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N-Acetylcysteine improves oocyte quality through modulating the Nrf2 signaling pathway to ameliorate oxidative stress caused by repeated controlled ovarian hyperstimulation

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ABSTRACT

Context. *N*-acetyl-cysteine (NAC) is a potent antioxidant that can be used for many gynecological diseases such as polycystic ovary syndrome and endometriosis. Controlled ovarian hyperstimulation (COH) is a critical step in infertility treatment. Our previous clinical studies have shown that repeated COH led to oxidative stress in follicle fluid and ovarian granulosa cells. **Aims.** In this study, we investigated whether NAC could inhibit oxidative stress in mice caused by repeated COH and improve the mitochondrial function of oocytes. **Methods.** Female Institute of Cancer Research (ICR) mice were randomly assigned into three groups: normal group, model (repeated COH) group, NAC group. We examined the morphology, number and quality of mitochondria. The mechanism of regulation of nuclear factor erythroid 2-related factor 2 (Nrf2) by NAC to ameliorate oxidative stress was also investigated. **Key results.** Repeated COH caused oxidative damage in ovaries and oocytes and decreased oocyte quality, while NAC prevented oxidative damage and increased oocyte mitochondrial function. In *in vitro* experiments, it was verified that NAC can promote the nuclear translocation of Nrf2, which transcriptionally activates the expression of superoxide dismutase and glutathione peroxidase, which removed excessive reactive oxygen species that causes mitochondria damage. **Conclusions.** The results suggest that NAC raises mitochondrial function in oocytes and improves oocyte quality through decreasing oxidative stress in mice with repeated COH. The underlying mechanism is related to the regulation of the Nrf2 signaling pathway. **Implication.** This study provides a meaningful foundation for the future clinical application of NAC during repeated COH.

Keywords: assisted reproductive technology, controlled ovarian hyperstimulation (COH), infertility, mitochondria, *N*-acetylcysteine (NAC), Nrf2, oocyte quality, oxidative stress.

Introduction

According to epidemiological infertility surveys, there are more than 1.86 million infertile couples worldwide, with a global average prevalence of about 9% (Izzo *et al.* 2015). Controlled ovarian hyperstimulation (COH) is a technique that simultaneously promotes the development of multiple follicles in the ovaries by injection of exogenous hormones and is a widely used treatment in *in vitro* fertilisation-embryo transfer (IVF-ET). However, the pregnancy rate in a single IVF cycle is about 29.5%, resulting in the need for multiple rounds of COH (Smith *et al.* 2015), which can lead to decreased ovarian responsiveness and/or result in impaired follicular development (Martin-Johnston *et al.* 2009). Therefore, there is an urgent need to improve the quality and developmental potential of the oocytes of subjects undergoing repeated COH.

Oxidative stress refers to imbalance of the redox system, with free radicals such as reactive oxygen species (ROS) accumulating to a level that exceeds the clearance capability of the endogenous antioxidant system (Schieber and Chandel 2014). It has been revealed that ROS play a fundamental role in the female reproductive system,

especially during folliculogenesis, oocyte maturation, and embryonic development (Agarwal *et al.* 2012). However, accumulation of ROS results in oxidative stress and several reproductive disorders (Costello *et al.* 2007; Augoulea *et al.* 2009). Mitochondria are the main sites of ROS production (Bentov and Casper 2013). The excessive accumulation of ROS-induced mitochondrial damage causes a rise in oxidative products, and the relatively high sensitivity of mitochondrial DNA (mtDNA) expression leads to further destruction of the organelle. This state poses a threat to the integrity of the mitochondrial electron transport chain, resulting in less ATP production, a decrease in mitochondrial membrane potential (MMP), higher oxidant levels, and higher ROS levels in oocytes (Shigenaga *et al.* 1994).

