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# Evaluation of the interaction effect of low-dose estrogen on morphine-induced ovarian polycystic in rats

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## Abstract

**Background and Objective:** Polycystic ovary syndrome (PCOS) is a relatively common problem among women of reproductive age. Morphine can disrupt the reproductive axis and cause ovarian cysts. Estrogen can reduce the incidence of benign cysts and ovarian cancer. We investigated the interfering effect of low-dose estrogen on morphine-induced PCOS in rats.

**Materials and Methods:** Female Wistar rats (weighing 200 to 250 g) were purchased from Pasteur Institute of Iran and adapted to standard conditions in an animal care center. In the diestrous phase, they were randomly divided into four groups: single morphine (5 mg/kg), alone estrogen (0.03, 0.06 and 0.09 mg/kg), estrogen + morphine, and control (saline, 1 mL/kg). Seventy-two h after injection (i.p.), blood samples were taken from animals under anesthesia to analyze serum factors. After surgery, the uteri and ovaries were examined biometrically, then collected in formalin and examined. Statistical analysis was performed by analysis of variance (ANOVA).

**Results:** The ovaries of morphine-treated rats showed polycystic feature, however, the number of cysts was significantly reduced in the presence of estrogen (especially at a very low dose of 0.03 mg/kg). Morphine also reduced LH level, which was improved by co-injection of low-dose estrogen (0.03 mg/kg).

**Conclusion:** Administration of estrogen in low doses may have a protective effect on morphine-induced PCOS in rat.

**Keywords** Polycystic ovary, Low dose Estrogen, Morphine, Rat

## 1. Introduction

Infertility is one of the health problems in the world (1). Aging and some diseases may contribute to infertility (1, 2), but the main reason is lack of ovulation (3). Polycystic ovary syndrome (PCOS) is one of the causes of ovulation failure (1). It is actually one of the endocrine disorders among women of fertile age. Although, estrogen levels are physiologically very low in postmenopausal women (4,5), but due to PCOS, the levels of the two main sex hormones, estrogen and progesterone, are drastically reduced (4). The most common treatment for PCOS in the clinic is the administration of ovulation-stimulating drugs such as clomiphene citrate and daily injections of gonadotropins (6), which cause follicular growth and ovulation. Because one of the disadvantages of gonadotropin therapy is excessive ovarian stimulation, physicians may prefer to treat this complication by suppressing the opioid system (7). The opioid drug morphine with the chemical formula C12H19NO3 is a major opium alkaloid. It is the most potent opioid analgesic that is occasionally prescribed during the estrous cycle. Drug use during the period of sexual

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differentiation and puberty causes dysfunction of the reproductive system. Studies on the effect of morphine on the ovulation stages of rats show that this substance can prevent ovulation process (8). Other studies have shown that morphine inhibits LH secretion before ovulation (9, 10). Morphine also has an inhibitory effect on TSH secretion and stimulates prolactin secretion in laboratory animals (11). Despite all the studies, we are not aware of all the negative effects of morphine on the reproductive system. Whether low-dose estrogen injection reduces ovarian cysts induced by intraperitoneal administration of morphine in rats is one of the most important questions to understand the mechanism of morphine-induced PCOS.

## 2. Materials and Methods

### 2.1. Animals

Female Wistar rats were subjected to receive peripheral (intraperitoneal) injection of morphine (5 mg/kg) for once (12) in order to become a suitable model for the study of PCOS complication. The intact rats were first kept in standard cages at the animal care center under ethical approval (IR.SHAHED.REC.1398.101) and fed with adequate water and food *ad libitum*. After diagnosis of estrous cycle (13), they were randomly classified into four groups and examined (single morphine, alone estrogen, estrogen + morphine, and saline). Estrogen dose range was selected using previous studies (14, 15).

The tails of rats in the estrogen dose groups (0.03, 0.06 and 0.09 mg/kg) were marked differently with black, blue and red colors. And each dose included at least 6 rats. The animals were studied at a certain stage of the dark/light cycle. Injections were given at certain times of the day (between 9 a.m. and 11 a.m.).

### 2.2. Intraperitoneal injection

This procedure was carried out in a calm and stress-free environment. With one hand (left), the skin of the animal's head and back were kept firmly, so that the rat was under our control. The tip of the injection needle must pass through the peritoneum, so then after, the injection should be done slowly. In this type of injection, there is no skin swelling over the abdominal cavity of the rat.

