

Pharmacological effects of low doses of some commonly used NSAIDs on ovulation and corpus luteum in mature female mice *Acellular and Hormonal comparative study *

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Abstract: Ovulation constitutes the central event in ovarian physiology, and ovulatory dysfunction is a relevant cause of female infertility. Non-steroidal anti-inflammatory drugs (NSAIDs), widely used due to their analgesic and anti-inflammatory properties, consistently inhibit ovulation in all mammalian species investigated so far, likely due to the inhibition of cyclooxygenase that is the rate limiting enzyme in prostaglandin (PG) synthesis. Short-term administration of low-dose (aspirin, mefenamic acid, ibuprofen & meloxicam) to female mice at proestrous cause a highly significant decrease in the ovulatory rate as compared to controls. Greater effect were produced by meloxicam.

I. Introduction

The NSAIDs are a group of chemically dissimilar agents that differ in their antipyretic, analgesic, and anti-inflammatory activities (1) the most frequently consumed drugs worldwide. NSAIDs derive much of their anti-inflammatory properties from their capacity to inhibit the synthesis of prostaglandins, have important roles in normal physiology that might best be described as protective in nature, released in response to injury and have the capacity to provoke vasodilatation, erythema, and hyperalgesia (2). Prostaglandin formation is mediated by cyclooxygenase. COX catalyzes the formation of prostaglandins and thromboxane from arachidonic acid (itself derived from the cellular phospholipid bilayer by phospholipase A₂) (3). There are 2 forms of cyclo-oxygenase (COX), COX-1 (the constitutive form), and COX-2 (the induced form in the presence of inflammation), inhibition of COX-2 is therefore thought to be responsible for at least some of the analgesic, anti-inflammatory, and antipyretic properties of NSAIDs (4). The process of ovulation has all the signs of an acute, self-controlled inflammatory reaction, including hyperaemia, edema, leukocyte extravasation and induction of proteolytic and collagenolytic activities (5,6). Prostaglandins, which play a central role in inflammation, stimulate many periovulatory processes including expansion of the cumulus granulosa cells (7,8), and enhancement of protease activities which degrade extracellular matrix (9). These and other periovulatory processes are essential for successful oocyte release and, therefore, fertility. In periovulatory follicles, the COX-2 isoform is responsible for prostaglandin production. COX-2 expression by granulosa cells increases after the ovulatory luteinizing hormone (LH) surge (10,11). Inhibition of COX-2 activity limits follicular prostaglandin production, prevents follicle rupture and blocks oocyte release supporting a key role for COX-2 in the ovulatory process (11,12). More recently, genetic studies in mice (13,14) have confirmed the obligatory role of COX-2/prostaglandins in ovulation. The present study was designed to compare between the effects of some NSAIDs (aspirin, mefenamic acid, ibuprofen and meloxicam) on ovulatory process and reproductive tract of female mice.

II. Material and methods:

All experiments were started and performed on mature female Swiss white mice with a body weight ranging from 25-30g, provided by Animal breeding at the National Center for control and pharmaceutical research. Drugs used are: Aspirin (Acetyl Salicylic Acid) The preparation used was Aspegic®, each vial contains 0.5g of A.S.A. for the preparation of the doses used in the study (2.5mg / Kg. B.W.), S/C, for oral used the final solution of s.c but double volume. Mefenamic acid the preparation used was fenamic® suspension contain 50 mg / 5 ml for the preparation of the doses used in the study (8.33mg/Kg). Ibuprofen the preparation used was profinal® suspension contain 100 mg / 5 ml for the preparation of the doses used in the study (10mg/Kg). Meloxicam The preparation used was mobic® ampoule, each ampoule containing 15 mg / 1.5 ml for the preparation of the doses used in the study (0.0033 mg/kg.B.W.) distilled water used for dilution.

1.Dose Calculation:

In order to determine the dose of (Mefenamic acid & Ibuprofen) which is comparable to aspirin 2.5 mg/kg (minimal inhibitory dose) (15) multiple doses were tried and the minimal inhibitory doses used in all experiments were as follows : Aspirin(2.5 mg/kg), Mefenamic acid(8.33mg/Kg), Ibuprofen(10mg/Kg) and meloxicam(0.0033mg/kg) depending on the outcome of the effects of these drugs on ovulation. A pilot study was applied on mature female mice, NSAIDs doses were administered three times daily, starting at proestrous phase of estrous cycle(8am 4 pm&12 am) for two days. Mice were killed at metestrous phase and ova flushed from the oviduct to determine the ovulatory rate.

