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Naloxone affects reproductive system in a rat model with polycystic features

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ABSTRACT

Objective: To make interaction between morphine and naloxone in the rat model of PCOS, we evidenced the opioid receptors involvement in this efficacy. **Methods:** A total of 48 female animals (Wistar rats weighing 200-250 g) were kept diestrous before experimental procedure began. They were grouped in single L-arginine (50 mg/kg, *i.p.*, once a day for 9 days), naloxone (0.4 mg/kg, *i.p.*, once daily for 9 days), morphine (5 mg/kg, *i.p.*, once a day for 9 days), and naloxone (0.4 mg/kg) pre-treated to L-arginine (50 mg/kg), morphine (5 mg/kg) pre-treated to L-arginine, morphine pre-treated to the collective naloxone-L-arginine. Control group solely received saline (1 mL/kg, once daily for 9 days). At the end of the treatment period all animals were surgically studied. The rats' ovaries and uteri were examined both biometrically and pathologically. **Results:** The ovaries of rats treated with L-arginine showed polycystic characteristics and their uteri illustrated inflammation changes to the controls. The samples obtained from rats pre-treated with naloxone revealed a decrease in sign of inflammation compaired with L-arginine received speciments, the signs got worse in the presence of the morphine. **Conclusion:** Aspect of rat reproductive system may be linked with the cystic characteristic of ovary. This study involves opioid receptors in the naloxone efficacy on reproductive agents of rat with polycystic aspect.

1. Introduction

Abuse of drug by women creates health risk to reproductive system and fetus. Opioid drugs are known with an increased risk of low weight infants at birth, preterm delivery, intrauterine growth restriction (IUGR), neonatal abstinence syndrome (NAS) and sudden infant death syndrome (SIDS)[1]. Endometrial alterations in laboratory animals are associated to histological and cytological lesions induced by morphine administration. These abnormalities are discussed as uterine factor infertility leading to implantation failure[2].

The naloxone plays role in dropping of cyst development in rat experimental model of polycystic ovary syndrome (PCOS)[3]. The anti-inflammatory morphine antagonist, naloxone, reduces cystic appearance in rat with polycystic ovaries likely by antagonizing the actions of endogenous opioids through blocking of peripheral opioid receptors.

The PCOS which is typified by polycystic features of ovaries along with the hyperandrogenism and ovulatory dysfunction [4,5] can be demonstrated with a higher exhibition of pro-inflammatory agents such as nitric oxide (NO)[3,6]. Chronic administration of L-arginine, a precursor of NO, induces the disorder (PCOS) in Wistar rats [3], a model seems as not appropriate for PCOS because no evidence of high androgen levels, insulin levels, and luteinizing hormone (LH) levels have been shown. However, it had a high level of low-density lipoprotein (LDL) showing the involvement of inflammatory factors through affecting the endocrine and metabolic events. They are properly relevant

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to long-term altered steroid hormone production and cardiovascular / glucose levels associated with PCOS.

To bring the role of inflammation and oxidative stress in this disorder ought to be mentioned that women with PCOS have evidence of oxidative stress[7] and increased follicular reactive oxygen species (ROS)[8].

Since little has been researched on the naloxone efficacy on rat reproductive system we aimed to make interaction between morphine and naloxone in the rat model of PCOS. The Wistar rats were treated chronically L-arginine. Also, the experimental animals' uteri were exactly examined to provide valuable data representing an opioid receptor as well as the NO involvement in pathophysiology of the disorder.

2. Materials and methods

2.1. Animals

In this experimental study, Wistar rats (body weight 200-250 g) purchased from Pasteur Institute of Iran were colonized in breeding room under standard conditions (21 ± 3 ℃ and 12-h light/dark cycle) with food and water *ad libitum*. The female pups after being weaned were kept as virgin in a separate animal room before experimental procedure began. We know that the female rats with 4-5 day sexual cycle are always in diestrous[7,8] unless in contacting with the male rats. Each animal was used only once. All experiments were approved in accordance to the Helsinki's animal welfare by local Ethical committee at the University (document No: 357, Oct 2013).

