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Original Article

Intra-ventromedial Hypothalamus Blockade of Dopamine Type 2 Receptors by Sulpiride Ameliorates the Fertility-Disrupting Effects of Morphine in Female Wistar Rats

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Abstract

Background and Objective: Polycystic ovary syndrome (PCOS) is a reproductive disorder that causes infertility. Morphine, when injected into the ventromedial hypothalamus (VMH), is effective in inducing PCO, but the mechanism of this effect, particularly concerning dopamine (DA), remains unclear. The study aims to understand how DA and its receptors contribute to this disruption.

Materials and Methods: Virgin animals weighing 220-250 g and aged 8-9 weeks were randomly divided into control, morphine alone, sulpiride alone, and sulpiride + morphine groups that received sulpiride before an effective dose of morphine. Morphine (0.1-0.4 µg/rat) was injected into the VMH (AP coordinates: -1.92; ventral: 9 mm; lateral: 0.5 mm). Sulpiride (0.1-0.4 µg/rat) was injected intra-VMH alone or before morphine (0.4 µg/rat, intra-VMH). The control group received saline merely (1 µL/rat, intra-VMH). The animals were anesthetized three days later, and after surgery, the ovaries, uterus, and brain were collected and examined in 10% formalin. All data were statistically analyzed.

Results: Ovaries of rats treated with morphine and not in the sulpiride single groups showed PCO features compared to the control group; however, the number of cysts was significantly reduced in the sulpiride + morphine samples. Morphine and sulpiride did not significantly induce uterine inflammation. Moreover, no significant effect on VMH neurons was observed in any of the groups.

Conclusion: These results indicate that morphine disrupts follicular growth and that this effect is partially reversed by blocking DA type 2 receptor signaling.

Keywords: Dopamine, Morphine, Polycystic ovary, Rat, Ventromedial hypothalamus

Background

Polycystic ovary syndrome (PCOS) is a problem affecting 5-10% of women of reproductive age worldwide. It is characterized by hyperandrogenism, chronic anovulation, irregular menstrual cycles, acne, hirsutism, metabolic abnormalities, and insulin resistance. The morphology of PCO includes an increased number of preantral follicles [1]. Defects in the hypothalamic-pituitary-gonadal (HPG) axis and abnormal changes in gonadotropin-releasing hormone (GnRH) cause irregular cycles and anovulation [2]. Although the role of GnRH in the reproductive axis cannot be ignored, it should not be overlooked that this factor is regulated by multiple agents. As previously indicated, if estrogen receptor alpha (ER-alpha) is not expressed in GnRH neurons, estrogen feedback regulation is disrupted [3].

Considering the potential mechanisms of neurotransmission modulation mediated by opioid and dopamine (DA) receptors in the brain, it was found that opioids bind to hypothalamic opioid receptors and reduce GnRH secretion, which in turn reduces luteinizing hormone (LH) secretion by the pituitary gland [4-6]. It has been demonstrated that opioid peptides, together with gamma-aminobutyric acid (GABA) and neuropeptide K, exert an inhibitory mechanism on hypothalamic GnRH secretion, while glutamate, norepinephrine, neuropeptide Y and nitric oxide (NO) stimulate GnRH secretion [7].

Despite our previous studies of the cystogenic effects of morphine in the ovaries of female rats, both by chronic peripheral administration [8] and by acute injection of morphine into the VMH [9], the mechanism is not fully understood. Previous studies have reported that fertility levels are reduced in the presence of morphine and that a neurotransmitter mediates this effect and reduces GnRH secretion. The inhibitory effect of morphine on hypothalamic gonadotropin secretion may be altered in the presence of a type 2 DA receptor blocker (sulpiride), which could interfere with the inducing effects of morphine by interfering with DA function.

Objectives

In order to obtain these findings from a mechanistic perspective, this study aimed to determine whether DA receptor blockade by prior injection of sulpiride into the VMH reduces ovarian cysts in the ovaries of recipient animals. Indeed, the precise mechanism by which morphine exerts its effects on GnRH-secreting neurons is unknown, and this issue is addressed in the present research.

