles, and a Graafian follicle with its different layers were seen. The nucleus of the oocyte was clearly observed, with the zona pellucida surrounding the oocyte. No atretic follicles were seen throughout the section (Figure 3A).

In the Piroxicam group (B), ovarian sections showed many degenerative follicles having characteristics of a corpus luteum. However, few viable follicles were observed. The medullary region also demonstrated numerous blood vessels (Figure 3B).

In the ibuprofen group (C), sections of the ovary showed that there were several atretic follicles seen, with obvious scarring in some of the follicles, which had probably matured into a Graafian follicle before degenerating. A few primordial follicles (arrows) were seen as well. At high magnification, the zona pellucida of a secondary follicle was observed as being very thin compared to those seen in the control groups (Figure 3C).

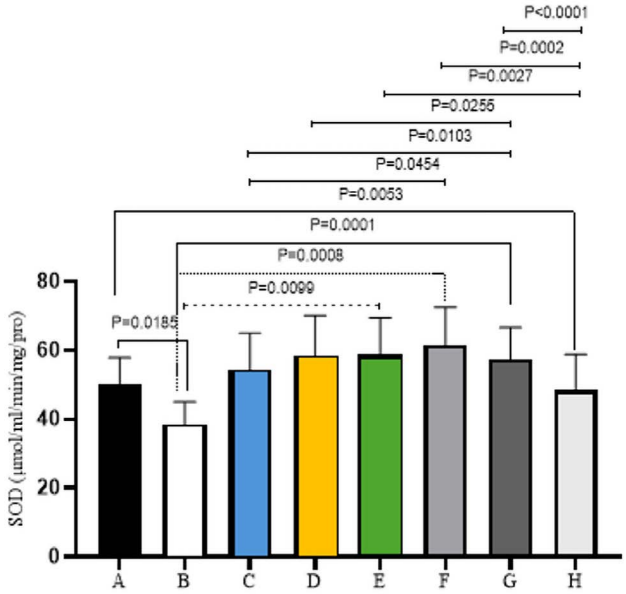
For the diclofenac group (D): several degenerative (atretic) follicles at different stages of follicular maturation were seen, with scarring occurring in some follicular cells; nuclear fading (karyolitic) was additionally observed. At high magnification, degeneration of the zona pellucida could be observed, with a break in the membrane (arrow). Interstitial stromal hyperplasia could also be observed (Figure 3D).

In the piroxicam and ibuprofen group (B and C): several atretic follicles could be seen in this section of the ovary. Oocytes were found in large follicles, which should have been Graafian follicles. However, no antrum was seen in the follicles. At high magnification, a poorly formed zona pellucida could be seen, with a degenerating oocyte inside. Several vacuoles were also seen in the follicles (Figure 3E).

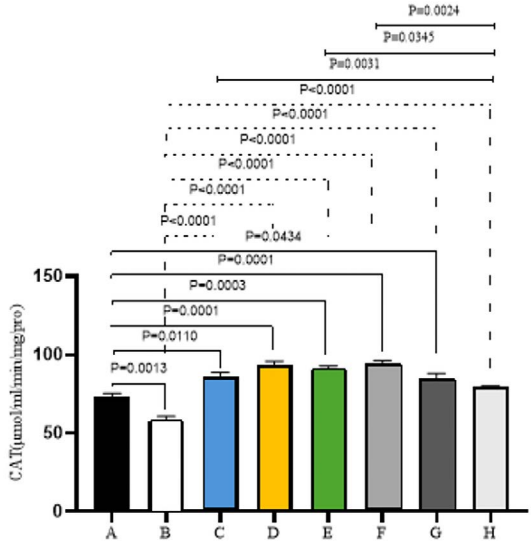
In the piroxicam and diclofenac group (B and D): atretic follicles, which appear highly vacuolated, were seen in this section. Atretic follicles (arrow) showed normally degenerating follicles with a persistent zona pellucida. However, at high magnification, a primary follicle with disintegrated zona pellucida and demonstrable scarring of the oocyte was observed. Also, the granulosa cells of the multilaminar primary follicle, normally cuboidal, showed degenerative changes, despite the fact that there was no corpus luteum formation. Granulosa cells do not become theca lutein cells; these are distinct populations. Theca cells develop separately around granulosa cells. Both can become lutein cells after ovulation, remaining distinct (Figure 3F).

