Original Article

Effects of Metformin on IL-17, IFN-γ, and Sperm Quality in a Rat Model of Chronic Non-bacterial Prostatitis

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ABSTRACT

Background: Chronic nonbacterial prostatitis (CNP) is a common, debilitating prostate disorder with no standardized treatment. Metformin (MET), an anti-diabetic medication, exhibits anti-inflammatory effects by reducing the production of cytokines.

Objectives: This study aimed to examine whether MET can modulate inflammatory responses and reproductive parameters in a rat model of CNP induced by carrageenan.

Methods: A total of 24 8-week-old male Wistar rats were selected and divided into 4 groups: The mock group (group I), CNP-control group (group II), CNP-Cernilton group (group III), and CNP-MET group (group IV). For CNP induction, 1% carrageenan (0.1 mL) was injected intraprostatically into groups II, III, and IV. The rats in groups III and IV received Cernilton (100 mg/kg) and MET (100 mg/kg) orally for three weeks. Prostatic index (PI), serum prostate-specific antigen (PSA), interleukin-17 (IL-17), interferon-γ (IFN-γ), and sperm parameters (counts, motility, and viability) were compared between groups. Data were analyzed using one-way ANOVA with Tukey post-hoc test for normal distributions and the Kruskal-Wallis with Mann-Whitney U test for non-normal distributions. Significance was set at $P<0.05$.

Results: There was a significant increase ($P<0.05$) in PI, serum levels of PSA, IL-17, and IFN-γ in group II. At the same time, the sperm quality parameters were significantly decreased ($P<0.05$) in this group. On the other hand, in group IV, a significant decrease ($P<0.05$) in PI, PSA, IL-17, and IFN-γ levels was observed, and sperm quality parameters improved compared to group II.

Conclusion: MET markedly reduced inflammation and enhanced reproductive parameters in a rat model of CNP. Through lowering PI, inflammatory cytokines (IL-17, IFN-γ), and PSA levels, and at the same time enhancing sperm quality, MET proves to be a therapeutic candidate for CNP management.

Keywords: Anti-inflammatory properties, Cytokine suppression, Interleukin-17 (IL-17) modulation, Sperm motility improvement, Intraprostatic intervention

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Introduction

The prostate gland, weighing about 20 g, is the most prominent accessory sex gland in men. This gland is located at the base of the bladder and surrounds the upper part of the urethra. The prostate gland consists of epithelial and stromal cells. The prostate's epithelial cells do their secretory job. Its stromal cells are connective tissue cells in the prostate. Two ejaculatory ducts open into the urethra. The structure's location is behind the prostate gland (Al-Shahrani et al., 2013). Research in epidemiology over the past decade shows that prostatitis is a significant health problem. It is often blamed on the interaction between the treating doctor and the patient. Previous studies have reported prostatitis rates ranging from 2.2% to 8.9%, with an average of 7.8%. Consequently, prostatitis is one of the most common urological problems in young men and a significant issue for men over 50 (Lu et al., 2011). Infections in the reproductive system cause male infertility. They include prostatitis, epididymitis, and testicular swelling. They account for about 12% of male infertility factors.

The National Institutes of Health (NIH) classifies prostatitis into 4 groups: acute and chronic bacterial prostatitis, chronic prostatitis or chronic pelvic pain syndrome, and asymptomatic inflammatory prostatitis. These groups are commonly used in clinical examinations to identify different types of prostatitis. The third form of prostatitis is divided into two subcategories: inflammatory and non-inflammatory (Yang et al., 2014). In chronic prostatitis or pelvic pain syndrome, levels of pro-inflammatory cytokines, such as interleukin (IL)-17 and interferon (IFN)-γ, rise considerably. This upsurge is a big reason for using anti-inflammatory drugs to treat it (Yoon et al., 2013). The etiology and pathophysiology of chronic prostatitis or chronic pelvic pain syndrome have not been understood. Despite its varying symptoms, it almost always comes with pelvic pain. It also causes urinary and genital pains, as well as symptoms related to the lower urinary tract. Unfortunately, the underlying cause, history, and appropriate patient treatment have not been adequately identified. Still, common treatments include non-steroidal anti-inflammatory drugs, antibiotics, muscle relaxants, and alpha-receptor blockers. However, no definitive treatment has been found for chronic nonbacterial prostatitis (CNP) (Chen et al., 2014).