Materials and Methods

Animals Used

A total of 40 female Wistar rats weighing (200-250 g) were kept in standard temperature (21±3°C) conditions and a 12-hour light/dark cycle in the Center for Animal Care and Breeding of Shahed University in transparent polycarbonate cages in groups of two without access to male animals, with sufficient water and food, ad libitum. According to the experimental design, after completing skill acquisition courses with experimental animals, they were randomly divided into control, saline-receiving, and experimental groups to receive the drug. All protocols were confirmed by the local ethics committee.

Restraining of Animal

The rat must first be handled and restrained. Restraining a rat is more difficult than a mouse due to the animal's large size. There is a risk of biting. For this purpose, thick gloves should be used. If gloves are not available, the animal's eye can be covered with a hand towel, and then it can be grasped over the skin of the neck along the back. In this case, the animal will be ready for intraperitoneal (i.p.) injection.

Intraperitoneal Injection

Intraperitoneal (i.p.) injection is the most common method of injection in laboratory animals. This should be performed in a calm and stress-free environment; otherwise, the animal will become stressed and nervous. Using the index and middle fingers, we grasped the skin of the neck and gently pulled the skin of the back area with the remaining fingers so that the rat was under our control. Then, an insulin syringe was used for an intraperitoneal (i.p.) injection. In these injections, no swelling was observed on the skin of the internal cavity (coelom).

Equipment and Materials Required

The Stereotaxis device was purchased from Stoltings, U.S.A., and the animal scale (with an accuracy of 1 g) was purchased from Mobtakeran Co., Ltd., Tehran, Iran. Pen drill with 2- and 3-mm drill bits, guide cannula (needle Gauge 23), dental needle (Gauge 30), acropars liquid cooling acrylic powder and hardener (Acropars Co., Iran), morphine sulfate (Temad Co., Tehran, Iran), sulpiride (Merck, Germany), as well as ketamine and xylazine (Veterinary Organization, Tehran, Iran) were purchased.

Stereotaxic Surgery

The animal was carefully weighed on an animal scale and anesthetized with an i.p. injection of ketamine 100 mg/kg and xylazine 20 mg/kg. After anesthesia, the animal was placed in the stereotaxic apparatus using ear bars placed inside the ear (behind the eardrum) and an incisor bar used to position the upper incisors. Three graduated systems based on vernier calipers perpendicular to each other were planned in the device to locate specific points on the skull accurately and to inject into the nucleus (VMH) based on the Paxinos and Watson's rat brain Atlas [10] at AP: -1.92 (Figure 1). Brain samples were examined by injecting one microliter of methylene blue into the VMH, and only samples with correct targeting were followed up for further histological examinations. After surgery, the rats were recovered for seven days; they were under daily care during the recovery period. Afterward, various experiments were conducted, and after 72 h, the animals were sacrificed for the isolation of brain and ovarian tissues. The collected tissues were then stored in 10% formalin for at least 48 to 72 h before sectioning.

In vivoInjection of Drug

Using the injection set, saline (control group) or the desired substance (experimental group) was injected into the VMH through the guide cannula at a volumetric concentration of 1 µL. The length of the guide cannula was 1 mm shorter than that of the injection cannula; however, the length of the injection cannula was precisely proportional to the desired injection site (Figure 2).

Hematoxylin-Eosin Staining

Staining of uterine and ovarian tissue with a thickness of 4-5 µm using the hematoxylin-eosin method (purchased from Farzaneh Arman Iran Company) was performed as follows: the tissue samples were first deparaffinized twice in xylene (first for 30 min and second for 10 min). They were hydrated with alcohol: 96% alcohol, 70% alcohol, 50% alcohol (each for 3 to 5 min). The slides were washed with distilled water and stained with hematoxylin (for 40 min), washed with distilled water, and immersed in eosin stain (for 20 min). They were then rinsed with running water and passed in 1% glacial acetic acid (to

create color differentiation). Then, the slides were dehydrated with 50%, 70%, and 96% alcohol clarified twice in xylene (3-5 min each) and finally mounted with Entellan (purchased from Merck, Germany) and covered with coverslips.

