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Effect of short-term administration of morphine on epidermal growth factor receptor level and iron deposition in rat ovarian tissue

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Abstract

Background and Objective: Morphine is one of the most powerful analgesic substances that is widely used in the clinic. This substance increases nitric oxide (NO) levels and blood flow to the ovary and ruptures the follicle wall. Damaged tissue can be repaired under the influence of epidermal growth factor (EGF). This study aimed to investigate the effect of short-term administration of morphine on the intensity of EGF receptor (EGFR) and iron deposition in ovarian tissue in Wistar rats.

Materials and Methods: In this study, 16 female Wistar rats were randomly divided into two groups. The control group (n=8) received 1 ml/kg of saline twice, once a day intraperitoneally (i.p.). The experimental group received 5 mg/kg morphine sulfate (i.p.) once a day for two consecutive days. 24 hours after the last injection, rats were anesthetized with ketamine and xylazine, and the ovaries and uterus were isolated for histological study. Iron deposition was investigated with the help of potassium ferricyanide staining and EGF receptor (EGFR) density was determined by immunohistochemistry method.

Results: Hemochromatosis in the form of blue iron deposits was shown significantly in the group receiving morphine as compared to the control group. Also, a higher concentration of EGFR was observed in the group treated with morphine than in the control group, which indicates the involvement of EGF in ovarian hyperemia.

Conclusion: Short-term use of morphine can cause hemochromatosis as a result of iron deposition in the ovary which is associated with hyperemia due to high NO levels induced by EGF.

Keywords: Morphine, Iron deposition, Epidermal growth factor, Receptor, Ovary

1. Introduction

Opium extracted from poppy secretions has long been used as a pain reliever (1). Morphine, the main alkaloid extracted from the poppy, is a powerful sedative (2). It has side effects on different parts of the body, including the reproductive and nervous systems (3). One of the mechanisms of action of morphine is the stimulation of the pro-inflammatory nitric oxide (NO) system (4). This gaseous molecule can be produced everywhere in the body by metabolic reactions and has a pro-inflammatory role (5). Inflammation is a rapid and protective response to irritation, injury, and infection, mediated by various signaling molecules. Ultimately, it promotes regeneration and prevents further harm to the injured tissue (6). Studies show that in acute inflammation, vascular permeability increases and leukocytes migrate to the site of inflammation. Then the level of inflammatory mediators such as reactive oxygen species (ROS) and free radicals such as hydrogen peroxide, superoxide and hydroxyl radicals as well as cytokines increases (7). NO is one of the important inflammatory mediators involved in acute and chronic inflammation. Clinical features of inflammation include redness, edema, warmth and pain in the inflamed area (8). Each cell can respond to specific signals. These signals determine differentiation, proliferation and programmed cell death. How the cell responds to these extracellular signals depends on the type of cell receptor and the

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cell signaling system. Epidermal growth factor (EGF) is a polypeptide that stimulates the growth of epidermal and epithelial cells (9). The uterus and ovaries are the target organs of EGF (10). Epithelial, stromal and myometrial cells of the uterus have EGF receptors (EGFR) (11). The unitary smooth muscle of genital organs also has EGFR receptors, and it seems that the activation of these receptors can cause rhythmic contractions and peristalsis (12). Also, studies show that EGF increases the production of prostaglandins by increasing intracellular calcium levels (13).

Whether morphine causes ovarian hemochromatosis due to high NO levels or whether EGF mediates the consequences of this phenomenon has not been investigated. Therefore, the aim of this study was to investigate the effect of short-term administration of morphine on EGFR density and iron deposition in ovarian tissue in female rats treated with morphine.

2. Materials and Methods

2.1. Animals

In this research, 16 female Wistar rats weighing 220 to 250 g were used. They were kept in the animal care center of Shahed University under standard conditions (free access to food and water and 12-hour light/dark cycle). The temperature was kept at 25ºC and the humidity was 60-70%.

2.2. Code of ethics

The ethical approval code of this research was IR.SHAHED.REC.1399.114.078.