Upon oxidative stress, the nuclear factor erythroid 2-related factor 2/antioxidant response element (Nrf2/ARE) pathway, one of the important anti-oxidant signaling pathways, is activated. Phosphorylated Nrf2 binds to the ARE sequence and transactivates the expression of downstream antioxidant proteins (Shaw and Chattopadhyay 2020), including heme oxygenase-1 (HO-1), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) (Adelusi *et al.* 2020). Nrf2 knockout mice are more susceptible to induced ovarian failure (Hu *et al.* 2006). Nrf2 protects against atrazine-induced oxidative damage to the ovaries and protects the reproductive function of female rats (Zhao *et al.* 2014). Repeated COH adversely affects mouse oocyte quality by increasing oxidative stress (Miyamoto *et al.* 2010). Elucidation of the mechanism by which repetitive COH causes oxidative stress is of great importance.

N-Acetylcysteine (NAC), a derivative of L-cysteine, is a frequently used antioxidant that can scavenge the generated ROS in numerous diseases (Yamada *et al.* 2010; Sharma *et al.* 2016) and xenobiotics (Subramanian *et al.* 2011). NAC has antioxidant functions as a precursor of glutathione (GSH) (Pei *et al.* 2018). NAC can improve embryo quality after intracytoplasmic sperm injection into oocytes (Cheraghi *et al.* 2016), delay oocyte aging in mice (Liu *et al.* 2012), and protect porcine oocytes against oxidative stress and apoptosis induced by heat stress (Hu *et al.* 2020). NAC can also increase SOD activity and preserve DNA integrity in human ovarian tissue by reducing the degree of DNA fragmentation after cryopreservation (Fabbri *et al.* 2015). These observations suggest that NAC can improve oocyte quality. However, there have been few studies regarding the beneficial effects of NAC on oocytes during repeated COH.

Thus, we speculated that NAC could improve ovulation during repeated COH. Consequently, we investigated the degree of oxidative stress *in vivo* and *in vitro*, as well as the beneficial effects of NAC on oocyte maturation and the underlying mechanisms.

Materials and methods

Animal model and treatment

Animal housing and procedures used in this study were approved by the local Animal Care and Use Committee of Hebei University of Chinese Medicine (No. DWLL2018034), in accordance with the guidelines of the National Research for the Care and Use of laboratory animals. ICR female mice (6–7 weeks and 24–26 days old) and male mice (7–8 weeks old) were purchased from Charles River Experimental Animal Center (Animal Certificate No. SCXK 2021-0006, Beijing, China). The mice were housed in a temperature-controlled room (23±2°C) with 12 h darkness-light cycles, with free access to pellet food and water throughout the study. The estrous cycle was monitored by vaginal smear.

The procedure of COH was modified as described (Kalthur *et al.* 2016). In brief, mice at dioestrus were injected intraperitoneally (IP) with 10 IU of gestational mare serum gonadotropin (PMSG, G4877, Sigma), followed by IP injection of 10 IU human chorionic gonadotropin (hCG, Sigma, 230734) 48 h later. As shown in Fig. 1, the mice were randomly allocated into three groups: Normal, model, and NAC. In the model group, the mice received three COH procedures. In the NAC group, mice were exposed to NAC

