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Effect of nitric oxide synthase inhibition on ovarian cystogenesis induced by morphine injection into ventro-medial nucleus of hypothalamus (VMH) of female Wistar rats

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Abstract

Background and Objective: Polycystic ovary (PCO) causes infertility and morphine use has been shown to induce this disorder. Microinjection of L-NAME, a competitive inhibitor of nitric oxide synthase (NOS) into the ventro-medial hypothalamus (VMH) may interfere with this effect as our aim

Materials and Methods: Female Wistar rats with a weight range of 200 to 250 g received morphine (0.001-0.4 µg/rat) intra-VMH with AP coordinates= -1.96. L-NAME (0.1-0.4 µg/rat, intra-VMH) was used alone or as a pre-injection to the effective dose of morphine. L-arginine (0.1-0.4 µg/rat, intra-VMH) was also injected alone or cumulatively with the inhibitor to elucidate the mechanism. Control group received saline (1 µL/rat), intra-VMH. Finally, the animals were anesthetized with ketamine and xylazine and the ovaries and uterus were dissected and examined pathologically. All data were analyzed using ANOVA (α= 0.05).

Results: Ovaries of morphine-treated rats showed a polycystic appearance, but the number of cysts in rats receiving L-NAME, intra-VMH, was significantly reduced. L-NAME/L-arginine intervention in the presence of morphine also had a reducing effect on cyst formation.

Conclusion: Induction of PCO by morphine injection in VMH may be involved in opioid receptors signaling and NOS enzyme activation, which is slowed by L-NAME intervention.

Keywords: Morphine, L-NAME, L-Arginine, Ventro-medial hypothalamus, Polycystic ovary

1. Introduction

Polycystic ovary syndrome (PCOS) is the most common disorder of the endocrine system that occurs in 5-7% of postmenopausal women (1-5), which has various symptoms such as anovulation leading to infertility, hairiness (hirsutism), acne, obesity, hyperandrogenism (excessive increase of androgens), insulin resistance and hyperinsulinemia, abnormal bleeding, pelvic pain, increased LH and decreased FSH secretion (2-7). Nitric Oxide (NO) is one of the main and fundamental triggers of this syndrome (8). This molecule is produced from L-arginine by nitric oxide synthase (NOS) through a catalytic reaction that converts arginine to citrulline without the use of energy and releases NO (8-10). With follicular growth, NO synthesis increases in the ovary (8). The high concentration of NO in the follicular fluid reduces the secretion of estradiol and decreases the quality of the ovaries. Likewise, the release of NO in the follicular granulosa cells inhibits estrogen production (11). NO regulates ovulation by influencing LH, and stimulates ovulation through the production of prostaglandins (12).

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Morphine with the chemical formula (C_17 H_19 NO_3) is the most abundant opium alkaloid (nearly 10%) (13). Opioids have previously been shown to decrease GnRH secretion by binding to hypothalamic opioid receptors. These conditions decrease the production of LH. But FSH is minimally affected. The authors have shown that the narcotic drugs reduce the negative feedback of sex steroids on pituitary LH secretion by suppressing hypothalamic GnRH levels (14), which may cause reproductive disorders. But which factor mediates the effects of morphine? NO is a pro-inflammatory agent (8). Is it possible that NO mediates the effect of morphine at the central level (*i.e.* hypothalamus)? Understanding the central effect induced by morphine on the HPG axis and especially NO modulation is worth studying, which is our goal in this study.

2. Materials and Methods

2.1. Animals

In this research, female Wistar rats were purchased from Pasteur Institute of Iran in the weight range of 200-250 g. These animals were kept in standard cages in groups of four rats in each cage with sufficient water and food (purchased from Pars Animal Feed co., Tehran, Iran) under a temperature of 23±2 ºC and a light/dark phase of 12/12. After adaptation, they were randomly divided into two groups: control (n=8) and experimental (n=8 in each dose). Tests were carried out at certain times (between 09 a.m. and 02 p.m.) according to the experimental design. All work was conducted in accordance with the ethical principles and approval was granted by the local ethics committee.