Recently, researchers have determined that patients with prostatitis experience oxidative stress, and it is well-accepted that inflamed areas of the prostate generate free radicals such as nitric oxide and various oxygen species. The overproduction of oxygen species, nitrogen radical species, and prostaglandins is characteristic of the inflammatory process. It is well-accepted that inhibitors of free radical formation can reduce the production of oxygen species, prostaglandins, and nitric oxide, thereby reducing inflammation. Thus, enhancing the activity of antioxidant enzymes can be very useful in treating chronic bacterial prostatitis (Yang et al., 2014). Metformin hydrochloride (1,1-dimethylbiguanide hydrochloride [MET]) is a first-line medication for the treatment of type 2 diabetes (T2DM) that exhibits multiple pleiotropic effects, including anti-inflammatory, anti-cancer, anti-aging, antimicrobial, anti-atherosclerotic, and immunomodulatory properties.

MET alone and in combination with other drugs restores ovarian function in women with polycystic ovary syndrome (PCOS) and improves embryo growth, pregnancy outcomes, and fertility. Studies have shown that MET treatment enhances the efficacy of laboratory fertilization, and experts consider it a complementary drug in fertility-assisting technologies. Prescribing MET has a positive effect on steroidogenesis and spermatogenesis in men with metabolic disorders, suggesting that MET therapy is a promising approach to enhancing reproductive function and fertility in men (Shpakov, 2021). This study explores the innovative use of MET in addressing CNP. It focuses on its impact on inflammatory markers such as interleukin (IL)-17, interferon-γ (IFN-γ), and prostate-specific antigen (PSA) in a rat model, despite the well-known benefits of MET for conditions such as T2DM and PCOS, including its anti-inflammatory, antioxidant, and immunomodulatory properties, its potential for modulating CNP has not been investigated, making this research a promising approach for managing this common and poorly understood condition.

Materials and Methods

Chemicals

The study employed several chemicals, including a rat PSA enzyme-linked immunosorbent assay (ELISA) kit, an IL-17 ELISA kit (MyBioSource, Inc.), and a DRG IFN-γ rat ELISA kit. MET (Amin Pharmaceutical Co., Tehran, Iran). Carrageenan was sourced from Sigma-Aldrich (St. Louis, MO, USA), and Cernilton (Swedish flower pollen from source naturals) was purchased from Amazon website. The study also involved various analytical chemicals.

Animal experiment

Twenty-four male adult Wistar rats were obtained from the lab's animal reproduction and maintenance section. At 7 weeks old, the rats weighed between 200 and 230 g. To adapt to the conditions, they were kept under the same temperature, humidity, light, diet type, and number of feedings for one week. They were also on a 12-h light/dark cycle. The rats were fed pellets explicitly prepared for the laboratory and had access to water at all times.

Experimental design

The study included 24 eight-week-old male Wistar rats. The animals were randomly divided into four groups, each containing 6 individuals. The groups included were the mock group (group I), the CNP-control group (group II), the CNP-Cernilton group (group III), and the CNP-MET group (group IV). To conduct experiments, we surgically visualized the prostatic tissues of all the rats. In the CNP-control, CNP-MET, and CNP-Cernilton groups, 100 µL of carrageenan (1%) was injected into the prostate of each rat. The mock group rats received equal amounts of physiological saline (0.9% sodium chloride solution; Lu et al., 2011). After 7 days, rats in the CNP-cernilton and CNP-MET groups were administered Cernilton (100 mg/kg; Chabot et al., 2021) and MET (100 mg/kg) orally for 3 weeks. Concurrently, the rats in both the mock and CNP-control groups were given saline for an equivalent period. The overall physical condition of every rat was constantly tracked during the test period. A rat varicocele model was used to determine the oral MET dose of 100 mg/kg. This dosage effectively improves oxidative stress (OS) markers and minimizes histological damage in testicular tissues, making it the most effective option reported (Karimi et al., 2023). Following the final dose on the 28th day, the rats underwent a 12-h fasting period before being weighed and promptly euthanized with chloroform under anesthesia. Blood samples were collected from the cardiac ventricles to prepare serum for biochemical analysis. The animals were euthanized, and their prostate samples were then extracted, cleansed with phosphate-buffered saline (PBS) to remove any attached blood and fat tissues, and weighed to calculate the prostatic index (PI).