For the ibuprofen and diclofenac group (C and D), ovarian section revealed many degenerating follicles at different stages of maturation. Additionally, several other follicles seemed to have lost their cellular integrity. At high magnification, degeneration of the zona pellucida of follicles was seen, as there is a thinning out of the pellucida (Figure 3G).

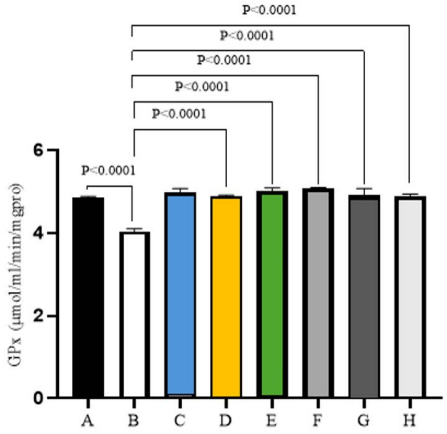
In the piroxicam, ibuprofen and diclofenac group (A, B and C): stromal hyperplasia was observed throughout the cortex of the animals. Follicles were not readily seen, as they seem to have blended with the rest of the stroma. Severe vacuolation was observed in the stroma and some follicles with a pyknotic nuclei (karyorrhexis) (Figure 3H).



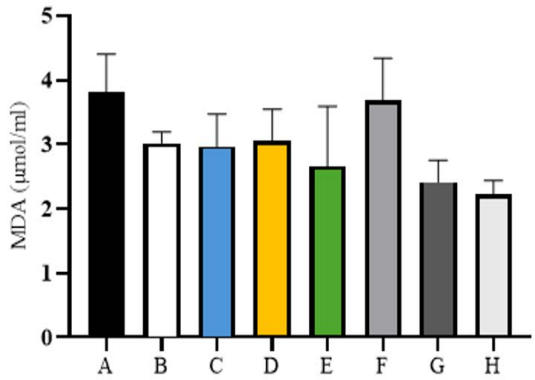
(A) Representative graph showing superoxide dismutase (SOD) concentration



(B) Representative graph showing catalase (CAT) concentration



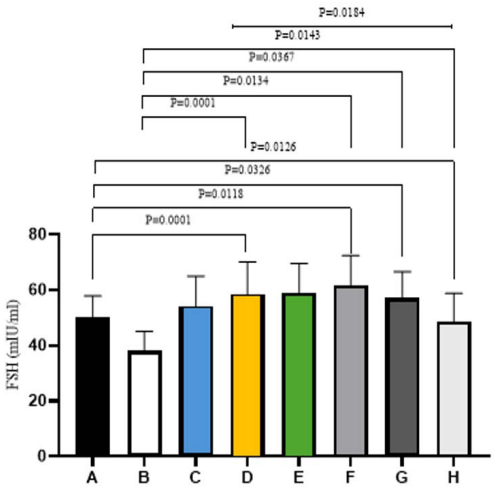
(C) Representative graph showing glutathione peroxidase (GPx) concentration



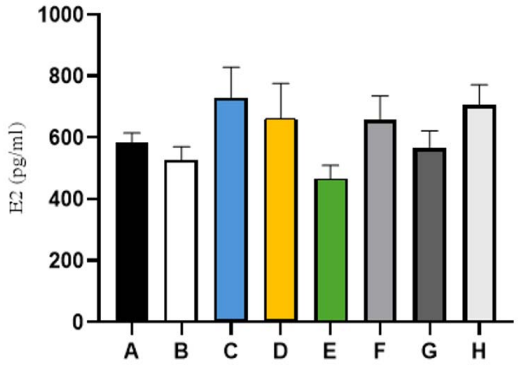
(D) Representative graph showing malondialdehyde (MDA) concentration