2.2. Used drugs

Morphine sulfate was provided by Temad co., Tehran, Iran. N_G -nitro-L-arginine methyl ester (L-NAME) was purchased from Biomedical Inc., U.S.A. L-Arginine was obtained from Merck (Germany). Ketamine and xylazine were provided from Veterinary Organization of Iran, Tehran. Hematoxylin and eosin were purchased from F Arman co., Tehran, Iran.

2.3. Stereotaxic surgery

Animals were deeply anesthetized by ketamine (100 mg/kg) and xylazine (20 mg/kg) and underwent surgery using Stereotaxic apparatus (Stoelting, U.S.A.), with the coordinates of Bregma and Lambda points (AP= -1.96). The desired point was drilled with a pen drill and the guide cannula was placed in the point using a stereotaxic device. After surgery, rats were allowed to recover for one week, after which they received different substances according to the grouping scheme listed below. After performing the experiments, the ovaries and uterus of the animals were surgically removed under deep anesthesia. The collected tissues were kept in 10% formalin for at least 72 hours before starting the pathological study.

1. Control group (n=8) that received saline (1 µL/rat, intra-VMH).

2. Single morphine dosage (0.001, 0 .01, 0.1, 0.2, and 0.4 µg/rat, intra-VMH, n=8 per dose-group).

3. Single L-NAME dosage (0.1, 0.2, and 0.4 µg/rat, intra-VMH, n=8 per dose-group).

4. Single L-arginine dosage (0.1, 0.2, and 0.4 µg/rat, intra-VMH, n=8 per dose-group).

5- Pre-injection of L-NAME (0.1, 0.2, and 0.4 µg/rat, intra-VMH, n=8 per dose-group) to the effective dose of morphine (0.4 µg/rat).

6. Pre-injection of L-NAME (0.1, 0.2, and 0.4 µg/rat, intra-VMH, n=8 per dose-group) to L-arginine before morphine effective dose (0.4 µg/rat).

2.4. Drug injection

After a week of recovery, the animals were given drug. Morphine sulfate (0.001 to 0.4 µg/rat) was microinjected into the ventro-medial nucleus of the hypothalamus (VMH: AP: -1.96). L-NAME (0.1 to 0.4 µg/rat) alone or in a cumulative manner with morphine and/or L-arginine was microinjected into the VMH: first L-NAME (0.1 to 0.4 µg/rat) and then morphine (0.4 µg/rat) were administered. The precursor of NO, L-arginine (0.1 to 0.4 µg/rat) was also microinjected into the nucleus alone and collectively with L-NAME in the morphine-treated groups at a concentration of 0.4 µg/rat.
The control group received only saline (1 µL/rat, intra-VMH). After the experiments, the animals were anesthetized and surgically, the ovaries and uterus were collected in 10% formalin and then examined.

2.5. Histological studies

Right and left ovaries and uterus were removed and fixed in 10% formalin. After 72 hours, the tissues were prepared for cutting and staining.

2.6. Hematoxylin-eosin (H & E) staining

Samples were cut into 4-5 µm sections using a microtome (Leica, Italy) and stained with H&E. At the end, the samples were assembled with Entellan glue (Merck, Germany). Tissue samples were studied biometrically and histopathologically and observed using a microscope (Olympus, Japan) to examine cysts.

2.7. Statistical analysis

After the Kolmogorov–Smirnov test, the data were calculated using analysis of variance (ANOVA) under

α=0.05. In case of significant difference, further analysis was followed by Tukey HSD *post hoc* to show differences between groups. Color figures were also analyzed with the help of an Image J program (free Java software).

3. Results

3.1. Dose response of morphine in ovarian cyst formation

This response is shown relative to the control group that received 1 µL of saline directly into the VMH. All doses of morphine (0.001-0.4 µg/rat, intra-VMH) were injected once within the nucleus of the VMH. Based on *post hoc* analysis, the dose of 0.4 µg/rat of morphine was more effective in inducing ovarian cysts ($P<0.05$) (Figure 1). It should be noted that morphine in concentrations of 0.001 to 0.4 µg/rat, intra-VMH, did not have a significant effect on the diameter of the ovary (Figure 2) and the size of the uterus (Figure 3).