2.3. Studied groups

Rats were randomly divided into two groups. The first group or control group received saline (1 ml/kg) intraperitoneally (i.p.) once a day for two days. The treated group was administered morphine (5 mg/kg, i.p.) once daily for two days. All injections were between 8 and 10 am.

2.4. Used substances

Morphine sulfate (provided by Temad Company, Tehran, Iran), 10% ketamine and 2% xylazine were purchased from the Veterinary Organization (Tehran, Iran). Potassium ferricyanide with catalog number 104982 and hydrochloric acid with catalog number 100314 were purchased from Merck, Germany and used for iron staining in ovarian tissue. For immunohistochemical staining, the EGFR pharmDx kit (provided by Dako Co., catalog number K1492) was used.

2.5. Sample separation

24 hours after the last injections, rats were anesthetized with an overdose of ketamine-xylazine. Then, by making a longitudinal incision on the surface of the rat's abdomen, the ovary and uterus were removed from the abdominal cavity, and after removing the fat tissue, their dimensions were measured using calipers. These tissues were fixed in 10% formalin for 48-72 hours. Then, with the help of a microtome (Leica, Italy), we prepared slices at a thickness of 3-4 µm. Then, after staining with Hematoxylin-Eosin, the slices were examined histologically by light microscope (Olympus, Japan).

2.6. Potassium ferricyanide staining

Potassium ferricyanide staining is prepared to check the parts where blood cells have accumulated. Small amounts of ferric iron are normally found in the spleen and bone marrow. Excessive amounts of ferric iron are accumulated due to hemochromatosis (14). The protein bonds in the hemosiderin molecules in tissue sections are broken when exposed to hydrochloric acid, and as a result, ferric ions (Fe^{3+}) are released. These ions combine with potassium ferricyanide to form ferric ferrocyanide, which is a light blue and insoluble substance. This process is one of the most sensitive histochemical tests and can detect even a grain of iron in blood cells (14). To perform this staining, we first fixed the slides in 10% formalin. To prepare an aqueous solution (20%) of hydrochloric acid, we combined 20 ml of distilled water with 5 ml of HCl. To prepare an aqueous solution of potassium ferricyanide, we mixed 25 ml of distilled water with 2.5 g of potassium ferricyanide trihydrate. Then we took an equal proportion of the aforementioned solutions and mixed them. We floated the slides in this new solution for 20 minutes. Then we washed the slides with distilled water in three steps (10 seconds each step). After that, we poured fast red violet dye on the slides, after five minutes, we rinsed the slides twice with distilled water for two minutes each time. Then we put them in alcohol (96%) and finally put them in xylene twice (three minutes each time). Then, we added Entellan (Merck, Germany) and placed the coverslips (14).

2.7. Immunohistochemical staining of EGFR

We deparaffinized the slides in two steps in xylene (30 minutes each) and then immersed them twice in alcohol (100%) for five minutes each. After that, we flooded the slides twice (each for two minutes) in descending degrees of 96, 90 and 80% alcohols. Then, the slides were washed with distilled water for five minutes. In the next step, we recovered the slides in citrate buffer with pH 7.4. To minimize evaporation, the dish was covered with perforated cling film and then placed in a microwaved at 600 W for 20 minutes. To prevent the slides from drying, we placed them in fresh buffer. Then we left the container at room temperature for 20 minutes. The slides were then placed in two steps (five minutes each) in TBS and

Triton X-100, mixed, and then placed in 10% normal serum with 1% BSA in TBS for two hours at room temperature. In the next step, the slides were washed for 3 minutes in TBS and Triton X-100 0.03% solution. Then we put the slides in a weak hydrogen peroxide solution for 10 minutes in a dark room. In the next step, we put the slides in the primary antibody for four hours at 37°C and then overnight at 4°C. After that, the slides were washed in three steps and each step for five minutes with TBS and Triton X-100 0.03% solution. Then, biotinylated buffer was added for 10 minutes and then slides were incubated with secondary antibody for one hour. After that, the slides were washed three times for one minute each time with TBS and Triton X-100 0.03% solution. Then, the slides were stained with streptavidin-HRP. Next, the slides were washed three times for one minute each time with TBS and Triton X-100 0.03% solution. In the next step, DAB chromogen was added and then, as in the previous step, the slides were washed three times for one minute with TBS and Triton X-100 0.03% solution. Then, the slides were dehydrated with ascending degrees of 80, 90 and 96% alcohols for five minutes. After that, they were placed in xylene three times for five minutes each time and finally mounted using Entellan (Merck, Germany) and covered (15).