PI determination

The Equation 1 was used to compute the PI:

1. Prostate weight (mg)/body weight (g) in normal 8-week-old rats

, this index is less than 1 mg/g.

Evaluation of serum PSA, IL-17, and IFN-γ

The serum was separated from the blood specimens by centrifuging for 10 minutes at 3000 rpm. PSA, IL-17, and IFN-γ serum levels were estimated using ELISA kits (MyBioSource, San Diego, CA, USA) and DRG Diagnostics GmbH (Marburg, Germany). The ELISA's detection limits for quantifying IL-17 and IFN-γ were 2 pg/mL and <13 pg/mL, respectively. The measurements were taken according to the manufacturer's instructions (MyBiosource, 2019).

Epididymal sperm analysis

The caudal epididymis (vas deferens) from each rat across all 4 groups was meticulously excised and placed in a sterile Petri dish containing 2 mL of warm normal saline at 37 ˚C. The epididymis was then macerated using sterile scissors, resulting in a semen suspension (Noori Alavije et al., 2022). Sperm quality was assessed based on three essential parameters, as recommended by the World Health Organization (WHO), including sperm count, viability, and motility. Sperm count was performed using a hemocytometer according to established counting methods (Saber et al., 2016). Specifically, 200 µL of semen was mixed with 800 µL of normal saline in a microtube (achieving a dilution factor of 1:5), to which a few drops of 40% formalin were added to immobilize the sperm.

After thorough mixing, the sperm count was performed using an improved hemocytometer counting chamber. The sperm concentration per mL was calculated by multiplying the total sperm count in four squares by 125×10^3. Sperm motility was evaluated by randomly counting over 200 spermatozoa in selected fields using a 40x light microscope. The motility percentage was determined by tallying both motile and non-motile sperm, with results expressed as a percentage (Saber et al., 2016). The eosin-nigrosin staining method was used to assess sperm viability (Merck, Darmstadt, Germany). One drop of the semen suspension was mixed with two drops of 1% eosin, followed by three drops of 10% nigrosin blue after a 30-second interval. The mixture was then spread onto a clean glass slide and allowed to air dry. Live sperm appear colorless, whereas dead sperm exhibit a red hue. The percentage of live sperm was determined by examining 200 sperm cells from each rat.

Data analysis

Statistical analyses were conducted using SPSS software, version 23 (SPSS Inc., Chicago, IL). Results are presented as Mean±SD. The Shapiro-Wilk test was ap-

plied to evaluate the normality of each variable's distribution. Variables that satisfied the normality criterion (P>0.05) were analyzed using one-way ANOVA, followed by Tukey post hoc comparison. For variables that did not meet the normality assumption (P<0.05), the Kruskal-Wallis test was used, with subsequent pairwise comparisons performed using the Mann-Whitney U test. A P<0.05 was regarded as statistically significant.

Results

Effect on PI

The PI was calculated by dividing the prostate weight by the rat's body weight (mg/g). As shown in Figure 1A, group II demonstrated a markedly elevated PI value (2.6±0.32; P=0.00) relative to Groups I (1.28±0.28), III (1.56±0.32), and IV (1.2±0.47). The comparison between groups III and IV, however, revealed no significant difference (P=0.75).

Effect on PSA levels in serum

Serum PSA levels were measured to determine the effects of MET on inflammatory prostatic indices in carrageenan-induced CNP rats. The effects of MET on the levels of PSA are presented in Figure 1B. The control group showed a PSA concentration of 0.25±0.075 ng/mL. In the CNP-control group, however, PSA levels rose markedly to 0.61±0.19 ng/mL (P=0.041 vs group I). Both the CNP-Cernilton and CNP-MET groups exhibited significantly reduced PSA levels compared with the CNP-control group (0.24±0.14 and 0.20±0.11 ng/mL, respectively; P=0.038 and P=0.027 vs Group II). Although the PSA values did not differ significantly between groups III and IV (P=0.4497), the decrease was more substantial in the CNP-MET group (Figure 1B).