2.2. Drugs

We used L-arginine (Merck Co., Germany), naloxone hydrochloride (Tolid Daru Co., Iran), Morphine sulfate (TEMAD Co., Iran), ketamine & xylazine (Veterinary Organization, Iran). All drugs were injected intraperitoneally (*i.p.*). The vehicle (saline at 1 mL/kg, *i.p.*) was administered in control group.

2.3. Female cycle test

The female rats were in diestrous (virgin) phase throughout the drug taking procedure as has been mentioned elsewhere[3]. The animals' vaginal smears were examined by Papanicolaou smear test during the research to verify the female cycle phase of the experimental animals.

2.4. Drug administrations

Animals were randomly divided into the single L-arginine (50 mg/kg, *i.p.*, once a day for 9 days), naloxone (0.4 mg/kg, *i.p.*, once daily for 9 days), morphine (5 mg/kg, *i.p.*, once a day for 9 days), and naloxone (0.4 mg/kg) pre-treated to L-arginine (50 mg/kg), morphine (5 mg/kg) pre-treated to L-arginine, morphine pre-treated to the collective naloxone-L-arginine. Control group only received saline (1 mL/kg, once a day for 9 days). Each group (n=6) was intra-peritoneally (i.p.) injected the drug or saline only once daily during the study period.

2.5. Surgery procedure

By the end of the treatment, the rats were anesthetized. Then midline incisions in the rats' lower abdomen areas were performed. The ovaries and uteri were examined and dissected out. They were collected in 10% formalin for histological examination.

2.6. Histological investigation

The collected tissues were processed (3-5 μm) and stained by hematoxylin and eosin (H&E)[9]. The thin sections were then dehydrated, and clearated, and eventually were mounted with entellan (Merck Co., Germany). The permanent slides were evaluated with light videophotomicroscope (Olympus) at 4-40×. The records were assessed in areas of 100-μm^2 with aid of Image Tool program (UTHSCSA, version 2.03, USA), the free image processing and analysis program for Microsoft Windows.

2.7. Statistical analysis

We evaluated the biometrical data by Kolmogorov-Smirnov (K-S) to show normality for analysis by variance (ANOVA). The ANOVA was performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL) followed by Tukey's Post hoc test to clear the between groups differences. Statistical significance was considered at $P< 0.05$. All data are expressed as mean ± SEM. The photos were examined in 100-μm^2 units by using the Image Tool program.

3. Results

3.1. Histology

The ovaries obtained from the L-arginine treated group (50 mg/kg, chronically) showed cystic formations (Figure 1B) compared with the control samples (Figure 1A). The samples of the group pretreated naloxone evidenced a significant decrease in numbers of cysts (Figure 1C). This characteristic, however, got worse when the morphine was injected prior to the naloxone (Figure 1D). The findings were quantified ($F_{3,20}$ = 48.571, P<0.0001) to improve the achievement.

The uteri samples obtained of rats received chronically L-arginine (50 mg/kg) (Figure 1F) presented the inflammation to those belonged to the controls (Figure 1E). The uteri wall revealed the aspects of distension, proliferation and angiogenesis. These characteristics were absent in the samples with naloxone pretreatment (Figure 1G) but not in the presence of morphine (Figure 1H).

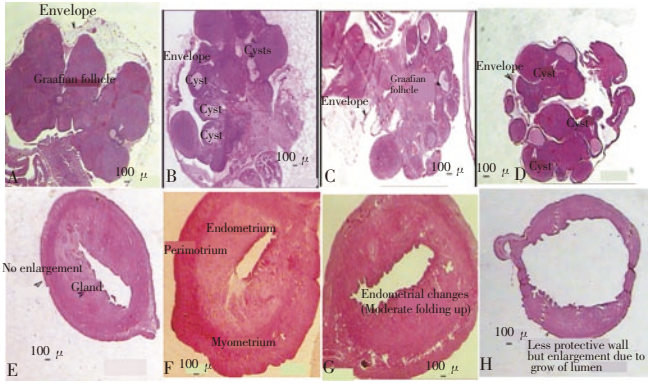

Figure 1. Pictures of ovaries and Uteri from control (1A, 1E), and L-arginine treated (1B, 1F) rats.