Bars represent mean number±SEM; wherein significance is at p-value<0.05, with the actual p-value displayed on the bars. When not significant there is none; based on a one-way ANOVA, with Tukey's multiple comparisons test Legend: A=Distilled water (5 mL/kg), B=Piroxicam (0.3 mg/kg), C=Ibuprofen (6.7 mg/kg), D=Diclofenac (1.7 mg/kg), E=Piroxicam (0.3 mg/kg)+Ibuprofen (6.7 mg/kg), F=Piroxicam (0.3 mg/kg)+Diclofenac (1.7 mg/kg), G=Ibuprofen (6.7 mg/kg)+Diclofenac (1.7 mg/kg), H=Piroxicam (0.3 mg/kg)+Ibuprofen (6.7 mg/kg)+Diclofenac (1.7 mg/kg)

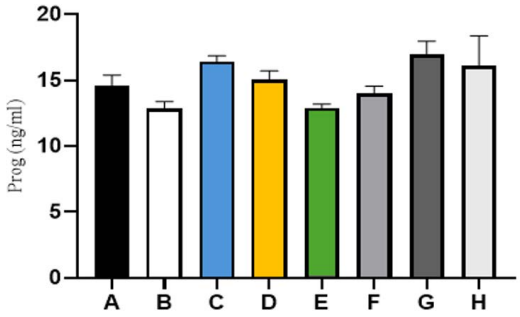
Figure 1 (A-D) The ovarian antioxidant and oxidative stress concentrations following a comparative NSAIDs oral administration in Wistar rats



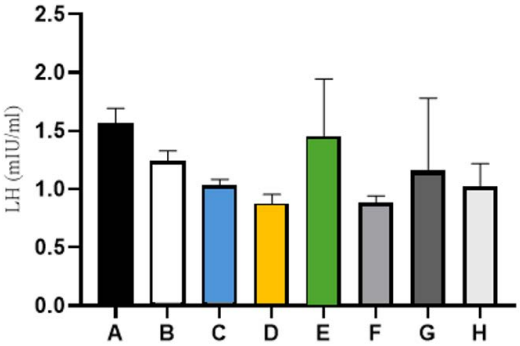
(A) Representative graph showing follicle-stimulating hormone (FSH) concentration



(B) Representative graph showing estradiol (E2) concentration



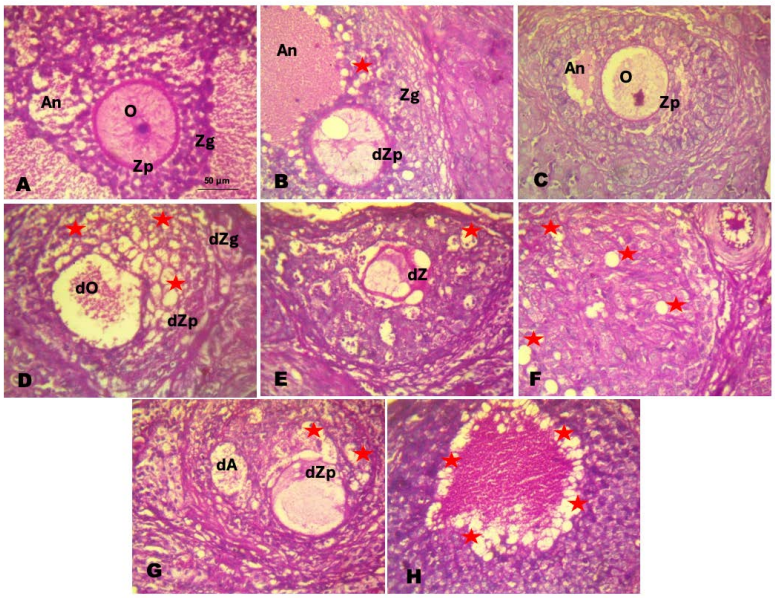
(C) Representative graph showing progesterone (P4) concentration