### 2.3. Blood sampling and preparation of blood serum

After deep anesthesia (with a mixture of 80 mg/kg ketamine and 20 mg/kg xylazine), each animal was placed on a table and blood was drawn from the animal's heart using a 2 mL syringe. To prepare the blood serum, after blood sampling, the sample was first placed in the laboratory for some time to coagulate and then centrifuged (3000 rpm for about 15 min) and the serum was collected in an eppendorf tube.

### 2.4. Surgery

To obtain ovarian and uterine tissue (for biometric studies), the rat was euthanized (by carbon dioxide gas) and the ovarian and uterine tissue were first biometrically studied and then dissected and stored in formalin 10%.

### 2.5. Tissue collecting and histology and hormonal analyses

With abdominal surgery, uterine branches and ovaries were observed and examined biometrically and then isolated and placed in 10% formalin. Then, after 48 h (up to 72 h), the tissues were cut into sections (4-5 µm) and stained with hematoxylin and eosin (H&E) and examined by photomicroscope (Olympus). Hormonal analyzes were performed by ELISA kits.

### 2.6. Statistical analysis

All data were analysed using analysis of variance (ANOVA) ($\alpha = 0.05$) to examine differences in variances after the normality test and Tukey's *post hoc* test was used to examine differences between groups if the result of ANOVA was significant.

## 3. Results

### 3.1. Effect of morphine injection (once) on ovarian cystogenesis

Morphine (5 mg/kg) was injected once intraperitoneally (i.p.) and the control group received saline (1 mL/kg) i.p. After that (72 h), the animals were anesthetized, ovarian and uterine tissues were removed, and placed in formalin 10%. Then tissue sections were prepared and stained by H&E method. Based on histological observations, the dose of morphine (5 mg/kg, i.p.) was more effective in inducing cysts than the control group. The ovaries of the control group (Figure 1) showed follicles at different stages of growth, while the number of these follicles (naturally growing and mature follicles) showed a decrease in morphine-receiving rats (Figure 2). (The scale line in all images is 100 µ).

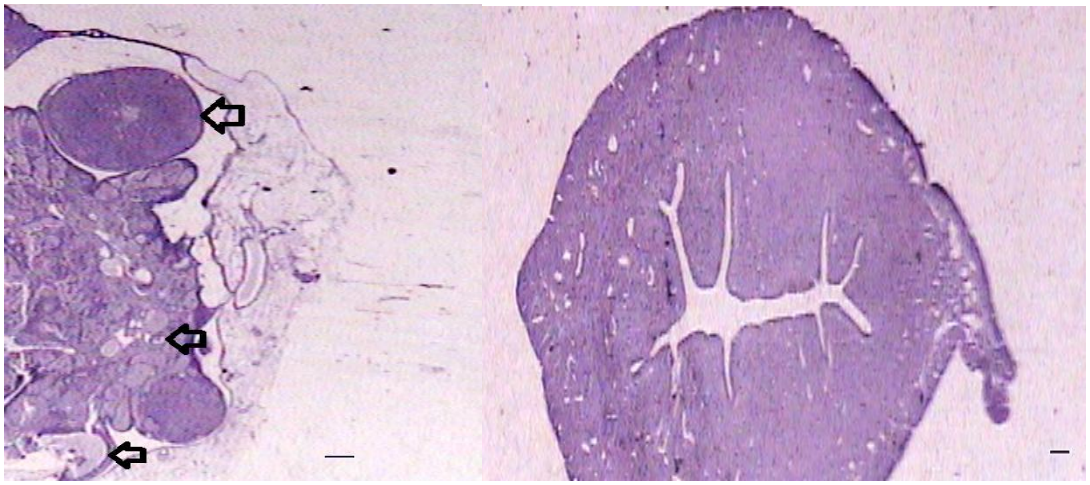

**Fig 1.** Tissue images of ovaries and uterus of control rat. The control group received saline intraperitoneally (i.p.). Later (72 h), the tissue samples were collected and examined. The ovaries of the control rats showed follicles at different stages of development as can be seen (see arrows). Uterus layers (perimetrium, myometrium, and endometrium) are normal.

