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Original article

Synergistic protective effects of α-tocopherol and zinc sulfate on superoxide dismutase activity and p53 expression in ovarian granulosa cells of lead-exposed female Wistar rats (*Rattus norvegicus*)

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ABSTRACT

This study aimed to evaluate the protective effects of α-tocopherol and zinc sulfate on oxidative stress parameters in ovarian granulosa cells of female Wistar rats (*Rattus norvegicus*) exposed to lead acetate ($Pb(C_2H_3O_2)_2$). A total of 25 rats were randomly divided into five groups: Control (C), lead exposure only (T0), α-tocopherol + lead (T1), zinc sulfate + lead (T2), and α-tocopherol + zinc sulfate + lead (T3). Treatments were administered orally for 21 days. Superoxide dismutase (SOD) levels were measured spectrophotometrically, and p53 expression was analyzed using immunohistochemistry. The results showed that SOD levels significantly decreased in group T0 compared to the control ($p < 0.05$), while treatment with either α-tocopherol (T1), zinc sulfate (T2), or their combination (T3) significantly improved SOD levels compared to T0. Moreover, p53 expression was markedly elevated in T0, indicating enhanced oxidative stress and potential apoptosis, whereas all antioxidant-treated groups showed reduced p53 expression, with T3 demonstrating values comparable to the control group. In conclusion, the combination of α-tocopherol and zinc sulfate provided a synergistic antioxidant effect, effectively enhancing SOD levels and suppressing p53 expression in granulosa cells of lead-exposed rats. This suggested a promising therapeutic potential of these compounds in mitigating lead-induced ovarian toxicity.

Keywords: α-tocopherol, granulosa cells, lead acetate, oxidative stress, p53, zinc sulfate

INTRODUCTION

Lead (Pb) is an environmental hazard known for its toxicity and carcinogenic effects, along with its ability to biomagnify and bioaccumulate. It is frequently detected in environmental media such as water, fruits, and vegetables. High levels of lead could adversely affect various organs (Collin *et al*., 2022). Lead could permeate biological membranes, leading to accumulation in soft tissues like those in the ovaries (Osowski *et al*., 2023). Prolonged exposure could cause lead buildup in the body, resulting in toxicity that impacted the

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reproductive system, potentially leading to issues like infertility, miscarriage, premature birth, fetal demise, and diminished fertility (Kumar *et al*., 2018; Dumitrescu *et al*., 2015). Lead ions possessed lipophilic characteristics, facilitating their penetration of cells through passive diffusion (Virgolini *et al*., 2021), thereby degrading to release Pb2+ ions. Pb2+ ions interacted with ligands present in enzyme proteins by substituting endogenous ions, forming metalloenzyme bonds that led to reduced enzyme activity (Cangelosi, 2017).

A study conducted on 15 cows through fecal examinations revealed an average lead concentration of 5.5651 ppm, indicating lead contamination in cow feces (Salundik *et al*., 2012). According to the World Health Organization (WHO, 2022), based on data from 2019 in the report titled Public Health Impact of Chemicals: Knowns and Unknowns, lead exposure was responsible for an estimated 21.7 million disabilities and deaths globally due to its long-term health effects. The body's defense system, including endogenous antioxidants such as catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD), could counteract oxidative stress caused by free radicals triggered by lead; however, the body's ability to manage free radicals was limited, necessitating supplementation with exogenous antioxidants (Jomova *et al*., 2023). Non-enzymatic exogenous antioxidants, such as vitamin C, vitamin E, and beta-carotene, could be used to protect cells from oxidative stress; however, these antioxidants had certain limitations. Vitamin C and beta-carotene were easily damaged and highly prone to oxidation (Pruteanu *et al*., 2023). Antioxidants were essential substances in the body to neutralize free radicals and prevented damage caused by lead. They functioned by donating electrons to free radicals, thereby halting cellular destruction (Jena *et al*., 2023).

