Ovarian stimulation induces high expression of interleukin-1β and disrupts the histological features of the fallopian tube

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Abstract: The objective of this study was to investigate whether there is an association between ovarian stimulation and interrupted gamete interaction in the rat fallopian tube in vivo. We evaluated the protein expression of interleukin-1β (IL-1β) via immunohistochemistry and assessed the key histological features of the ovaries, fallopian tubes, and uteri of the rats following treatment with mild (15 IU) and high (30 IU) doses of gonadotropins compared to an unstimulated control group. The serum concentration of estradiol was also determined by immunoassay. The results revealed that high levels of circulating estrogen were associated with higher expression of the IL-1β protein in the ovaries and fallopian tubes of highly stimulated rats than in those of the mildly stimulated and unstimulated control groups. In contrast, endometrial IL-1β immunoexpression was not affected by ovarian stimulation. The histological features of the fallopian tubes were also dramatically altered upon ovarian stimulation, reducing the efficiency of the fallopian tube. In conclusion, the resulting high circulating estrogen level disrupts the histological features of the fallopian tubes and can lead to higher expression of IL-1β, thus negatively affecting gamete interactions in vivo. These results provide useful information regarding the lower fertilization rate observed following ovarian stimulation.

Keywords: Ovarian stimulation, interleukin-1β, immunohistochemistry, fallopian tube

1. Introduction
Multiple ovulation and embryo transfer (MOET) programs have been widely implemented in dairy cattle worldwide. High viability of the embryos obtained via MOET is observed compared with the viability of embryos produced in vitro (1). Despite the promising results of the application of MOET technology, it is thought that approximately 20% of cows produce the majority of the viable embryos (2). The underlying reasons for such low success rates are often multifactorial, but the ovarian stimulation protocol is recognized as a major contributor, as it plays an integral role in all assisted reproductive technologies (ARTs) (3). Although an exaggerated ovarian response resulting in a large number of retrieved oocytes may be alluring, the supraphysiological serum estradiol levels resulting from the growth of multiple follicles might explain the significant reduction of good-quality oocytes, fertilization rates (4), and implantation rates (5,6) and the increased occurrence of perinatal disorders (7,8).

The failure of fertilization following ovarian stimulation is a multifactorial process, and although there is much to be discovered about the factors involved, it is evident that gamete interaction is a far more complicated process than previously thought. The fallopian tube and its specific fluid provide the necessary environment for sperm capacitation, final oocyte maturation, fertilization, and regulation of early embryo development (9,10). For these processes to occur, diverse types of communication between the fallopian tube and early embryo are required, and any disruption of this tightly controlled environment may directly lead to infertility. Cytokines are among the molecules that are involved in such communication (11). The epithelium of the fallopian tube contains both ciliated and nonciliated secretory cells. The movement of the active cilia helps transport gametes along the oviduct (12). In contrast, embryo transportation and development are regulated by different cytokines and growth factors secreted by another subtype of fallopian tube epithelial cells (13).

It has been reported that interleukins (IL-1 and IL-2) have adverse effects on sperm motility and fertilization when applied at very high concentrations in vitro (14,15) and can lead to the degradation of sperm and their elimination by the cilia of the epithelium in vivo (16).
In contrast, reduced interleukin-1β (IL-1β) levels allow sperm to survive in sperm-storage tubules (16). Tanaka et al. (17) reported that aged bovine fallopian tube epithelial cells secrete a greater amount of IL-1β than young cells, which may accelerate oviduct cell senescence, elevate the inflammatory response, and lead to infertility by reducing oviduct function. Furthermore, elevated IL-1β levels were found to negatively correlate with embryonic implantation and pregnancy outcomes (18–20) and can cause several complications, including increased susceptibility to infectious diseases and inflammation (21). As suggested by Lekovich et al. (22), elevated IL-1β levels 1 week after embryo transfer may be associated with ectopic (tubal) pregnancy and can be used as a marker of tubal inflammation. Given the major role of IL-1β in fertilization and the regulation of early embryo development, it would be reasonable to hypothesize that IL-1β might also play a role in abnormal fertilization following ovarian stimulation in the application of MOET technology.

Despite the central role of the fallopian tube as the site of fertilization and early embryogenesis, the effect of ovarian stimulation on the functions of the fallopian tube has not been previously studied. Using the rat as a model animal, the objectives of the present study were to investigate the effect of ovarian stimulation on the regulation of tubal IL-1β secretion and the key histological features of the ovaries, fallopian tubes, and uteri of the rats.