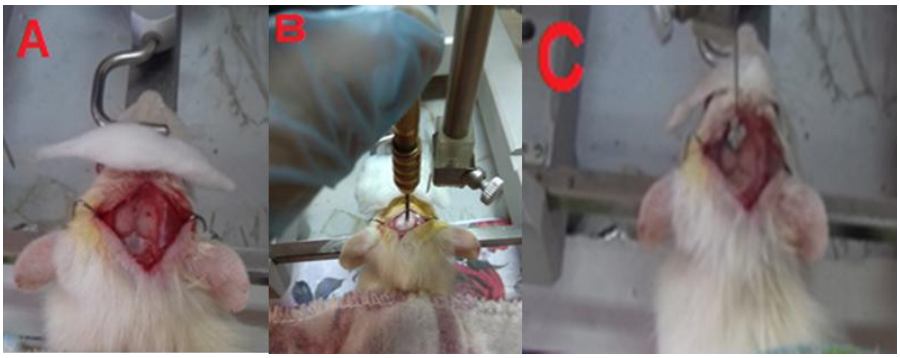

Fig 1. Stereotaxic rat surgery under deep anesthesia (A). We drilled the desired point at the skull (AP: -1.92; ventral: 9 mm; lateral: 0.5 mm) while the animal was restrained in the tool (B). Using device manipulation, the guide cannula was inserted into the VMH (C).

Fig 2. *In vivo* drug injection into the VMH nucleus of a living rat. The injection set consisted of an injection cannula (length exactly for the target point) connected by a polyethylene tube (inner diameter 0.6 mm) to a 1 µL Hamilton syringe. The substance was injected into the target nucleus at a volumetric concentration of 1 µL over 1 min, remained in the same position for 30 s, and then slowly withdrawn. During this time, the animal was completely unstressed and calm.

Staining of Brain Tissue Using the Cresyl Violet Method

Brain slides were placed twice in xylene (first for 30 min and then for 10 min) and subsequently in 96%, 70%, and 50% alcohol (each for 5 min) and then washed in distilled water. Afterward, they were placed in cresyl dye (1% in distilled water, purchased from Merck, Germany) for 40 min. They were then washed in distilled water and immersed in 50%, 70%, and 96% alcohol. They were then cleared twice (each for 3 min) in xylene, mounted with Entellan (purchased from Merck, Germany), and covered with coverslips. After drying, the prepared slides were examined under a microscope to verify the criteria.

Statistical Analysis

After analyzing the data using the Kolmogorov-Smirnov test to confirm the parametric nature of the data, they were statistically analyzed using one-way ANOVA or, if necessary, two-way ANOVA. If the aforementioned analysis was significant, the subsequent Tukey test was also used to follow up on differences between groups. $P<0.05$ was considered significant.

Results

Morphine Dose Response for Ovarian Cystogenesis

Morphine doses used were 0.1, 0.2, and 0.4 µg/rat, and the drug was injected directly into the VMH at a volume concentration of 1 µL. This response was dose-dependent compared to the control that received 1 µL of saline, intra-VMH. Based on the results, the dose of 0.4 µg/rat was completely effective in the development of ovarian cysts compared to the control group, which was subsequently used in cumulative injection with sulpiride doses (Figure 3). Sulpiride was used at different doses (0.1, 0.2, and 0.4 µg) alone and prior to morphine effective dose (0.4 µg/rat) into the VMH. This substance had no significant effect on cyst growth alone; however, it reversed the effect of morphine (Figure 3).