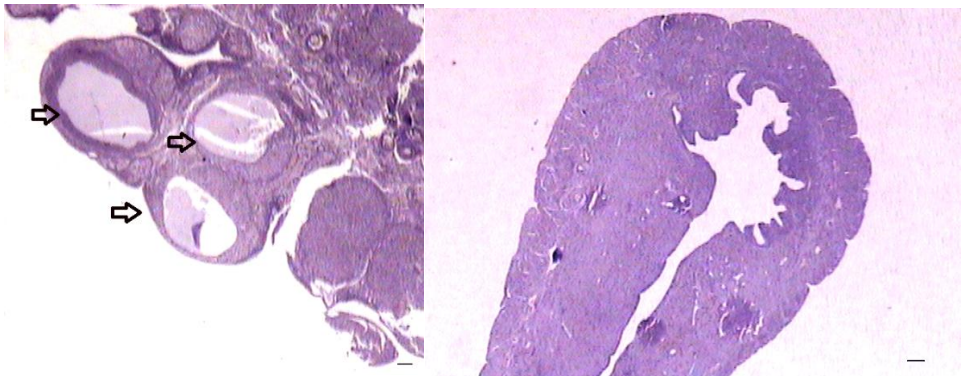

**Fig 2.** Tissue images of the ovaries and uterus of morphine-receiving rat. Morphine (5 mg/kg) was injected once i.p. alone, and 72 h later, the animal was examined histologically.

The dose of morphine was effective in the formation of ovarian cysts (thick-walled and marginal cysts), so that the ovary showed a polycystic feature. Uterine diameter is larger than normal (inflammation).

## 3.2. The effect of single estrogen injection at low doses on the ovaries

Single estrogen (0.03, 0.06, 0.09 mg/kg) was injected i.p. once and compared with the control group receiving 1 mL/kg saline, i.p. After 72 h, ovarian and uterine tissue were removed and examined. According to the findings (Figure 3), single estrogen does not cause cysts at these low doses. The uterus showed no change compared to the control.

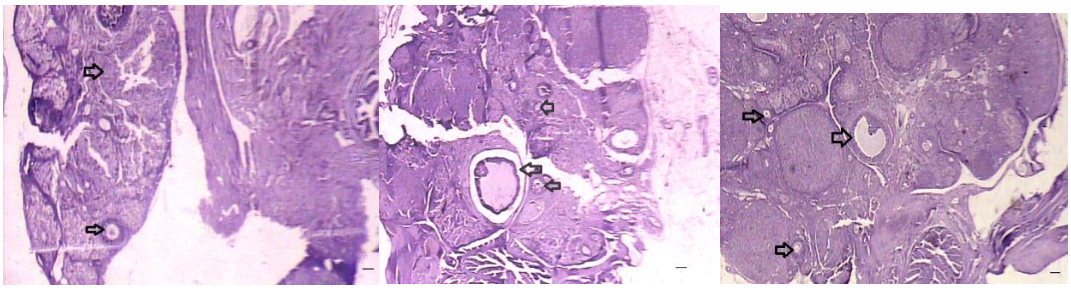

**Fig 3.** Ovary image of estrogen-receiving rat. Doses of estrogen (0.03, 0.06, 0.09 mg/kg) were injected once i.p. The control group only received saline (1 mL/kg, i.p.). Tissue was collected 72 h later. It had no cyst production effect on the ovaries (Images, from left to right, represent the effects of three doses of estrogen, 0.03, 0.06, and 0.09 mg/kg, respectively).

### 3.3. The effect of injection of estrogen + morphine on ovarian cystogenesis

Estrogen doses (0.03, 0.06, 0.09 mg/kg) were injected once i.p. before (20 min) morphine (estrogen + morphine groups); the control group received only 1 mL/kg of saline (i.p.). 72 h later, the tissues samples were removed and examined histologically. Histological observations showed that estrogen before injection reduced the number of cysts, especially at the lowest concentration and reversed uterine inflammation (Figure 4).