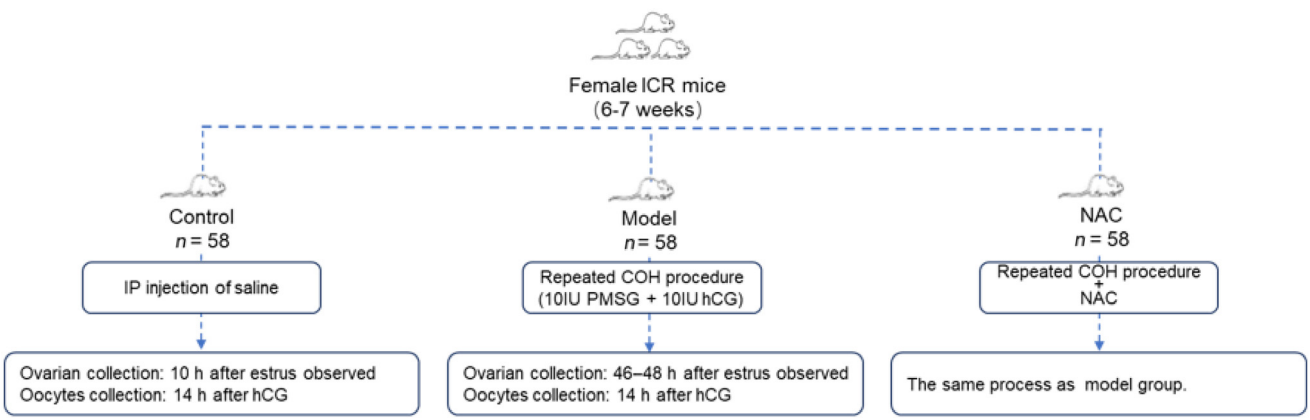

Fig. 1. Flow chart of animal handling and sampling. Female ICR Mice were randomly allocated into three groups: normal, model, and NAC.

(15 mg/100 g, A7250, Sigma) by oral administration during three COH procedures. In the normal group, mice received saline IP injections for the same time and volume as those applied in the model group. All the procedures were repeated three times with an interval of 4 days. To observe the pregnancy rate, the female mice were kept overnight with males (1:1) after receiving hCG or saline. Mice with vaginal plugs are recorded as the first day of pregnancy (D1). The pregnancy rate was calculated at D8. Fourteen hours after hCG injection, the mice were subjected to anesthesia with 3% isoflurane and the blood was collected from the orbit. The mice were then sacrificed by CO_2 asphyxiation and both ovaries and oviducts were surgically removed. The cumulus-oocyte complexes (COCs) were collected from the oviduct ampulla. After incubation with 1 mg/mL hyaluronidase, oocytes were collected and centrifuged at 7000*g* for 10 min. The oocytes were picked under the microscope, the discharge rate of the first polar body (PB1) was observed and counted.

In vitro maturation (IVM) of oocytes

After the ICR female mice (24–26 days) were injected with PMSG for 48 h, the ovaries were isolated and the larger follicles were picked under a stereo microscope to obtain COCs. The COCs were transferred into IVM culture medium (α-MEM medium + 10% FBS + 100 mIU/mL rFSH + 1.5 U/mL hCG + 3 ng/mL EGF + 100 IU/mL penicillin + 100 μg/mL streptomycin) and incubated at 37°C, 5% CO_2. 100 μM H_2O_2 was added to the model group and 10 μM NAC was added to the NAC group. The COCs were cultured for 6 h and oocytes were collected for testing after hyaluronidase digestion.

Morphological observation

Mouse ovaries were fixed with 4% paraformaldehyde and the tissue was embedded in paraffin. 5 μm-thick sections were dewaxed to water, sequentially stained with hematoxylin and eosin, dehydrated and sealed, microscopically examined and images were acquired for analysis. After hematoxylin-eosin (HE) staining, ovarian tissues were observed under the optical microscope to evaluate structural characteristics and follicle number.

ELISA assay

Freshly collected blood samples from the mice were stood at room temperature for 2 h and centrifuged (1000*g*, 4°C) for 10 min. Serum hormones follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E2) were detected using the ELISA kit (CSB-E06871m, CSB-E12770m, CSB-E07280m, Cusabio, Wuhan, China) in strict accordance with the instructions. The absorbance was measured at 450 nm. The ovarian and oocytes sample lysis fluid were diluted to the optimal concentration to meet the standard curve for detection. Biomarkers related to oxidative stress including total antioxidant capacity (T-AOC), 8-hydroxy-2 deoxyguanosine (8-OH-dG), advanced oxidation protein products (AOPP), molondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) were detected by ELISA assay kit (A015-1-2, A001-3-2, A005-1-2, Jiancheng Bioengineering Institute, Nanjing, China; ab201734, ab242295, ab118970, Abcam, USA) following the manufacturer's instructions. The absorbance values were measured using a microplate reader (VersaMax, Molecular Devices, USA).

Analysis of ROS levels in oocytes.

ROS levels in oocytes were detected using 2,7-dichlorofluorescein diacetate (DCFH-DA) assay (S0033, Beyotime, China). Briefly, oocytes were incubated 30 min with 10 μM DCHF-DA in prewarmed M16 media droplets maintained at 37°C and 5% CO_2. Oocytes were then washed three times in M16 medium and observed under the green fluorescence filter of a fluorescence microscope (EVOS® FL, Thermo, USA). Fluorescence intensity was estimated using Image J software.