2.Hormonal Assay:

Luteinizing hormone (LH) & follicular Stimulating hormone (FSH) progesterone and estradiol were assayed by measuring their levels in serum using prepared kits of Radio – Immunoassay (RIA) (16).

3.Detection of Phases of Estrous Cycle:

Phases of estrous cycle of the mice were detected and recorded using vaginal smears. Smears were then spread on a clean slide and fixed on a flame before staining with 1% aqueous methylene blue for 3-5 minutes. Stained smears were then washed with tap water, air dried and examined using a light microscope at 10X magnification to determine phases of the estrous cycle (17).

4.Flushing of ova:

The dissection of the ovaries from the oviduct was carefully performed with a fine surgical scissors, and then a tuberculin syringe (30 gauges) with blunt ended needle filled with 1 ml of D.W was inserted into the fimbriated end of the oviduct and held in place with fine surgical forceps. The ova were then flushed and recovered into a Petri dish with D.W delivered from the syringe. Ova obtained were identified and counted using a dissecting microscope under 2.5X, 16X magnification (18).

5.Preparation of Blood Sera:

Blood was collected from treated animals & controls by cardiac puncture from anesthetized animals with diethylether. Blood left to clot for 30 minutes at room temperature then, centrifuged using conical test tubes for 15 minutes at 2500 rpm. Prepared sera were then transferred into a plastic vial and stored in a deep freezer (-20°C) until used for hormonal determination (19).

6.Animals:

Animals of the present study (n =36female) were subdivided into 6 groups (6 animals/group). The 1st group received aspirin s.c, the 2nd group received aspirin oral, the 3rd group received mefenamic acid, the 4th group received ibuprofen, the 5th group received meloxicam. doses mentioned above, and the 6th group received D.W as a control group. In all cases NSAIDs doses and the distilled water were administered three times daily, starting at proestrous phase of estrous cycle (8 am&4 pm&12am) on the same day. Before killing the anesthetized animals at metestrous phase, blood was collected and sera prepared and stored at -20°C for subsequent hormonal assay. The whole reproductive system was quickly removed after killing, and was immersed in a Petri-dish filled with an in vitro D.W kept at 37°C. Both ovaries were quickly dissected out using fine surgical scissors, cleared from surrounding non-ovarian tissue under the dissecting microscope and weighed (after drying by filter papers) using electronic precision balance. The uteri were then quickly dissected out slightly at the tubouterine junction from one end and immediately close to the internal orifice of the cervix from the other end, they were cleared from the surrounding non-uterine tissue.

III. Results

Morphological change :

Ovaries :of the control group that received distilled water showed slight congestion larger in size with smooth surface while animals that received 2.5 mg/kg of aspirin seemed to be enlarged, congested with cystic appearance compared to those received 8.33 mg/kg of mefenamic acid and 10 mg/kg of ibuprofen that showed more obvious cystic appearance with the same enlargement and congestion, while those received 0.0033 mg/kg of meloxicam showed very obvious cystic appearance with the same congestion as the previous three groups as in fig 1,2,3,4 and 5.

Uteri :of the control group that received distilled water showed congestion with very slight prominent blood vessels while animals that received 2.5 mg/kg of aspirin seemed to be congested with prominent blood vessels and lumen filled with fluid compared to those received 8.33 mg/kg of mefenamic acid and 10 mg/kg of ibuprofen and those received 0.0033 mg/kg of meloxicam that showed the same changes as in fig 1,2,3,4 and 5..

Change in ovarian and uterus weight:

Ovaries: No significant changes ($P > 0.05$) were seen in the ovarian weight between control and animals that received aspirin (s.c or oral) and mefenamic acid as in table 1,2,3,respectively , and significant decrease($P < 0.05$) were seen in the ovarian weight between controls and each of animals that received ibuprofen& meloxicam as in table 4,5,respectively. While there is no significant changes($P > 0.05$) were seen between animals that received aspirin(s.c) and those received mefenamic acid , ibuprofen, and meloxicam as in table 6.