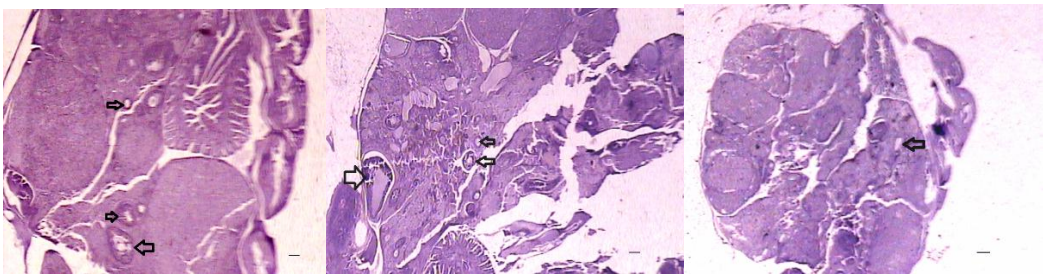

**Fig 4.** Ovary image of rat receiving estrogen prior to morphine. Estrogen doses were injected i.p. once before (20 min) morphine; and the control group received only saline (1 mL/kg, i.p.). 72 h later, the tissues specimens were removed and placed in formalin 10%. In these samples, estrogen prescription reduced the cyst production due to morphine, especially at the lowest dose, and the uterus did not show any inflammation (Left-to right images are for prior injection of estrogen doses, 0.03, 0.06 and 0.09 mg/kg, respectively).

### 3.4. Serological findings

Morphine at a dose of 5 mg/kg was injected once i.p. alone. Estrogen (0.03, 0.06, 0.09 mg/kg, i.p.) was injected both singly and before morphine (20 min). The control group received only saline (i.p.). 72 h later, the animals' blood samples under anesthesia were provided and examined with ELISA kit. Levels of LH, FSH, estrogen, progesterone, 17OH progesterone, and prolactin were obtained and shown (in case of a significant difference) (Fig. 5-14).

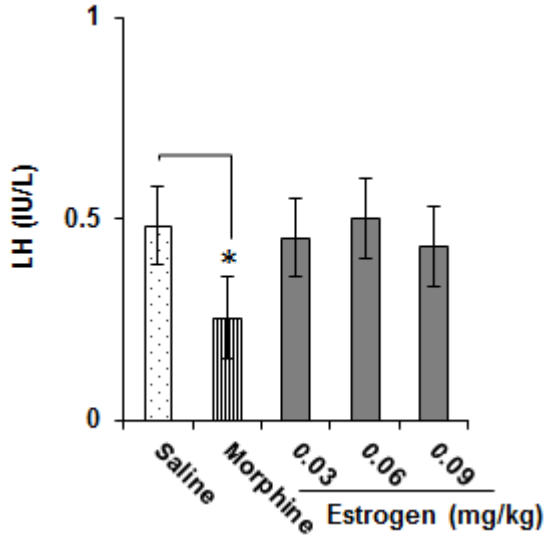


**Fig 5.** LH levels in the single estrogen-dose groups and morphine compared with the control group (saline). The effect of morphine was significant compared to the control group. Data are based on mean and S.E.M. Tukey's *post hoc* test showed differences between groups * P <0.05.

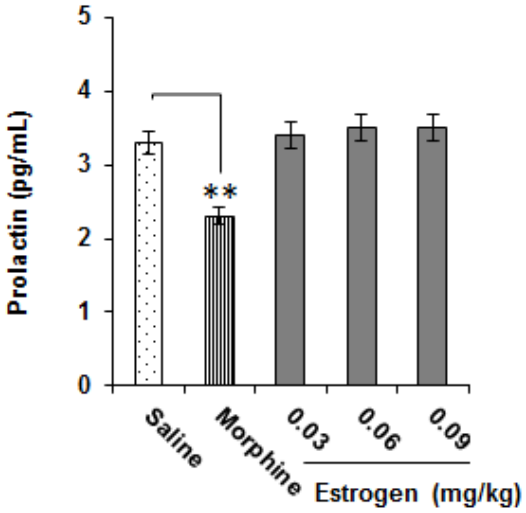


**Fig 6.** Prolactin levels in estrogen and alone morphine-dose groups compared to saline control. Morphine changes were significant in association with the control group. Data are based on mean and S.E.M. ** P <0.01 was obtained based on Tukey's *post hoc*.

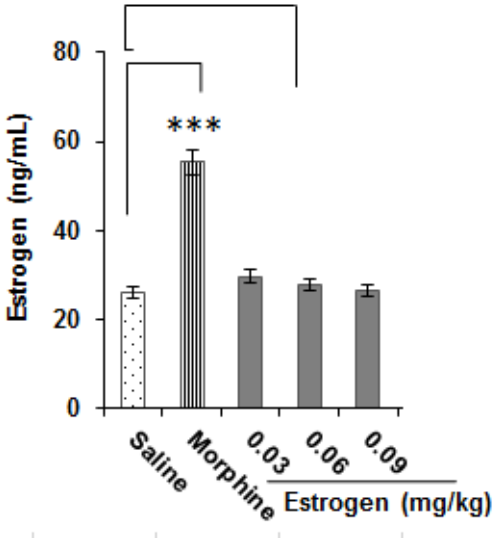


**Fig 7.** Estrogen levels in single dose-groups of estrogen and morphine compared with control (saline). The morphine changes were meaningful in comparison to the control group. Results are shown based on mean and S.E.M. (Tukey's *post hoc*: ***P <0.001).

