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Rat's Polycystic Ovary Due to Intraventromedial Hypothalamus Morphine Injection

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Abstract

Background and Objective: The most important alkaloid of opium family, morphine, may show unfavorable effect on the reproductive organs. This research investigated the effect of microinjection of morphine into the rat's ventromedial hypothalamus (VMH) on cystic genesis in the ovary and the health of neurons of VMH. **Materials and Methods:** Female rats (Wistar, weighing 200-250 g) kept under standard conditions were cannulated under anesthesia by Stoelting stereotaxic instrument at the coordinates anterior-posterior: −1.92, ventral: 9, lateral: 0.5. After being recovered, they were microinjected single morphine (0.1-0.4 μg/rat, once intra-VMH) and/or naloxone hydrochloride (0.1-0.4 μg/rat, once intra-VMH) using a 5-μL Hamilton syringe with the polyethylene tubing. The control group solely given physiological saline (1 μL/rat, intra-VMH). Three days after the experiment, both ovary and brain samples were collected from the control and the experimental groups, and they were studied histopathologically. The brain samples were checked out with the aid of the cresyl violet, and the ovaries were stained by the hematoxylin and eosin. The samples were also biometrically examined to compare the ovaries' cystic formations. Also, the number of healthy or injured neurons in the nuclei was compared. **Results:** The ovaries of morphine-treated rats showed polycystic characteristics in comparison with the control samples. In addition, the brain slices of the morphine-treated rats illustrated a significant decrease in intact neurons. Both mal effects were resolved in the presence of naloxone. **Conclusion:** These results may show that the morphine induces anovulatory infertility probably by hypothalamus–pituitary–ovary axis dysfunction.

Keywords

polycystic ovary, morphine, naloxone, ventromedial hypothalamus, rat

Introduction

Morphine, a main psychoactive alkaloid compound found naturally in the opium poppy plant *Papaver somniferum* L, is used clinically for the treatment of acute and chronic pain.[1,2] However, the use of the alkaloid along with medical benefits is associated with abuse and addiction and side effects. There is problem with long-term use of morphine[3] and it is associated with several adverse effects and toxicities, such as peripheral edema, immune suppression, hyperalgesia, sleep apnea, unfavorable effects on reproductive organs, and changes in endocrine function, many of which are not fully appreciated.[4,5]

Morphine, found as much as 10% in opium,[5] can stimulate the release of nitric oxide (NO) in different tissues.[6] This is known as a paracrine messenger involving in the central and peripheral physiological and pathophysiological events.[7] This free radical is produced from the oxidation of terminal guanidino nitrogen of arginine by the action of NO synthase (NOS) enzyme.[7] The NO is known as a local inflammatory element that is responsible for the ovulatory processes.[8] It has also been involved in the polycystic ovary syndrome (PCOS).[9] The PCOS is an endocrine disorder that affects up to 10% of women during their reproductive ages.[10] Furthermore, the opioid morphine is associated with the inflammatory indication of PCOS as reported in previous finding.[11]

According to the previous documents, pro-opiomelanocortin (POMC) neurons in the ventromedial arcuate nucleus project to the medial preoptic area (POA) from which some of these neurons emit to luteinizing hormone (LH)-releasing hormone neurons.[12] In view of the above-mentioned subject use, an animal PCOS model depends on the intactness of a POMC connection between the POA and the arcuate nucleus to maintain normal positive feedback in the animal. Thus, while the fact is considered, we evaluate that the morphine

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microinjection intra-ventromedial hypothalamus (VMH) induces the polycystic ovary in Wistar rats.

Materials and Methods

Animals

The subjects were 8-week-old female Wistar rats (weight: 200-250 g). The rats were kept under standard conditions in accordance with the Guide for the Care and Use of Laboratory Animals.[3] The animals were maintained in a standard temperature (21°C ± 3°C) and 12-hour light/dark cycle with food and water ad libitum. All experiments were approved by the local ethical committee at Shahed University.

Drugs

Morphine sulfate was bought from Temad Co (Tehran, Iran), after approval from the Food and Drug Administration of Ministry of Health of Iran. Naloxone hydrochloride was obtained from Tolid Daru Co (Tehran, Iran). Ketamine and xylazine were provided by the Veterinary Organization of Iran. Cresyl violet, and hematoxylin, and eosin were purchased from Merck Co (Darmstadt, Germany).