Electron microscopy

Oocytes were prepared for transmission electron microscopy (TEM) to observe mitochondrial ultrastructure. Briefly, oocytes were cut into 70 nm ultrathin sections, stained with uranyl acetate and lead citrate, and then examined by TEM (Hitachi HT7800/HT7700, Japan). Morphology of mitochondrial ultrastructure was determined by electron micrographs at 5000-fold magnification. To assess mitochondrial morphology, five different regions of each oocyte were observed.

Analysis of mitochondrial distribution

Metaphase II (MII) oocytes were stained with a staining kit-CytoPainter Mitochondrial Staining Kit-Red Fluorescence (ab112145, Abcam) to reveal mitochondrial distribution. Briefly, oocytes were transferred to prewarmed droplets of M16 medium supplemented with staining buffer (1:1). Oocytes were incubated at 37°C, 5% CO_2 for 30 min and examined by a laser scanning confocal microscope (Olympus, Japan) equipped with helium-neon lasers at excitation/emission = 585/610 nm. Labeled mitochondria were categorised as homogeneous or heterogeneous as previously described (Torner *et al.* 2004).

Analysis of oocyte MMP

MII oocytes were incubated in complete medium with JC-1-Mitochondrial Membrane Potential Assay Kit (ab113850, Abcam, US) at a concentration of 10 μg/mL at 37°C, 5% CO_2 for 30 min and analysed using a laser scanning confocal microscope (Leica, Germany). The images of oocytes were acquired with both the fluorescein isothiocyanate (FITC) and the rhodamine isothiocyanate (RITC) channel. Acquired

images were analysed using Image-Pro Plus Software. Membrane potential was expressed as the ratio of the intensity of RITC to FITC.

Analysis of oocyte ATP

The ATP content of oocytes was measured using an assay kit (S2007, Beyotime, Shanghai, China) following the manufacturer's instructions and detected with the microplate reader (VersaMax, Molecular Devices, USA). The amount of ATP in oocytes was calculated according to a standard curve of relative luminous intensity.

Assessment of mitochondrial DNA number

The mitochondrial DNA number of oocytes was detected according to the methods previously described (Iwata *et al.* 2011). Briefly, oocyte zona pellucida were digested with 0.5% pronase (Sigma, US), and polar bodies were removed by pipetting. Oocytes were lysed using the QIAamp DNA Micro Kit (QIAGEN). In this study, the PCR products of the corresponding genes were cloned into plasmids using Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA, USA) and the DNA copy number was calculated using the formula DNA copy number (Copies/μL) = $(6.02 \times 10^{23}) \times (ng/\mu L \times 10^{-9})$/(number of DNA bases × 660). The cloned genes were sequenced for confirmation before use. The standard curve was prepared using the standard as a template. Real-time quantitative PCR was conducted using a SYBR Premix Ex Taq II (Takara, Japan) following the manufacturer's instructions. The following primer sequences were used: 5′ ACGAA ATCAA CAACC CCGTA TTAA 3′ and 5′ CTCGG TTATC AACTT CTAGC AGTC 3′.

RNA preparation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRIzol (Invitrogen, USA) and reverse transcribed into cDNA using a reverse transcription system (Invitrogen, USA) following the manufacturer's instructions. We performed real-time PCR analysis with a Platinum SYBR Green qPCR SuperMix UDG Kit (Invitrogen, USA) and an ABI 7500 FAST system, following the manufacturer's instructions. The final data were analysed using $2^{-\Delta\Delta Ct}$. All PCRs were conducted in triplicate. Primer sequences are shown in Table 1.