2. Materials and methods

2.1. Animals, management, and experimental design

Adult female albino rats (Rattus norvegicus) weighing 250–300 g (aged 2.5–3 months) were obtained from the Lab Animal House of the Faculty of Veterinary Medicine, Suez Canal University (Ismailia, Egypt). All animals were housed under a 12/12 h light/dark cycle at a temperature of 25 ± 2 °C, with food and water available ad libitum. The current study was designed to minimize the number of animals used as well as the pain and suffering of all treated animals. The experimental techniques were approved by the Institutional Laboratory Animal Care and Use Committee of the Faculty of Veterinary Medicine, Suez Canal University (Approval No. 2018054) and were performed in accordance with its guidelines.

The animals (n = 15) were divided equally into 3 groups (n = 5 each). The controls were left unstimulated to allow spontaneous ovulation cycles to occur. Two hormone regimens were used for the induction of ovarian stimulation: 15 IU (mild dose) and 30 IU (high dose). A mild or high dose of FSH (Fostimon, Institut Biochimique SA, Switzerland) was administered intraperitoneally to the experimental rats, followed by the same dose of hCG (Chorimon, Institut Biochimique SA, Switzerland) 46–48 h later. Six hours after injection, females were checked for

Figure 1. Determination of the rat estrous phase via assessment of the vaginal opening reveals that the vaginal opening becomes less pink, less moist, and less swollen.

Figure 2. Vaginal cytology representing the estrous phase of the rat. Cornified epithelial cells are identified (white arrowheads).
signs of estrus based on visual observation of the external genitalia (Figure 1), in addition to the assessment of vaginal smears and the presence of nonnucleated cornified epithelial cells in the examined smear (Figure 2).

2.2. Determining the estrus phase
A vaginal swab was collected by gently turning and rolling a clean cotton-tipped swab moistened with physiological saline (NaCl 0.9%) along the wall of the rat's vagina. The cotton tip from each animal was rolled along the length of a dry glass microscope slide. The slide was then air-dried and stained with approximately 150 mL of crystal violet for 1 min. Next, the slides were rinsed with distilled water, overlaid with a coverslip, and viewed immediately under a high-power field (200× magnification) using an Olympus BX41 optical research microscope. The estrus phase was identified based on the presence or absence of leukocytes, nucleated epithelia, and cornified epithelial cells (23).

2.3. Sample collection and estrogen (E2) assay
Upon decapitation, blood samples were individually collected into heparinized collection tubes from the medial canthus of the eye, after which they were centrifuged at 3000 rpm for 20 min at 4 °C, and the obtained sera were stored at –20 °C until analysis. The concentration of E2 in the serum was analyzed using a Roche Elecsys 1010/2010 Cobas e 411 (Roche Diagnostics GmbH, Germany).

2.4. Histological and immunohistochemical procedures
Animals that showed signs of estrus were sacrificed via cervical dislocation. The ovaries, fallopian tubes, and uteri of the rats were collected in 10% neutral buffered formalin (NBF) or a 4% paraformaldehyde solution (PFA) for histological or immunohistochemical evaluation, respectively. All collected specimens were processed for routine histological procedures. First, the samples were dehydrated in an ascending graded series of ethanol (70%, 90%, and absolute alcohol), cleared in three xylene washes (2 h each), and embedded in hard paraffin. The prepared NBF paraffin blocks were cut into microtome slices 5–7 μm thick, mounted on glass slides, and subjected to hematoxylin and eosin staining for general histomorphological evaluation (24).

For IL-1β immunohistochemistry, the prepared PFA paraffin sections were deparaffinized through three washes of xylene (5 min each), rehydrated in a descending alcohol series, and then washed with distilled water. Antigen retrieval was performed via heat-mediated pretreatment for 20 min with sodium citrate buffer, followed by treatment with 3% H₂O₂ to inhibit endogenous peroxidase activity. Following pretreatment, the sections were incubated for 1 h with a primary rabbit polyclonal antibody (anti-IL-1β, ab9722, diluted 1:100) at 4 °C, then for 1 h with a goat antirabbit biotinylated secondary antibody (diluted 1:200) at 4 °C. The slides were subsequently incubated with an avidin-biotin-peroxidase complex (diluted 1:2000) at room temperature for 1 h. To observe the reaction, the localization of peroxidase activity was visualized by treating the sections with a DAB chromogen for 20 min. Phosphate-buffered saline with Triton X-100 (PBST) was used to dilute the reagents employed during the immunohistochemical steps and to wash the sections after each step. The sections were then counterstained with Harris hematoxylin, followed by alcohol dehydration (ascending grades), clearing via three changes of xylene, and mounting with DPX. A positive IL-1β signal was detected as a dark precipitate within the tissues.