Uteri:No significant changes ($P > 0.05$) were seen in the uterine weight between controls and each of animals that received aspirin(s.c or oral) as in table1,2 respectively and significant decrease($P < 0.05$) were seen in the ovarian weight between controls and each of animals that received mefenamic acid ,ibuprofen& meloxicam as in table3,4,5 respectively. While there is no significant changes($P > 0.05$) were seen between animals that received aspirin(s.c) and those received mefenamic acid , ibuprofen, and meloxicam as in table 6,.

Number of flushed ova:

A highly significant decrease($P < 0.05$) were seen in the number of ova flushed was seen between the control and female mice that received aspirin (s.c and oral) ibuprofen, and meloxicam table1,2 ,4,5 respectively and highly significant decrease ($P < 0.001$) were seen in the number of ova flushed was seen between the control and female mice that received mefenamic acid as in table3 while no significant difference ($P > 0.05$) was seen between each of animals that received aspirin(s.c) and those received mefenamic acid , ibuprofen, and meloxicam, as in table 6.

Hormonal changes:

No significant changes ($P > 0.05$) were seen in the levels of LH FSH Progesterone nor Estradiol collected from the treated animals between controls and each of animals that received aspirin, mefenamic acid , ibuprofen and meloxicam) as in table1,2,3,4,5 respectively and no significant difference ($P > 0.05$) was seen between each of animals that received aspirin(s.c) and those received mefenamic acid , ibuprofen, and meloxicam, as in table 6.



Figure(1):Genital system of mature female mouce at metestrous phase, (control group)



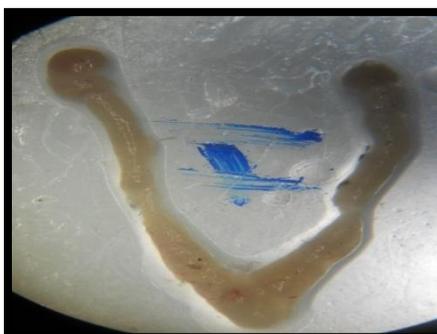
Figure(2):Genital system of mature female mouce at metestrous phase, (Aspirin group)



Figure(3):Genital system of mature female mouse at metestrous phase, (Mefenamic acid group)



Figure(4):Genital system of mature female mouse at metestrous phase, (Meloxicam group)



Figure(5):Genital system of mature female mouse at metestrous phase, (Ibuprofen group)

Table (1): Changes in weight of the ovaries uteri and difference in number of ova flushed & changes of LH , FSH , Progesterone , & Estradiol levels (cycling female mice treated with aspirin s.c, on day of proestrous phase).

Parameter	Control	Aspirin s.c	P value
Uterine wt	52.35 ± 0.76	31.10 ± 0.1	0.13°
Ovarian wt	7.06 ± 0.07	5.93 ± 0.06	0.27°
Number of ova flushed	9.83 ± 0.872	4.16 ± 0.872	0.001**
#LH	4.31 ± 0.066	4.17 ± 0.232	0.57 °
#FSH	8.38 ± 0.083	8.32 ± 0.122	0.70 °
##Prog.	17.19 ± 0.383	16.88 ± 0.131	0.46 °
###Estradiol	20.85 ± 0.174	20.39 ± 0.43	0.27 °

Table (2): Changes in weight of the ovaries uteri and difference in number of ova flushed & changes of LH , FSH , Progesterone , & Estradiol levels (cycling female mice treated with aspirin oral, on day of proestrous phase).

Parameter	Control	Aspirin oral	P value
Uterine wt	52.35 ± 0.76	29.21 ± 0.14	0.19°

Ovarian wt	7.06 ± 0.07	5.53 ± 0.01	0.29°
Number of ova flushed	9.83 ± 0.872	4.33 ± 0.843	0.001**
# LH	4.31 ± 0.066	4.31 ± 0.093	0.96°
# FSH	8.38 ± 0.083	8.43 ± 0.119	0.76°
##Prog.	17.19 ± 0.383	16.79 ± 0.105	0.34°
###Estradiol	20.85 ± 0.174	20.47 ± 0.307	0.31°

Table (3): Changes in weight of the ovaries uteri and difference in number of ova flushed & changes of LH , FSH , Progesterone , & Estradiol levels (cycling female mice treated with mefenamic acid, on day of proestrous phase).