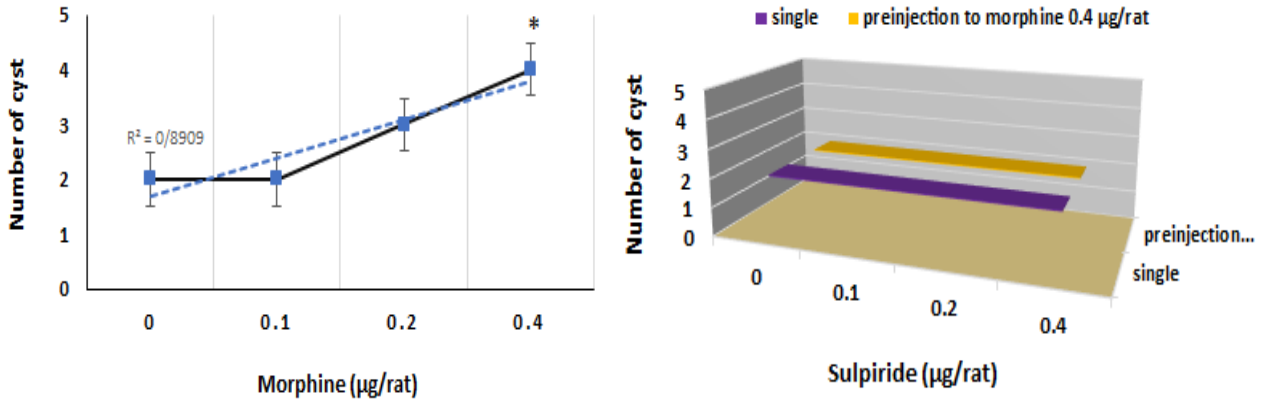

Fig 3. Dose-response curve in rats treated with morphine (intra-VMH) after recovery. The trend clearly indicates that there is a significant relationship between ovarian cystogenesis and morphine dose. Sulpiride (0.1–0.4 µg/rat) was injected alone and with morphine at an effective dose of morphine (0.4 µg/rat) once into the VMH nucleus. The zero point represents the control group that received 1 µL/rat of saline. All data are based on the mean ± standard error of the mean. The asterisk is based on Tukey's post-test (*P<0.05 *vs.* Control).

Findings Related to Ovarian Cysts

According to the histological results, the thickness of the cyst walls showed the pattern of PCO cysts. The ovaries of the control group showed follicles at different stages of development, while the number of these follicles (normally developing and mature) was reduced in the morphine-treated rats (Figure 4). Moreover, there is a positive correlation between morphine and cystic features of the ovary.

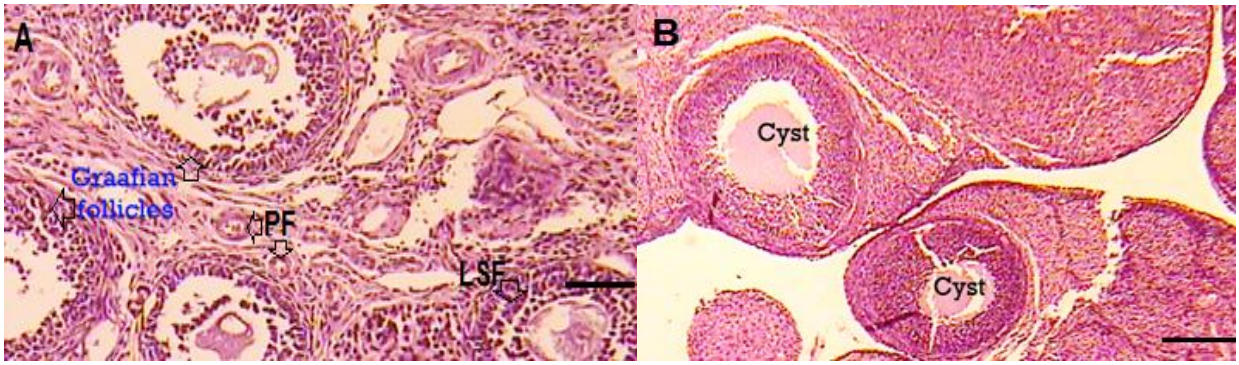

Fig 4. Histological images of the ovary. The effect of morphine treatments on ovarian cystogenesis can be seen (B *vs.* Control: A). Morphine (0.1 to 0.4 µg/rat) was injected once into the VMH nucleus and indicated a polycystic ovary structure at an effective dose (B) (0.4 µg/rat), (PF: primary follicle; LSF: late secondary follicle). The scale bar is equal to 100 µm.