The percentages of ovarian, tubal, and uterine IL-1β expression in all experimental groups were detected by counting the cells showing high expression in five representative selected high-power fields (objective lens 40×) per slide in each experimental group. The percentage of IL-1β expression was further assessed as the ratio of the number of immunoreactive cells/total nucleated cells × 100%, graded from 0 to 3 according to the number of cells counted (0 = absent, 1 = 1–10 cells, 2 = 10–20 cells, and 3 = more than 20 cells). The total area of tissue measured in each subject is presented in square microns. The specimens were examined using an Olympus BX41 research optical photomicroscope fitted with an Olympus DP25 digital camera. ImageJ digitized calibration software was employed for cell counting (ImageJ Basics, Ver. 1.38).

2.5. Statistical analysis:
The quantitative data obtained in the present study were analyzed through one-way analysis of variance (ANOVA) according to Snedecor and Cochran (25) to test differences between the experimental groups, along with the presentation of descriptive measures such as the means, standard errors, and standard deviations of the tested parameters. Mean separation was conducted using the least significant difference (LSD) test and Duncan’s multiple range test (26). Statistical analyses were conducted with SPSS 20 for Windows and Statistical Analysis System 2003 software. The results were considered significant at a probability level of less than 0.05.

3. Results
3.1. Estrogen immunoassay and IL-1β immunohistochemistry
As shown in the Table and Figures 3–6, there were highly significant differences (P < 0.01) between the three experimental groups in terms of the E2 immunoassay and IL-1β immunohistochemistry. The highest values obtained in both the E2 immunoassay and the IL-1β immunohistochemistry assay came from the high dose-stimulated group, showing grade 3 IL-1β immunohistochemistry, while grade 2 IL-1β immunohistochemistry was recorded in the mild
Table. Means, standard errors (SEs), and standard deviation (SDs) with significance test for estradiol immunoassay and IL-1β (ovarian, tubal, and uterine sections) in the control and stimulated groups (mild and high).

| Studied group Parameter | Control   | Mild dose | High dose | P-value |
|-------------------------|-----------|-----------|-----------|---------|
|                         | Mean ± SE | SD        | Mean ± SE | SD      | Mean ± SE | SD      |
|                         | 13.29c ± 0.21 | 0.48 | 15.14b ± 0.36 | 0.81 | 20.03a ± 0.16 | 0.35 |
| Estradiol immunoassay   | 22.46c ± 0.71 | 1.59 | 31.78b ±1.11 | 2.49 | 49.98a ± 0.47 | 1.05 |
| IL-1β in ovarian sections | 13.18c ± 1.17 | 2.63 | 27.86b ± 0.79 | 1.76 | 42.12a ± 0.97 | 2.17 |
| IL-1β in tubal sections  | 10.94c ± 0.38 | 0.86 | 13.26b ± 0.59 | 1.25 | 15.76a ± 0.39 | 0.87 |
| IL-1β in uterine sections | 22.46c ± 0.71 | 1.59 | 31.78b ±1.11 | 2.49 | 49.98a ± 0.47 | 1.05 |

Within the same row means with different superscripts are significantly differ (P < 0.01). **: Highly significant at the 0.01 level of significance (P < 0.01).

Figure 3. Mean values obtained from the estradiol immunoassay in the control and stimulated groups (mild and high).

Figure 4. Mean values of IL-1β immunoexpression in ovarian sections from the control and stimulated groups (mild and high).

Figure 5. Mean values of IL-1β immunoexpression in tubal sections from the control and stimulated groups (mild and high).

Figure 6. Mean values of IL-1β immunoexpression in uterine sections from the control and stimulated groups (mild and high).
dose-stimulated group and grade 1 was observed in the unstimulated control group.

3.2. Effect of ovarian stimulation on ovarian, tubal, and uterine histological features

When H&E-stained sections were examined (Figures 7A–7D, 8A–8C, 9A–9C), the ovarian, tubal, and uterine specimens collected from control animals were found to show a normal histoarchitecture (Figures 7A, 8A, 9A).