Parameter	Control	Mefenamic Acid	P value
Uterine wt	52.35 ± 0.76	29.88 ± 0.59	0.04*
Ovarian wt	7.06 ± 0.07	6.56 ± 0.01	0.7°
Number of ova flushed	9.83 ± 0.872	3.33 ± 0.421	< 0.001**
# LH	4.31 ± 0.066	4.10 ± 0.076	0.06°
# FSH	8.38 ± 0.083	8.20 ± 0.103	0.19°
##Prog.	17.19 ± 0.383	16.40 ± 0.178	0.09°
###Estradiol	20.85 ± 0.174	20.12 ± 0.331	0.08°

Table (4): Changes in weight of the ovaries uteri and difference in number of ova flushed & changes of LH , FSH , Progesterone , & Estradiol levels (cycling female mice treated with Ibuprofen, on day of proestrous phase).

Parameter	Control	Ibuprofen	P value
Uterine wt	52.35 ± 0.76	24.25 ± 0.71	0.02*
Ovarian wt	7.06 ± 0.07	4.41 ± 0.06	0.02*
Number of ova flushed	9.83 ± 0.872	4.33 ± 0.988	< 0.002*
# LH	4.31 ± 0.066	4.27 ± 0.196	0.84°
# FSH	8.38 ± 0.083	8.45 ± 0.127	0.64°
##Prog.	17.19 ± 0.383	16.92 ± 0.165	0.53°
###Estradiol	20.85 ± 0.174	20.80 ± 0.349	0.91°

Table (5): Changes in weight of the ovaries uteri and difference in number of ova flushed & changes of LH , FSH , Progesterone , & Estradiol levels (cycling female mice treated with Meloxicam, on day of proestrous phase).

Parameter	Control	Meloxicam	P value
Uterine wt	52.35 ± 0.76	25.78 ± 0.8	0.03*
Ovarian wt	7.06 ± 0.07	4.60 ± 0.08	0.04*
Number of ova flushed	9.83 ± 0.872	5.1 ± 0.872	< 0.004*
# LH	4.31 ± 0.066	4.32 ± 0.123	0.96°
# FSH	8.38 ± 0.083	8.50 ± 0.108	0.40°
##Prog.	17.19 ± 0.383	16.91 ± 0.168	0.52°
###Estradiol	20.85 ± 0.174	20.87 ± 0.275	0.94°

Table (6) : P value according to multiple comparisons test for changes in weight of uteri , ovaries and changes in n. of flushed ova and changes of LH , FSH , Progesterone , & Estradiol levels comparison with Aspirin s.c

Parameter	Aspirin s.c Vs. Aspirin oral	Aspirin s.c Vs. Mefenamic acid	Aspirin Vs. Ibuprofen	Aspirin Vs. Meloxicam
Uterine Weight.	0.91°	0.92°	0.60°	0.69°
Ovarian Weight.	0.7°	0.63°	0.14°	0.24°
Number of ova flushed	0.89°	0.41°	0.90°	0.43°
#LH	0.57°	0.77°	0.75°	0.58°
# FSH	0.56°	0.45°	0.47°	0.30°
##Prog.	0.61°	0.054°	0.84°	0.88°
###Estradiol	0.87°	0.58°	0.42°	0.31°

Values are mean ± standard error (SE) ° = non significant difference
 * = significant difference ** = highly significant difference
 # levels of hormones in (mIU/ml) ## levels of hormones in (ng/ml)
 ### levels of hormones in (pg/ml)