Findings of Ovarian Diameter as well as Uterine Horn Diameter and Length

The effect of morphine compared to control was a relative increase in ovarian and uterine diameter and a slight change in uterine length. The relative inflammatory effects were reversed by prior injection of sulpiride (Figure 5).

Effect on Animal Body Weight

The effect on the body weight of the animals was not significant (Figure 6).

Effect on Neurons in the VMH area

Morphine had no significant effect on hypothalamic nucleus neurons (Figure 7).

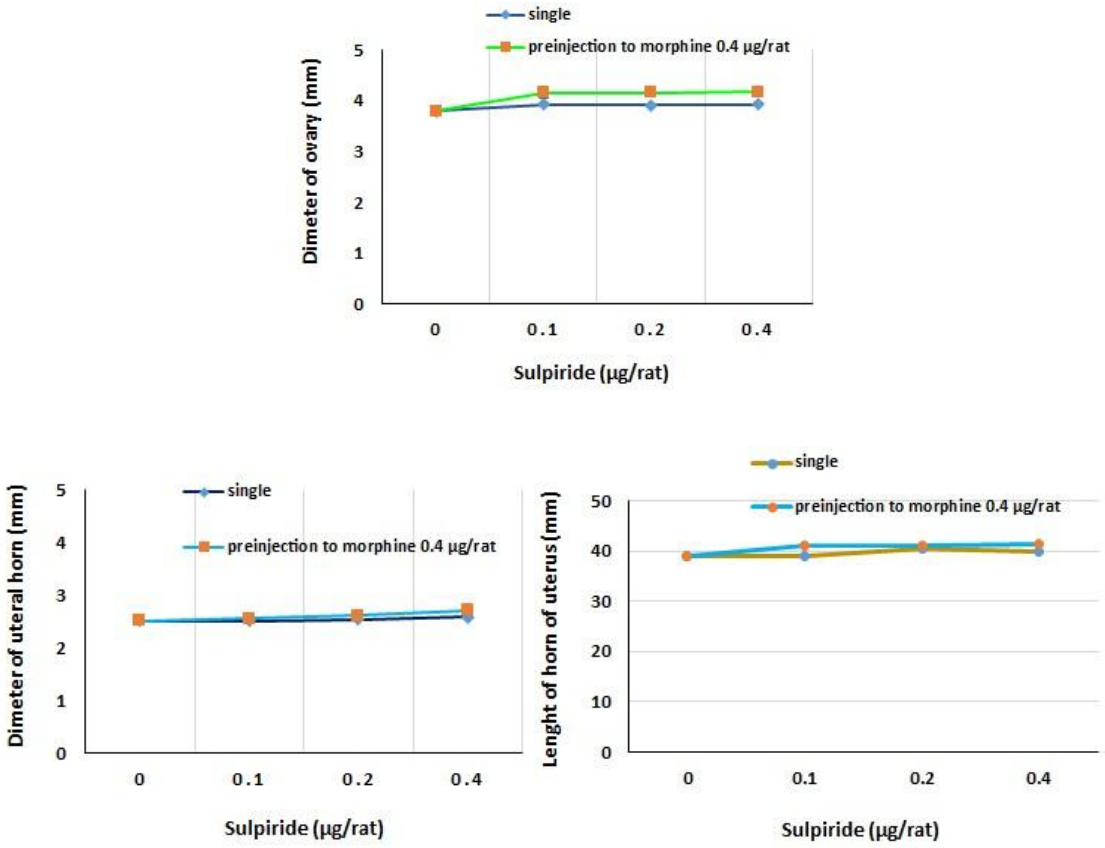

Fig 5. Sulpiride (0.1-0.4 µg/rat) injection alone and before 0.4 µg/rat morphine injection has been indicated. Morphine (0.1-0.4 µg/rat) injected once into the VMH nucleus showed effects on ovarian diameter, uterine horn diameter, and uterine length at the high dose (0.4 µg/rat) that were reversed by cumulative injection of sulpiride with this drug.

