

RESEARCH ARTICLE

Protective effect of silver nano particles against ovarian polycystic induced by morphine in rat

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ABSTRACT

Objective: Morphine can cause harmful effects in the ovaries. But its effect on reproductive (hypothalamus-pituitary-gonad) axis is not clearly known. Today, silver nanoparticles are widely used in healthcare products because of antimicrobial properties. These particles can pass the blood-brain barrier and change in the nitric oxide level. We studied the effect of pre-injection of Ag-NPs prior to morphine (intra-VMH) in order to investigate the protective effect on the rat's polycystic ovary induced of morphine. The effects were also compared with those observed in the morphine addicted animal.

Materials and Methods: The rats (200-250 g) were housed under standard conditions and fed *ad libitum*. They were randomly divided into addicted to morphine (10-100 mg/kg, i.p., 7 days, twice daily) or receiving the drug (0.001 to 0.4 µg/rat, once, intra-VMH) (AP:-1.92), a week after stereotaxic surgery. Ag-NPs (0.01, 0.001 and 0.0001 µg/rat) were administered for once (intra-VMH) alone or prior to the morphine effective dose (0.4 µg/rat). Control group was given only saline. At the end, the animals' ovaries and/or brain both histopathologically and by NADPH-diaphorase were examined.

Results: All rats' ovaries treated morphine showed polycystic features compared with the saline group (p<0.05). The activation of the NO system in the ovaries was also evidenced by NADPH-diaphorase. However, with pre-injection of the Ag-NPs, (intra-VMH) to morphine, the number of cysts was reduced and the VMH neurons were protected.

Conclusion: The Ag-NPs may inhibit the morphine- induced PCO by decreasing the level of NO in the ovary through protection of hypothalamic neurons.

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INTRODUCTION

Morphine, an extremely potent opiate analgesic drug that acts by binding to µ-opioid receptors[1, 2], is used to treat of acute and chronic pain. However, the morphine abuse is associated with disadvantages and side effects. Long-term use of the opioid[3,4] is coupled with maleffects and toxicities, e.g. peripheral edema, immune suppression, hyperalgesia, sleep apnea, and changes

in endocrine function. Also, there are unfavorable effects on reproductive organs[5-7].

Morphine, the most important alkaloid of opium family which found as much as 10% in opium[6,8], can stimulate the release of nitric oxide (NO) in different tissues[9]. From the past[10] we know that it is an important paracrine messenger. It participates in numerous physiological and pathophysiological procedures in the endocrine

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system. This free-radical molecule is formed from the conversion of arginine to citrulline by the NO synthase enzyme (NOS)[10]. The molecule is well documented both as a restricted inflammatory generator and included with the factors involved in the ovulatory processes[11,12]. In our recent study[13], we induced the polycystic ovary by injecting morphine into the ventromedial hypothalamus of (VMH) rat. In addition, the slices of morphine-treated rat revealed a significant decrease in the VMH neurons. The Polycystic ovary syndrome (PCOS) is introduced as familiar endocrine disorder that affects approximately 10% of women during their reproductive ages[14]. But so far no solution is presented to prevent this complication. In order to investigate this, we focused on the protective effect of silver nanoparticles (Ag-NPs). The use of Ag-NPs in the industry is increasing, especially in the detergent business. But in use these particles on neural stem cells (in vitro) they showed neurotoxicity[15]. As a reason, when a large number of electrons from a metal nanoparticle are removed from equilibrium, different types of plasmon are produced[16]. This may lead to more permeability of nanoparticles through the cell membrane alive, which can have protective effects. So, we here work on the hypothesis that morphine addiction induces ovarian polycystic, and it also affects the hypothalamus-pituitary-gonad (HPG) axis after intra-VMH infusion. We examine the protection of ovary as well as brain to morphine by Ag-NPs.

MATERIALS AND METHODS

Animals

In this investigational study, the animals were adult female Wistar rats (weight: 200-250 g). Animals were kept under standard temperature ($21 \pm 3^\circ\text{C}$) and 12 hours light / dark cycle with food and water *ad libitum*. All experiments were conducted according to National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). We also considered the Guide for the Care and Use of Laboratory Animals[3] and the local Ethical Committee at Shahed University approved it (Approval Notifications 04/17/2014-2017).

Drugs

Morphine sulphate provided by TEMAD co., Tehran, Iran, after confirmation. Ketamine (100 mg) and xylazine (20 mg) were bought of Veterinary Organization of Iran. Nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotide phosphate

(NADPH) and violet cresyl were bought from Merck co, Germany. Hematoxylin and eosin were taken of Farzaneh Arman Co., Iran. Silver nano particles (Ag-NPs at sizes 50-60 nm) were provided at this local center with sonoelectrochemistry.

