Pharmacological effects of low-dose of aspirin on Corpus Luteum functions in mature cycling female mice

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ABSTRACT

Objective: To investigate long-term effect of aspirin in low-dose on the corpus luteum functions and its hormonal changes associated with ovarian and uterine structural changes.

Design: Prospective study.

Setting: Institute of the Embryo Research and Infertility Treatment- University of Baghdad.

Materials and methods: In the treatment group, 24 mature cycling female mice underwent subcutaneous administration of aspirin at a dose level of (7.5mg/Kg b.w) twice daily at the beginning of diestrous phase of the estrous cycle. In the control group, 24 mature cycling female mice underwent subcutaneous administration of a placebo(distilled water) twice daily at the beginning of diestrous phase of the estrous cycle.

Main outcome measure: Uterine and ovarian morphological changes, uterine and ovarian weight changes, serum level of (FSH, LH & Progesterone) and ovarian and uterine structural changes.

Results: There was statistically significant increase in progesterone level, number of corpora lutea, diameter of granulosa cells, and a significant decrease in gonadotropins (FSH/LH), number of growing follicles, uterine weight, endometrial living cell height, endometrial and myometrial thickness, diameter of endometrial glands.

Conclusion(s): long-term administration of a low-dose of aspirin to mice at the beginning of diestrous phase, causes the following changes: significant decrease in uterine weight with development of hemorrhagic spots on the external surface of uterine horns of only those animals that receive treatment for 30 days, and a significant decrease in serum level of both gonadotropins (FSH/LH) associated with significant increase in progesterone level, number of corpora lutea, diameter of granulosa cells, congestion in the uterus, ovary and prolongation of the luteal phase in all 4 periods of treatment.

Keywords: Low-dose aspirin, Long-term treatment, uterine morphology, ovarian morphology.

Aspirin is one of the most famous, cheapest, available and widely used drugs in the world in patients with a wide range of therapeutic uses for the treatment of inflammatory joint diseases, prevention of thrombosis and many other causes for its anti-inflammatory, analgesic antipyretic and antiplatelets effects (1,2). And on the reproductive system, however, there are several reports in women indicating that the use of aspirin before pregnancy associated with decrease risk of ectopic pregnancy (3). Also low-dose of aspirin may improve ovarian responsiveness, uterine and ovarian blood flow velocity, implantation and pregnancy rates in patients undergoing IVF (4). Also, low-dose aspirin (100 mg/day) may improve uterine perfusion in women with unexplained infertility and impaired uterine blood flow (5). The life span of the corpus luteum has been linked with prostaglandins (PG) since late sixties and early seventies when it was found that PGF2α of endometrial origin is responsible for luteolysis in several mammalian species (6,7). Moreover PGF2α.
administration could terminate pregnancy in several laboratory animals (8,9). In primate, PGF2α causes decreased progesterone production by primate granulosa cells in vitro (10,11). In the present investigation effect of low-dose of aspirin on corpus luteum activity is detected. Prostaglandin synthesis has linked to aspirin action since the late sixties of the last century (12,13).

**MATERIALS AND METHODS**

All experiments were performed on mature female Swiss White mice, 15-16 weeks old with a body weight ranging from 25-30g. The mice were obtained from the colony of the animal house of the institute for embryo research and infertility treatment, University of Baghdad. They were kept in an air-conditioned room (22-24°C) with an automatically controlled photo-period (14 hours light and 10 hours darkness). Mice were fed the standard balanced pelleted diet presented with tap water "ad libitum". Before experimentation all mice were left for at least three weeks for adaptation. Mice that showed at least three consecutive regular cycles were included in the study (n=48 mice). Experimental animals (n=24 animals) were injected subcutaneously with aspirin (Aspegic®, Laboratories synthelabo, France) at a dose level of 7.5mg/Kg. b.w given twice daily starting on the first day of diestrous phase. Treated animals (n=6 mice/group) were killed after 5, 10, 20 and 30 days post-treatment. Changes in vaginal smear were also measured daily. At the end of each period of treatment and before killing of the anaesthetized animals (24 hours after the last injection), blood was collected through cardiac puncture and sera were prepared and kept in deep freezer (-20°C) for the hormonal assay. After the killing the whole reproductive system was quickly removed, and was immersed in a Petri-dish filled with in vitro medium (IVF) (Universal IVF medium, Medicult, Denmark) kept at 37°C. Both ovaries were quickly dissected out, cleared from surrounding non-ovarian tissue and weighed using electronic precision balance (Sartorius - Switzerland). 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 surrounding non-uterine tissue and were dried from IVF fluid using filter paper and they were then weighed using electronic precision balance (14). And the organs were then taken for subsequent histological examination (both ovaries and pieces from uterine horns). Animals (n=24 mice) were given distilled water and exposed to the same protocol of injection, duration, blood collection and histological examination served as control for the experimental group. The following parameters were used to evaluate ovarian and uterine changes: Number of growing and Graffian follicles; number of corpora lutea and the diameter of the granulosa lutein cells; endometrial lining cell height; thickness of the endometrium and myometrium; and diameter of the endometrial glands. In all these histological measurements ocular and stage micrometers were used.