Effect on IL-17

Serum IL-17 concentrations were assessed to determine the impact of MET in carrageenan-induced CNP rats (Figure 1C). In the mock group, IL-17 levels measured 23.94±3.26 pg/mL, but they rose markedly to 31.5±8.54 pg/mL in the CNP-control group (P=0.027 vs group I). Treatment with Cernilton or MET significantly lowered IL-17 levels (21.26±4.19 and 20.93±1.86 pg/mL, respectively; P=0.003 for both compared with group II). Although the difference between groups III and IV was not statistically significant (P=0.072), the MET-treated group showed a slightly greater reduction in the MET-treated group.

Effect on IFN-γ

Serum IFN-γ concentrations were measured to assess the effect of MET on prostatic inflammation in carrageenan-induced CNP rats. As illustrated in Figure 1D, IFN-γ levels were 8.05±4.68 pg/mL in the mock group and rose sharply to 23.86±4.56 pg/mL in the CNP-control group (P=0.000 vs group I). Administration of Cernilton or MET markedly decreased IFN-γ levels to 8.03±1.97 and 8.43±3.02 pg/mL, respectively (P=0.000 for both compared with group II). Importantly, there was no significant difference between groups III and IV (P=0.142).

Sperm parameters evaluation

The results of the analysis of sperm parameters in the tested rats are shown in Table 1. The sperm quality parameters (counts, motility, and viability) in the CNP-control group decreased significantly (P<0.05) compared to the mock group. In the CNP-cernilton and CNP-MET groups, all sperm quality parameters were significantly improved and increased compared to the CNP-control group (P<0.05).

Discussion

CNP is one of the most common and debilitating prostate syndromes in men. It reduces the quality of life by causing urinary symptoms (Wang et al., 2016). The present study investigated the protective effects of MET on CNP rats. There is currently no research on the impact of MET in reducing CNP-induced inflammation. Additionally, the effects of MET have only been studied on prostate cancer (Hou et al., 2019). Our study will provide insight into how MET can protect against CNP. There is no current research on the effects of MET on prostatitis in either humans or animals (Maniar et al., 2017). Despite exhibiting antimicrobial, antiviral, and antifungal properties, MET is being developed to create innovative antimicrobial compounds (Chen et al., 2018). Therefore, MET may lower the incidence of prostatitis due to its anti-inflammatory, antimicrobial, and anti-glycemic properties. Although there is insufficient data to prove the effect of MET on prostatitis, its antimicrobial and anti-inflammatory properties may be beneficial. By blocking the insulin-like growth factor-1 pathway, MET can reduce the risk of benign prostatic hyperplasia (BPH). Some research suggests that MET may reduce the risk of prostate cancer (Tseng, 2022).

Table 1. MET's effect on sperm parameters in carrageenan-induced CNP in rats

Groups	Mean±SD		
	Sperm Counts (×10⁶/mL)	Sperm Motility (%)*	Sperm Viability (%)*
The mock group (I) (n=6)	88±14.69[a]	82.5±9.57[a]	77.5±5[a]
CNP-Control-group (II) (n=6)	17.6±10.18[b]	42±4.47[b]	40±7.07[b]
CNP-Cernilton group (III) (n=6)	52.8±7.56[a]	70±7.07[a]	58±4.47[a]
CNP-MET-group (IV) (n=6)	53.8±3.89[a]	68±13.04[a]	64±11.4[a]

*A minimum of 5 microscopic fields were assessed to evaluate sperm motility and viability on at least 200 spermatozoa for each animal.

Note: Group 1: The mock group (control); Group II: CNP-control-group; Group III: CNP-cernilton group (treated with cernilton); Group IV: CNP-MET-group (treated with MET). Differing letters indicate statistically significant differences (P<0.05), whereas identical letters denote no significant change.