Panels show the ovaries and uteri of two other groups of rats: naloxone pretreated to L-arginine (1C, 1G), and morphine pretreated to naloxone plus L-arginine (1D, 1H). The figure 1B illustrates the polycystic aspect of the rat ovary treated L-arginine compared to the control 1A. The feature was repaired by naloxone pre-treatment as seen in Figure 1C (the graafian follicles were appeared). But, the morphine pre-injection reversed the effect and the cysts were grown (1D). The Figure 1F, furthermore, shows the inflammation of the rat uterus compared with the 1E as well as 1G. A swelling in the rat uterus (Figure 1H) is seen due to morphine pre-injection. Bars beside the samples show the μm values. The arrows also indicate the desired characteristics.

3.2. Biometrical measurement (diameters of uteri)

The uteri diameters were calculated in all groups. They showed increased width in L-arginine treated group to those obtained from saline group. The uteri of rats treated L-arginine provided statistical significant value when compared with the control group (P<0.05) (Figure 2).

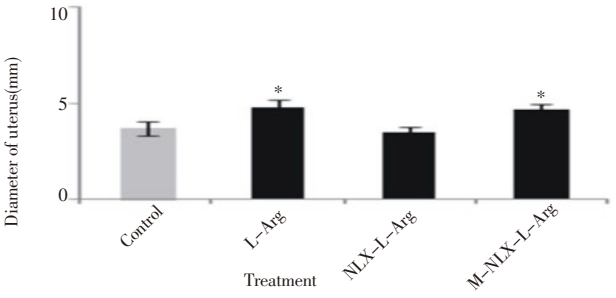

Figure 2. Figure indicates the diameters of uteri in rats.

X axis denotes the control and experimental groups (n=6). Control was injected solely saline (1 mL/kg, *i.p.*, once a day for 9 days). The experimental rats received only L-arginine (50 mg/kg, *i.p.*, once a day for 9 days) or naloxone (NLX) (0.4 mg/kg, once a day for 9 days) prior to L-arginine (50 mg/kg, *i.p.*, once daily for 9 days) or morphine (M) (5 mg/kg, *i.p.*, for 9 days/daily once) prior to the cumulative naloxone –L-arginine.

Values are mean ± SEM. *P<0.05 vs. control (based on Post hoc test).

3.3. Female cycle evaluation

The animals' vaginal smears were assessed by Papanicolaou smear test and determined as diestrous (Figure 3). The large quantities of the leukocytes were seen both in the controls' and the treated rats' vaginal smear.

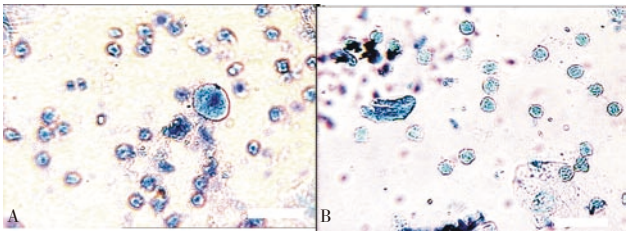

Figure 3. Vaginal smear Papanicolaou (PAP) stain of female virgin control rats (A) and those treated L-arginine for 9 days (B) (detailed in Materials and Methods).

The great quantities of leukocytes in the smears samples signify that the cycle phases are diestrous.

4. Discussion

We aimed to make interaction between morphine and naloxone in the rat model of polycystic ovary syndrome

(PCOS). Based on our data, the treatment of rats chronically with a nitric oxide (NO) precursor, L-arginine, induces the polycystic formation of ovary as well as the uteri inflammation. An opioid receptor mechanism affects the rat reproductive system with polycystic ovary.