2.8. Statistical analysis

We analyzed the data using SPSS (version 19) under $\alpha = 0.05$. The percentage of IHC response was also determined by Image J (Java, free).

3. Results

3.1. Hematoxylin & Eosin staining results

As can be seen in figure 1, there is a significant difference between the control group (saline 1 ml/kg, i.p.) and the rats treated with morphine. A number of developing follicles were present in the ovaries of saline-treated rats, but a number of follicular cysts were observed in the ovaries of rats receiving morphine.

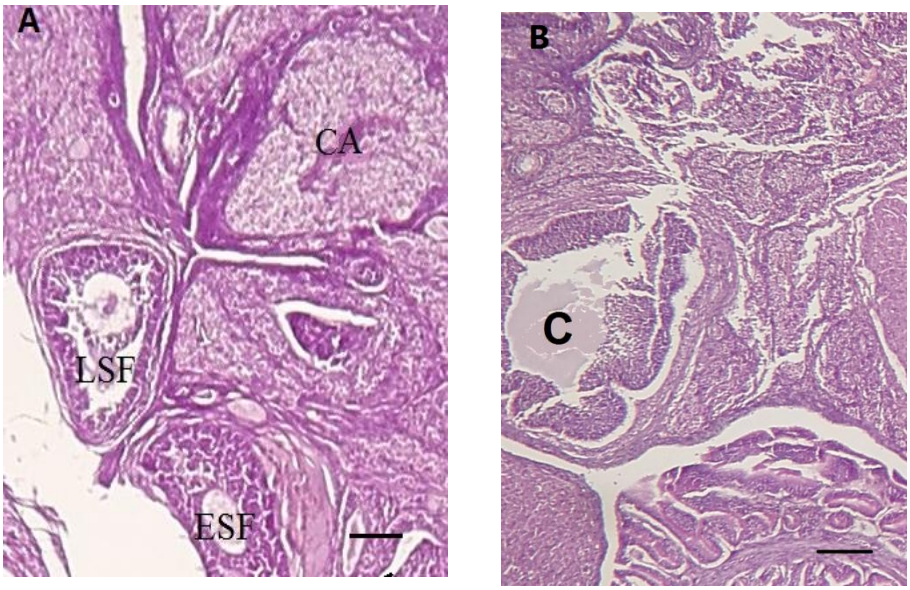

Fig. 1: Ovaries of control rats (A) and rats that received morphine 5 mg/kg (B) are compared. Cysts (cyst: C) are evident in the morphine treated group. (Late secondary follicle: LSF, Early secondary follicle: ESF, Corpus albicans: CA). Scale bar is 50 µm.

3.2. Findings of potassium ferricyanide staining

In this staining, the place of iron deposition is clearly defined (blood iron deposition). As can be seen in the figure 2, there is a significant difference in the control rats (1 ml/kg saline, i.p.) and the experimental group treated with morphine. In the ovaries of rats treated with morphine, more iron deposits are shown.

