Original Article

Comparative study of the effects of some NSAIDs on ovulation in female mice.

Mohammed Q. Y.Al-Atrakji* **MBChB, MSc**
Ahmad M. Al-Zohyri** **BSc, VM&S, MSc, PhD**
Adnan S. Al-Janabi*** **BSc, V.M&S, PhD**

Summary:

Background: Ovulation constitutes a central event in ovarian physiology, and ovulatory dysfunction which is a relevant cause of female infertility. Mammalian ovulation is comparable to aninflammatory reaction since many of the molecules responsible for inducing the inflammatorycascade including PGs, leukotrienes and various cytokines have been describedin the ovary.

Objectives: This study was designed to compare between the effects of some NSAIDs(aspirin,diclofenac sodium and meloxicam)on the ovulatory process and the reproductive tract of female mice.

Materials and methods: Twenty four female mice were subdivided into 4groups (6 animals/group).The first received distilled water serve as control group, the second receivedaspirin (7.5 mg/kg.B.W) ,thethird received diclofenac sodium(2mg/kg.B.W) and the fourth received meloxicam(0.001mg/kg.B.W) subcutaneously at proestrous phase,Blood was collected at metestrousfor subsequent hormonal assay. The whole reproductive tract was excised out, examined, weighed and ova were flushed.

Results : Female mice that received 7.5 mg/kg.B.W of aspirin,showedenlarged, congested ovaries with cystic appearance and congested uteri with prominent blood vessels.Mice that received 2mg /kg.B.W.diclofenac and those received 0.01 mg/kg.B.W. meloxicam showed more congestion and more obvious cystic appearance of ovaries with uteri more bulky compared to controls that showed no congestion and no cystic appearance of the ovaries with thin non congested uteri.Highly significant ($P < 0.001$) increase in the ovarian weight and highly significant decrease in number of ova flushedwhile no significant changes ($P > 0.05$) were seen in the uterine weight nor in hormonal levels of LH and FSH between controls and each of animals that received aspirin, diclofenac and meloxicam respectively.

Conclusion: Short -term administration of low-dose (aspirin, diclofenac& meloxicam) to female mice at proestrous cause a highly significant increase in the ovarian weight with morphological changes of ovaries and uteri and a highly significant decrease in the ovulatory rate as compared to controls .Greater effect were produced by meloxicam with a lower dose compare to diclofenac and aspirin.

Keywords:NSAIDs, reproductive system, ovulatory rate.

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Introduction:

Most NSAIDs act as nonselective inhibitors of the enzymecyclooxygenase (COX), inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) is oenzymes(1). COX catalyzes the formation of prostaglandins and thromboxane from arachidonic acid (1). Prostaglandins act as messenger molecules in the process of inflammation. The hypothesis that mammalian Ovulation is comparable to an inflammatory reaction was first proposed in 1980 (2)as many of the molecules responsible for inducing the inflammatory Cascade including PGs, leukotrienes, bradykinin, ,platelet activating factor and various cytokines have been described in the ovary (3). The role of COX enzymes and PGs in the inflammation of ovulation is unclear, since inhibition of COX enzyme with NSAIDs inhibits ovulation completely (3).Undoubtedly, the discovery that the (COX) enzyme induced by the LH surge in granulosa cells prior to ovulation was a COX-2, represented an important landmark in the field.