The PI in the CNP-control group showed a significant increase compared to the mock group (P<0.05). Also, a significant decrease (P<0.05) in the PI of the MET-treated and CNP-Cernilton group. MET and Cernilton showed a protective effect on improving CNP. The related semen parameters, like weight loss, increased testicular weight, and reduced testicular cell apoptosis, were enhanced by MET treatment (Yan et al., 2015). Using MET in patients with BPH had a beneficial effect on PI (Yang et al., 2024). Streptozotocin-induced diabetic male rats treated with MET significantly improved PI (Koroglu et al., 2022)

As shown in Figure 1B, MET has significantly reduced PSA levels in the CNP-MET group compared to the CNP-control group. The results of similar studies were

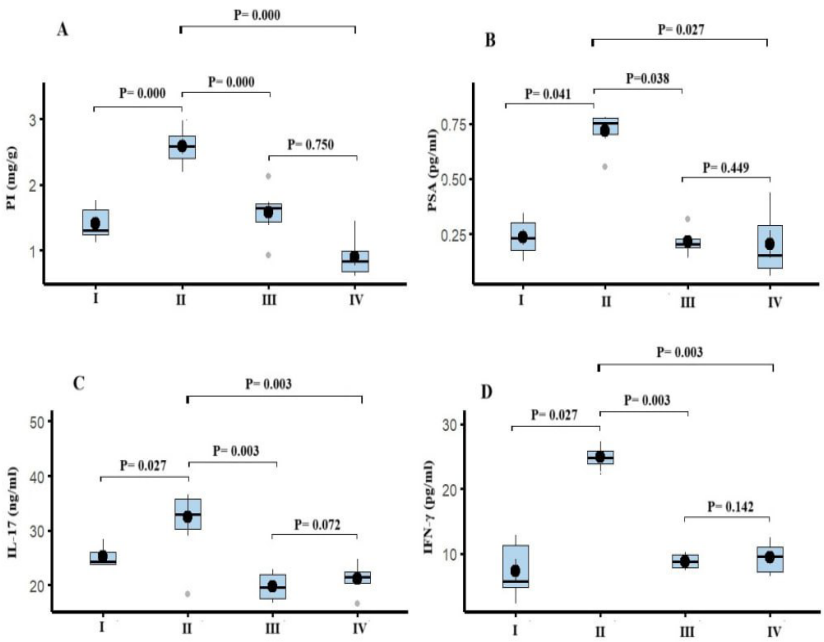

Figure 1. Boxplot representation of (A) PI, (B) serum PSA, (C) IL-17, and (D) IFN-γ levels in 4 groups: I – Mock, II – CNP-control, III – CNP-Cernilton, and IV – CNP-MET

Note: In each plot, the central horizontal line within the box represents the median, the upper and lower borders of the box represent the first (Q1) and third (Q3) quartiles, and the whiskers indicate the minimum and maximum values within 1.5 inter-quartile range (IQR). Outliers are shown as individual grey dots. The black circles within the boxes represent the mean values for each group. Exact P for pairwise comparisons between groups are indicated above the respective comparisons. A P<0.05 was considered statistically significant.

consistent with the results of the present study (Atalay et al., 2021). A survey on the effect of MET on PSA reported that the level of PSA in rats treated with MET is lower than that in untreated rats (Park et al., 2017). Another study reported a correlation between MET use and serum PSA levels, with an average PSA level 30% lower among MET users, which aligns with our findings (Jayalath et al., 2016). According to some research, MET decreases *PSA* gene expression (Besla et al., 2013; Wang et al., 2015). In two human prostate cancer cell lines, LNCaP and C4-2, a study demonstrated a significant reduction in PSA by MET, which involved upregulating the protein level of the small heterodimer partner-interacting leucine zipper, thereby inhibiting androgen receptor function (Lee et al., 2009). Past studies have reported that MET treatment reduces the expression of cancer cell genes (Besla et al., 2013; Wang et al., 2015) and PSA levels, thereby improving survival. The current research team presented similar results in their previous studies on the effect of Cernilton and pentoxifylline (PTX) on CNP (Hajighorbani et al., 2017; Yousefi et al., 2018).