We know that the pro-inflammatory NO participates in endocrine physiological and pathophysiological events [10] including PCOS. However, circulating NO levels are unchanged or low in women with PCOS, resulting in impaired cutaneous vasodilation compared with similarly obese controls[11].

To discuss why increased NO induced follicle arrest, we may refer to evidence of the detrimental effect of NO on follicle development[12]. Likewise, it should be argued that dietary arginine supplementation during early pregnancy enhances embryonic survival in rats[13].

It is worthy to bring in mind that the NO is metabolically produced by the activation of enzyme nitric oxide synthase (NOS)[10]. The molecule NO is well introduced as a local inflammatory generator[14] and the present work provides support for the NO role in the ovarian and uterine inflammatory events. It highlights that the hyperactivity of enzyme NOS due to constant usage of the L-arginine induces polycystic formation in treated rats' ovaries. In accord, the presence of large cysts due to treatment by NO producer, L-arginine, has been deal with common characteristics of PCOS[15].

The uterus of the L-arginine treated rats beside of significant change in feature of ovary showed swelling compared with the saline control group suggesting a pro-inflammatory role for the NO in uteri as well as ovaries. We may propose a metabolic pathway to involve the short-lived cytotoxic mediator NO[16] in the events. An increased uteri's diameter of the L-arginine-treated rats may also participate the local inflammatory processes in reproduction at all levels from the follicles to the uterus.

The NO function in activation of NOergic neurons of the pelvic plexus has been shown previously[17]. This metabolic agent has also been involved in the control of uterine smooth muscle via NOergic terminals[18].

We further offer an opioid system involvement in the ovary and uterus inflammatory processes. Because, the naloxone pre-treatment improved the inflammation changes of the rats' uteri treated by L-arginine. This advantage, however, got worse in the presence of the morphine. Additionally, we did not observe a blocking consequence due to naloxone when co-administered with morphine. This result though evidences an opioid receptor- mediated mechanism, but, the exact manner remains to be explored in future. The authors may propose a hypothalamic morphine-opioid receptor signaling pathway.

To interpret the naloxone efficacy we may notify that the antagonist is locally blocking the opioid receptors to prevent PCO. Therefore, we may consider the opioids as the pro-inflammatory substances. They can increase oxidative stress in tissues[19]. Even though how L-arginine induces or promotes peripheral opioid secretion remain elusive.

By considering of the aspect of rat reproductive system this study may clearly involve the opioid receptors in the naloxone efficacy on reproductive agents of rat with polycystic feature.

In summary, this study identifies the pathological evidence in reproductive system of Wistar rats. In the current study both the NO and the opioid receptor involvement in pathophysiology of the PCOS is clear. Morphine alone induces the cystic characteristic as shown; the opioid drug when giving together with naloxone lessens the antagonist efficacy independently to the dose effect. This finding also provides that the naloxone in interaction with opioid receptor causes effectiveness on rat reproductive system with polycystic feature.

We may conclude that the activation of the L-arginine-related metabolic pathway may cause the polycystic ovary as well as the swelling of uterus, the aspects assisted by the anti-inflammatory naloxone involvement.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Mawhinney S, Ashe RG, Lowry J. Substance abuse in pregnancy: opioid substitution in a Northern Ireland maternity unit. *Ulster Med J* 2006; **75**(3): 187.

[2] Dehghan M, Jafarpour M, Mahmoudian A. The effect of morphine administration on structure and ultrastructure of uterus in pregnant mice. *Iran J Reprod Med* 2010; **8**(3): 111-118.

[3] Hassani F, Karami M, Jalali Nadoushan MR, Eftekhari Yazdi P. Nitric oxide-induced polycystic ovaries in the Wistar rat. *Int J Fertil Steril* 2012; **6**(2): 111-116.