Methods to enhance antioxidant levels in the body included the intake of exogenous antioxidants from dietary sources, such as α-tocopherol (Lobo, 2014). According to research by Saleh (2015), α-tocopherol had beneficial effects in female reproductive physiology, particularly concerning treatments that induced oxidative stress. The use of vitamin E as a source of α-tocopherol alleviated lead toxicity. α-Tocopherol exhibited anticoagulant effects that ensured a normal blood supply to the ovarian follicles (Hamed *et al*., 2014). Additionally, α-tocopherol provided protective effects by neutralizing free radicals, thereby preventing cellular damage to ovarian tissue exposed to lead (Beredugo *et al*., 2022). Furthermore, the administration of α-tocopherol significantly increased the activity of SOD when exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Wurlina *et al*., 2022).

Zinc was an essential component in several processes that regulated germ cell growth, fertility, and successful fertilization. Zinc played a pivotal role in controlling ovarian function, follicular development, and assisting in oocyte maturation (Garner *et al*., 2021). Moreover, zinc was involved in hormonal metabolism within the female reproductive system and protected lymphocytes from damage caused by free radicals, as evidenced by increased SOD enzyme activity (Prasad and Bao, 2019). Redox balance also required the role of zinc as an antioxidant, providing protective effects against reactive oxygen species (ROS) and working synergistically with other antioxidants such as α-tocopherol (Nasiadek, 2020).

In light of the aforementioned background, there was a need for research exploring the protective mechanisms of α-tocopherol and zinc sulfate on the levels of SOD and the expression of p53 in granulosa cells of rats (*Rattus norvegicus*) exposed to lead acetate.

MATERIALS AND METHODS

This research was conducted at the Animal Laboratory of the Faculty of Health, Medicine, and Life Sciences, Universitas Airlangga, in Banyuwangi, from August to September 2022. Data observations were carried out at the Instrument Laboratory of the same faculty in

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Banyuwangi, while histopathological preparations and superoxide dismutase (SOD) measurements were performed at the Satwa Sehat Indonesia Laboratory in Malang. Prior to the study, medical ethical testing was conducted to ensure that all procedures involving the experimental animals adhered to standard operating protocols. The ethical review was carried out at the Faculty of Dental Medicine, Universitas Airlangga, Campus C, Surabaya, under certificate number 884/HRECC/XII/2022.

In this study, the experimental unit consisted of 25 female Wistar strain rats (*Rattus norvegicus*), aged 4 months, with body weights ranging from 250 to 300 grams, obtained from the Faculty of Pharmacy, Universitas Airlangga, Surabaya. During the study period, the rats were provided with commercial feed and drinking water ad libitum. The rats were acclimatized for 7 days before being randomly divided into five groups. Group C (control) received 0.5 mL of distilled water, followed by 0.5 mL of distilled water and 0.5 mL of corn oil four hours later. In group T0, the rats received 0.5 mL of distilled water and 0.5 mL of corn oil, followed by lead acetate at a dose of 1.5 mg/rat in 0.5 mL of distilled water four hours later.

In group T1, the rats were given α-tocopherol at a dose of 100 mg/Kg bw in 0.5 mL of corn oil, followed by lead acetate at a dose of 1.5 mg/rat in 0.5 mL of distilled water four hours later. In group T2, the rats received zinc sulfate at a dose of 0.54 mg/rat in 0.5 mL of distilled water and 0.5 mL of corn oil, followed by lead acetate at 1.5 mg/rat in 0.5 mL of distilled water four hours later. Group T3 rats received zinc sulfate at 0.54 mg/rat in 0.5 mL of distilled water, along with α-tocopherol at a dose of 100 mg/Kg bw in 0.5 mL of corn oil, followed by lead acetate at a dose of 1.5 mg/rat in 0.5 mL of distilled water four hours later. These treatments were administered orally every day for 21 days.

On the 22nd day, at the conclusion of the treatment period, the experimental animals were handled using gloves for the collection of final body weight data through weighing. The initial and final body weight data were utilized to obtain homogeneous results and ensure normal data distribution. After weighing, each female rat was euthanized by cervical dislocation. Following euthanasia, the ovaries were excised for the assessment of SOD level and p53 expression analysis in granulosa cells. The ovaries were then rinsed with physiological saline and placed in a 10% formalin solution for further examination.