(D) Representative graph showing luteinizing hormone (LH) concentration

Bars represent mean number±SEM; wherein significance is at p-value<0.05, with the actual p-value displayed on the bars. When not significant there is none; based on a one-way ANOVA, with Tukey's multiple comparisons test

Figure 2 (A-D) The ovarian hormonal concentrations following a comparative NSAIDs oral administration in Wistar rats



Red asterisk=Vacuolations and lesions; Degenerated spermatogenic lineage cells; Legend: A=Distilled water 5 mL/kg bw, B=Piroxicam (0.3 mg/kg), C=Ibuprofen (6.7 mg/kg), D=Diclofenac (1.7 mg/kg), E=Piroxicam (0.3 mg/kg)+Ibuprofen (6.7 mg/kg), F=Piroxicam (0.3 mg/kg)+Diclofenac (1.7 mg/kg), G=Ibuprofen (6.7 mg/kg)+Diclofenac (1.7 mg/kg), H=Piroxicam (0.3 mg/kg)+Ibuprofen (6.7 mg/kg)+Diclofenac (1.7 mg/kg), Zp=Zona pellucida, Zg=Zona granulosa, A=Antrum, dO=degenerating Ovary, dZp=degenerating Zona pellucida, dAn=degenerating Antrum, dZg=degenerating Zona granulosa

Figure 3 (A–H) Photomicrographs of transverse sections of the ovary (H&E) stained x 400

Discussion

Dysmenorrhea is a very common gynecological health condition, with over 90% of females at reproductive age (15–49 years) affected in different populations of the world[5].

From the findings of this study, no weight loss was observed in any of the groups; in contrast, all NSAIDs-administered groups experienced a spike in weight. This increase in weight could be because of PG modulating the functions of ion and water transport at the level of renal tubules. PG inhibition by the NSAIDs leads to increased sodium reabsorption, resulting in fluid retention and peripheral edema. This aligns with the reports by Gorczyca et al.[26], who independently noted that NSAIDs cause weight gain by mechanisms of sodium and water retention. However, this difference in weight across the various groups was not statistically significant.

Aside from group B, all other experimental groups had significantly increased CAT and GPx concentrations compared to group A, with higher concentrations occurring in group F. CAT and GPx are antioxidant enzymes that help to protect cells from oxidative damage. When cells are exposed to oxidative stress, they can increase the production of these enzymes to counteract the damage. This implies that NSAIDs, such as ibuprofen and diclofenac, used singly or concomitantly, induced oxidative stress.

MDA concentrations were reduced in all experimental groups in comparison to group A, with the lowest concentration observed in group H, which was administered with a piroxicam, ibuprofen, and diclofenac combination. However, no statistically significant difference was observed. Studies have reported that NSAIDs exhibit a dual nature regarding oxidative stress. At lower doses or short-term use, they may show antioxidant properties. However, higher doses or prolonged use can induce oxidative stress. This paradox depends on specific NSAIDs, dosage, duration, and individual patient factors; this could be the reason for the observed decrease in MDA concentrations across the different test groups[27].

A mature Graafian follicle contains its own potential sources of ROS, including large numbers of macrophages, neutrophils, and metabolically active granulosa cells[28]. Increased ROS levels can be cytotoxic, while lower levels are necessary for regulating key physiological mechanisms, including cell differentiation, apoptosis, cell proliferation, and the modulation of redox-sensitive signal transduction pathways. However, elevated levels can lead to ROS-induced damage, including cell death, mutations, chromosomal aberrations and carcinogenesis. ROS pathways in ovarian tissue damage involve mitochondrial dysfunction, lipid peroxidation, protein oxidation, and DNA damage. These mechanisms trigger cellular stress, disrupt follicular function, and induce apoptosis. ROS-independent pathways include: direct activation of apoptotic mechanisms, gene expression alterations, and hormonal signaling disruption. Both pathways contribute to follicular atresia and reduced ovarian reserves by compromising cellular integrity, metabolic processes, and reproductive potential. Understanding these complex interactions is crucial for developing targeted interventions to protect ovarian function and fertility[6,29].