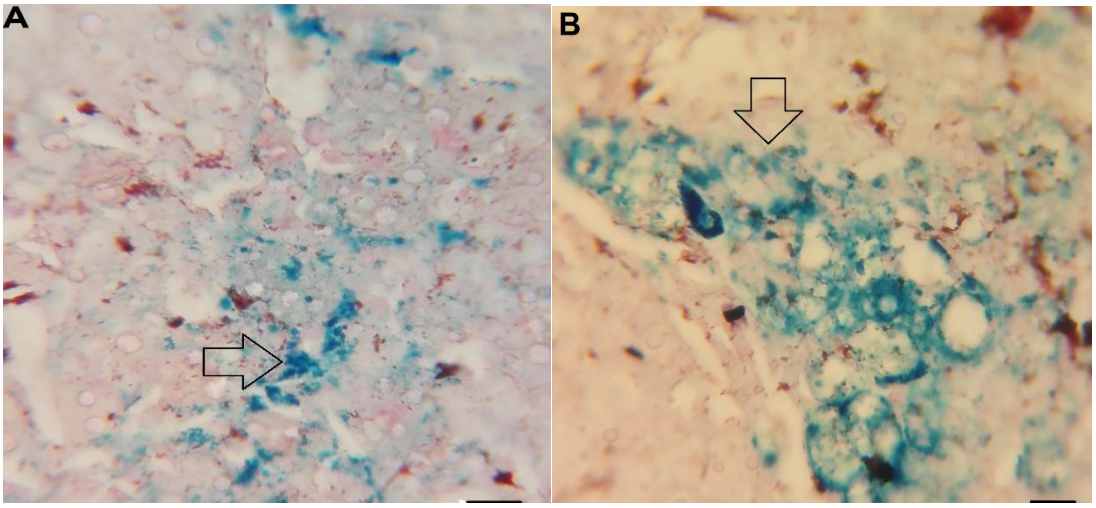

Figure 2: There is a significant difference in the control rats (1 ml/kg saline, i.p.) and the experimental group treated with morphine. In the ovaries of morphine-treated rats, the positive reaction to the staining is more evident.

3.3. Findings of EGF immunohistochemical staining

As seen in the figure 3, there is a significant difference in the control rats (saline 1 ml/kg, i.p.) and morphine-treated rats. In the group receiving morphine, the presence of extensive brown areas was observed, which indicates the level of EGFR protein expression. In the ovarian tissue in the control group, brown spots were shown to a lesser extent.

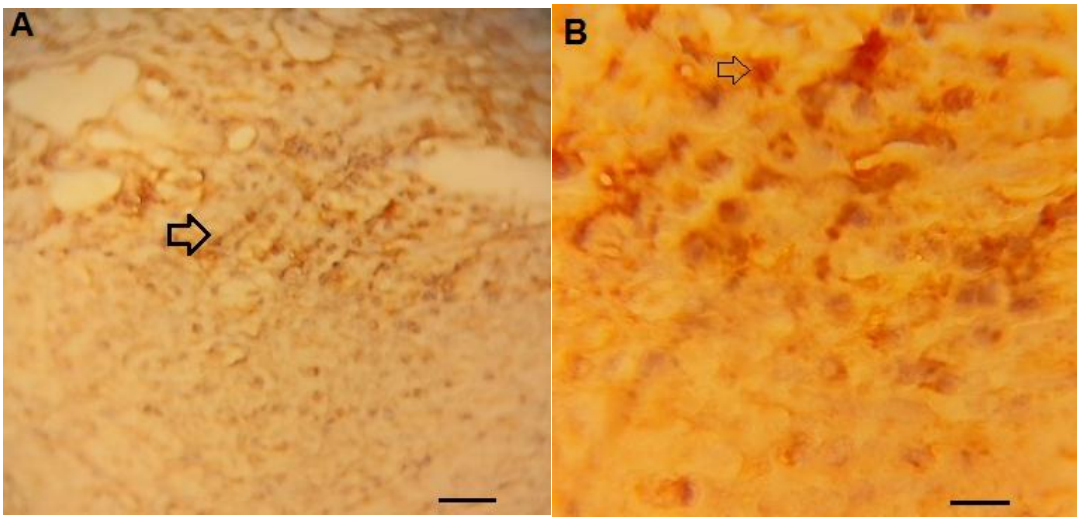

Fig. 3. This figure shows the ovaries of the control rats compared to the ovaries of rats that received morphine. The difference is intensity of brown colored zones (positive reaction) is obvious. The scale bar is 50 µm.

3.4. Immuohistochemical (IHC) percentage

In this study, the density percentage of EGFR was determined. As can be seen in the figure, there is a significant difference between control rats (saline 1 ml/kg, i.p.) and those treated with morphine (Figure 4).