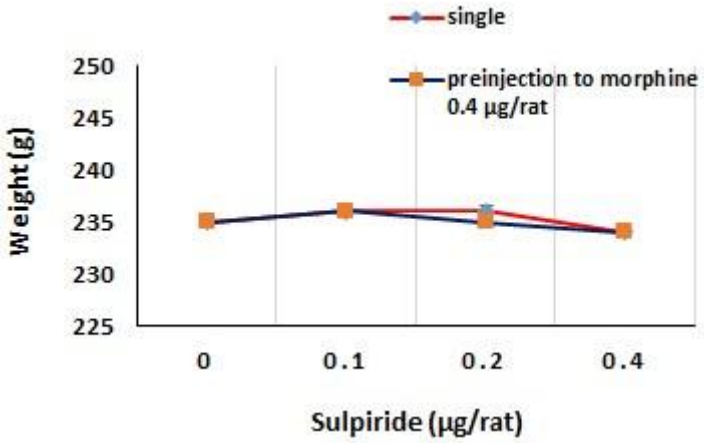

Fig 6. Animal body weight. As can be seen in the graph, there is no significant difference between sulpiride alone and combined with morphine.

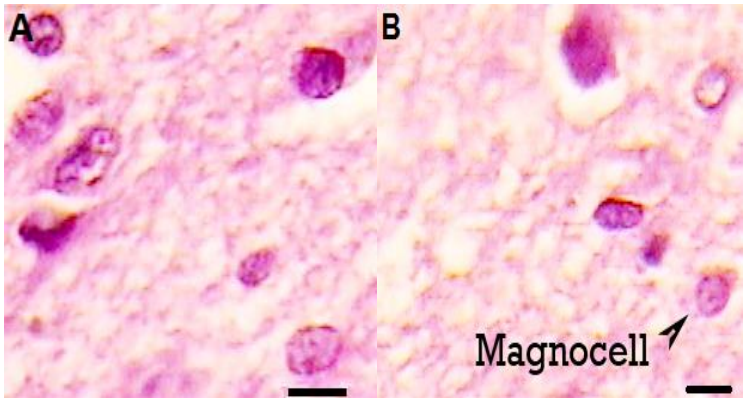

Fig 7. VMH nucleus. Morphine was injected once into the VMH nucleus; its effect on the VMH nucleus neuron population (B) was negligible compared to the control (A).

Discussion

In the present study, the inhibitory effect of sulpiride on cystogenesis induced by microinjection of morphine into the ventral median nucleus of the hypothalamus was investigated. According to the findings, cystic structure was observed in the ovaries of morphine-injected rats. A dose of 0.4 µg/rat of morphine was significantly more effective in the development of ovarian cysts than the control group. Additionally, the thickness of the cyst wall exhibited the characteristic pattern of PCO cysts. In rats treated with this dose of morphine, not only did the number of ovarian follicular cysts increase compared to the control group, but it also had a relative effect on the diameter of the ovary and the dimensions of the uterus. While in the ovaries of morphine-treated animals pre-injected with sulpiride, the number of cysts did not increase compared to the control. Sulpiride before morphine injection showed a somewhat protective effect on the reproductive accessory organ or VMH neuronal population compared to the control group. This antagonist, alone with no cystogenic effect, did not show an inflammatory effect on the reproductive organs and was also ineffective on the neuronal population of the nucleus.

Given that one of the common problems in society is infertility and its treatment, and since one of the causes of infertility is PCO, in this experiment, we first used morphine to induce this disorder. We then examined the mechanism of the disorder and studied the modulatory effect of DA as a neuronal substance.