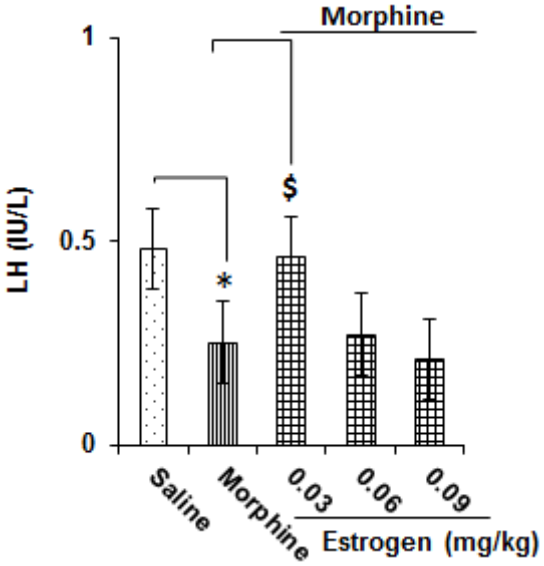


**Fig 9.** LH levels in morphine alone and saline (three groups were estrogen + morphine, which estrogen was injected before morphine). LH levels in morphine alone showed a significant difference compared to saline. This figure also clearly shows a significant difference in the very low dose (0.03 mg/kg) of estrogen + morphine.

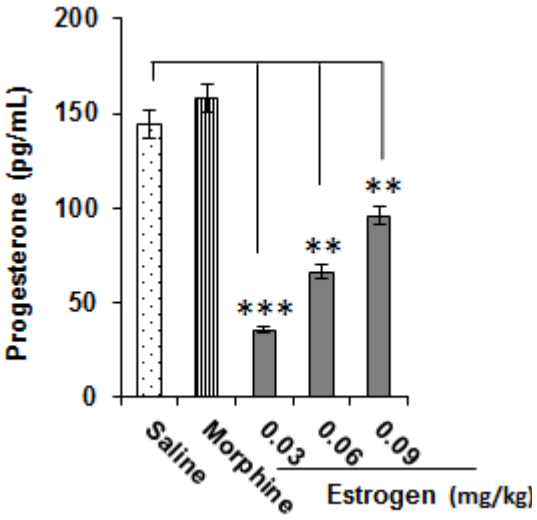


**Fig 8.** Progesterone levels in single dose-groups of estrogen and morphine compared with saline control. The effects of estrogen groups were significant compared to the control. Data are based on mean and S.E.M. (**P <0.01 and ***P <0.001 are obtained based on Tukey's *post hoc*).

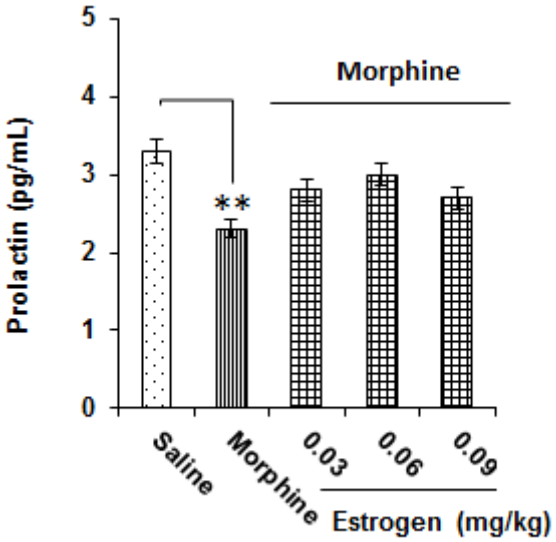


**Fig 10.** Prolactin levels in three groups: saline, morphine alone, and doses of estrogen before morphine injection. Levels in the morphine group showed a significant difference compared to saline. The star is based on Tukey's *post hoc*.