Female Cycle Test

Female rat as been proposed if it is not adjacent to or mated with male rat is always in diestrous[9,10]. The rats thus were kept as virgin to avoid the phase change. Also, the animals' vaginal samples were examined by vaginal washing between 11:00 am and 12:00 pm and they were screened during the experiment by means of Papanicolaou (PAP) stain. Three types of the epithelial cells, the round and nucleated; and the irregular unnucleated, and the cornified cells, in PAP stained smears were found: The little round cells, the leukocytes were illustrated too. In view of the proportion among them we determined the estrous cycle phases.

Drug administration

Animals (48 rats) were administered morphine progressively (10-100 mg/kg, intra-peritoneally) twice per day for 7 days[17] otherwise the rats were given only saline (control group: 1 mL/kg) during the experimental period. By the end of the treatment, the animals' ovaries were dissected under deep anesthesia by ketamine (100 mg) and xylazine (20 mg). The collected ovaries were studied histopathologically. They were further analyzed by the nicotinamide adenine dinucleotide phosphate (NADPH) - diaphorase reaction. In using Ag-NPs in 2nd set of animals (72 rats), a week after skull cannulation at anterior-posterior -1.92[18], they were injected morphine (0.001-0.4 $\mu\text{g}/\text{rat}$) and/or Ag-NPs (0.0001-0.01 $\mu\text{g}/\text{rat}$, intra- ventromedial hypothalamus (intra-VMH). The control was microinjected saline (1 $\mu\text{L}/\text{rat}$, intra-VMH). After treatments, the animals' ovaries and brain were collected in 10% formalin to provide evidence.

Surgery procedure

The treatment groups were deeply anesthetized by ketamine (100 mg) and xylazine (20 mg) used in the ratio of 5 to 2. Then, an incision was created in the lower abdominal part. The ovaries were dissected and examined biometrically. They were then collected in 10% formalin for histological examination. The brain samples after decapitation of rats under deep anesthesia were completely separated; they were finally cut at 3-4 μm after tissue processing.

Histological investigation

To investigate histologically, the ovaries were collected in the 10% formalin solution and they were processed with a tissue processor through paraffin embedding. Serial sections (3-4 μm) were then prepared with a rotary microtome. The ovary slides were stained in the end using the hematoxylin and eosin (H&E) staining method[11] and the brain samples were identified with violet cresyl. The mounted slides were evaluated with a light photomicroscope (Olympus, U.S.A) at 4X-100X.

NADPH-diaphorase reactivity

The enzyme's ((NOS) activation in the ovarian tissue was shown using the NADPH-diaphorase at the histochemical level. Rats were anesthetized 24 hours after the last injection of morphine addiction dose, the ovaries then were excised and trimmed from periovarian fat and bursae[10,19]. The tissue samples were thus cleared in xylene, hydrated in descending series of ethanol (96%, 95%, 70%, 50%) and rinsed with water. The staining was followed by keeping the slides (24 hours at 37°C) in a solution containing equal parts of nitro-blue tetrazolium (NBT, 0.2 mg/mL in phosphate buffer) and NADPH (1 mg/mL in phosphate buffer). Upon reduction by NADPH-diaphorase, NBT yields a blue formazan that is visible by light microscopy[10,20]. Control specimens were assessed using the same procedure except that the specimens were placed in an incubation bath devoid of the marker. No

reaction to the NADPH was observed in the control samples. The tissue samples were then dehydrated in ascending series of ethanol (50% 70% 95% 96%), and mounted in Entellan (Merck co., Germany) after being cleared xylene.

Statistical analysis

At first the data were tested by Kolmogorov - Smirnov (K - S) to determine a normal distribution. The statistical comparing was done by SPSS software (version 13.0; SPSS, Inc., Chicago, IL). T-test was used to show differences in average cyst number between groups (control and experimental) in addicted group. The analysis of variance (ANOVA) was applied in experiments with more than two groups. If ANOVA meant it was followed by Tukey's *post hoc*. Statistical significance was measured at $\alpha < 0.05$. All data are expressed as mean \pm SEM.

RESULTS

Female cycle

The sexual cycle phase of the female rat was indicated diestrous because there was a plethora of round nucleated epithelial cells in the PAP smears.