The principle of measuring SOD with Nitroblue Tetrazolium (NBT) involved a reaction between xanthine and xanthine oxidase, leading to the generation of superoxide radicals. Subsequently, these superoxide radicals reacted with NBT, resulting in the formation of purple-formazan. The SOD present in the tissue competed with NBT for reaction with the superoxide radicals, thereby inhibiting the formation of the colored compound. The materials and equipment used included phosphate-buffered saline, xanthine (25 mm, 100 µL), xanthine oxidase (1 unit, 100 µL), NBT (25 units, 100 µL), a balance, petri dishes, a mortar, and Eppendorf tubes (Erdemli *et al*., 2019).

Initially, the ovaries were weighed to a maximum of ≤ 100 mg for each group, and then placed in a dish containing phosphate-buffered saline (PBS) to remove blood and blood clots. The ovaries were then finely chopped and homogenized using a mortar. Afterward, 1 mL of PBS was added to the sample, which was transferred to an Eppendorf tube and vortexed. Xanthine (25 mm, 100 µL), xanthine oxidase (1 unit, 100 µL), NBT (25 units, 100 µL) and the sample (100 µL) were added to the tube. The mixture was vortexed again and incubated at room temperature (30°C) for 30 minutes. Following incubation, the mixture was centrifuged at 3500 rpm for 10 minutes at room temperature. Absorbance was measured using a spectrophotometer at a wavelength of 580 nm (Erdemli *et al*., 2019).

The level of SOD, as an endogenous antioxidant enzyme, was measured in the ovaries of rats (*Rattus norvegicus*) by recording the absorbance using spectrophotometric methods at

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a wavelength of 580 nm. The principle of measuring SOD involved the reaction between xanthine and xanthine oxidase, which produced superoxide radicals. These radicals then reacted with NBT to form purple formazen. In the tissue, SOD competed with NBT for reaction with the superoxide radicals, thereby inhibiting the formation of color (Erdemli *et al*., 2019).

Immunohistochemical examination method

Paraffin blocks containing tissue were sectioned to a thickness of 3-4 µm and placed on polylysine-coated slides. The slides were then incubated overnight at 45°C in an incubator. Deparaffinization was performed using xylene three times for 3 minutes each, followed by washing with PBS three times for 5 minutes each. The sections were immersed in 3% hydrogen peroxide (H_2O_2 in methanol) for 20 minutes, rinsed with distilled water, and washed with PBS three times for 5 minutes each. Antigen retrieval was performed by immersing the preparations in citrate buffer (pH 6.0) in a microwave. After cooling for 20-30 minutes, the sections were washed with PBS three times for 5 minutes each.

The preparations were incubated in normal mouse serum for 5 minutes. The normal mouse serum was removed without washing, and the preparations were treated with primary monoclonal anti-p53 antibody for 60 minutes or overnight in a refrigerator at 8°C. Afterward, the sections were washed with PBS three times for 5 minutes each and incubated with biotinylated secondary antibody for 5 minutes each. The sections were then washed again with PBS three times, for 5 minutes each and treated with streptavidin-peroxidase for 5 minutes, followed by three PBS washes, each for 5 minutes. After rinsing with tap water for 10-15 minutes, the sections were counterstained with hematoxylin for 3-4 minutes and rinsed again with tap water for 10-15 minutes.

Graded dehydration was performed using absolute ethanol, 95% ethanol, 80% ethanol, and xylene twice, followed by mounting under coverslips. The expression of p53 in ovarian cells from all experimental groups was analyzed using a light microscope (Nikon Eclipse E200) at a magnification of 400x. Cells expressing p53 appeared brown in the cytoplasm, while those without p53 expression exhibited a purple tint in the cell membrane. The assessment of p53 expression was performed semi-quantitatively using the Remmele scoring index method, as outlined in Table 1.