Female Cycle Test

The female Wistar rats were kept as virgin with no nearby to or mated with male rats. This process will keep them always in diestrus.[6,7] Also, the animals' vaginal smears obtained by vaginal washing between 11:00 AM and 12:00 PM were examined during the experiment by means of Papanicolaou (PAP) stain. Three types of the epithelial cells, the round and nucleated, the irregular unnucleated, and the cornified cells, in PAP-stained smears were found. The little round cells, the leukocytes, were also illustrated. In view of the proportion among them, we could determine the estrous cycle phases

Drug Administration

Once after recovery, animals received morphine (0.1-0.4 μg/rat, intra-VMH) subsequently to the skull cannulation by the coordinates in accordance with the rat's skull atlas.[13] The control group was given only saline (1 μL/rat, intra-VMH) during the experimental period. To survey on the opioid receptor involvement, the naloxone hydrochloride (0.4 μg/rat, intra-VMH) was pre-microinjected to the morphine dose (0.4 μg/rat, intra-VMH). By the end of the treatment, the animals' ovaries as well as brains were dissected under anesthesia and studied histopathologically. They were further analyzed biometrically.

Surgery Procedure

The treatment groups were anesthetized by ketamine (100 mg/kg) and xylazine (20 mg/kg, intraperitoneal injection). Then, a midline incision in the lower abdomen area was performed. The ovaries were biometrically examined and dissected out. They were collected in 10% formalin for histological examination. The brain samples were also saved after decapitation of the animals in 10% formalin for at least 72 hours before processing.

Histological Investigation

For histological investigations, both ovaries and brains were fixed in a 10% formalin solution and processed with a tissue processor through paraffin embedding. Serial sections (3 μm) were prepared using a rotary microtome. The ovary slides were then stained using the hematoxylin and eosin staining method[8] and cleared with xylene. After mounting, the slides were evaluated with a light photo-video-microscope and photo-video-binocular (Olympus, Tokyo, Japan) at the desired magnification. The brain slices were studied by cresyl violet (0.1%) and passed clearation with xylene and mounting. The slices were then examined with the aid of a light photo-video-microscope and a photo-video-binocular (Olympus) at the desired magnification.

Statistical Analysis

The data were initially evaluated by Kolmogorov-Smirnov to determine a normal distribution. The analysis of variance was performed using SPSS software (version 13.0; SPSS, Inc, Chicago, Illinois). Tukey-Kramer post hoc test was used to show differences between groups. Statistical significance was considered at $P < .05$. All data are expressed as mean ± standard error of the mean (SEM).

Results

Female Cycle

The rats' female cycle phases were indicated diestrus since the round nucleated epithelial cells were abundant in the Papanicolaou smears.

Histology

By the end of the experiments, all animals' ovaries as well as brains were dissected and studied histopathologically. The control rats' ovaries (Figure 1A) taken of the saline-treated group had mature follicles. But, in contrast to the control, those collected from the morphine-injected group (0.1-0.4 μg/rat, intra-VMH, for once) had large cysts with thickened granulosa cell layer or large cystic follicles with scant granulosa cells (Figure 1B). The morphine effective dose (0.4 μg/rat) that was preinjected with naloxone (0.4 μg/rat) illustrated the significant ($P < .05$) decrease in the cyst formation (Figure 1C), though the single naloxone sample (0.1-0.4 μg/rat, intra-VMH, for once) exhibited the mature follicles (Figure 1D).

Taken as whole, the morphine-treated rats' ovaries showed polycystic characteristics when compared with the control samples unless they were pre-microinjected by naloxone.