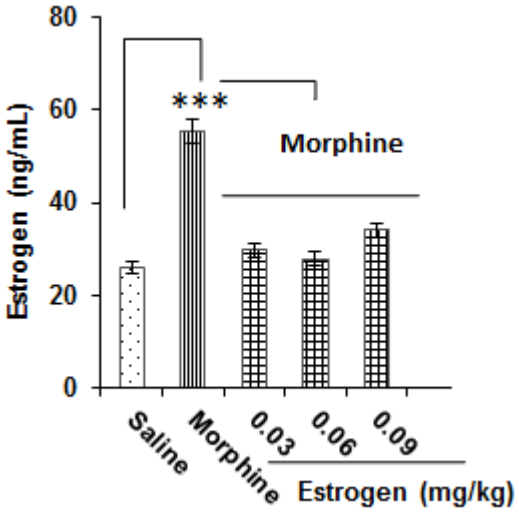


**Fig 11.** Estrogen levels in three groups: single morphine, and saline, and estrogen doses + morphine. The single morphine compared with the saline shows a significant difference. The star is shown based on Tukey's *post hoc* test.

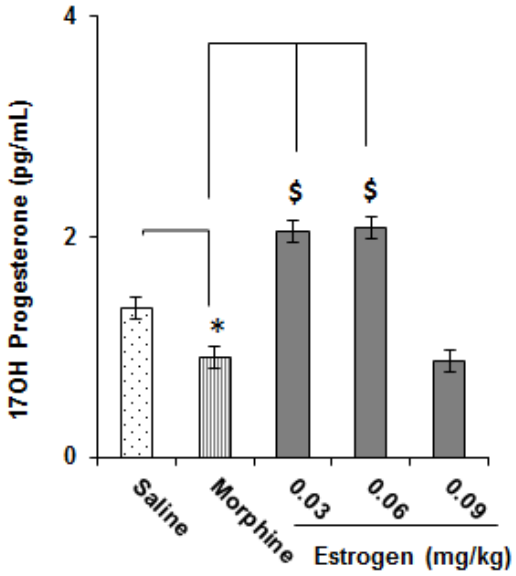


**Fig 13.** 17OH Progesterone levels in saline, morphine alone, and estrogen single doses. Morphine is significantly different from saline, and also there are differences between estrogen lower doses than morphine that obtained by Tukey's *post hoc*.

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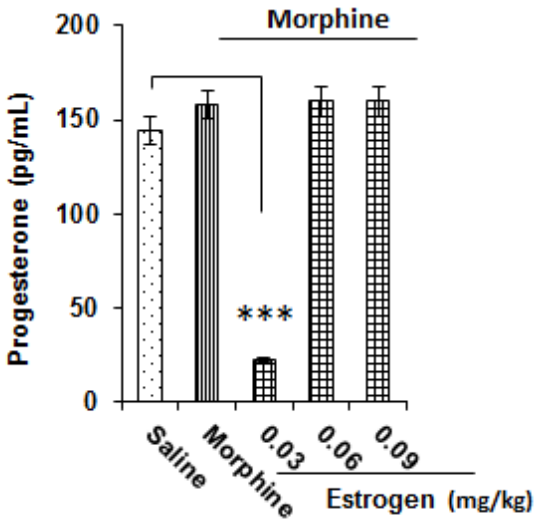


**Fig 12.** Progesterone levels in different groups: morphine alone, and saline, and estrogen + morphine, very low dose estrogen levels (0.03 mg / kg) compared to saline is very significant as obtained by posttest.

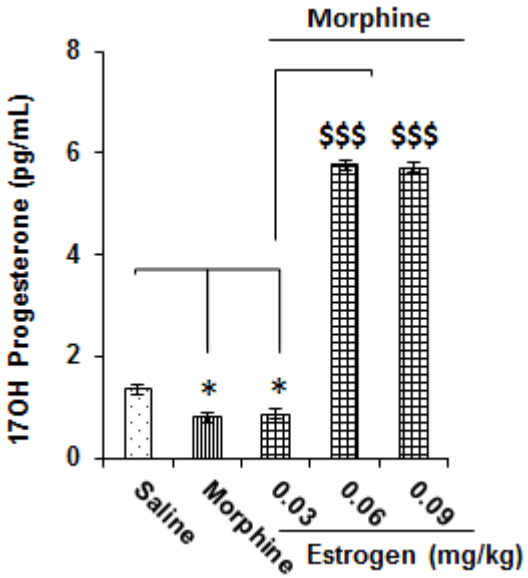


**Fig 14.** Levels of 17OH progesterone in saline and morphine alone as well as estrogen before injections. Levels show significant differences. The difference between the groups is based on the posttest.