Table 1 Remmele index scale (Fedchenko and Reifenrath, 2014)

A (percentage of positive cells)	B (intensity of color reaction)
score 0: no positive cells	score 0: no color reaction (not stained)
score 1: positive cells less than <10%	score 1: weak color intensity (not continuous)
score 2: positive cells 11%-50%	score 2: medium color intensity (continuous incomplete)
score 3: positive cells 50%-80%	score 3: high color intensity (continuous and complete
score 4: positive cells > 80%	

Data analysis

The data obtained from the administration of α-tocopherol, zinc sulfate, their combination, and exposure to lead acetate were analyzed using the Statistical Product and Service Solutions (SPSS) software version 20 for Windows. The analysis was conducted using One-Way Analysis of Variance with a significance level set at $p < 0.05$. To determine the differences between groups for each treatment variable, the Least Significant Difference test and Duncan's test were employed.

RESULTS

The SOD level in the group T0 were significantly lower ($p < 0.05$) than that in the control (C) group. In contrast, the SOD levels in

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the T1, T2, and T3 groups were significantly higher ($p < 0.05$) compared to the T0 group. However, the SOD levels in the T3 group remained significantly lower ($p < 0.05$) than that in the control group. Regarding p53 expression, the T0 group exhibited significantly higher level ($p < 0.05$) compared to the control group. Treatments administered in the T1, T2, and T3 groups significantly reduced p53 expression ($p < 0.05$) compared to the T0 group. Notably, the p53 expression level in the T3 group was not significantly different ($p > 0.05$) from that observed in the control group (Table 2).

Table 2 SOD levels (Mean ± SD) and p53 expression (Mean ± SD) after administration of zinc, α-tocopherol, and lead acetate

	SOD levels	p53 expression
C	53.52 ± 8.60 ^{d}	1.60 ± 0.24 ^{a}
T0	21.35 ± 2.59 ^{a}	5.57 ± 0.84 ^{c}
T1	27.75 ± 1.72 ^{b}	2.91 ± 0.77 ^{b}
T2	29.57 ± 3.45 ^{bc}	2.33 ± 0.26 ^{ab}
T3	35.01 ± 2.64 ^{c}	1.97 ± 0.31 ^{a}

Superscripts (a, b, c, d) indicate significant differences ($p < 0.05$); C: rats received distilled water, followed four hours later by an additional dose of distilled water and corn oil; T0: rats received distilled water and corn oil, followed by lead acetate; T1: rats received α-tocopherol (100 mg/kg body weight in corn oil) and distilled water, followed by lead acetate; T2: rats received zinc sulfate (0.54 mg/rat in distilled water) and corn oil, followed by lead acetate.; T3: rats received a combination of zinc sulfate and α-tocopherol, followed by lead acetate; lead acetate (1.5 mg/rat in distilled water) was administered four hours after the other treatments; all treatments were administered orally in a volume of 0.5 mL per day for 21 consecutive days.