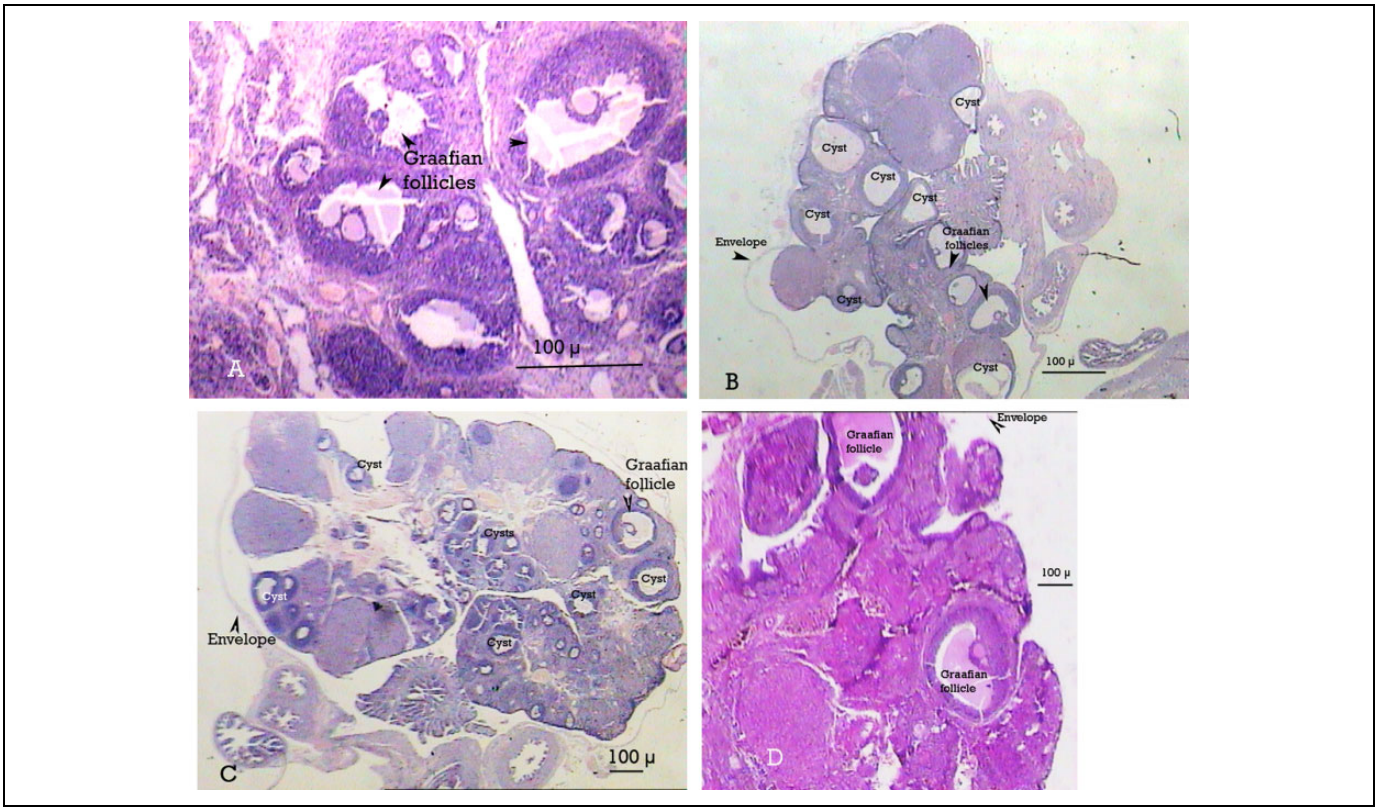

Figure 1. The ovaries of different groups of rats: control saline microinjected (intraventromedial hypothalamus; A), morphine microinjected into ventromedial hypothalamus (VMH; 0.1-0.4 µg/rat; B), naloxone (0.4 µg/rat) pre-microinjected into morphine addicted (0.4 µg/rat; C), single naloxone (0.1-0.4 µg/rat; D). Bars beside the samples show the values. The arrows and notes in each picture signify the desired characteristics.

Cyst

The number of the cyst in all samples was comparatively analyzed. In the single naloxone sample (0.1-0.4 µg/rat, intra-VMH), it was not significantly different to that of the control (saline solution). However, there was a significant difference ($P < .05$) among the drug (morphine) with the saline control and the naloxone-pretreated groups (Figure 2A-C).

Neurons of VMH

The neurons of the VMH in all samples were additionally comparatively analyzed. In the single naloxone sample (0.1-0.4 µg/rat, intra-VMH), there was no significant change when compared to that of the control (saline solution). Conversely, there was a significant difference ($P < .05$) among the drug (morphine) with the saline control or the naloxone pretreated groups (Figure 3A-C).