Histology

Finally, all animals' ovaries were dissected, they were then studied histopathologically. The control rats' ovaries taken from saline - treated group had mature follicles (Fig. 1A) compared with those collected from the morphine addicted group

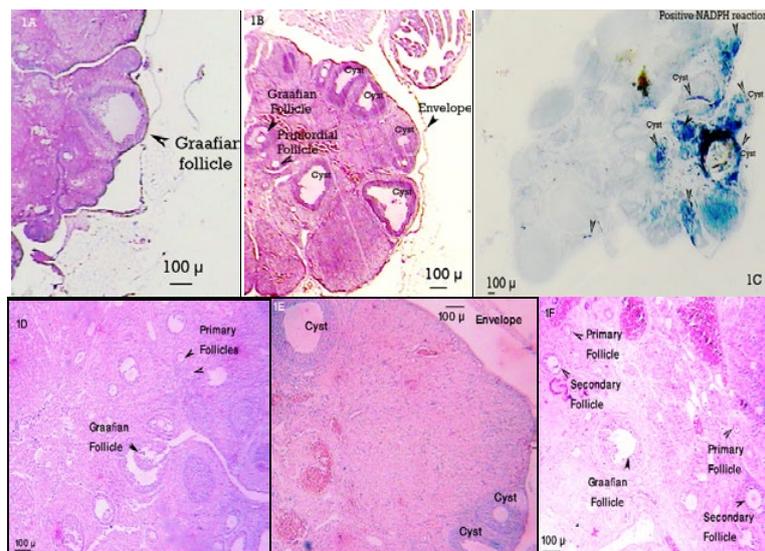


Fig. 1. Panels show the ovaries of different groups of rats: control (1A), morphine - addicted (10 - 100 mg/kg) - (1B), morphine addicted (10 - 100 mg/kg) which was given NADPH-diaphorase test (1C), saline microinjected into the VMH (1D), morphine microinjected into the VMH (1E), Ag-NPs microinjected into the VMH prior to (along with) morphine (1F). The Bars on the pictures show the μm values. The arrows and notes also signify the desired characteristics.

(receiving morphine 10-100 mg/kg, i.p., twice per day during the 7- day period). They exhibited large cysts with thickened granulosa cell layer or large cystic follicles with scant granulosa cells (Fig. 1B). The morphine samples passed the NADPH-diaphorase illustrated the significant ($p < 0.05$) activation response for NO (Fig. 1C). The cyst's wall exhibited the blue reaction to the NADPH-diaphorase in contrast to the solely saline group. The above results were also quantified and analyses as significant ($p < 0.05$).

The ovaries which gathered from rats that were treated morphine intra-VMH also showed polycystic characteristics when compared with the control saline (1 μ L/ rat, intra-VMH) samples. However, in the cumulative injection with Ag-NPs, the number of ovarian cysts decreased to control sample size (Fig. 1D-F).

Cyst

The cysts no in all ovaries samples were counted. After analysis, it was shown a significant difference

between the control (saline treated) and morphine administered group ($p < 0.05$). But, in Ag-NPs pre-injection sample, the number of cyst decreased significantly (Fig. 2a-2c).

Neurons of VMH

The VMH's neurons in morphine microinjected samples were additionally studied. Single Ag-NPs (0.0001-0.01 μ g/rat, intra-VMH) samples showed no significant change compared with the control (saline group). Conversely, more damaged neurons were observed in morphine receiving samples than control. This feature was shown regarding the Ag-NPs sample too($p < 0.05$) (Fig. 3A-3C).

DISCUSSION

Regarding that the use of opioid substances is addictive, non-clinical consumption of these drugs is called abuse. In this study, we investigated the effect of morphine on addicted rat ovaries. We also examined the morphine-receiving (intra-VMH) animal brain to investigate the protective role of

