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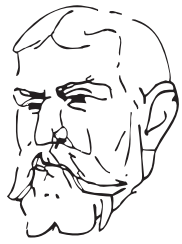

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ORIGINAL ARTICLE

REACTIVE OXYGEN SPECIES GENERATION BY BLOOD LEUCOCYTES OF RATS WITH POLYCYSTIC OVARY SYNDROME UNDER THE CONDITIONS OF INTERMITTENT COLD EXPOSURE

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ABSTRACT

The aim of this study was to determine the level of ROS production by blood leukocytes of rats with PCOS under the conditions of intermittent cold exposure.

Materials and methods: In the study, 40 immature female rats of the WAG population at the age of 27 days with a body weight of 80-90 g were used. Five groups were formed (8 animals in each group). Group 1 was represented by intact rats that were not subjected to any manipulations. Group 2 was represented by rats that were injected subcutaneously with 0.2 ml of purified and sterilized olive oil daily for 25 days. Group 3 was represented by rats that were exposed to intermittent cold for 25 days. Group 4 was represented by rats that were modeled with PCOS. Group 5 was represented by rats, which were simulated PCOS against the background of intermittent cold exposure. ROS production was estimated in leukocytes isolated from rats of all groups by flow cytometry using the fluorescent probe of 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA).

Results: The experimental study revealed an intracellular excessive production of ROS by leukocytes in rats with polycystic ovary syndrome. The use of intermittent cold exposure normalized the production of reactive oxygen species by leukocytes in rats with polycystic ovary syndrome.

Conclusions: The effectiveness of intermittent cold exposure, proven by the authors, allows recommending its use as one of the methods of prevention and treatment of the polycystic ovary syndrome.

KEY WORDS: leukocytes, rats, reactive oxygen species, polycystic ovary syndrome, intermittent cold exposure, oxidative stress

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INTRODUCTION

Polycystic ovary syndrome (PCOS), the major endocrinopathy among reproductive-aged women, is not yet perceived as an important health problem in the world. It affects 4-20% of women of reproductive age worldwide [1]. This pathology is associated by hyperandrogenism, hirsutism, menstrual disorders, ovulatory dysfunction, polycystic ovaries and metabolic disorders [2, 3]. In the development of PCOS, according to many scientists, one of the key roles is played by oxidative stress, in which there is an increase in the production of reactive oxygen species (ROS) [4, 5].

It is now known that free radical reactions involving free radicals (a part of a molecule or a molecule having an unpaired electron) can lead to the formation of ROS and reactive nitrogen species. Oxidative stress reflects a disturbance in the balance between the formation and elimination of ROS. ROS include both free radical and non-free radical molecules, in particular hydrogen peroxide, hydroxyl radical, singlet oxygen, etc. [4-6]. In a healthy body, there is a balance between oxidant and antioxidant systems (enzymatic and non-enzymatic) [4]. The study of pathophysiological mechanisms of the oxidative stress development in PCOS is necessary for the development of modern effective methods of preclinical correction, prevention and rehabilitation of reproductive pathology.

In patients with PCOS, a combination of low-grade chronic inflammation, vascular endothelium damage, excessive production of ROS and oxidative stress development is common [4-8]. PCOS patients demonstrated significantly higher concentrations of circulating inflammatory cells, such as lymphocytes, neutrophils, eosinophils, monocytes etc. [7]. It is extremely important to search the sources, i.e. cells that can produce an excessive amount of ROS causing the development of PCOS.

THE AIM

The aim of the study was to determine the level of ROS production by blood leukocytes of rats with PCOS under the conditions of intermittent cold exposure.

MATERIALS AND METHODS

In the study, 40 immature female rats of the WAG population at the age of 27 days with a body weight of 80-90 g were used. Five groups were formed (8 animals in each group).

Group 1 was represented by intact rats that were not subjected to any manipulations.

Group 2 was represented by rats that were injected subcutaneously with 0.2 ml of purified and sterilized olive oil daily for 25 days.

Group 3 was represented by rats that were exposed to intermittent cold for 25 days. Intermittent cold exposure was modeled by keeping animals daily for 4 hours in a chamber where the light regime and temperature + 4°C were maintained. The animals were kept under normal conditions for the last 20 hours of the day. The animals were exposed to intermittent cold for 25 days.