**Statistical Analysis**

Collected data were analyzed using SPSS version 10.0 for windows (SPSS, Chicago, Illinois, USA). Differences of means between groups were examined by student t-test, P. value <0.05 was considered as statistically significant.

**Table 1.** Changes in the weight of ovaries and uteri of those animals that received long-term administration of Aspirin.

<table>
<thead>
<tr>
<th>Weight (mg)</th>
<th>Duration of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 days</td>
</tr>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Ovary</td>
<td>1.88±0.12</td>
</tr>
<tr>
<td>Uterus</td>
<td>24.44±0.34</td>
</tr>
</tbody>
</table>

* Values are mean ± standard error (SEM), (n=6 animals/group).
* * = Significant changes (P < 0.001).
RESULTS

Ovarian and uterine changes

A - Macroscopic morphological changes:

Ovaries

Examination of the ovaries of experimental mice revealed the following morphological changes: congestion, cystic appearance due to the presence of many corpora lutea. On the other hand, the ovaries of the control animals were pale with a smooth surface associated with presence of few number of corpora lutea (Plates 1, 2).

Uteri

Examination of uteri of experimental animals, showed: congestion and lack of luminal fluid. However, those treated for 30 days and killed at the 31st day, showed presence of hemorrhagic spots on the uterine surface of both uterine horns (i.e., at the site of uterine wall vascularization). Uteri of control animals, on the other hand, appeared congested and blood vessels over the area of uterine horns were less prominent; as it was seen with treated animals (Plates 1, 2).

B - Ovarian and uterine weight changes:

Ovaries

Ovarian weight of experimental mice revealed a non significant changes as compared to the control animals. (Table 1) and (Figure 1).

Uteri

Experimental animals that received aspirin for 5, 10, and 20 days and killed on day 6, 11, 21 showed a non significant changes in uterine weight when compared to the control animals. On the other hand, when aspirin is administered for 30 days and killing on the 31st day, resulted in a significant decrease ($P = 0.003$) in uterine weight as compared to the control animals. (Table 1) and (Figure 1).

Hormonal changes

All aspirin treated mice for: 5, 10, 20 and 30 days
Table 2. Hormonal changes associated with long-term administration of Aspirin to mature cycling female mice at the diestrous phase.

<table>
<thead>
<tr>
<th>Hormones</th>
<th>5 Days</th>
<th>10 Days</th>
<th>20 Days</th>
<th>30 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>LH mIU/mL</td>
<td>3.01±0.21</td>
<td>1.02±0.39*</td>
<td>3.08±0.17</td>
<td>0.81±0.05*</td>
</tr>
<tr>
<td>FSH mIU/mL</td>
<td>1.76±0.18</td>
<td>1.11±0.06*</td>
<td>1.65±0.15</td>
<td>1.09±0.21*</td>
</tr>
<tr>
<td>Progesterone ng/ml</td>
<td>22.05±0.39</td>
<td>24.12±0.51*</td>
<td>22.09±0.83</td>
<td>24.07±0.28*</td>
</tr>
</tbody>
</table>

- Values are mean ± standard error (SEM), (n=6 animals/group).
* = Significant changes.

and killed at the: 6th, 11th, 21st and 31st days respectively, showed a significant decrease in the level of both gonadotropins: LH and FSH when compared to the control animals. However, a significant increase in the level of progesterone was seen in aspirin treated mice as compared to the control (Table 2) and (Figure 2).

Structural changes of both ovaries and uteri

Mice treated with aspirin showed the following structural changes in the ovaries and uteri:

Ovaries

A highly significant increase, in the number of intact corpora lutea; in the diameter of the granulosa lutein cells as compared to the control animals.

A highly significant decrease, in the number of the growing follicles as compared to the control animals. On the other hand, no regressed corpora lutea and no Graffian follicles were seen in treated mice as compared to the control animals. (Tables 3, 4, Figures 3, 4, and Plates 3-6).

Figure 2. Hormonal changes associated with long-term aspirin administration at the beginning of diestrous phase.
Table 3. Structural changes of the ovaries and uteri associated with long-term administration of Aspirin to mature cycling female mice at the diestrous phase.