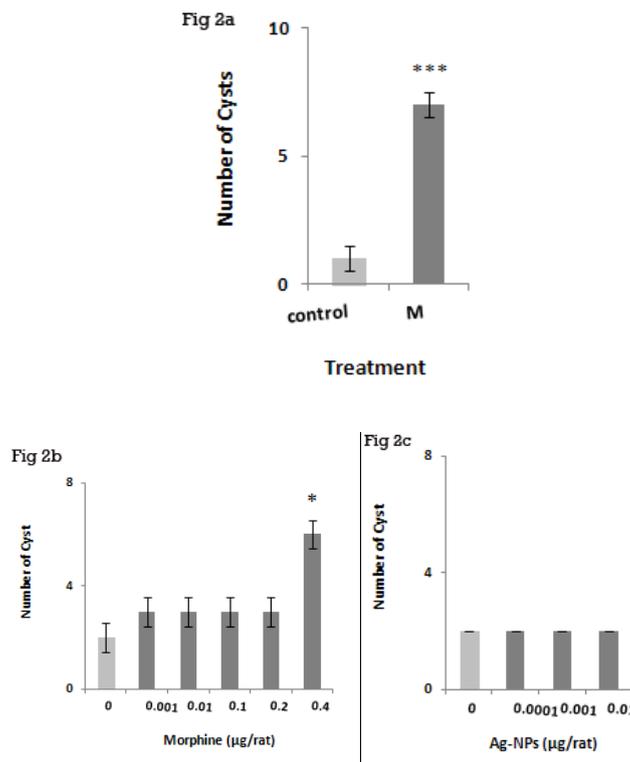


Fig. 2. This Figure illustrates the numbers of cysts both in the control and experimental rats. Fig 2a: The control group was injected saline (1 mL/kg, i.p., 7 days / twice per day). The experimental group (M: morphine addicted) was administered morphine (10-100 mg/kg, i.p., 7 days / twice daily). Fig 2b: rats were single injected morphine intra-VMH (0.001-0.4 μ g/rat, intra-VMH). Fig 2c: the animals received Ag-NPs (0.0001-0.01 μ g/rat, intra-VMH) along with morphine effective dose (0.4 μ g/rat, intra-VMH). Values are indicated as mean \pm SEM.

* $p < 0.05$ and *** $p < 0.01$ according to Tukey's *post hoc* indicate the difference to control (0 group).

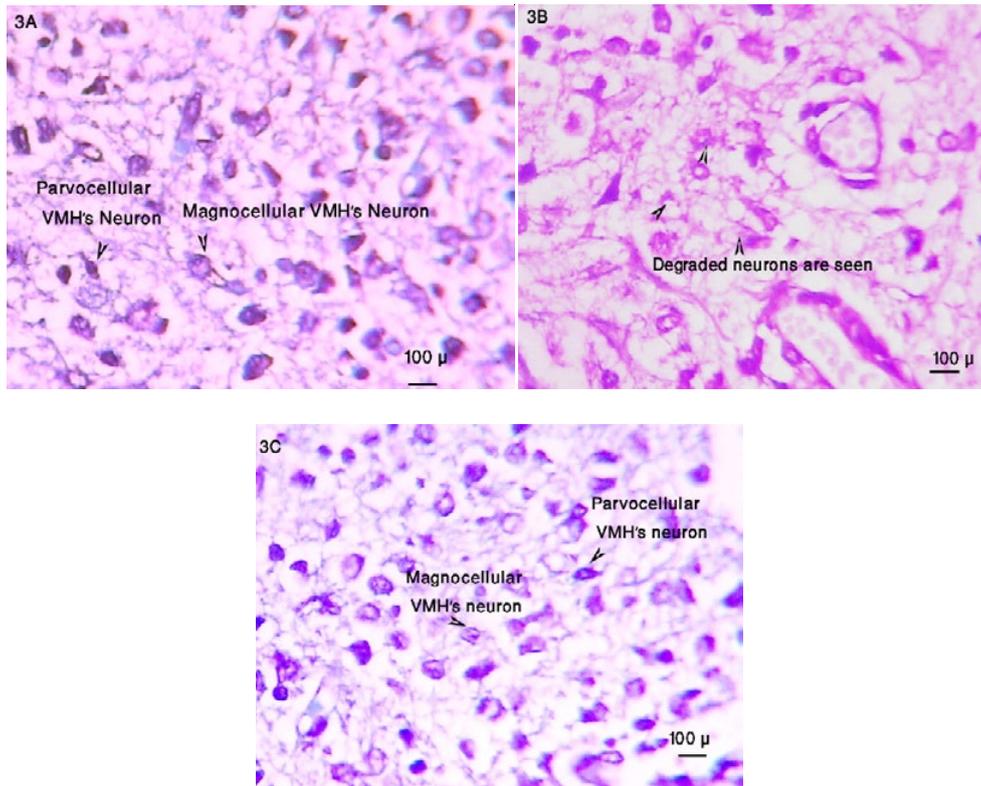


Fig. 3. This figure indicate the VMH's neurons both in the control saline microinjected (intra-VMH: 3A) and morphine injected groups (3B), and Ag-NPs injected group (3C). Bars beside the samples indicate the values. The arrows and notes further show the preferred characteristics.

Ag-NPs. Based our finding the ovarian aspect of the morphine addicted rats is similar to ovarian polycystic (PCO). We also showed the role of NO system in inducing this effect by the NADPH-diaphorase reaction.