Group 4 was represented by rats that were modeled with PCOS. PCOS was modeled by daily subcutaneous administration for 25 days of dehydroepiandrosterone (DHEA) at a dose of 8 mg per 100 g of animal body weight, dissolved in 0.2 ml of purified and sterilized olive oil.

Group 5 was represented by rats, which were simulated PCOS against the background of intermittent cold exposure. In this group, the methods for modeling of intermittent cold exposure and PCOS were similar to those used in groups 3 and 4.

Rats of all groups receiving the same diet were removed from the experiment by cervical dislocation on the 26th day. After decapitation of animals of groups 1-5 blood samples were collected to vacutainer tubes with ethylenediaminetetraacetic acid dipotassium salt (EDTA) (Guangzhou, China).

Preparation of samples (blood) for research on a flow cytometer according to the protocol took place at the Institute of Experimental and Clinical Medicine of the Kharkiv National Medical University (Ukraine).

The method used in this study for determining the generation of ROS in leukocytes was described by us in a previously published article [9].

Suspension of leukocytes was prepared. Blood from each animal lysed and washed twice with Pharmalyse solution (BD, USA) and phosphate-buffered saline. Blood samples in the amount of 100 µl were placed in a 12×75 mm polystyrene tube (Falcon, Mexico), and 2 ml of 1x FACSLyse solution (BD FACS™ Lysing, San Jose, USA) was added. We mixed and incubated in the dark at 23°C for 15 minutes. Then, for 5 min, centrifugation was carried out at 500 g. Supernatant liquid was discarded and 2 ml of sodium phosphate buffer was added.

Leukocyte suspensions were used for further evaluation of ROS levels in leukocytes. The fluorescent probe of 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) was employed for detection the intracellular ROS concentration. It is cleaved by intracellular esterases to form 2,7-dichlorodihydrofluorescein, which is transformed by ROS into highly fluorescent 2,7- dichlorofluorescein (DCF).

The flow cytometer BD FACSCanto ™ II (BD Biosciences, USA) was used. The mean fluorescence intensity (MFI) of DCF was analyzed using BD FACSDiva™software (Becton Dickinson, USA) for quantitative assessment of ROS production by leukocytes.

Statistical processing of the obtained data was performed using the Graph Pad Prism 5 program (Graph Pad Software, USA). The indicators were compared using the non-parametric Mann-Whitney U test. The results in groups were presented in the form of median (Me) and interquartile ranges. The differences at $p<0.05$ were considered statistically significant.

RESULTS

In groups 1-5, flow cytometry allowed us to assess the generation of ROS by analyzing the fluorescence inten-

Table I. The analysis of the fluorescence intensity of DCF in leukocytes of peripheral blood of rats of groups 1-5.

Group number	MFI of DCF in leukocytes	
	Median	[25% percentile; 75% percentile]
Group 1	407.1	[355.8; 425.9]
Group 2	416.5	[374.8;456.0]
Group 3	361.6	[297.2;409.5]
Group 4	539.4 [1,2,3]	[494.5; 632.4]
Group 5	377.0 [4]	[347.5; 404.8]

Note: 1 – significant ($p<0.05$) difference compared to the indicator of group 1; 2 – significant ($p<0.05$) difference compared to the indicator of group 2; 3 – significant ($p<0.05$) difference compared to the indicator of group 3; 4 – significant ($p<0.05$) difference compared to the indicator of group 4.