IV. Discussion:

The morphological changes observed in cycling female mice treated with aspirin, mefenamic acid, ibuprofen and meloxicam on day of proestrus namely: intense organ congestion, cystic appearance of the ovaries and enlargement of the organs are clear indication of pronounced inflammatory response. Vascular changes induced by aspirin, mefenamic acid, ibuprofen and meloxicam (vasodilation & increased permeability) (20) as well as the normal physiological morphological changes associated with ovulatory process (21) have a combined effect to give such outcome that observed on ovarian and uterine morphological changes. All these inflammatory changes occurring during ovulation are thought to be mediated mainly by: PGs (PGE₂), (PGI₂) and histamine (22). Moreover, PGF₂ α is a well known factor in terminating inflammatory changes associated with ovulation (23), and its synthesis is stimulated by gonadotropins (FSH/LH) (24) which are known to increase during this period. These interactions suggest that inhibition of PGF₂ α by NSAIDs administration may block or prevent the termination of the inflammatory process and allowing it to extend longer than normal and the slight difference observed between the different NSAIDs may reflect the difference in potency of PGF₂ α inhibition and anti-inflammatory action between the different NSAIDs according to the difference in their selectivity towards COX enzyme selectivity and in their pharmacokinetic properties (25). The cystic appearance of ovaries of experimental animals was due to failure of ovulation of certain number of graafian follicles which has been turned into luteinized unruptured follicles. It has been reported that, presence of PGF₂ α is critical factor in ovulation (5,26). Inhibition of PG synthesis in the preovulatory follicles by the four NSAIDs treated subgroups hinders completion of the ovulatory process leading to the failure of the follicular rupture and hence formation of cysts (27) which gave rise to the cystic appearance of ovaries in treated animals. The vasodilation and the consequent slow blood movement caused by NSAIDs in these organs due to the suppression of several substances including PGF₂ α . Also as it is well known that several platelet substances such as: collagen, thrombin, TXA₂, adenosine diphosphate (ADP) and dense and alpha granules produce platelet activation (28). Moreover activated platelets, release calcium from the dense granules into the cytoplasm (29) and calcium causes platelets contraction with a further release of serotonin, ADP, and arachidonate. Arachidonic acid, then converted into TXA₂ by the COX enzyme, this enzyme could be irreversibly inhibited by low-dose aspirin treatment & reversibly inhibited by low-dose mefenamic acid, ibuprofen and meloxicam thus, vasoconstriction and platelet aggregation may be avoided (30). The above process may improve blood flow to these organs, uteri & ovaries and this lead to congestion. Such a disturbance in the vascular blood supply of the uterine wall may explain the significant decrease in the uterine weight. Significant decrease in ovarian weight in animal that received NSAIDs may be due to decrease number of ova in ovary compare to control that received distilled water it could be due to the balanced changes in the main components of ovarian weight i.e.: number of corpora lutea, growing and Graafian follicles. Regarding reduction in ovulation rate (number of flushed ova) which was reduced significantly in treated animals compared to control group, the underlying cause could be due to incomplete central inhibition of gonadotropin release (FSH and LH) which are known to be a key hormones for ovulation (31). Aspirin administration in a very high dose caused a complete cessation of ovulation with a significant decrease in both FSH and LH, this decrease was thought to be due to suppression of PGE₂ by aspirin centrally in hypothalamus and locally on the ovarian level PGs (32). Results of the present study indicate that low doses of NSAIDs (2.5 mg/kg.bw of aspirin, 8.33 mg/Kg mefenamic acid, 10 mg/Kg ibuprofen and 0.0033 mg/kg.bw of meloxicam) were unsuccessful in inducing any significant changes in the serum level of (FSH, LH, progesterone, and estradiol), as well as, the low doses of aspirin were unsuccessful in inducing any significant changes in the serum level of both gonadotropins (FSH/LH) (33) and this effect seems to have been mainly on the ovarian level. Yen et al, have shown that PGs have little direct effect on gonadotropin secreted from the pituitary, while NSAIDs seems to suppress these hormones at the hypothalamic level by inhibition on GnRH release (34,35), and this what happened when high doses of aspirin are used. PG levels are elevated in the mature ovarian follicles due to the ovulatory LH surge (35). Several reports have shown that, ovulation can be inhibited by NSAIDs despite of undetectable changes in several key hormones of ovulation (FSH, LH, E₂ & P₄), suggesting that local ovarian factors are the predominant driving force in ovulation (26). Many authors reported that, there is a marked increase in intrafollicular levels of PGs (E and F series) shortly before ovulation (36). Moreover it has also been reported that, low dose of aspirin partially reduced follicular level of PGE₂ and PGF₂ α and in order to abolish ovulation totally, NSAIDs must be administered in high dose (37). Women taking anti-PGs drugs, suffer from luteinization of graafian follicle, to produce a syndrome referred to as luteinized unruptured follicle syndrome (38). Thus failure of the dose levels of the four subgroups of NSAIDs that are used in present study to induce any significant changes in gonadotropins (FSH/LH) may be due to several causes: NSAIDs doses used was too low to induce suppression of hypothalamic PGE₂ (39). the other possibility is that NSAIDs acted locally at ovarian level without any effect on gonadotropins (FSH/LH) (40).

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