### 3.5. Ovarian biometric findings

Morphine at a dose of 5 mg/kg, once alone (i.p.), estrogen once alone and once 20 min before morphine in the dose groups (0.03, 0.06 and 0.09 mg/kg) were administered. The control group received saline only. 72 h later, the animals underwent surgery under anesthesia and the ovaries and uterus were examined and the remarkable results (diagram) are as follows:

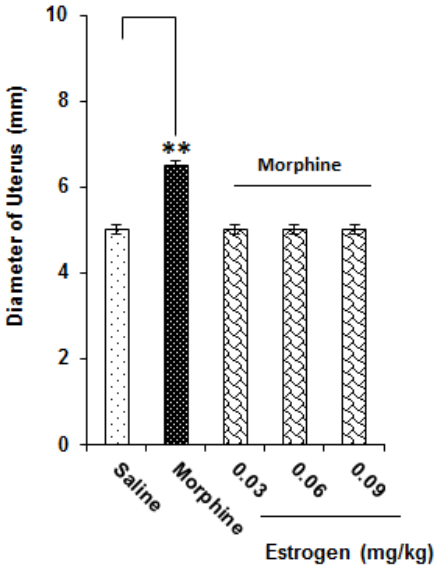


**Fig 15.** Diameter of the uterus in the estrogen + morphine, morphine alone and saline groups. Uterine diameter in the morphine group was significantly different from the control group (increase in uterine diameter). The Tukey's *post hoc* shows the significant difference (**P < 0.01).

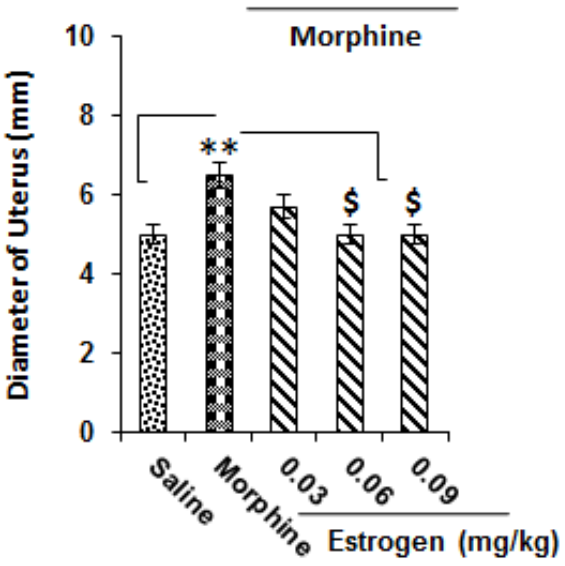


**Fig 16.** Uterine diameter in estrogen + morphine, morphine alone and saline. Uterine diameter was significantly increased in morphine group compared to control. Also, the diameter of the uterus in the estrogen + morphine group with doses of 0.03 and 0.06 mg/kg was significantly different from the morphine group.

### 3.6. Weight study findings

The weights of all animals were measured before and after the experiment and no significant differences were observed (Figures are not show).

## 4. Discussion

The aim of this study was to evaluate the effect of low doses of estrogen on morphine-induced polycystic ovaries in rats. Based on the findings, the cystic feature was evident in the ovaries of morphine-treated rats (5 mg/kg). In rats injected with single estrogen (doses of 0.03, 0.06, and 0.09 mg/kg, i.p.), the ovary was normal and follicles were observed at different stages (e.g, primary, secondary, antral, and gravid). Cysts were completely absent following estrogen injection (low dose) relative to morphine (in estrogen + morphine groups).

We know that polycystic ovary syndrome (PCOS) is a disorder that has a set of symptoms and is often caused by an increase in androgens and insulin in the blood and obesity. Elevated serum LH and decreased FSH are seen in people with PCOS. Increased LH stimulates ovarian cells to produce high androgens. In this syndrome, the ovaries also contain numerous follicular cysts on the periphery (marginal side) that originate from the ovarian follicles and are covered by several layers of granulosa cells (16).