3.2.1. Ovary

The ovaries of the unstimulated control rats showed normal ovarian follicles at different developmental stages, from primary to Graafian follicles, and were covered by a smooth ovarian surface consisting of a single layer of low cuboidal cells. The newly formed corpus luteum was observed within the cortical tissue and mainly consisted of small-sized deep basophilic cells. The vascularized ovarian medulla, represented by the central zone of its stroma, was composed of bundles of smooth muscle cells and stromal cells, mostly consisting of fibroblasts (Figure 7A). The ovaries of the rats treated with the mild dose exhibited an atypical ovarian surface. Occasional abnormal invaginations were observed on the ovarian surface. In addition, an increase in the number and size of ovarian follicles was observed. Evidence of atretic follicles was observed in the cortical region, along with congested and dilated blood vessels in the ovarian medulla (Figure 7B). In the rats treated with the high dose, the atypical ovarian surface pattern was pronounced, with deeper cortical invaginations and an obvious increase in ovarian follicles. In addition to inflammatory cell infiltration and numerous cystic follicles within the cortical tissues, severely dilated and congested vessels were observed within the medulla (Figures 7C, 7D).

3.2.2. Fallopian tube

The fallopian tubes of the unstimulated control group rats were lined with undulant mucosa covered by a single layer of mostly ciliated columnar cells (Figure 8A). In the mild dose-treated group, increased proliferation was observed within the tubal epithelium, along with pseudostratification of the epithelial cells and loss of their ciliated border in some areas (Figure 8B). Increased proliferation and multilayering of the tubal epithelium with the appearance of a micropapillary pattern were frequently observed in the fallopian tubes of high dose-treated rats (Figure 8C). Inflammatory cell infiltration and congested blood vessels were observed in both mild and high dose-treated animals (Figures 8B, 8C).

3.2.3. Uterus

The endometrium of the unstimulated control group was covered with pseudostratified columnar cells, with an underlying highly cellular and vascularized connective tissue stroma. Endometrial glands were embedded within the connective tissue stroma and were lined with a single layer of high cuboidal-to-low columnar epithelial secretory cells (Figure 9A). A notable increase in dilated blood vessels, along with shrunken uterine glands, was observed within the highly cellular stroma of both the mild and high dose-treated groups (Figure 9B, 9C). Undulation of the uterine mucosa was observed in the mild dose-treated group (Figure 9B), and the mucosa was highly folded in the high dose-treated group (Figure 9C).

3.3. Effect of ovarian stimulation on ovarian, tubal, and uterine IL-1β expression

In IL-1β immunostaining (Figures 10A–10D, 11A–11D, 12A–12D), negative results were determined immunohistochemically in a negative control section of ovarian, tubal, and uterine specimens (Figures 10A, 11A, 12A), respectively.

3.3.1. Ovary

In the unstimulated control group, immunostaining was almost completely restricted to the subepithelial cortical tissue, and there was a negative reaction in follicular cells (Figure 10B). However, IL-1β immunostaining extended to the follicular cells in both the mild and high dose-stimulated groups (Figures 10C, 10D).

3.3.2. Fallopian tube

Moderate IL-1β immunostaining was observed within the underlying connective tissue of the fallopian tube in the unstimulated control group (Figure 11B). IL-1β immunohistoexpression was high in the underlying connective tissue and the micropapillary projections in both stimulated groups (mild and high) (Figures 11C, 11D).

3.3.3. Uterus

The endometrial expression of IL-1β was similar in all groups, both the unstimulated control and the mild and high dose-treated groups (Figures 12B, 12C, 12D).

4. Discussion

Ovarian stimulation induces an abnormal physiological environment within the female genital tract due to the supraphysiological levels of circulating steroids. The supraphysiological serum E2 levels resulting from the growth of multiple follicles following ovarian stimulation is considered the main factor contributing to the inefficiency of ARTs. However, the mechanism by which high levels of estradiol affect the fertilization process remains largely unknown. In the present study, we attempted to elucidate the underlying mechanism leading to a lower fertilization rate following ovarian stimulation.

Fertilization is a unique mechanism involving successive interactions between two gametes that are genetically distinct. In mammals, fertilization occurs in the fallopian tube. During this period, the fallopian tube undergoes a transition and reaches an appropriate
Figure 7. H&E-stained ovarian sections from the unstimulated control (A), mild dose-treated (B), and high dose-treated (C) groups and the (D) ovarian surface (red arrow), ovarian cortex (C), ovarian medulla (M), ovarian follicles (F), corpus luteum (CL), atretic follicles (white arrow), congested blood vessels (black arrows), occasional abnormal invaginations (green arrow), deeper cortical invagination (black arrowhead), inflammatory cell infiltration (white arrowhead), and cystic follicles (CF).