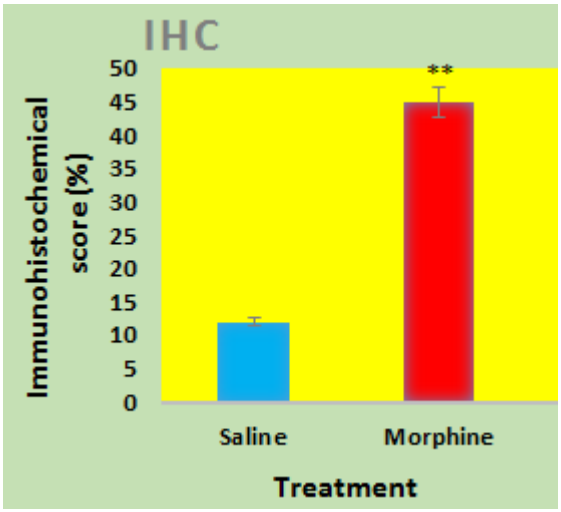

Fig. 4. Quantitative difference of EGFR in control rats and morphine-treated rats. * indicates 99% difference between morphine-treated and control groups by Tukey's *post hoc* test.

4. Discussion

Nitric oxide (NO) is a gaseous molecule synthesized by NO synthase (NOS) in many organs, including the reproductive organs. NOS is an enzyme that converts L-arginine and O2 to L-citrulline and NO (16). The molecule NO increases the production of prostaglandins, which increase the contraction of smooth muscles (17). It has a very high affinity with iron found in non-heme and heme proteins, one example of which is COX (18-20). In uterine tissue, endogenous NO directly activates COX, leading to increased prostaglandin production. As mentioned, one of the functional effects of morphine is the stimulation of the NO pro-inflammatory system (4). Ovary and uterus are two female sex organs that are target sites of epidermal growth factor (EGF) (10). Studies have shown that granulosa cells of patients with polycystic ovary syndrome (PCOS) express higher levels of the EGF receptor (EGFR) than patients with normal ovaries and patients treated with gonadotropins, regardless of the cause of infertility (21). Also, a recent study has shown that short-term use of morphine can also cause polycystic ovary (PCO) (22). In our study, ovarian tissue of rats receiving morphine showed a significant increase in EGFR density compared to the control group. On the other hand, according to the sources, there are also differences of opinion. Some studies have shown that prostaglandins inhibit the effects of EGF on vascular smooth muscle contraction (23). Other findings show that both EGF and IL-1 increase the concentration of prostaglandins in the female organ and vascular contractions (24). Other studies have demonstrated that EGF stimulates the synthesis of NO and induces the synthesis of prostaglandins in the rat uterus of (25). Therefore, in the present study, there may be a logical relationship between morphine consumption and stimulation of inflammatory and growth factor systems. Based on previous studies, uterine epithelial and stromal cells have EGFRs. Therefore, EGFRs may be expressed in more cell types in the reproductive system, including the ovary, and EGF may stimulate the growth of one or more cell types in that organ. But is the ovarian hemochromatosis in this study due to the induction effects of morphine on increasing the level of NO? If so, does morphine increase NO levels and mediate these effects? Is this agent also effective in inducing growth factors (such as EGF) and inflammatory mediators? Does this growth factor (EGF) play a role in supporting damaged tissue? Some of these questions cannot be answered now because we did not measure NO levels. Also, the level of different growth factors and cytokines were not measured, and these are all limitations of the current study, but these studies can be postponed to the next works, which will certainly resolve many of these uncertainties in the future. The authors mentioned the increase of prostaglandin synthesis by EGF due to the increase of NO levels (26), so if morphine has increased the level of NO, the effect of inflammatory factors cannot be neglected.

Conclusion

From the present study, it is concluded that short-term injection of morphine increases the density of epidermal growth factor receptor (EGFR) in the ovary. The increase of EGFR in the ovary may cause the increase of prostaglandins and hyperemia and inflammation in the ovarian tissue. As a result, blood vessels may rupture and iron deposits may concentrate in the ovarian tissue. Also, the presence of EGFR in the ovarian tissue may indicate that EGF has a supportive role on the damaged ovarian tissue caused by hemochromatosis.

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