Histological images on ovarian cysts can also be seen below. The thickness of the cyst wall showed PCO pattern. Ovaries of control animals showed follicles at various stages of development, while the number of these follicles (normally developing and mature) showed a significant decrease in morphine-treated rats (Figure 4A-4B).

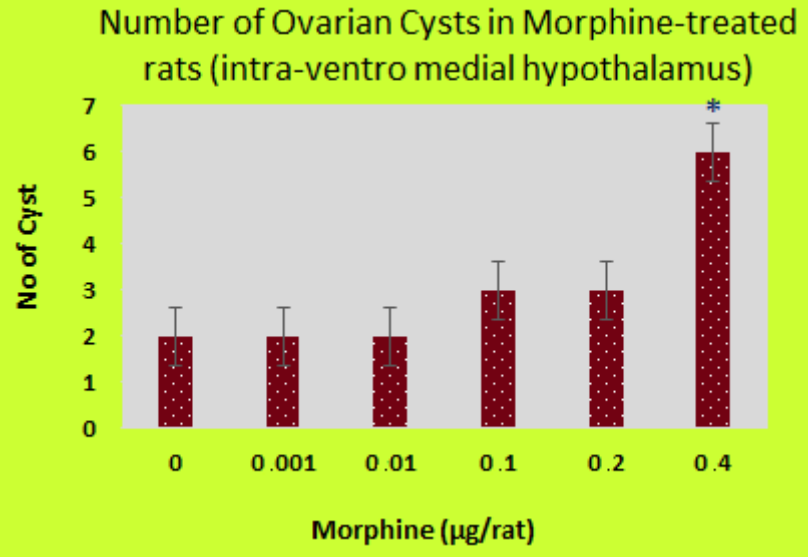

Fig. 1: Morphine (0.001-0.4 µg/rat) was injected once into the VMH nucleus. The zero point represents the control group that received one µL of saline inside that nucleus. Morphine at the effective dose significantly induced cyst formation. Data are based on mean and standard error. Asterisk (*$P<0.05$) is based on Tukey's *post-hoc* test.

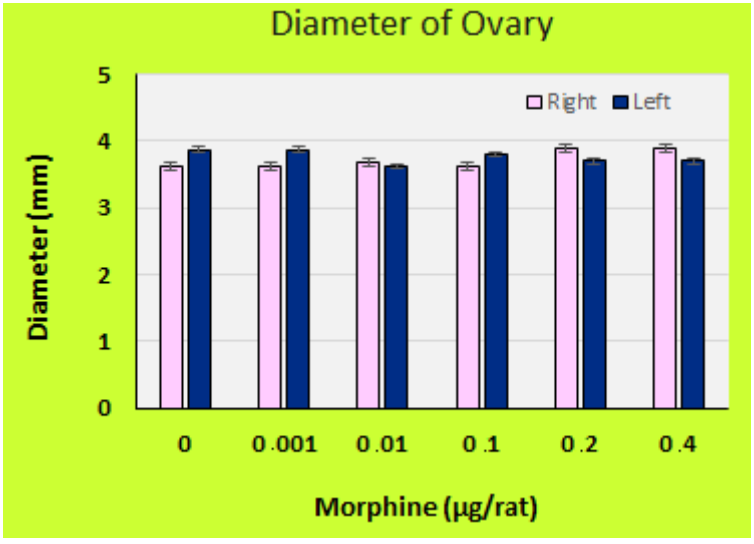

Fig 2: Morphine (0.001 to 0.4 µg/rat) was injected once in the nucleus of VMH, but no significant effect was shown on the diameter of the ovaries.