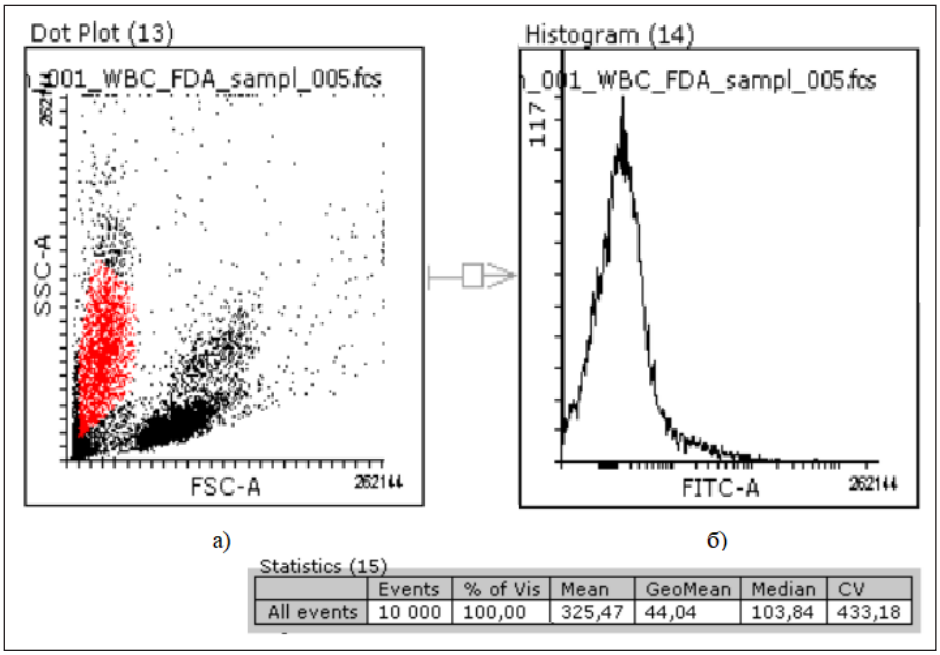

Fig. 1. Representative cytogram (A) and histogram SSC/FL1 (2,7 – dichlorofluorescein) (B) of leukocytes of rat No. 2 of group 1. Mean fluorescence intensity of DCF is 325.47.

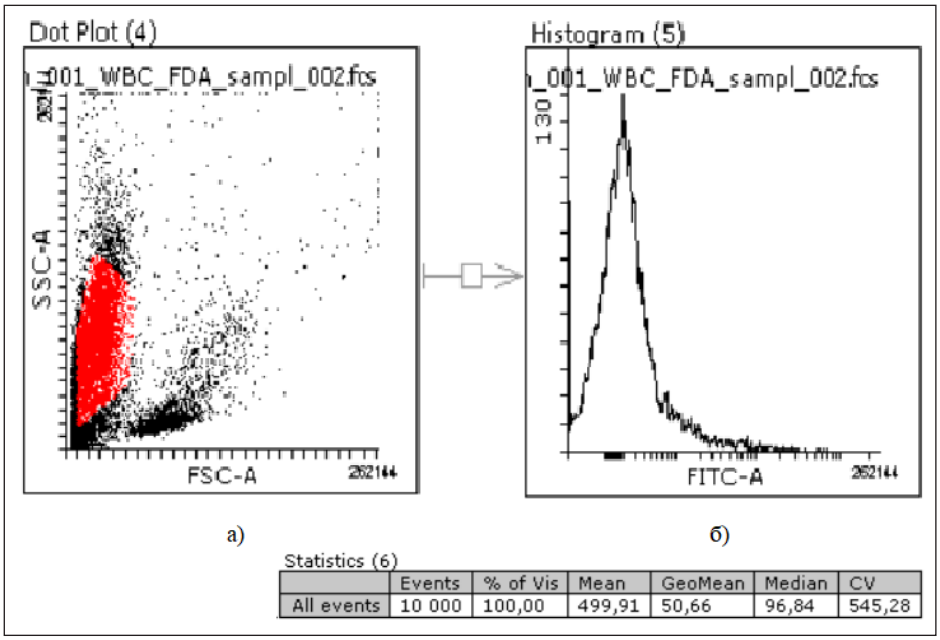

Fig. 2. Representative cytogram (A) and histogram SSC/FL1 (2,7 – dichlorofluorescein) (B) of leukocytes of rat No. 1 of group 2. Mean fluorescence intensity of DCF in leukocytes is 499.91.

sity of DCF in a population of leukocytes. The results obtained are presented in table I.

The MFI of DCF in group 1 coincides with the results obtained by us earlier [9] and will be used in this study as a standard indicator. Figure 1 shows the generation of ROS in leukocytes of rat No. 2 of group 1.

In group 2 the MFI of DCF in leukocytes did not significantly ($p>0.05$) differ from the indicator of group 1 (Table I). Figure 2 presents the results of evaluating the fluorescence intensity of DCF in leukocytes in rat No. 1 of group 2.