Ovarian follicular development lies with the interaction between pituitary gonadotropins, folliclest imulating hormone and luteinizing hormone and intraovari anfactors suchassteroid, cytokines ando the growth factors (4). Amongthese, prostaglandins (PGs)possess vasoactive, mitogenic, and differentiating properties and are implicated in various femalere productive functions (5).Inparticular, PGshave been shown to play key rolesin ovarian physiology, the periovulatory period, and female eproduction(6). Several studies have suggested arole for PGsin them aintena ncean dfunction of the cumulus – oocytecomplex (COC) (7,8). PGE2 was one of the earliest subs tances shown toinducecu mulusex pansion invitro(9).Additionally, COC sobtained

**Department of Pharmacology / College of Medicine / University of Baghdad / Baghdad.*
***Department of Pharmacology/ College of Medicine / University of Baghdad*
**** Dept. of / College of / Al-NhrainUniversity.*

from rats and mice after superovulation synthesize PGE1, PGE2 and PGF2 (10).Therefore, any drug that block ssyn thesis of PGs would affect femal ere productive function.
Infact, itisre ported that high doses of indome tha canc an block ovulationinrats (6,11). In addition, treatment of COCs with indome tha cingreatly Reduces the invitr of ertilization rateo foocytes, and this effect isreversed if PGE1 and PGE2 are added to the mediain the presence find omethacin (11).
These data suggest that PG smay be critical for maintaining an optimal microenvironment for oocyte survival and fertilization. However, although the useo flow- dose indomethacin can aboli shovarian PGE2 synthesis, low - dose indome tha cinfailed to affect ovulation (11).In contrast, high – dose indome tha cinresulted insignificant ant inhibition of ovulation (11). The present study was designed to compare between the effects of some NSAIDs (aspirin ,diclofenac sodium and meloxicam) on ovulatory process and reproductive tract of female mice.

Materials and methods:
All experiments were started and performed on mature female Swiss white micewith a body weight ranging from 25-30g.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 (7.5mg / Kg. B.W.), 8mg of the powdered drug was dissolved in 1mL distilled water, the final solution will contain (4mg/ mL) and each mouse was given 0.1mL S/C .(Diclofenac sodium):The preparation used was olfen® ampoule ,each ampoule containing 75 mg / 2 ml. For the preparation of the doses used in the study (2 mg/kg.B.W.) distilled water used for dilution.(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 (0.01 mg/kg.B.W.) distilled water used for dilution .
Dose Calculation:In orderto determine the dose of (diclofenac&meloxicam) which is comparable to aspirin 7.5 mg/kg (minimal inhibitory dose)(13),multiple doses were tried and the minimal inhibitory doses used in all experiments were as follows :Aspirin(7.5 mg/kg), diclofenac sodium(2mg/kg) and meloxicam(0.01mg/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 s/c twice daily ,starting at proestrous phase of estrous cycle(9am & 2 pm) on the same day .Mice were killed at metestrous phase and ova flushed from the oviduct to determine the ovulatory rate.
Hormonal Assay: Luteinizing hormone (LH) & follicular Stimulating hormone (FSH) were assayed by measuring their levels in serum using prepared kits of Radio – Immunoassay (RIA) (14).Detectionof 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 (15).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 IVF medium 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 IVF medium delivered from the syringe. Ova obtained were identified and counted using a dissecting microscope under 2.5X,16X magnification (16).
Preparation of Blood Sera:Blood was collected from treated animals & controls by cardiac puncture fromanasthetized animals with Nembutalintraperitonially. 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 (17).
Animals:Animals of the present study (n =24 female) were subdivided into 4groups (6 animals/group).The 1st group received distilled water serve as control group ,the2nd group received aspirin, the3rd group received diclofenac sodium and the 4th group received meloxicam in doses mentioned above. In all cases NSAIDs doses and the distilled water were administered twice daily, starting at proestrous phase of estrous cycle (9 am&2 pm) on the same day S/C. Before killing the anaesthetized animals at metestrousphase, 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 medium (IVF), 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 (17).
Statistical Analysis:Collected data were analyzed using SPSS version 12.0 for windows (SPSS, Chicago, Illinosis, USA). Differences of means between groups were examined by student t-testand ANOVA, P. value < 0.05 was considered as statistically significant.