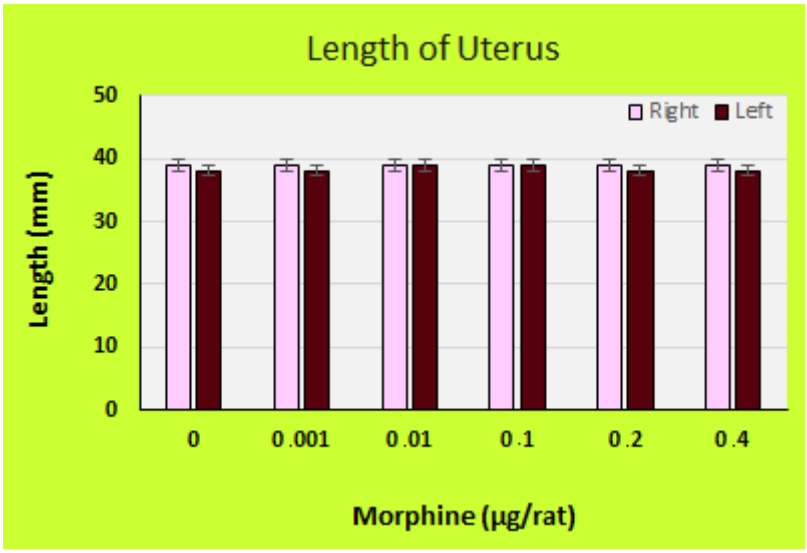

Fig 3: Morphine (0.001 to 0.4 µg/rat) was injected once into the VMH nucleus, but no significant effect on uterus size was shown.

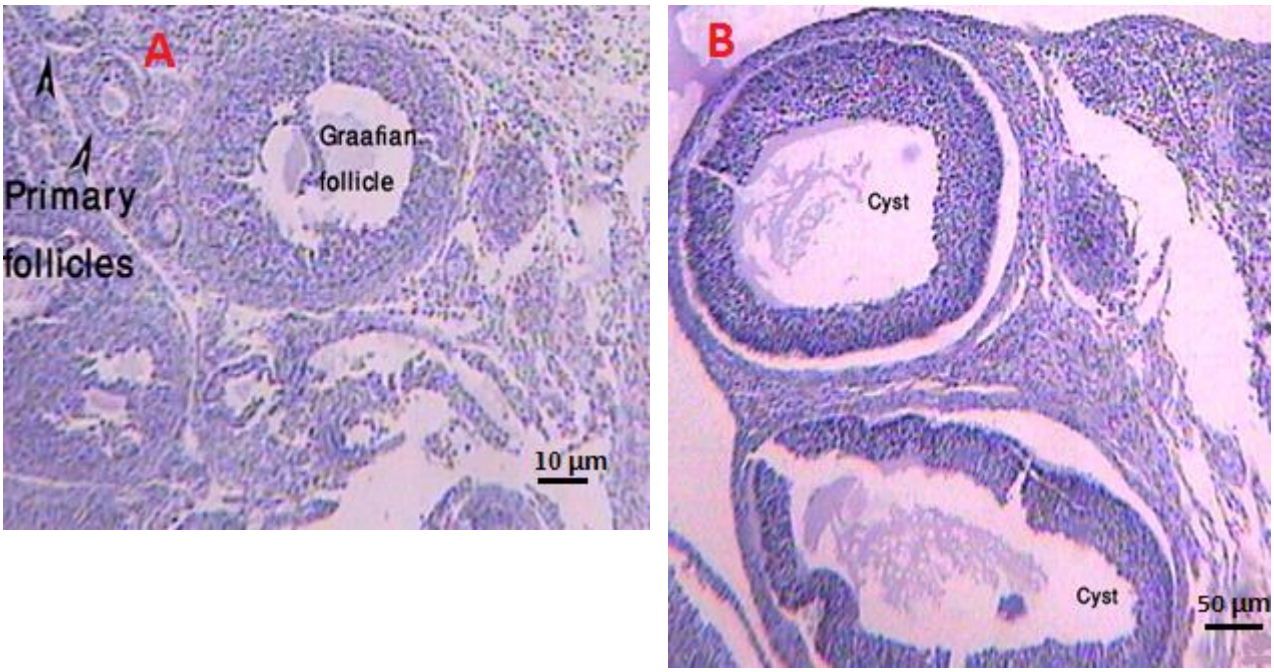

Fig 4: Ovaries of rats in the control group (A) show follicles in different stages of development. However, the morphine-treated sample (B), showed the follicular cysts in the ovary. Scale bars are also shown.