The MFI of DCF in leukocytes in group 3 was characterized by a tendency ($p>0.05$) to decrease compared to the indicator of group 1 (Table I). Figure 3 shows the results of the fluorescence intensity assessment of DCF in leukocytes in rat No. 4 of group 3.

In group 4 the MFI of DCF was significantly ($p<0.05$) higher compared with the corresponding indicators of groups 1-3 (Table I). The results obtained in this group indicated the formation of a larger number of ROS compared to groups 1-3. Figure 4 shows the results of evaluating the fluorescence intensity of DCF in leukocytes in rat No. 3 of group 4.

In group 5 the MFI of DCF in leukocytes decreased ($p<0.05$) compared with the corresponding indicator of group 4 and did not significantly ($p>0.05$) differ from the corresponding indicators of groups 1-3 (Table I). Figure 5 presents the results of evaluating the

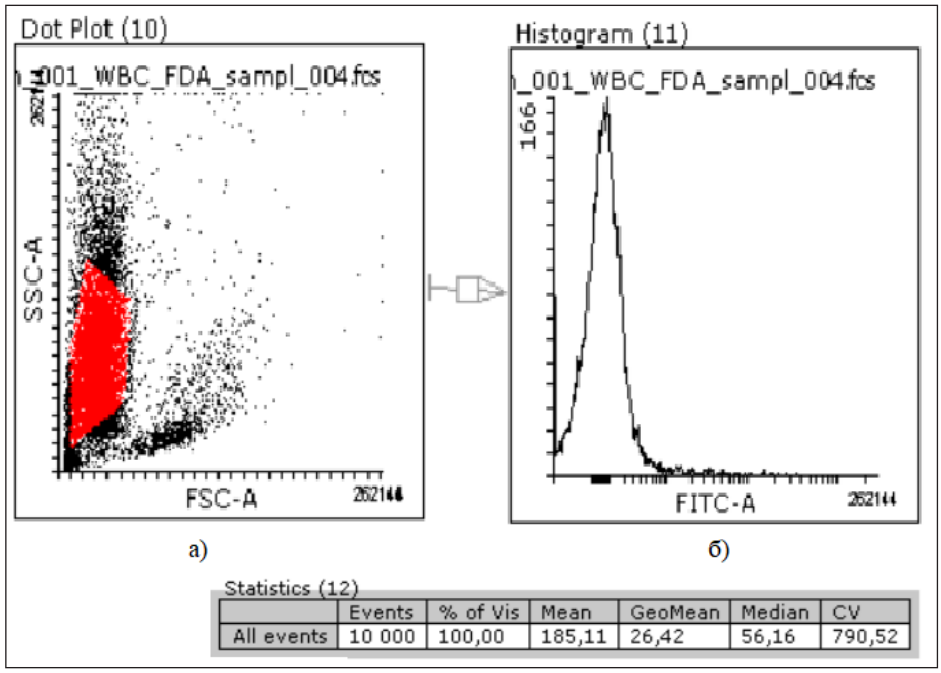

Fig. 3. Representative cytogram (A) and histogram SSC/FL1 (2,7 – dichlorofluorescein) (B) of leukocytes of rat No. 4 of group 3. Mean fluorescence intensity of DCF in leukocytes is 185.11.