Figure 8. H&E-stained fallopian tube sections from the unstimulated control (A), mild dose-treated (B), and high dose-treated (C) groups, showing the undulant mucosa (white arrows), ciliated columnar epithelium (E), congested blood vessels (white arrowheads), inflammatory cell infiltration (I), and mucosal micropapillary patterning (black arrows).
Figure 9. H&E-stained uterine sections from the unstimulated control (A), mild dose-treated (B), and high dose-treated (C) groups, showing the endometrial epithelium (E), vascularized connective tissue (white arrowheads), endometrial glands (G), and highly cellular stroma (HC).

Figure 10. Ovarian expression of IL-1β (white arrowheads) determined immunohistochemically in a negative control section (A), the unstimulated control group (B), the mild dose-treated group (C), and the high dose-treated group (D).
morphological and functional state under the influence of ovarian steroids, facilitating the union of two gametes. It has been reported that E2 acts as a proinflammatory factor that may enhance the immune response (27,28), resulting in the activation of inflammatory cells such as neutrophils and macrophages (29). Histological analysis of the fallopian tubes of the rats in the stimulated groups revealed a stronger inflammatory reaction, as demonstrated by the presence of a large number of inflammatory cells and their infiltration, in addition to the loss of a ciliated border in some areas. The fallopian tubes exhibited more signs of disturbed histoarchitecture than the uterus, suggesting that the lower rate of viable embryos obtained following the application of MOET technology is more closely related to the disturbed gamete interaction than to uterine receptivity. Moreover, this study defined substantial changes in fallopian tube histoarchitecture induced by ovarian stimulation. The unstimulated control group showed a significant reduction of disturbed fallopian tube histology compared with that of either stimulated group. The fallopian tubes of the rats in the unstimulated control group appeared to be more intact, showing fewer histological changes than those in the stimulated groups. Such changes were more obvious after the application a high dose of gonadotropins.

In addition, there is sufficient evidence that the stability of the fallopian tube epithelium is essential to support embryonic development by reducing reactive oxygen species and secreting growth and embryotropic factors into the medium (30). We found that higher expression of IL-1β disrupts the fallopian tube epithelium as well as other histoarchitectural features. These findings are supported by a previous study showing that collagen, a major structural component of various connective tissues, was negatively affected by the higher expression of IL-1β through inhibition of its production and promotion of its degradation (31).

IL-1β is a potent proinflammatory cytokine involved in numerous cellular activities, such as cell proliferation,
differentiation, and apoptosis (32). IL-1β exhibits a wide spectrum of functions in the female reproductive tract. It plays an important role in sperm survival and fertilization (16) as well as in early embryo development (22) and can be considered the earliest sign of pregnancy in pigs (detected 2–3 days after mating) (33). As a result of high E2 levels, a variety of cell types release inflammatory cytokines, including IL-1β (34). In this study, the higher expression of IL-1β is mainly attributed to the high circulating level of E2 resulting from ovarian stimulation. In the present study, the expression level of IL-1β in the fallopian tubes was found to be higher in the stimulated groups than in the unstimulated control group. Several studies have indicated that higher levels of IL-1β negatively affect the fertilization process (14–16), embryonic implantation, and pregnancy outcomes (18–20), and can cause several different complications during pregnancy (21) and can be employed as a marker for tubal pregnancy (22). The elevated inflammatory response observed within the fallopian tube due to higher IL-1β levels, accompanied by the disturbed histoarchitecture of the fallopian tube, might directly affect the fertilization process.

It can be suggested that the main reason for the failure of fertilization following ovarian stimulation is the high level of circulating E2, which may lead to significant alterations in genetic and structural features of the fallopian tube that are required for successful fertilization. Based on the observed changes, the present study suggests that the disturbed fallopian tube transformation status hinders, or even prevents, gamete fusion. Overall, our findings contribute to the understanding of the mechanisms involved in fertilization failure following the application of MOET technology and may be of particular importance in clinical practice to ensure that the maximal fertilization rate is achieved for all ARTs.

Based on the findings of the present study, it can be inferred that high E2 levels resulting from ovarian stimulation may impair gamete interaction in fallopian

Figure 12. Uterine immunohistochemical expression of IL-1β (white arrowheads) in a negative control section (A), the unstimulated control group (B), the mild dose-treated group (C), and the high dose-treated group (D).
tubes in an indirect manner. High E2 levels can lead to high expression of IL-1β, which induces a greater inflammatory response and disrupts the fallopian tube histoarchitecture, ultimately rendering it nonfunctional. Both mild and high doses of gonadotropins have deleterious effects on the fallopian tube environment, with a more negative impact induced by high doses. Intensive work should be performed to establish effective ovarian stimulation protocols for improving MOET technology.

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