[4] Amowitz LL, Sobel BE. Cardiovascular consequences of polycystic ovary syndrome. *Endocrinol Metab Clin North Am* 1999; **28**: 439-458.

[5] Dokras A. Cardiovascular disease risk factors in polycystic ovary syndrome. *Semin Reprod Med* 2008; **26**: 39-44.

[6] Apridonidze T, Essah PA, Iuorno MJ, Nestler JE. Prevalence and characteristics of the metabolic syndrome in women with polycystic ovary syndrome. *J Clin Endocrinol Metab* 2005; **90**(4): 1929-1935.

[7] Kocer D, Bayram F, Diri H. The effects of metformin on endothelial dysfunction, lipid metabolism and oxidative stress in women with polycystic ovary syndrome. *Gynecol Endocrinol* 2014; **30**(5): 367-371.

[8] Saller S, Kunz L, Berg D, Berg U, Lara H, Urra J, et al. Dopamine in human follicular fluid is associated with cellular uptake and metabolism-dependent generation of reactive oxygen species in granulosa cells: implications for physiology and pathology. *Hum Reprod* 2014; **29**(3): 555-567.

[9] Manneras L, Cajander S, Holmang A, Seleskovic Z, Lystig T, Lonn M, et al. A new rat model exhibiting both ovarian and metabolic characteristics of polycystic ovary syndrome. *Endocrinology* 2007; **148**(8): 3781-3791.

[10]Zackrisson U, Mikuni M, Wallin A, Delbro D, Hedin L, Brännström M. Cell-specific localization of nitric oxide synthases (NOS) in the rat ovary during follicular development, ovulation and luteal formation. *Hum Reprod* 1996; **11**(12): 2667-2673.

[11]Murri M, Luque-Ramírez M, Insenser M, Ojeda-Ojeda M, Escobar-Morreale HF. Circulating markers of oxidative stress and polycystic ovary syndrome (PCOS): a systematic review and meta-analysis. *Hum Reprod Update* 2013; **19**(3): 268-288.

[12]Battaglia C, Regnani G, Mancini F, Iughetti L, Flamigni C, Venturoli S. Polycystic ovaries in childhood: a common finding in daughters of PCOS patients. A pilot study. *Hum Reprod* 2002; 17(3): 771-776.

[13]Zeng X, Wang F, Fan X, Yang W, Zhou B, Li P, et al. Dietary arginine supplementation during early pregnancy enhances embryonic survival in rats. *J Nutr* 2008; **138**(8): 1421-1425.

[14]Nakamura Y, Kashida S, Nakata M, Takiguchi S, Yamagata Y, Takayama H. Changes in nitric oxide synthase activity in the ovary of gonadotropin treated rats: The role of nitric oxide during ovulation. *Endocrinol J* 1999; **46**(4): 529-538.

[15]Legro RS. Polycystic ovary syndrome: the new millenium. *Mol Cell Endocrinol* 2001; **184**: 87-93.

[16]Drapier JC, Wietzerbin J, Hibbs JB Jr. Interferon-γ and tumor necrosis factor induce the l-arginine-dependent cytotoxic effector mechanism in murine macrophages. *Eur J Immunol* 1988; **18**: 1587-1592.

[17]Burnett AL, Lowenstein CJ, Bredt DS, Chang TSK, Snyder SH. NO: A physiologic mediator of penile erection. *Science* 1992; **257**: 401-403.

[18]Brännström M, Janson PO. The role of leukocytes and cytokines as paracrine regulators in the mechanisms of ovulation. In: Fujimoto S, Hsueh AJW, Strauss IH. (eds.) *Frontiers in Endocnnology*. Vol 13. New York: Ares-Serono Symposium Pub; 1995, p. 225-233.

[19]Skrabalova J, Drastichova Z, Novotny J. Morphine as a potential oxidative stress-causing agent. *Mini Rev Org Chem* 2013; **10**(4): 367-372.