Western blot analysis

Proteins were extracted from oocytes as described (Wang *et al.* 2021). The same amount of total protein in each well (15–20 μg) was separated by 10% SDS-PAGE and transferred to the PVDF membrane (Millipore, Germany) afterelectrophoresis. The PVDF membrane was blocked in 5% skimmed milk powder in TBST for 2 h and incubated with different primary antibodies overnight at 4°C. The primary antibodies and their concentrations used in this study were 1:400 protein kinase C (PKC) (ab32376, Abcam, USA), 1:200 Nrf2 (ab92946, Abcam, USA), 1:200 kelch-like ECH-associated protein 1 (Keap1) (ab119403, Abcam, USA), 1:400 GSH-Px (ab22604, Abcam, USA), 1:200 MnSOD (ab68155, Abcam, USA). The PVDF membrane was washed with TBST three times and incubated with the secondary antibody (SA00001-2, Protein tech, USA), and incubated at room temperature for 1 h. After the PVDF membrane was washed three times with TBST, antibody-antigen complexes were visualised using a Chemiluminescence Plus Western immunoblot analysis kit (Millipore, USA). The images were collected by chemiluminescence imager (Image Quant LAS 4000, USA) and quantitatively analysed by Image J software.

Immunofluorescence staining

The treated oocytes were fixed with 4% paraformaldehyde for 20 min, dropped with 1% Triton for 15 min, sealed with 10% goat serum for 30 min, and then incubated with Nrf2 antibody (1:150) at 4°C overnight. After the oocytes were incubated with fluorescence-labeled secondary antibodies at room temperature for 2 h, the oocytes were stained with DAPI for 10 min. Finally, it was observed under a laser scanning confocal microscope (Leica, Germany).

Statistical analysis

Statistical differences were analysed using the SPSS ver. 23.0 software (SPSS Inc., Chicago, IL, USA). The normal

Table 1. Primers for oocytes gene expression for transcript levels quantified by quantitative real-time PCR in mice.

Mice mRNA	Forward primer	Reverse primer	Amplified product length (bp)
PKC	CCGTCCTCCATTTCTGTCATTC	TATGCAGCACTTTCTACATGCC	209
Keap1	GCTATGATGGCCACACTTTTCT	GTTGTCAGTGCTCAGGTATTCC	193
Nrf2	GTTGGGGAGCTTGGAGAGAT	ACATGGGGAGTAGATGCTGG	224
MnSOD	TCTTTGGCTCATTGGGTCCT	CAGATAAACAGGGGCTTCGC	213
GSH-Px	GAACCTGACATAGAAACCCTGC	TTCATTAGGTGGAAAGGCATCG	213
GAPDH	GGAGAAAGTGGGGAAAAGCC	GAACAGGGAGGAGCAGAGAG	168

distribution and homogeneity variance were tested. One-way ANOVA was used to compare differences. Specifically, the l.s.d. method was used for data with equal variances, while Dunnett's T3 test was used for data with unequal variances. Categorical variables were compared with the use of chi-square analysis. Data are reported as mean ± s.d. $P < 0.05$ was considered statistically significant.

Results

Effects of NAC on ovarian morphology, the number of follicles, the first polar body discharge rate, serum hormone levels, and the pregnancy rate

To evaluate the effects of NAC on the ovaries and oocytes, we observed ovarian morphology and counted the follicles. Compared with the control group, the ovarian volume was smaller, ovarian interstitial lymphocytes were infiltrated, the number of preantral follicles was lower, the number of atretic follicles was increased, oocyte morphology was altered, and the granulosa cells were disorganised in the repeated COH model group. After NAC administration, the infiltration of ovarian interstitial lymphocytes was alleviated, oocyte morphology was recovered (the oocytes appeared regular and their size was moderately recovered), the number of oocytes was increased, and the granulosa cells were arranged neatly (Fig. 2*a*). As shown in Fig. 2*b*, repeated COH decreased the number of follicles significantly ($P < 0.01$), and NAC increased the number of follicles ($P < 0.05$). MII oocytes were obtained from COCs from the abdomen of the fallopian tube (Fig. 2*c*) and used to calculate the first polar body discharge rate. The first polar body discharge rate was reduced in the model group and elevated in the NAC group (Fig. 2*d*). According to the two-cell, two-gonadotropin theory, normal folliculogenesis in humans requires FSH and LH. LH stimulates theca cells to produce androgens, which in turn are aromatised by granulosa cells to estradiol in response to FSH. Both of them are indicators of the evaluation of ovarian endocrine function. The results showed that E2 levels were decreased after repeated COH ($P < 0.01$) and increased after NAC administration ($P < 0.01$) (Fig. 2*g*). FSH and LH showed no significant difference among groups ($P > 0.05$) (Fig. 2*e, f*), suggesting that NAC affects estrogen levels after repeated COH, which was confirmed by the recovery of follicular morphology and the increase in the number of granulosa cell layers after the application of NAC. We also evaluated the effects of NAC on pregnancy rates. Compared with the normal group, the pregnancy rate was reduced in the model group and significantly increased in the NAC group, suggesting that NAC can improve the pregnancy rate in mice treated with repeated COH (Fig. 2*h*). These results demonstrated that NAC improved ovarian and follicular morphology, increased the follicle count and the first polar body expulsion rate, elevated estrogen levels in serum and increased pregnancy rates in mice.