Discussion

Aside from the medical use of opioid drugs including morphine (eg, used to treat pain), they have psychological and physical side effects. Repeated administration of the drugs leads to the person's addiction. We know that the morphine is often used clinically to reduce pain to enhance recovery from injury or surgery.

The main problem in case of morphine is that the person is addicted after the long-term use of it. In addition to the problem, the reproductive system becomes inefficient due to opioid. In this research, the rats' ovaries and the rats' brains of the morphine-microinjected (intra-VMH) Wistar rats were investigated. It was found that the morphine (0.1-0.4 µg/rat, intra-VMH) causes a large follicular cyst with thickened granulosa cell layer or a large cystic follicle with scant granulosa cells (Figure 1B cf Figure 1A). The morphine dose effect (0.4 µg/rat) was reversed when naloxone (0.4 µg/rat) was pre-microinjected (Figure 1C).

The morphine-treated rats' ovaries showed polycystic characteristics when compared with the control samples (Figure 2A and B). In addition, the neurons of the VMH in the drug (morphine)-treated brain samples showed injuries and destructions in comparison with that of the saline control or the naloxone-pretreated groups (Figure 3A-C).

It has been indicated that the opioid abuse may even have unfavorable effects on reproductive organs.[5] The morphine disrupts ovarian cycles and reduces fertility. Also, chronic morphine influences noradrenergic mechanisms in the hypothalamus, which may be responsible for reduced reproductive activity.[14] Extensive preclinical and clinical data have