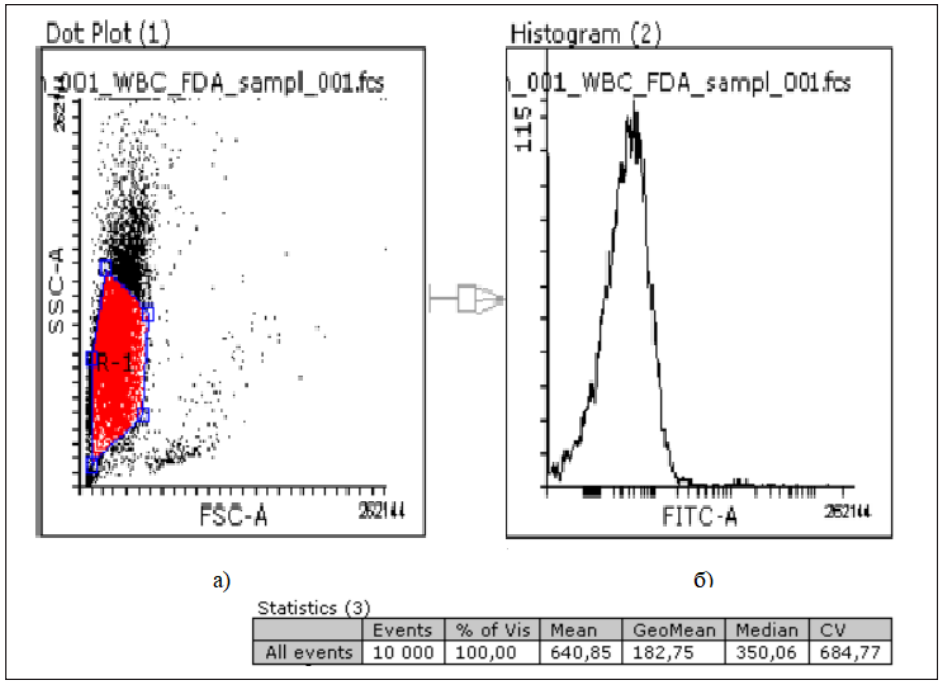

Fig. 4. Representative cytogram (A) and histogram SSC/FL1 (2,7 – dichlorofluorescein) (B) of leukocytes of rat No. 3 of group 4. Mean fluorescence intensity of DCF in leukocytes is 640.85.

fluorescence intensity of DCF in leukocytes in rat No. 6 of group 5.

DISCUSSION

Blood leukocytes provide the body's immune status. The most numerous populations of leukocytes are neutrophils (47-72%), which are microphages and carry out phagocytosis of infectious agents. Morphologically, these cells have a segmented nucleus, an endoplasmic reticulum that does not contain ribosomes. There are few mitochondria in the cells, but they contain many different granules with various enzymes (peroxidases, hydrolases, alkaline phosphatase), proteins – lactoferrin, lysozyme, cationic proteins and others. The source of energy is glucose, which is oxidized in the pentose phosphate pathway, glycolysis reactions (90%) or can be transformed and stored as glycogen in tissues. An increase in the intensity of metabolic pathways for the conversion of glucose accompanies phagocytosis. During phagocytosis, the intensity of oxygen uptake by neutrophils increases with the formation of ROS: 1) superoxide anion formed with the help of

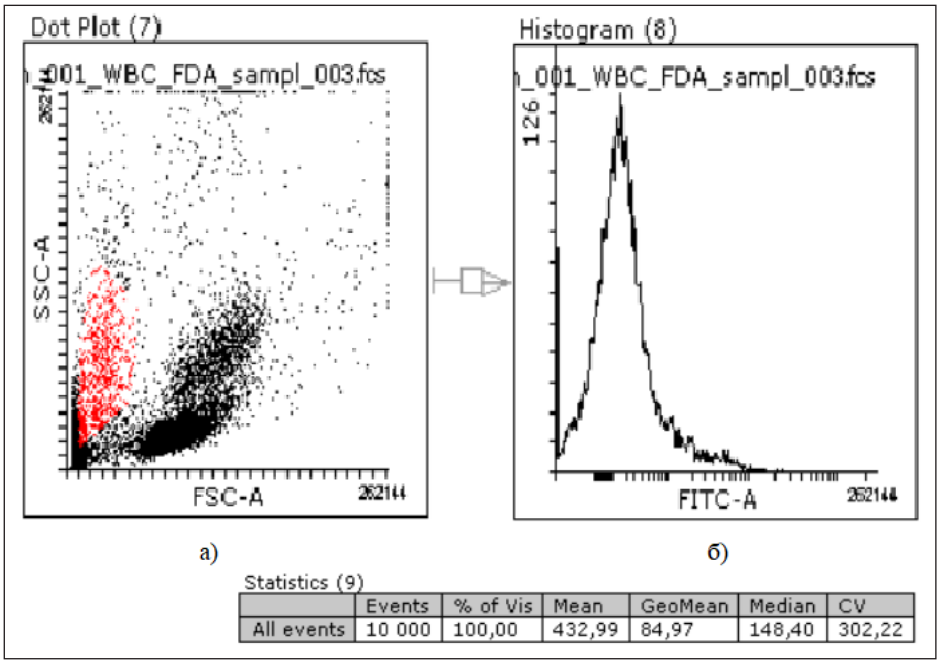

Fig. 5. Representative cytogram (A) and histogram SSC/FL1 (2,7 – dichlorofluorescein) (B) of leukocytes of rat No. 6 of group 5. Mean fluorescence intensity of DCF in leukocytes is 432.99.