In normal conditions, low concentrations of ROS are crucial for the proper functioning of the female reproductive system, ensuring normal oocyte maturation, corpus luteum formation, ovulation, fertilization, and ultimately implantation[30,31].

Elevation of antioxidant activities suggests that ovarian and uterine tissues were protected against oxidative damage that may have arisen due to NSAIDs administration. Maintaining a proper balance between ROS production and elimination is essential for the optimal function of the ovary and uterus, as oocyte maturation and cumulus-oocyte interactions could be impaired[31]. As CAT and SOD levels are highest in follicular fluid of small follicles, they may represent a dominant antioxidant defense in the initial stages of folliculogenesis. This implies that the activity of these antioxidants and H_2O_2 levels decrease significantly as follicle size increases[28].

In this current study, FSH concentrations were significantly decreased in group D compared to groups A and B, which had the highest concentrations of FSH. The other treatment groups showed a reduction in FSH concentrations, although the values were not statistically significant. This agrees with Ji et al.[32], who reported that NSAIDs, such as acetylsalicylic acid, diclofenac sodium, ibuprofen, mefenamic acid, and naproxen in zebra fish revealed altered plasma sex hormones, which caused reproductive dysfunction. The production of gonadotropin hormone has been reported to be disrupted due to a modulation of the gonadotropin-releasing hormones. This implies that such a reduction in FSH concentration, as observed in this present study, could result in anovulation or a delay in maturing of the follicles; thereby, leading to a delay in ovulation.

In a physiologically normal ovary, an increase in P4 concentrations is due to the presence of the corpus luteum,

which only regresses very close to the next menstrual cycle in the case of failed fertilization[33]. However, in the groups where an increase of P4 was recorded, changes in histology were not in consonance with physiology. This is seen in the fact that group C demonstrated atretic follicles in early stages of maturation; group G displayed atretic follicles at different stages of maturation, with persistent oocyte and/or antral cavity in the follicles, indicating that they were not corpus lutea. Lastly, group H also presented follicles in both the early and mid-stages of maturation with vacuolations. Groups B and D–F, had an increased number of corpora lutea showing decreased P4 concentrations instead. Therefore, alterations in P4 concentrations could be attributed as being the effect of the NSAIDs.

Increased estrogen concentration indicate that follicles are in the late stages of maturation. This is because estrogen is secreted by the granulosa cells of the follicles[33,34]. In groups C, D, F, and H, which showed increased estrogen concentrations, minimal viable secondary or Graafian follicles were observed with no definitive granulosa cells for estrogen concentration increase.

Physiologically, FSH and LH work synergistically in the maturation of ovarian follicles. Both concentrations peak at ovulation and decline sharply thereafter[33]. A surge in LH secretion occurs in the presence of very mature follicles ready to be ovulated. Reduced concentrations of LH indicate that follicles are in the early stages of maturation. Despite the presence of mature follicles and/or corpus lutea in all groups, there was a reduction in LH concentrations. The lowest concentrations were found in the diclofenac group, despite the presence of the most mature follicles; however, they were degenerative. The variation in the effects of various NSAIDs on the different hormones could be because the selected NSAIDs have different pharmacokinetics and pharmacodynamics. These properties lead to differences in inhibiting PG formation, affecting hormonal balance across different drugs. Notably, NSAIDs with longer half-lives and extended-release formulations may pose increased risks of adverse effects[35].

As a result, the different NSAIDs may inhibit PG formation at different lengths of time and in potency; thus, leading to differences in the effect on hormonal balance across the various drugs. It should be noted that NSAIDs with longer half-lives and extended-release formulations have been associated with an increased risk of adverse effects compared with immediate release formulations[35].

This study aligns with Matyas et al.[36], suggesting that NSAIDs when used during the follicular phase result in higher luteal P4 concentrations. In contrast, same analgesics do not significantly affect E2, LH or FSH concentrations. Similarly, European League Against Rheumatism[37] reported that use of over-the-counter drugs; such as NSAIDs, even for short periods of time leads to significant decreases in P4.