3.2. Dose response of L-NAME on morphine-induced ovarian cystogenesis

Different doses of L-NAME (0.1-0.4 µg/rat) were injected alone/or prior to the morphine (0.4 µg/rat) into the VMH. No ovarian cysts were observed in the single L-NAME groups ($P > 0.05$) (Figure 5).

The effect of NOS stimulator (L-arginine 0.1-0.4 µg/rat) was necessarily studied (Figure 5). The cumulative injection of L-NAME/L-arginine prior to morphine (0.4

µg/rat) was additionally shown to indicate the cyst reduction. Histological images were also presented to demonstrate the inhibition effect of L-NAME on the follicular cyst-genes (Figure 6).

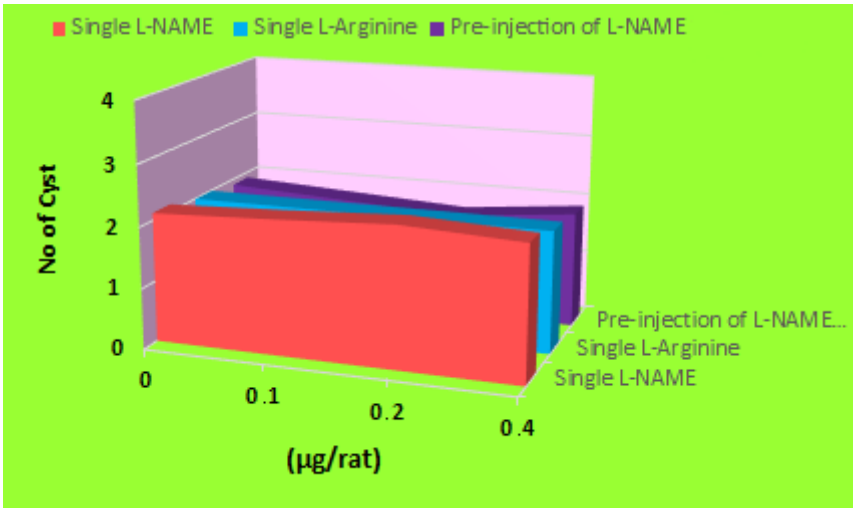

Fig 5: Effete of Single L-NAME (0.1-0.4 µg/rat, intra-VMH), and single L-arginine (0.1-0.4 µg/rat, intra-VMH), and L-NAME (0.1-0.4 µg/rat, intra-VMH) plus L-arginine (0.1-0.4 µg/rat, intra-VMH) in animals receiving morphine 0.4 µg/rat inovarian cystogenesis. No significant cysts were observed indicating an inhibitory effect of L-NAME.