The expression of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), IFN-γ, IL-17, and IL-1β, increases in CNP (Liu et al., 2021; Motrich et al., 2010; Liu et al., 2017; Zhao et al., 2020). MET reduces the expression of inflammatory cytokines, such as IFN-γ, IL-17, IL-1β, and TNF-α, thereby impacting the intensity and course of inflammation. In the present study, treatment with MET decreased levels of INF-γ and IL-17 compared to the CNP-control group (Rokni et al., 2021; Taher et al., 2023). Therefore, the analgesic effects of MET on CNP in the present study may arise from its impact on inflammatory cytokines. It has been suggested that the anti-inflammatory effect of MET is due to its antioxidant activities (Alhaidar et al., 2011). In a study, it was reported that in a rat model of periodontitis, MET restored the expression of markers of antioxidant genes such as superoxide dismutase (SOD), which neutralizes ROS and malondialdehyde (MDA). MET prevented the infiltration of inflammatory cells and the expression of pro-inflammatory factors, such as IL-6, IL-17, and IL-18. This study suggests a strong antioxidant effect, resulting in significant decreases in the expression of pro-inflammatory factors, such as IL-1β and TNF-α, as well as COX-2 immunostaining (Lin et al., 2023). MET may substantially reduce OS and prevent apoptosis (Naghibi et al., 2022). PTX facilitates carrageenan-induced CNP in our research team's previous investigation by preventing OS and releasing inflammatory cytokines, including TNF-α (Hajighorbani et al., 2017). The sperm quality parameters (count, viability, motility) improved in the CNP-MET group. It seems that the reason is the positive effect of MET on reducing inflammation and oxidative stress. In a study, it was shown that MET enhanced the antioxidant capacity of the testes and also enhanced the quality of sperm in diet-induced obese rats (Fang et al., 2012). The mean diameter of seminiferous tubules and sperm functions were significantly improved in rats (Ghasemnejad-Berenji et al., 2018). MET improves pig sperm viability in animals following a 24-hour storage period (Hurtado de Llera et al., 2018). The cryopreservation process improved the quality of frozen-thawed dog semen (Grandhaye et al., 2020). A study examining the impact of ginseng oil on the reproductive performance of adult male rats exposed to alloxan found that oral administration of ginseng oil improves the number, motility, and viability of the rats' sperm (Mhaibes et al., 2023). In a study, the effects of silymarin and MET on sperm and testicular changes in diabetic mice were investigated and reported. Both drugs improved sperm quality, including sperm DNA integrity, tubule diameter, and epithelial thickness (Pourheydar et al., 2021). Another study on the combined effects of forskolin and MET in diabetic rats examined the quality of sperm parameters and the concentration of testosterone and antioxidant enzymes. The results indicated that these two substances significantly increased serum testosterone concentration and levels of oxidant enzymes, thereby enhancing sperm quality parameters (Naghibi et al., 2022). A study demonstrates that the new GGC medium (green tea extract, glutathione, and vitamin C) is effective in stimulating sperm in vitro using the direct swim-up method, thereby improving sperm motility and reducing sperm DNA fragmentation in individuals with asthenozoospermia (Kadhim & Zwamel, 2023). A systematic review study investigating the effect of MET on sperm quality parameters reports that MET has a positive effect on sperm quality parameters (Fang et al., 2012; Adaramoye et al., 2014; Bertoldo et al., 2014; Nguyen et al., 2014). These improvements are because of the MET's ability to reduce OS and lipid peroxidation, increase 5'-AMP-activated protein kinase activity, and restore levels of pituitary-gonadal hormones (Banihani, 2016).

Conclusion

The present study's findings suggest that MET has significant potential in alleviating CNP in a rat model. It modulates inflammatory markers such as IL-17, IFN-γ, and PSA. Given MET's known anti-inflammatory, antioxidant, and immunomodulatory properties, this research is crucial in evaluating its potential to improve CNP. The results underscore MET's ability to reduce inflammation and address a condition often observed but

insufficiently studied. Further clinical studies are needed to confirm its efficacy in humans.

Ethical Considerations

Compliance with ethical guidelines

The experimental protocols were conducted in accordance with the guidelines of the NIH in the USA. This study was approved by the Animal Research Ethics Committee of Faculty of Veterinary Science, Semnan University, Semnan, Iran. All experimental procedures were conducted in full compliance with the ethical principles for the care and use of laboratory animals.

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Authors' contributions

Conceptualization and study design: Mahmood Ahmadi-hamedani, Hamid Reza Moslemi, Khatereh Kafshdouzan, and Salar Nik; Resources, data collection, analysis, review and editing: Mahmood Ahmadi-hamedani and Hamid Reza Moslemi; Writing the original draft: Mahmood Ahmadi-hamedani; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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