The histological slides obtained from the various NSAIDs groups showed several atretic follicles, degeneration of zona pellucida, scarring of follicles, stromal hyperplasia, pyknosis and vacuolation in the follicles in the ovary (Figure 3). Also, the pharmacokinetic characteristics of NSAIDs influence their safety and tolerability, the increased number of atretic follicles in the diclofenac group could be attributed to its longer half-life, leading to more severe effects[35].

Nuclear fragmentation and nuclear fading of ovarian follicular cells was observed in this present study as well as significant reduction in mitotic activity of the endometrial gland of the uterus. Inadequate removal of ROS or the extended production of same, results in the influx of Ca^{2+} into cells. This in turn leads to induction of further cell injury, and an increase in DNA breakage[30]. This could explain the reason for the disorientation of the various cell types found in the ovaries.

The changes in ovarian cytology is in tandem with Obayes et al.[38], who discovered that ovaries of mice

injected with isoxicam revealed a hyperplasia of germinal cells on the surface of the ovary, a tongue-like projection of primordial oocytes extending to the medulla. Multiple oocytes with disarrangement of follicles and a deficiency of follicular fluid associated with the disappearance of oocytes; vacuolation in the cortical layer of the ovary, compressed premature follicles, hypercellularity of follicular cells, degeneration of the germinal layer of the cortex surface, and hyperplasia of primordial oocytes. It is believed from their research that the use of NSAIDs could impose severe, adverse effects on female fertility. In addition, Rajaei et al.[30] reported that increases in the stromal and ovarian capsule thickness, hyperplasia of the theca interna cells, and ovulation are directly related to oxidative stress. In other words, indirectly related to the inhibition of gonadotropin hormone.

In addition, Colcimen et al.[39] reported that diclofenac sodium injected for 15 days at 1 mg⁄kg in pregnant Wistar rats caused reduced follicular intensity in the ovary of their pups. Similarly, a reduced intraovarian follicular number as well as development of intraovarian corpus luteum was observed as a side effect of piroxicam administration.

However, NSAIDs have been linked to reversible female infertility when used over a prolonged period[40]. Additionally, depending on the dose administered and length of exposure, NSAIDs can reduce spontaneous and induced ovulation rates; thus they can exert very damaging effects on female reproductive physiology. As a result, the histological features which demonstrate ovarian perturbations are not so surprising. However, over time, the specific role of PGs in the ovulatory process is not fully understood.

Conclusion

This study demonstrates that different NSAIDs have varying degrees of impact on ovarian structure and function in female Wistar rats. After a 3-day oral administration, diclofenac, ibuprofen, and piroxicam, both individually and in combinations, induced reproductive toxicities. These effects were evident in alterations of hormones, oxidative stress markers, and ovarian microanatomy.

The severity of these effects followed a distinct pattern, with diclofenac showing the most significant impact, followed by ibuprofen and then piroxicam. Combination treatments exhibited even more pronounced effects, with the trio of piroxicam+ibuprofen+diclofenac causing the most severe changes.

These findings highlight the potential risks associated with unsupervised use of NSAIDs for dysmenorrhea management. Even short-term use could lead to adverse effects on ovarian health, emphasizing the need for caution with over-the-counter NSAIDs and the importance of medical supervision. This research provides valuable insights into the comparative effects of common NSAIDs on ovarian health, contributing to informed clinical practice and women's health decisions.

Future research should investigate long-term NSAID effects on ovarian function, exploring mechanisms of oxidative stress and potential protective strategies. Studies should examine impacts on follicular development, oocyte quality, and fertility. Comparative human cell culture research, identification of biomarkers, and comprehensive assessment of NSAID-induced reproductive toxicity are essential for understanding and mitigating potential reproductive health risks.

Author contributions

BEK, IAE, IUU and EIB conceptualized and designed and supervised the study, BEK, AJP, EIE, and MAA performed the experiments, analyzed the data and co-drafted the manuscript with IAE, EIB and IUU.

Conflict of interest

The authors declare that there is no conflict of interest concerning this manuscript.

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