<table>
<thead>
<tr>
<th>Duration of treatment</th>
<th>Structural changes of the ovaries and uteri (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>5 Days</td>
<td></td>
</tr>
<tr>
<td>Endometrial lining cell height</td>
<td>21.23±0.80</td>
</tr>
<tr>
<td>Endometrial thickness</td>
<td>339.75±19.3</td>
</tr>
<tr>
<td>Diameter of the Endometrial glands</td>
<td>92.70±0.90</td>
</tr>
<tr>
<td>Myometrial thickness</td>
<td>243.90±3.25</td>
</tr>
<tr>
<td>Diameter of the granulosa lutein cells</td>
<td>14.08±0.52</td>
</tr>
</tbody>
</table>

- Values are mean ± standard error (SEM), (n=6 animals/group).
* = Significant changes (P < 0.001)

Uteri

A highly significant decrease, in the height of the endometrial lining cells; in the endometrial thickness and in the diameter of the endometrial glands as compared to the control animals.

On the other hand, myometrial thickness was significantly decreased in animals that received aspirin treatment for 30 days while it was not significantly changed for the remaining 3 groups i.e.: that were received treatment for: 5, 10, and 20 days respectively (Table 3, Figures 5, 6 and Plates 7-15).

Table 4. Structural changes of the ovaries, associated with long-term administration of Aspirin to mature cycling female mice at the diestrous phase.

<table>
<thead>
<tr>
<th>Duration of treatment</th>
<th>Structural changes of the ovaries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>5 Days</td>
<td></td>
</tr>
<tr>
<td>No. of functioning C.L</td>
<td>4.00±0.37</td>
</tr>
<tr>
<td>No. of growing follicles</td>
<td>12.83±0.60</td>
</tr>
</tbody>
</table>

- Values are mean ± standard error (SEM), (n=6 animals/group).
* = Highly Significant changes (P < 0.001)
DISCUSSION

The morphological changes of both ovaries and uteri seen in the experimental group, may be due to the vascular properties of aspirin. It is well known that several platelets substances such as: Collagen, thrombin, thromboxane A2 (TXA2), adenosine diphosphate (ADP) and dense and alpha granules produce platelet activation (15).

Activated platelets, release calcium from the dense granules into the cytoplasm (16).
Calcium causes platelets contraction with a further release of serotonin, ADP, and arachidonate, this in turn is converted into TXA2 by the cyclooxygenase enzyme, this enzyme could be irreversibly inhibited by low-dose aspirin treatment thus vasoconstriction and platelet aggregation may be avoided (17,18).

This in turn may improve blood flow to these organs (19). Animals receiving long-term administration of aspirin for 30 days, developed an areas of hemorrhagic spots, at the site of uterine wall vascularization and this may be due to blood vessels weakness induced by the prolonged program of aspirin-induced treatment (20). Such hemorrhagic spots may also develop due to cumulative effect of repeated daily administration of aspirin. In human, daily administration of 30-50 mg of aspirin, results virtually complete suppression of platelet thromboxane biosynthesis after 7 to 10 days (21-23). These changes in platelet biochemistry are associated with maximal inhibition of thromboxane - dependent platelets aggregation and prolongation of the bleeding time (23-25). Such a disturbances in the vascular blood supply of the uterine wall may explain the significant decrease in the uterine weight of experimental animals received aspirin for 30 days as compared to the control animals.

Absence of changes in ovarian weight experimental groups as compared to the control animals may be due to the balanced changes in the main component of ovarian weight i.e.: number of corpora lutea, growing and Graffian follicles in treated and control animals.

The prolongation of the diestrous phase and delayed luteolysis in aspirin treated mice in the 4 periods of the treatment may be attributed to inhibition of PGF2α more than other prostaglandins.

Prostaglandin F2α known to be the physiologic uterine luteolysin in non-primate species including mice (26,27). This luteotrophic effect of aspirin may have been augmented by PGE2 present in ovarian tissue which was not affected by administered aspirin. The luteotrophic activity of this type of prostaglandin is well known (28).

It has been shown that, repeated injection of PGF2α during the luteal phase (i.e.: diestrous phase) of the estrus cycle of some mammals hastened luteolysis, similar injection of PGF2 in indo-
-methacin treated animals failed to induce luteolysis (29). Also, it was demonstrated that treatment of hysterectomized pseudopregnant rats with indomethacin, results in both lengthening of diestrous phase and delayed luteolysis (30).