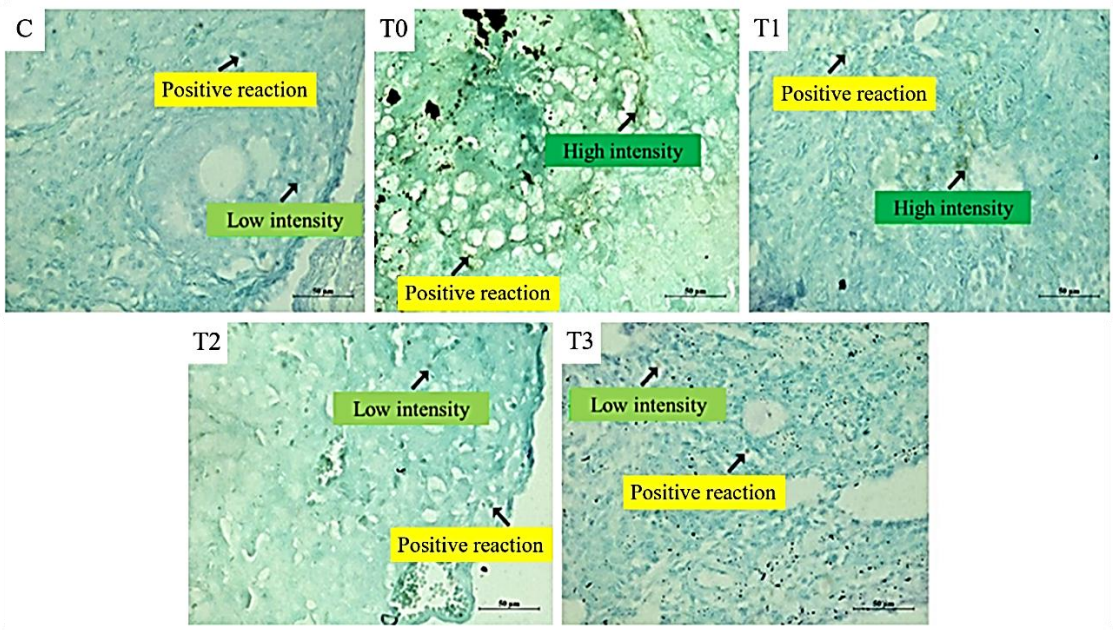

Figure 1 Histopathological representation of p53 expression in granulosa cells of Wistar strain rats (*Rattus norvegicus*). The examination was conducted using Nikon Eclipse E200 microscope at a magnification of 400x; C: granulosa cells from the control group exhibited the lowest level of p53 expression; T0: granulosa cells from the untreated group exhibited the highest p53 expression; T1: granulosa cells from the first treatment group showed reduced p53 expression compared to T0; T2: granulosa cells from the second treatment group exhibited lower p53 expression than both T0 and T1; T3: granulosa cells from the third treatment group showed p53 expression level slightly lower than T2 and comparable to the control group. The treatment

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protocol was as follows: group C received distilled water, followed four hours later by an additional dose of distilled water and corn oil; group T0 received distilled water and corn oil, followed by lead acetate; group T1 received α-tocopherol (100 mg/kg body weight in corn oil) and distilled water, followed by lead acetate; group T2 received zinc sulfate (0.54 mg/rat in distilled water) and corn oil, followed by lead acetate.; group Group T3 received a combination of zinc sulfate and α-tocopherol, followed by lead acetate; lead acetate (1.5 mg/rat in distilled water) was administered four hours after the other treatments; all treatments were administered orally in a volume of 0.5 mL per day for 21 consecutive days.

DISCUSSION

The administration of lead to female rats (*Rattus norvegicus*) showed adverse effects on SOD levels. This finding corroborated the study by Al Aziz and Marianti (2014), which suggested a reduction in SOD activity when lead acetate degraded inside cells, releasing Pb2+, which subsequently inhibited SOD activity by binding to proteins and replacing endogenous ions (Cu/Zn) within metalloenzymes, thereby rendering the SOD enzyme inactive as an antioxidant (Shalan, 2024). Uchewa *et al*. (2019) reported that exposure to lead acetate at a dosage of 1.5 mg/rat for 21 days led to a decrease in SOD levels and caused edema and necrosis in granulosa cells of ovarian follicles. The decline in the activity of SOD, CAT, and GPx contributed to the increased presence of ROS such as hydrogen peroxide (H_2O_2) and superoxide ($O2^-$), which induced oxidative stress in granulosa cells (Ansar *et al*., 2019).

Cells under oxidative stress may suffered membrane damage and underwent cell death, indicated by elevated malondialdehyde (MDA) levels and decreased SOD activity (Jena *et al*., 2023). Under oxidative stress conditions, superoxide radicals ($O2^-$) and hydrogen peroxide (H_2O_2) interacted to form hydroxyl radicals (OH^-) (Haber-Weiss reaction). Additionally, hydrogen peroxide (H_2O_2) reacted with Fe2+/Cu2 to produce OH^- (Fenton reaction) (Virgolini *et al*., 2021). The resulting hydroxyl radicals (OH^-) migrated to the nucleus of the cell, causing damage to genetic material and DNA fragmentation, which triggerred the release of p53. Elevated p53 levels activated Bcl-2 and Bcl-x, leading to a reduction in Bax, which facilitated the release of cytochrome c, culminating in apoptosis, or programmed cell death (Xu *et al*., 2007).