NAC inhibits the repeated COH-promoted oxidative stress responses in ovaries and oocytes

NAC has antioxidant effects on the ovary (Mahmoodi *et al.* 2015), but the effects on oxidative stress in the ovaries and oocytes of repeated COH mice have not been reported. DCFH-DA staining was used to detect ROS in oocytes (Fig. 3*a*), which is an important triggering factor for oocyte degeneration. As seen in Fig. 3*b*, the ROS levels were elevated in oocytes of the model group ($P < 0.01$) and decreased in oocytes of the NAC group ($P < 0.01$). Next, T-AOC, a marker of antioxidation capacity, was detected. T-AOC levels were reduced in oocytes of the repeated COH model group ($P < 0.01$) and restored in the NAC group ($P < 0.01$) (Fig. 3*c*). As shown in Fig. 3*d, e, f*, the levels of the oxidative stress products 8-OH-dG, AOPP, and MDA in ovaries of repeated COH mice were significantly higher than in the control group ($P < 0.01$). After NAC administration, 8-OH-dG, AOPP, and MDA levels were significantly lower than the model group ($P < 0.01$). The levels of the antioxidant enzymes SOD and GSH-Px in ovaries were significantly lower in the model group compared with the control group ($P < 0.01$), and they were significantly increased after NAC administration ($P < 0.01$) (Fig. 3*g*). We also detected the oxidative stress products and antioxidant enzymes in oocytes where similar results were obtained (Fig. 3*h, i, J*). Repeated COH increased the levels of oxidative stress products (8-OH-dG, AOPP, and MDA) and decreased the activity of antioxidant enzymes ($P < 0.01$). NAC administration exerted antioxidant effects, reducing the levels of oxidative stress products and improving the activity of antioxidant enzymes in the oocytes of repeated COH mice ($P < 0.01$) (Fig. 3*k*).

Effects of NAC on mitochondrial ultrastructure and distribution

Oxidative stress directly affects mitochondrial function (Dröge 2002). To determine whether NAC impacts the mitochondrial ultrastructure of oocytes, transmission electron microscopy (TEM) was used to examine oocytes after repeated COH. As shown in Fig. 4*a*, we observed mitochondrial vacuoles, narrowed intermembrane spaces, loss of cristae, and partial fusion between mitochondria in oocytes after repeated COH. After NAC administration, mitochondrial cristae were distinct, the matrix density was uniform, mitochondria were more numerous, and a few mitochondria were locally cavitated in oocytes. During oocyte maturation, mitochondrial distribution changes dramatically; they undergo a stepwise spatial redistribution. The general pattern is from a predominantly peripheral distribution in immature oocytes