Results:
Ovariesof the control group that received distilled water showed slight congestion small in size with smooth surface while animals thatreceived 7.5 mg/kg of aspirin seemed to be enlarged, congested with cystic appearance compared to

those that received 2 mg/kg of diclofenacand those thatreceived 0.01 mg /kg of meloxicam which showed more obvious cystic appearance with the same enlargement and congestion.

Uteriof the control group that received distilled water showed congestion with very slight prominent blood vessels while animalsthat received 7.5 mg/kg of aspirin seemed to be congested with prominent blood vessels and lumen filled with fluid compared to those received 2 mg/kg of diclofenac and those received 0.01 mg /kg of meloxicam that showed the same changes but with more bulky appearance.

Highly significant increase ($P < 0.001$) were seen in the ovarian weight between controls and each of animals that received aspirin, diclofenac and meloxicam respectively. The same highly significant changes ($P < 0.001$) were seen between animals that received diclofenac and each of animals that received aspirin and meloxicam respectively, while no significant changes ($P > 0.05$) were seen between animals that received aspirin from that received meloxicam. (Table 1and4) Uteri:No significant changes (P 0.05) were seen in the uterine weight between controls and each of animals that received aspirin, diclofenac and meloxicam respectively. (Table 1) Number of the flushed ova:A highly significant changes ($P < 0.001$) were seen in the number of ova flushed from different subgroups of treated animals. This highly significant decrease in number of ova flushed was seen between the control and each of female mice that received aspirin, diclofenac and meloxicam respectively, while no significant difference ($P > 0.05$) was seen between each of the animals that received aspirin from those that received diclofenac or meloxicam,and neither between those received diclofenac from those received meloxicam.(Table 2&4)

Hormonal changes:No significant changes ($P > 0.05$) were seen in the levels of LH nor FSH collected from the treated animals between controls and each of animals that received aspirin, diclofenac and meloxicam respectively.(Table 3).

Table (1) :Changes in weight of the ovaries& uteri of Experimental Group (cycling female mice treated with aspirin,diclofenac sodium &meloxicam on day of proestrous phase).

Parameter	Control	Aspirin	Diclofenac	Meloxicam	P value ANOVA
*Ovarian Weight.	2.4 ± 0.07	3.05 ± 0.07	3.46 ± 0.05	3.09 ± 0.05	< 0.001**
*Uterine Weight.	25.09 ± 0.06	25.14 ± 0.04	25.15 ± 0.04	25.21 ± 0.15	0.78º

Values are mean ± standard error (SE), (n =6 animals/group).
* weight (mg) / 10 g B.W, **=highly significant difference ($P < 0.001$), º =non significant difference ($P > 0.05$)

(Table 2): Difference in the number of ova flushed from Experimental Group (cycling female mice treated with aspirin,diclofenac sodium &meloxicam on day of proestrous phase).

Parameter	Control	Aspirin	Diclofen-ac	Meloxic-am	P value ANOVA
Number of ova flushed	7 ± 0.37	1.67 ± 0.33	2.17 ± 0.48	1 ± 0.37	< 0.001**

Values are mean ± standard error (SE), (n =6 animals/group).
**=highly significant difference ($P < 0.001$)

Table (3) : changes of LH & FSH levels in Experimental Group (cycling female mice treated with aspirin,diclofenac sodium &meloxicam on day of proestrous phase).

Parameter	Control	Aspirin	Diclofenac	Meloxicam	P valueANOVA
LH*	4.47 ± 0.06	4.43 ± 0.06	4.54 ± 0.05	4.58 ± 0.07	0.34º
FSH*	8.55 ± 0.08	8.43 ± 0.06	8.32 ± 0.03	8.44 ± 0.1	0.21º

Values are mean ± standard error (SE), (n =6 animals/group).
*levels of hormones in (mIu/ml), º =non significant difference ($P > 0.05$)

Table (4) :P value according to multiple comparisons test in Experimental Group (cycling female mice treated with aspirin,diclofenac sodium &meloxicam on day of proestrous phase).