Antioxidants were essential substances in the body for neutralizing free radicals and preventing damage, thereby halting cellular destruction (Jena *et al*., 2023). α-Tocopherol had protective effects by eliminating free radicals, thus preventing cellular damage to ovarian tissue (Beredugo *et al*., 2022). This assertion was supported by the results from treatment group which received the antioxidant α-tocopherol and showed improvements compared to those without treatment. These findings were further corroborated by the research of Wurlina *et al*. (2022), which demonstrated that administration of α-tocopherol significantly increased SOD activity. Additionally, research by Prasad and Bao (2019) highlighted the critical role of zinc in the repair of reproductive organs, as it was a component or cofactor of SOD, an enzyme that protected cells from oxidative stress-induced damage. Zinc activated enzymatic systems that neutralized free radicals. The combination of α-tocopherol and zinc sulfate administered to rats (*Rattus norvegicus*) exposed to lead acetate resulted in enhanced SOD levels. This enhancement was attributed to the synergistic effect of both α-tocopherol and zinc sulfate in increasing SOD activity. This study demonstrated that lead administration in female rats (*Rattus norvegicus*) negatively impacted p53 expression. This finding was consistent with the research by Ali (2018), which showed that lead acetate induced cytotoxicity through the production of ROS that caused DNA damage (Xu *et al*., 2007). This damage led to an increase in the expression of p53, Bax, and Bcl2 in response to DNA damage, ultimately resulting in

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apoptosis. Moreover, exposure to lead at a dose of 1.5 mg/day for 21 days resulted in reproductive disturbances and induced an increase in ROS levels (Uchewa *et al*., 2019).

In the study by Hamed *et al*. (2014), it was reported that α-tocopherol possessed abilities related to anti-apoptotic reactions, protective effects, and therapeutic properties through the stimulation and enhancement of hormone levels. This assertion aligned with the results of this study, which received the antioxidant α-tocopherol, showing improvements compared to the treatment group of lead acetate only. This was supported by the research conducted by Lebold *et al*. (2014), which indicated that α-tocopherol, a fat-soluble antioxidant, inhibited lipid peroxidation by breaking the chain of free radicals, thereby disrupting the sequence of lipid peroxidation. The study by Formigari *et al*. (2013) demonstrated that zinc modulated mitogenic activity through the p53 signaling pathway. Intracellular free zinc modulated the activity and stability of p53. Zinc imbalance led to a higher prevalence of p53 mutations, thereby triggering apoptosis. The treatment group which received zinc sulfate as an antioxidant and exhibited the second highest average p53 expression score after treatment group received α-tocopherol and zinc sulfate. This was further supported by research from Marriero *et al*. (2017), which indicated that zinc also induced the synthesis of metallothionein, a protein that effectively reduced hydroxyl radicals and sequesters ROS produced under stress conditions. The combination of α-tocopherol and zinc sulfate on p53 expression in the granulosa cells of rats (*Rattus norvegicus*) exposed to lead acetate reduced p53 expression. This effect was attributed to the synergistic interaction between the two antioxidants, α-tocopherol and zinc sulfate, in reducing p53 expression.

CONCLUSIONS

The administration of a combination of α-tocopherol (100 mg/Kg bw) and zinc sulfate (0.54 mg/rat) has been shown to be highly effective in improving oxidative stress parameters in female rats (*Rattus norvegicus*) exposed to lead acetate. Specifically, the treatment significantly increased SOD levels and reduced the expression of p53 in ovarian granulosa cells. These findings demonstrate that combined antioxidant therapy using α-tocopherol and zinc sulfate exerts a synergistic protective effect, mitigating oxidative damage and apoptotic signaling in lead-induced reproductive toxicity. This supports the potential role of these antioxidants as therapeutic agents in preserving female reproductive health under heavy metal exposure conditions.

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AUTHOR'S CONTRIBUTIONS

Iqbal Dwi Warsito (IDW), Suherni Susilowati (SS), Ragil Angga Prastiya (RAP), Erma Safitri (ES), Maya Nurwartanti Yunita (MNY), Amung Logam Saputro (ALS)

IDW, SS, RAP: conceived the idea, designed the mainframe of this manuscript, acquisition, analysis and interpretation of data, and manuscript drafting. ES, MNY, ALS: critically read and revised the manuscript for intellectual content. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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