oxidase (NADPH-dependent); 2) hydrogen peroxide, the formation of which depends on the activity of NADH-dependent oxidase; 3) hypochloric acid formed under the action of myeloperoxidase (hypochloric acid in the form of an anion reacts with hydrogen peroxide to form singlet oxygen); 4) free radicals, which have a powerful bactericidal effect, are unstable, active and interact with organic substances. Due to the formation of ROS in neutrophils, the bactericidal action of these cells is carried out, as well as the regulation of the functioning of the neutrophils themselves [9]. In the human body, the generation of ROS by neutrophilic leukocytes can have not only physiological or homeostatic, but also pathological significance [10].

ROS play a vital role in ovarian physiological activity as a secondary messenger for cellular signaling and are involved in the regulation of the ovarian cycle, including in meiosis, ovulation, corpus luteum maintenance, and regression [11].

Excessive generation of ROS and violation of the activity of antioxidant protection are the cause of oxidative stress. The latter, as is known, manifests itself as free-radical oxidation of lipids, proteins, and nucleic acids, i.e., initiates the lipid, protein, and nucleic mechanisms of cellular damage. Oxidative stress is the cause of the development of ovarian diseases including age-related ovarian dysfunction, ovarian cancer, PCOS, ovarian endometriosis [11, 12]. Overproduction of ROS may damage the oocytes and impair their fertilization capacity [13].

In this study, the author determined the excessive generation of ROS by leukocytes in experimental PCOS. Excessive production of ROS, from our point of view, is due to neutrophilic leukocytes, since these cells account for the largest percentage in the leukocyte formula. Excessive generation of ROS and the development of oxidative stress have also been noted in numerous studies by various scientists conducted on clinical and experimental material [14].

Cold exposure is one of the stressogenic factors. Literature data about the influence of cold on the processes of ROS generation and the occurrence of oxidative stress are debatable and contradictory. According to some scientists, cold exposure results in an elevation of metabolic rate in mammals, an imbalance in the antioxidant defense system, increased ROS production and oxidative stress. Elevation of ROS production correlated with an increase in energy generation [15]. The results of some studies indicate the activation of the antioxidant system (enzymatic and/or non-enzymatic) and a decrease in the production of ROS under conditions of cold exposure [16-18]. Contradictory data from the literature, from our point of view, are mainly caused by different characteristics of the cold factor (duration of action, degree of temperature reduction, etc.).

Our experimental study showed that the use of intermittent cold exposure (4 hours every day at a temperature of +4°C for 25 days) in rats with PCOS led to the normalization of the rate of ROS production by leukocytes. Taking into account the importance of hyperproduction of ROS in the etiopathogenesis of PCOS, the results allow us to recommend the intermittent cold exposure as one of the methods of prevention and treatment of the above ovary pathology.

In the literature of recent years, there are studies that use a technique similar to ours for modeling the PCOS and prove the effectiveness of using the cold exposure, aspirin, glutamine, vitamin D, vitamin C, marjoram, metformin, transplantation of brown adipose etc. as methods of treatment of this pathology [19-23]. In these studies, the effectiveness of the treatment methods was proven by studying various processes and mechanisms, including the oxidant and antioxidant systems.

CONCLUSIONS

The experimental study revealed an intracellular excessive production of reactive oxygen species by leukocytes in polycystic ovary syndrome. The use of intermittent cold exposure normalized the production of reactive oxygen species by leukocytes in rats with polycystic ovary syndrome. The effectiveness of intermittent cold exposure, proven by the authors, allows recommending its use as one of the methods of prevention and treatment of the polycystic ovary syndrome.

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Conflict of Interests
The Authors declare no conflict of interest.

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