Evidence indicates the adverse effects of opioid materials on reproductive organs[6]. The drug morphine may disrupt ovarian cycle. Also, long-term use of morphine reduces reproductive activity through noradrenergic mechanisms in the hypothalamus[21].The opioids can attenuate the secretion of sex hormone that increases the likelihood of irregular menstruation as well as infertility [22]. There is other publication which mentions the cellular consequences of the drugs of like nuclear membrane convolution of endometrial epithelial cells[6]. Authors have further demonstrated that morphine can stimulate the NO release in dissimilar endometrial glandular epithelial tissue cells[9,23,24]. The free radical NO is the major inflammatory mediator [25]. This molecule is formed with metabolic processes by the action of NOS [10]. Although its function

during menstruation is almost unclear, however, there is some information about the endometrial NOS expression and NO production during the menstrual cycle that strongly confirm its role in these trends[25]. The NO is commonly categorized as a major paracrine mediator of ovulation, and it has also a regulatory effect in the formation of corpus luteum[26,27]. This laboratory has already[28,29] involved this molecule as the pro-inflammatory element in the PCOS. Morphine abuses as this research indicates, furthermore, caused histological changes (cyst genesis) through the pro-inflammatory NO activation at the follicular walls of ovarian cysts (evidenced by NADPH-diaphorase). This finding is consistent with our previous results, although there was no morphine-addicted animal model[28,29].

Referring to other present finding we may show the unpleasant effect of opioids on the entire HPG axis parts accordingly with the previous report[24] suggesting that opioids can inhibit the functioning of the entire HPG axis by interaction with opioid receptors in the hypothalamus. Opioids also

directly bind and inhibit other members of the axis. For example, opioids bind μ -opioid type receptors in the pituitary gland, limiting the production of LH in women, thereby interfering with the menstrual cycle[30]. The aspects of PCOS may vary among individual women as hyperandrogenism, inappropriate gonadotropin secretion, and chronic anovulation are the characteristics of this disorder[31]. It is worth noting that having more than 3 follicular cysts in one or both ovaries is the most common feature. This symptom was induced in all treated animal specimens in the present research, whether addicted or morphine microinjected into the nucleus (VMH). But the important point is the effect of the Ag-NPs on this feature. Because, the polycystic ovarian did not view in the samples received Ag-NPs in the brain, while simultaneously receiving morphine intra- nucleus. Recalling that this work's goal was to show that relatively low concentration of Ag-NPs interferes with the undesirable effects of morphine on reproductive system, it seems that we achieved this, but we still do not know the underlying mechanism properly. Our finding is in contrast with the published data emphasizing the hazardous effect of the Ag-NPs on neuronal system[15]. It indicates in contrast that Ag-NPs have little poisoning effect at least at these low concentrations. The other opinion is that these particles may be converted into more motile forms of materials which are more diffusible through neuronal cell membrane and created an electrophysiologically evoked medium and/or metabolites. This was previously called plasmon formation[16]. More work on this topic will definitely lead us to more accurate results.

Basically, PCOS, a long lasting features, begins generally in adolescence with oligomenorrhea / amenorrhea and goes to problems e.g. infertility and metabolic complications and even cancer in the distant future[32,33]. But as present achievement shows the Ag-NPs may inhibit the morphine - induced polycystic by inhibiting the high NO expression in the end organ (ovary) through neuronal protection of hypothalamic axis. There are also suggestions for future study including i.p. administration of the Ag-NPs along with the morphine that can be pursued with seriousness and use of drugs coated with these nanomaterials intra-VMH as some researchers have done[34,35]. Briefly based on present results, we may finish the explanation that the drug abuse or even single use of morphine induces the female gland polycystic

probably by activation of pro-inflammation NO pathways and Ag-NPs can be candidate for improving these harmful effects.

CONCLUSION

The Ag-NPs may inhibit the morphine - induced PCO by decreasing the NO expression in the ovary through neuronal protection of the hypothalamic neurons.

CONFLICT OF INTEREST

There is no conflict of interest between authors.

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ABBREVIATIONS

Analysis of variance (ANOVA)
Hematoxylin and eosin (H&E)
Intra-peritoneally (i.p)
Kolmogorov - Smirnov (K - S)
Nicotinamide adenine dinucleotide phosphate (NADPH)
Nitric oxide (NO)
Nitroblue tetrazolium (NBT)
NO synthase enzyme (NOS)
Mean of standard error (SEM)
Papanicolaou (PAP)
Polycystic ovary syndrome (PCOS)
Silver nanoparticles (Ag-NPs)
Intra- ventromedial hypothalamus (intra-VMH)

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