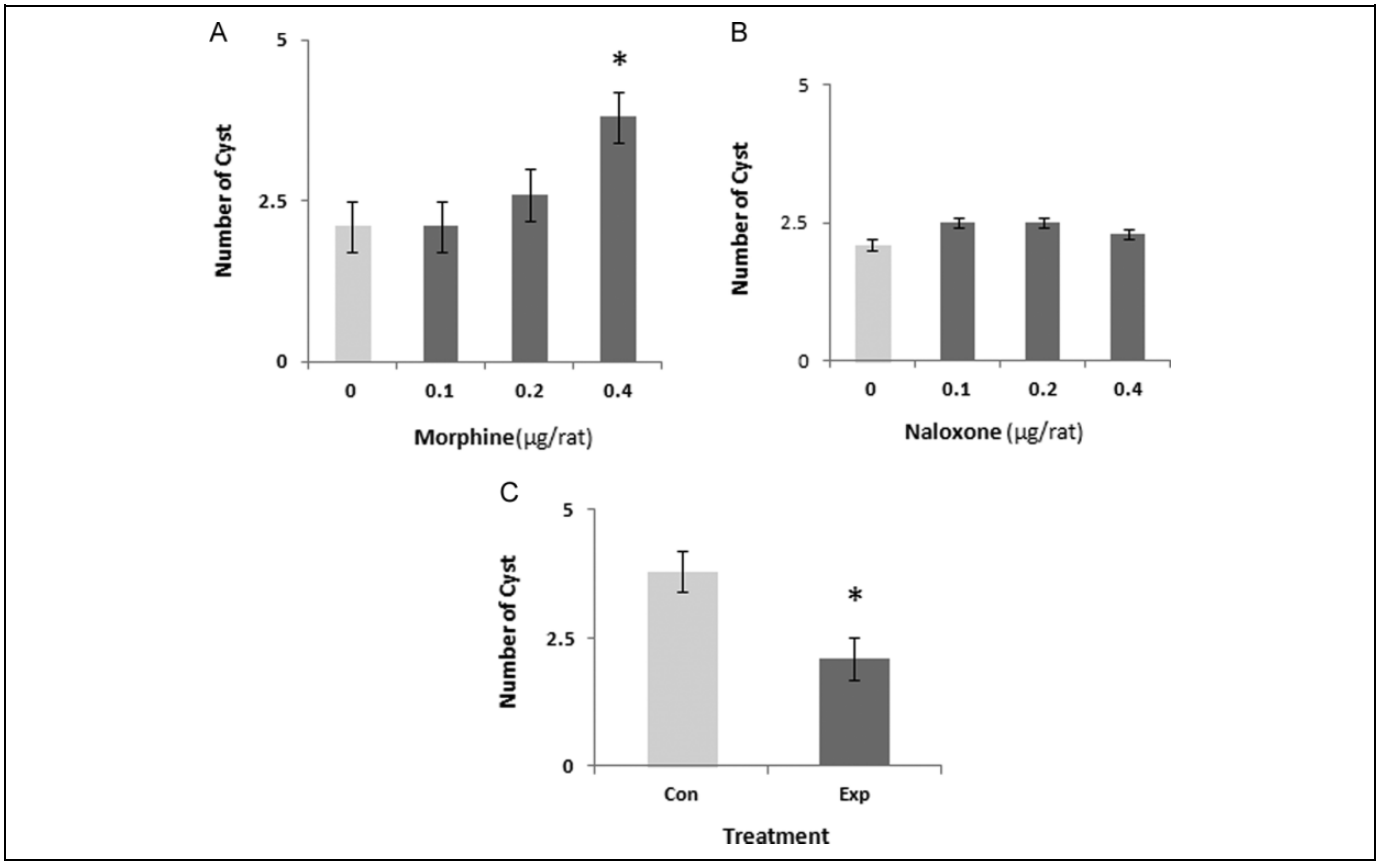

Figure 2. Numbers of cysts both in the control saline microinjected (intraventromedial hypothalamus; A) and experimental groups (B and C). The experimental groups were single morphine (0.1-0.4 μg/rat; A), administered or received single naloxone (0.1-0.4 μg/rat; B), or pre-microinjected naloxone (0.4 μg/rat) to morphine (0.4 μg/rat; C). Values are indicated as mean ± standard error of the mean (SEM).
Note. *$P < .05$.

demonstrated that opioids inhibit the functioning of the entire hypothalamic–pituitary–gonadal axis in large part by binding to opioid receptors in the hypothalamus, thereby decreasing the secretion of gonadotropin-releasing hormone.[15] This inhibitory effect of opioids on the catalyzing members of the endocrine pathway is then realized throughout the cascade, but opioids also directly bind and inhibit other members of the axis.

Also we know that the opioids bind receptors in the pituitary gland, limiting the production of LH in women, thereby interfering with the menstrual cycle.[16] It seems that the direct effects of opioids on the ovaries are to reduce sex hormone production, consequently leading to the risk of altered menstrual flow and probably reduced fertility.[17] This research, on one hand, not only showed the morphine-induced cystic changes in the rat ovary but also provided that the drug has damaging effect on the neurons of VMH. In one hand, the cystic genesis, such as thickening of follicular wall and distance between cell layers,[5] is visible. On the other hand, it seems that the effect of morphine is directly induced through the receptor connections at the VMH's cells. Morphine, as several times been previously identified, can stimulate NO release in different tissues such as endometrial glandular epithelial cells.[6,18,19] The NO is a major paracrine mediator of various biological processes, including vascular functions and inflammation.[20] This molecule is a free radical produced from the oxidation of terminal guanidino nitrogen of arginine by the action of NOS.[7] Although the precise role of NO during menstruation is still unclear and studies of NOS inhibitors on uterine bleeding are not yet available, the existing data on endometrial NOS expression and NO production during the menstrual cycle strongly suggest that NO plays a central role in controlling both the initiation and maintenance of uterine bleeding.[20] Nitric oxide is categorized as an important intraovarian mediator that affects the ovulatory process and regulates the functions of the corpus luteum.[21,22] This laboratory has also previously demonstrated that this molecule, as a pro-inflammatory element, may induce PCOS by activating inflammatory factors in ovaries.[9,23] The causes may vary among individual women with PCOS and hyperandrogenism, inappropriate gonadotropin secretion, and chronic anovulation, which are the hallmarks of this disorder.[24] Essentially, PCOS is a chronic condition with manifestations that begin most commonly in adolescence with oligomenorrhea/amenorrhea and transition into problems including infertility and metabolic complications and even cancer over time.[25,26] Based on the present results, we may conclude that the morphine