According to the above description of PCOS, in the present study, a set of symptoms related to this complication appeared, including the presence of thick-walled follicular cysts and increased E2 levels. To explain the consequences of increased estrogen levels, it should be noted that in a normal menstrual cycle, an increase in estradiol (E2) levels causes the release of GnRH, which in turn leads to an increase in LH and ovulation. Why a decrease instead of an increase in LH has been observed in the present study is currently unclear. It may have occurred due to the 72-h interval between injection and sampling. Also, morphine may have caused a significant increase in estrogen by another mechanism, which needs to be clarified. Despite this limitation in this study, it should be noted that FSH is responsible for changes in the follicular phase and estrogen production. Progesterone is another sex hormone which is released by the ovaries and adrenal glands. The progesterone plays a key role in maintaining pregnancy. Its levels are low during the follicular phase of the menstrual cycle but significantly goes up after corpus luteum production (17). In the present study, due to estrogen administration, progesterone levels were negatively reduced, albeit, this feedback was moderated in the

presence of morphine (in estrogen + morphine group). By examining the pathways that have been discussed and studied for morphine so far, we have not come across anything specific in this regard, however, due to the low level of LH, it may be related to the interaction of opioid receptors. Because it has previously been shown that increased extracellular levels of opioids in the hypothalamus cause a decrease in GnRH, which in turn reduces LH production, but FSH is minimally affected (9,10). By reviewing the findings of the articles, it seems that the use of morphine causes irregularity of the estrous cycle. Also, prescribing narcotics during the period of sexual differentiation causes disturbances in the function of the reproductive system during puberty. Studies on the effect of morphine on ovulation in rats also show that it can inhibit ovulation in rats during the proestrus phase (8). In comparison, the rats in the present study were in the diestrous stage and their ovaries became polycystic after morphine injection. Given the increase in estrogen levels in the estrogen + morphine group, it can only be concluded that estrogen helps to improve the condition. In order to elucidate the ligand-receptor interaction, it is necessary to investigate the activation or increased expression of selective estrogen receptors by immunohistochemistry, but at this stage these criteria have not been studied and this is a limitation of our work.

When a woman has a lot of androgens in her body, she cannot release eggs from the follicles. Therefore, follicles that are full of fluid do not open, do not empty, and remain in the ovary. In this case, the ovaries become cystic. In women, estrogen is released from the follicles during the normal cycle of the ovaries. Estradiol is one of the most effective natural estrogens that stimulate the female reproductive system and create feminine traits. It is generally stated that this hormone is the main cause of most female complications including endometriosis, fibroids and cancers (such as endometrial cancer). But estrogen regulates the flow and thickness of uterine mucosal secretions (4). Estrogen also reduces menstrual bleeding, prevents and eliminates anemia, prevents ectopic pregnancy, slows down pelvic inflammatory disease, and reduces the rate of benign cysts and ovarian cancer. According to the present findings, although estrogen therapy reduces progesterone, it has an increasing effect on 17-hydroxy progesterone as a natural stressor process (18).

However, in this work, morphine reduced prolactin levels, it had also a decreasing effect on LH levels. It should be noted that according to previous findings, increased prolactin is another factor involved in anovulation. This disorder is seen in 1% of women. The secretion of prolactin is regulated by the secretion of the dopamine from the hypothalamus (secretion inhibitor) (19).

The results of the present study are not consistent with prolactin levels (because it decreased in the morphine group), but may indicate that PCOS is not directly related to morphine decreasing or increasing effect on dopamine activity in the hypothalamus.

We also know that LH levels are particularly elevated in women with PCOS, and their ovaries produce androgen. Insulin levels and insulin-like factors such as IGF also increase in women with PCOS, which increases androgen synthesis in isolated cells and thereby enhances LH function (1). But as with prolactin, in this study, morphine had a negative effect on LH levels, however, due to the most effective dose of estrogen (0.03 mg/kg), this effect was stopped, so the possibility that the effect of morphine was mediated by indirect pathways is strengthened. It seems that this section needs more research.

Due to the low levels of LH (due to morphine) which improved with estrogen (0.03 mg/kg) and also the lack of change in FSH levels, there may be an interaction of morphine with opioid receptors in the ovary. In order to better clarify the results and explain the results of this study, the level of 17-hydroxy progesterone was measured, which increased to some extent in the single estrogen group and decreased in the morphine group, which confirms the discussion of the morphine-mediated effects. On the other hand, due to the low level of the same hormone in the minimum dose of estrogen (in the group of estrogen + morphine in which the best anti-cystic effect was obtained), the ambiguities of the effect of morphine remain unclear.

## Conclusion

According to this study, it is concluded that estrogen in low doses may have a protective effect on morphine cystogenesis in the ovary. As a future suggestion, more attention should be paid to the induction of PCOS by morphine, especially at estrogen-progesterone-mediated signalling levels.

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## Conflict of interest

Authors declare no conflict of interest

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