The hormonal changes seen in the present study, demonstrate that, the level of progesterone for the control animals are lower than that of treated animals and this implies the beginning of luteolysis in the control. In the process of luteolysis, a decrease in progesterone production marks the early phase of luteolysis, whereas structural involution occurs later (31-33). In human, luteolysis ensues with a prompt linear decline in circulating progesterone, estradiol and inhibin A levels during the last 4 or 5 day of the functional life of the corpus luteum (34,35). While it is immediate in the absence of mating in mice (36). During the luteal - follicular transition, waning luteal cells biosynthetic capacity results in the loss of both inhibin and steroids (progesterone and estrogen) production (34). Decreased circulating levels of inhibin allow an increase in FSH production, which in turn rescues the developing cohort of follicles (primarily through induction of aromatase activity). Increased GnRH pulsatility secondary to the loss of inhibitory steroidal feedback also contributes to the rise in both FSH and LH serum level (37,38). Conversely, a significant increase in progesterone level was seen in animals who have received long-term aspirin administration for (5, 10, 20 and 30 days) with no variation in the level of progesterone seen between them. This may mean that, aspirin administration persistently arrest luteolysis (i.e. intact and functional corpora lutea), which has been confirmed by daily vaginal smear, that reflect a cellular picture of diestrous phase during all the time of treatment.

The vaginal epithelium undergoes well marked changes during the estrous cycle in mice. Heat or estrous phase is characterized by marked squamification and cornification of cells and the disappearance of leukocytes (39). At the end of the estrous phase the cornified layer sloughs off and invasion of leukocytes occurs (40).

Inhibitors of PGF2α and synthesis (indomethacin and acetysalicylic acid) as the endogenous mediator of luteolysis, were shown to delay the regression of the corpora lutea and to prolong the luteal activity in pseudopregnant rats (41). The inhibition of prostaglandins by antiprostaglandins appear to be unrelated to produce any change in the secretion of progesterone of bovine corpora lutea (42). In mice, indomethacin administration did not affect...
progesterone synthesis (43). Also serum mid-luteal progesterone levels were unaffected by administration of Ibuprofen to normally cycling women during the luteal phase (44). These changes reflect the presence of functional corpus luteum with increasing ability to synthesize mainly large amount of progesterone and to a lesser extent estradiol, as well as, inhibin hormone which is secreted along with the steroid sex hormones by the granulose lutein cells of the active ovarian corpus luteum (45). All these hormones together have a combined negative feedback effect on the anterior pituitary gland and hypothalamus to cause suppression of both FSH and LH secretion (46). The continued secretion of low level of LH, with no variation between our four treated periods (in the present study) is essential for the production of progesterone from the corpora lutea and it's maintenance. The secretary activity of the corpus luteum and it's functional life-span are dependent on appropriate LH support (47-49). Which is known to play a major role in the sustenance of the corpus luteum function (50). The endogenous low pulses of LH have a role in the development and maintenance of the corpus luteum during the estrous cycle of the bovine female (51), while the FSH is not required for the maintenance of the corpus luteum (52); however, it's low level during the luteal phase is important to prevent the initiation of folliculogenesis (53). Moreover, it has been shown that inhibin A will reduce bioactive FSH blood level and prevent follicular development in monkeys (54). In monkey, progesterone from the corpus luteum support both antiatretic and pro-differentation of follicles, to promote follicular health and remodeling during the development of the corpus luteum (55). Thus, a possible explanation of the structural ovarian changes: significant decrease in the number of growing follicles with complete absence of Graafian follicles in the four treated groups of mice, as compared to the control, is that low-FSH level during the luteal phase (i.e.: diestrous phase) that inhibit folliculogenesis.

Collectively, the above observations, that revealed, the prevention of PGF2α - induced luteolysis, by low-dose aspirin administration and maintenance of corpora lutea function with persistence of significantly high level of progesterone associated with a significant suppression of both FSH and LH, may provide a strong support to the structural ovarian changes seen: significant increase in the number of corpora lutea and a significant increase in the diameter of the granulose lutein cells, with absence of regressed corpora luteal when compared to the control animals.

The structural uterine changes namely: the significant decrease in the endometrial lining cell height, endometrial thickness and diameter of endometrial glands in all 4 periods of aspirin treated mice (5, 10, 20 and 30 days), might be due to the strong anti-inflammatory effect of progesterone hormone secreted by the active corpora lutea, on the uteri of the treated mice (56). The other possibility is that, the effect exerted by progesterone hormone, cause prolonged suppression of the ability of estrogen to promote uterine growth (protein synthesis and cell division) and vaginal cornification (57). During the luteal phase, progesterone decrease the number of estrogen receptors and increase the activity of 17B-HSD (17-B hydroxy steroid dehydrogenase enzyme), which enhance the conversion of estradiol to estrone and of estrone sulfotransferase.

The net effect of all these actions of progesterone is to decrease the biological action of estradiol on the endometrium during the luteal phase (58).

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Received December 5, 2004; revised and accepted April 24, 2005