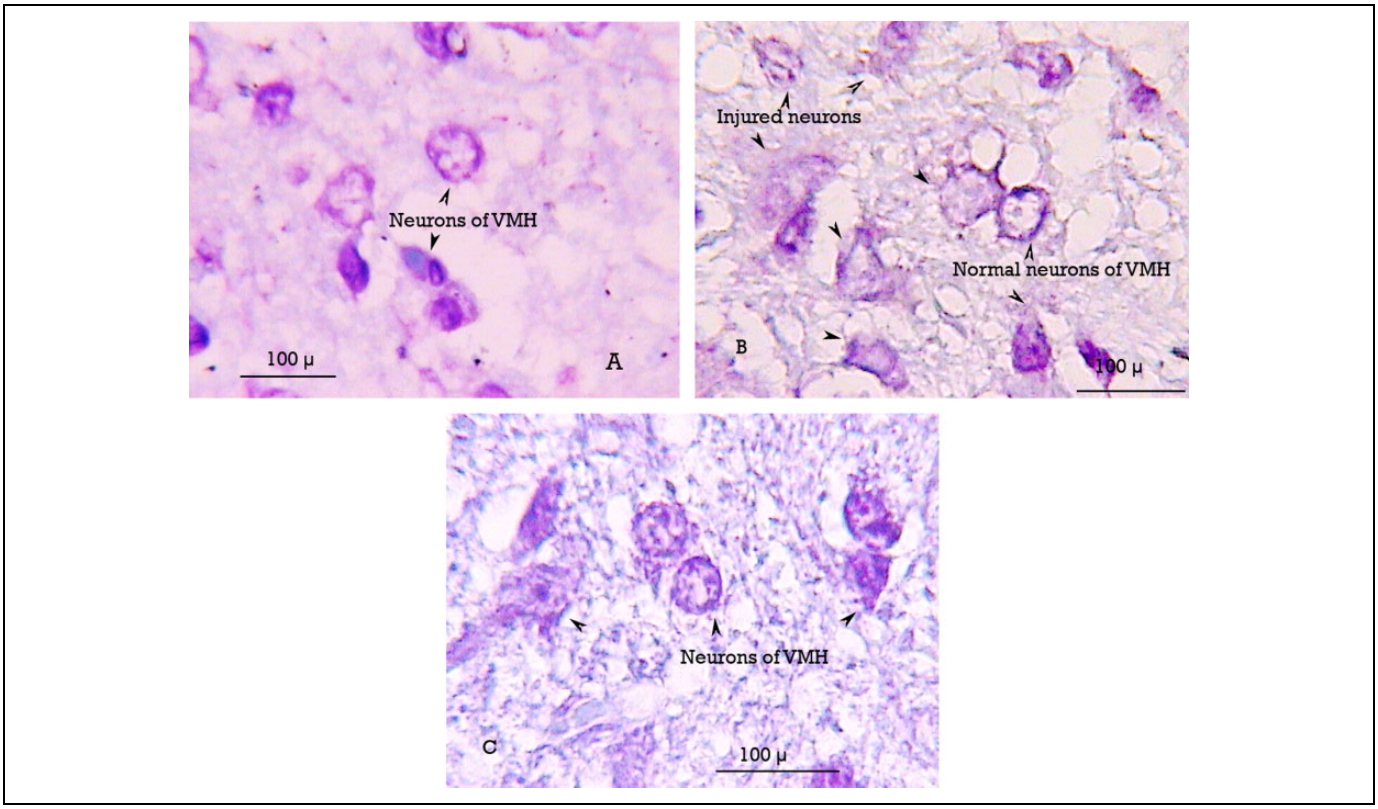

Figure 3. Neurons of ventromedial hypothalamus (VMH) both in the control saline microinjected (intra-VMH; A) and experimental groups (B and C). The experimental groups were single morphine (0.1-0.4 µg/rat; B) microinjected or preadministered naloxone (0.4 µg/rat) to morphine (0.4 µg/rat; C). Bars beside the samples show the values. The arrows and notes in each picture signify the desired characteristics.

microinjection into the VMH induces the infertility; maybe the cause is polycystic ovaries or it destroys the neurons of VMH. In the study of naloxone pre-microinjection, we showed that the injury of the neurons and the cysts in the ovary were resolved. So, there is a correlation between the harmful effects of morphine in the rats' reproductive axis with the opioid receptors. Maybe, there is a correlation between mal effect of morphine on the rat's reproductive system and the activation of a pro-inflammation NO pathway, which remains to be clarified.

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Declaration of Conflicting Interests

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