Parameter	Control Vs. Aspirin	Control Vs. Diclofenac	Control Vs. Meloxicam	Aspirin Vs. Diclofenac	Aspirin Vs. Meloxi-cam	Diclofenac Vs. Meloxi-cam
Number of ova flushed	< 0.001*	< 0.001*	< 0.001*	0.4º	0.2º	0.05º
Ovarian Weight.	< 0.001*	< 0.001*	< 0.001*	< 0.001*	0.7º	< 0.001*

*=highly significant difference ,º = non significant difference

Discussion:

The morphological changes observed in cycling female mice treated with aspirin,diclofenac and meloxicam on the day of proestrous in this study namely: intense organ congestion and bulky enlargement of the organs are clear indication of pronounced inflammatory response.It has been reported that vascular changes induced by aspirin,diclofenac and meloxicam (vasodilation &increased permeability) as well as the normal physiological and morphological changes associated with ovulatory process had a combined effect to give such an outcome that was observed on the ovarian and uterine morphological changes(18,19,20).All these inflammatory changes occurring during ovulation are thought to be mediated mainly by :PGs(PGE_2) , (PGI_2) and histamine (21). Moreover,$PGF_2\alpha$ is a well known factor in terminating inflammatory changes associated with ovulation (22), and its synthesis is stimulated by gonadotropins (FSH/LH)(23) which are known to increase during this period (23). These interactions suggest that inhibition of $PGF_2\alpha$ 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$PGF_2\alpha$ 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.

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 unrupturedfollicles. It has been reported that, presence of $PGF_2\alpha$ is critical factor in ovulation (24, 25) .It can be assumed that , (26)inhibition of PG synthesis in the preovulatory follicles by the three NSAIDs hindered the completion of the ovulatory process leading to the failure of follicular rupture and hence formation of cysts which gave the cystic appearance of ovaries in treated animals. Formation of these cysts has a positive impact on ovarian weight, since in ovulation, there is normally a release of not only the ova but also the escape of accumulated follicular fluid,and thus decreasing ovarian weight after ovulation. These factors may explain the significant increase in ovarian weight seen in aspirin, diclofenac and meloxicam treated mice with the difference in this increase according to the difference in COX enzyme inhibition selectivity and in kinetic properties. 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 gonadotopin release (FSH and LH which are known to be a key hormones for ovulation (27). 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 PGE_2 by aspirin centrally in hypothalamusand locally on the ovarian level PGs(27).Results of the present study indicate that low doses of NSAIDs (7.5 mg/kg.bw of aspirin , 2 mg/kg.bw of diclofenac and 0.01 mg/kg.bw of meloxicam) were unsuccessful in inducing any significant changes in the serum level of (FSH nor LH), and this effect seems to be mainly on the ovarinlevel.These finding might explain the differences in suppression of ovulation rate between the present study and Al-Bayati study in which high doses of aspirin were used (28). 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 (29,30) and this what happened when high doses of aspirin are used. PG levelsare elevated in the mature ovarian follicles due to the ovulatory LH surge (30). 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(31) . Many authors reported that , there is a marked increase in intrafollicular levels of PGs (E and F series) shortely before ovulation (30,31).Moreover it has also been reported that ,low dose of aspirin partially reduced follicular level of PGE_2 and $PGF_2\alpha$ and in order to abolish ovulation totally , NSAIDs must be administered in high dose(32,33). Women taking anti-PGs drugs, suffer from luteinization of graafian follicle, to produce a syndrome referred to as luteinized unruptured follicle syndrome (34) .Failure of the dose levels of the three NSAIDs that were used in present study to induce any significant changes in gonadotropins (FSH/LH) may be due to several causes : NSAIDs doses used were too low to induce suppression of hypothalamic PGE_2. The other possibility is that NSAIDs acted locally at ovarian level without any effect on gonadotropins (FSH/LH) (35).

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