Morphine injection causes a polycystic appearance in the ovaries. To explain this phenomenon, it can be stated that certain areas in the central nervous system (CNS) express mu and delta opioid receptors. Among these areas are the arcuate nucleus (ARC), the VMH, and the medial pre-optic area (MPOA) [11, 12]. Research has shown that these areas also play a role in the mating process [13]. Scientists have reported that, in humans and animals, opioids generally increase GH and prolactin and decrease LH [6]. Authors have also postulated that morphine injection into hypothalamic areas such as VMH inhibits the normal ovulation process in rats [14].

Considering that opioids bind to hypothalamic opioid receptors, causing a decrease in GnRH and LH production, we used morphine as a selective mu opioid receptor agonist. However, this study had limitations in serological analysis. This condition in women causes menstrual cycle disorders, reduced production of sex hormones, and infertility. Opioids reduce the negative feedback of sex steroids on pituitary LH secretion through hypothalamic GnRH [5]. This condition increases LH secretion [8]. In previous studies, the effect of peripheral injection of morphine on ovarian cystogenesis in Wistar rats was investigated [8], and it was observed that the ovaries of rats receiving morphine had follicular cysts. Given the findings on ovarian and uterine diameter, previous studies have shown that individuals with PCOS have higher T cell activity in follicular fluid compared to controls, suggesting a possible interaction between morphine and inflammatory factors in the ovary. Moreover, it was shown that morphine inhibits hepatic clearance and stimulates the biosynthesis of hepatic insulin-like substances, which causes hyperinsulinemia. Finally, stimulation of IGF-1 and IGF-2 receptors, as well as increased androgen production by the adrenal gland and ovary, ultimately leads to hyperandrogenism [15], which is also considered a factor in the development of PCOS [1]. It should be noted here that acute intracerebral administration of this low dose of morphine in the present study did not likely have these effects, as no statistically significant changes in the dimensional parameters were observed. Regarding the change in the target neuronal population, we did not find a significant effect in this study; however, this finding also likely

requires further investigation. Regarding the mechanism of sulpiride-morphine interaction, it can be argued that at this stage, the signaling pathways have not been investigated; however, most previous findings indicate the involvement of opioid receptors in triggering internal signaling that leads to the activation of NO [9]. Dopamine is a neurotransmitter involved in the control of reproductive function in the hypothalamus. In addition, DA receptors are present in both the pituitary and the ovary. At the hypothalamic level, DA has an inhibitory effect on GnRH secretion [16]. DA dysregulation in these areas may play a role in various ovulatory disorders, based on studies of DA, which is an inhibitor of the hormone prolactin. effect of sulpiride cannot be definitively attributed to the HPG axis and central DA inhibition (limitations of this study). To further explain, the ovaries of some animals contain mRNA for DA receptors [18, 19]. The presence of DA receptors in the ovary of rats suggests that DA may show a regulatory role in the growth of follicles [18]. This finding partially confirms the functional interaction between DA and morphine, which aligns with the results of the present study. Morphine also caused mild uterine inflammation in the animals compared to the saline-treated group, which was resolved by pre-administration of sulpiride. However, sulpiride alone had no inflammatory effect on the uterus. Furthermore, sulpiride did not show a significant effect on hypothalamic neurons. We suggest that this drug may affect the production of prostaglandins and cause ovarian inflammation; however, further studies are needed in the future. This study was limited by the inability to measure brain GnRH and circulating sex hormone levels, which prevented a discussion of feedback mechanisms. However, the strength of this study is in demonstrating DA as a key mediator in the reproductive axis.

Conclusions

The present work examined the role of DA D2 receptors in the disruption of the reproductive axis induced by morphine injection into the VMH. The findings indicated that sulpiride, a DA D2 receptor antagonist, can reduce the cystogenic effects of morphine, suggesting that dopaminergic pathways are involved in the cystogenic processes associated with morphine exposure.

Ethical Considerations

All ethical principles were observed in this research and were approved by Shahed University with number 962 on November 23, 2017.

Acknowledgments

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Authors' Contributions

M.K. proposed the research design. S.A. conducted the research. S.A. wrote the initial draft of the article. M.K. finalized the article. Both authors read and approved the final article.

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No specific grants were used in this research.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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