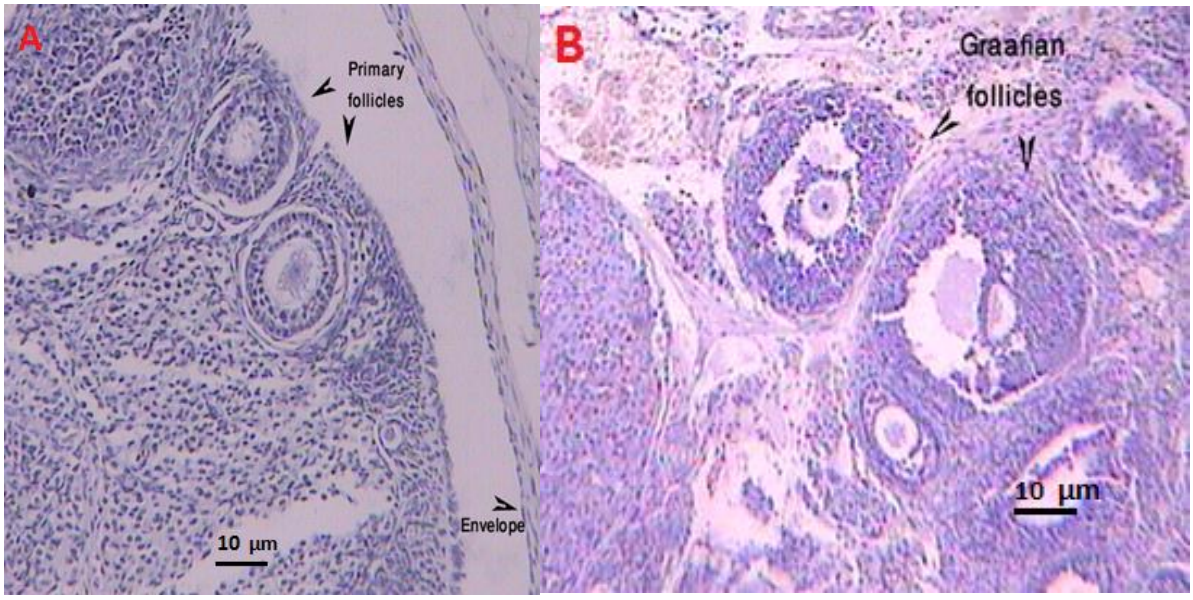

Fig 6: No significant effect on cyst formation was shown in the treated group (B) compared to the control (A). Scale bars are also shown..

4. Discussion

In this study, we found that in morphine-injected animals, the ovaries appeared polycystic, but using pre-injection L-NAME as a NOS inhibitor, the aforementioned effect was moderated and the number of cysts was reduced.

The hypothalamus-pituitary-gonadal (HPG) axis consisting of the hypothalamus, pituitary gland, and gonads controls the reproductive function. The hypothalamus sends gonadotropin-releasing hormone (GnRH) through the network of blood vessels that connects the hypothalamus to the pituitary gland, and stimulates the gonadotropic cells through specific receptors. As GnRH binds to these receptors on the surface of the pituitary gland, chemical events occur that causes the release of the hormones LH and FSH, which regulate the function of the gonads of both sexes (15). Ovaries are the end organs of the HPG axis, which are responsible for two important functions, one is ovulation and the other is the production of estrogen and progesterone (16-18). There is evidence that the NO acts as a transmitter in the hypothalamus, pituitary gland and other gonads (19). NO producing neurons are located near GnRH-secreting neurons in the hypothalamus and play the most important role in regulating GnRH secretion (20).

NOS is expressed in the granulosa and luteal cells of the ovary, and in this way, NO has a positive role in the ovulation process of rats and increases the secretion of progesterone and decreases the secretion of estradiol in the ovary. NOS inhibitors suppress ovulation-stimulating hormone in the ovaries (21). With these explanations, the results obtained in this study cannot be discussed by gonadal NO. However, from another point of view, the level of μ opioid receptors is high in VMH and arcuate nucleus. These nuclei control the mating and sexual behavior of rats. By acting on these receptors, morphine inhibits sexual behavior and lordosis in female rats (22,23).

These findings and other previously reported results (13,14) are appropriate to discuss the results of this research. By interacting with these receptors on the surface of these nuclei, this substance (morphine) can stimulate the NO system, and if NO is the mediator of cyst induction, it is enough to inhibit the enzyme that produces it. This was done in the present study. Therefore, it can be concluded that morphine can easily attack the ovaries and induce cysts through high levels of central NO. We suggest that morphine injection into the VMH may affect the hypothalamic opiate receptors and activate the NOS enzyme, as prior L-NAME injection blocks this effect.

Conclusion

This article investigated the interaction between morphine and L-NAME in ventro-medial hypothalamus (VMH) of female Wistar rats and showed that PCO induction by morphine in VMH may be involved in opiate receptors and NOS enzyme activation, because that phenomenon was stopped by inhibiting the enzyme.

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