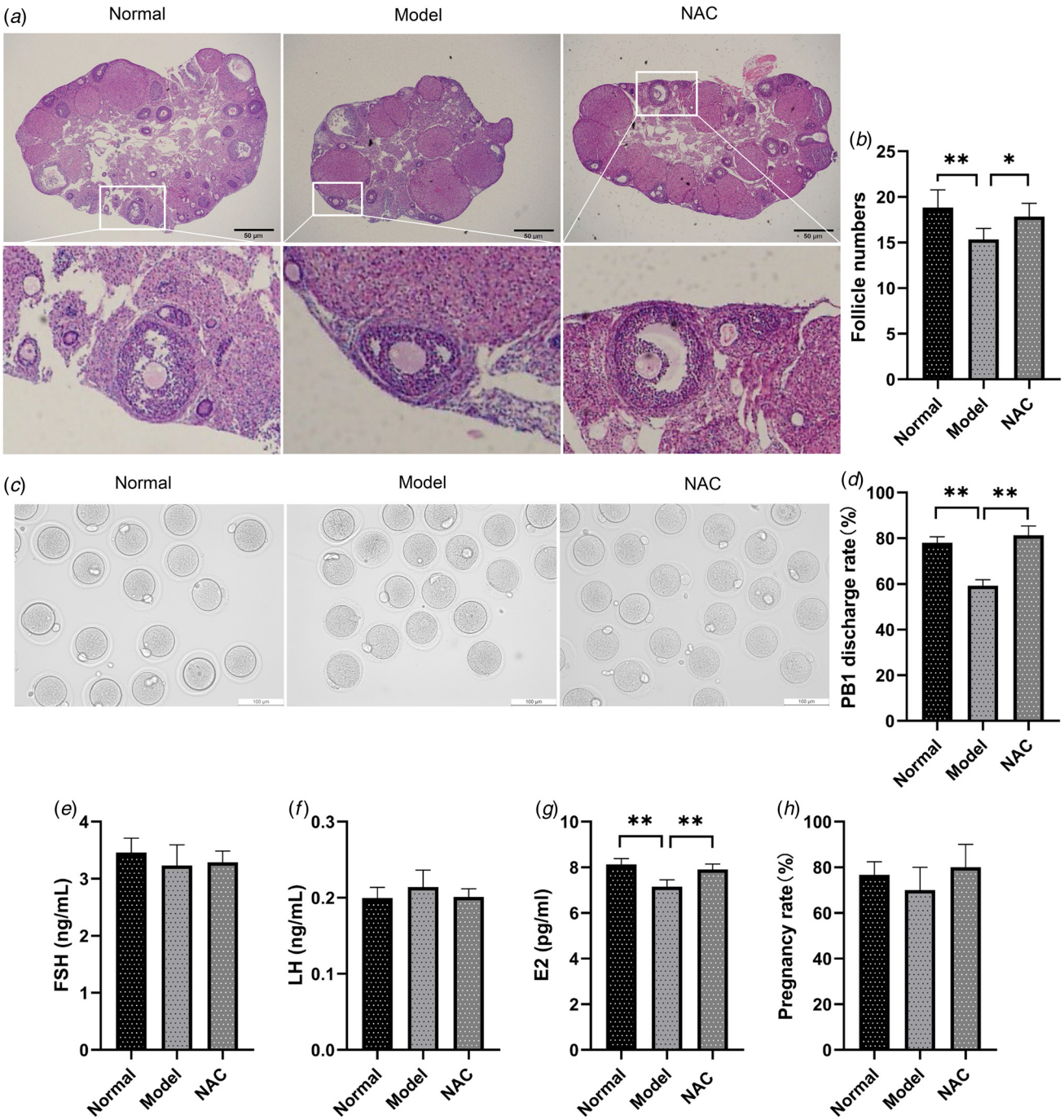

Fig. 2. Effect of NAC on the ovarian morphology, number of follicles, number of retrieved oocytes and serum hormone levels. Female ICR mice were randomly assigned into three groups: normal (saline, $n = 10$), model (repeated COH, $n = 10$), NAC (repeated COH + NAC, $n = 10$). (*a*) HE staining of ovarian tissues (×40). (*b*) Ovarian follicle count in mice. (*c*) MII oocytes under the microscope (×200). (*d*) PB1 discharge rates of oocytes after repeated COH. (*e*) Serum FSH levels in mice. (*f*) Serum LH levels in mice. (*g*) Serum E2 levels in mice. (*h*) Pregnancy rates after repeated COH. $^{*}P < 0.05$, $^{**}P < 0.01$. Data are shown as mean ± s.d.

to a progressive perinuclear or uniform distribution in mature oocytes (Sanchez *et al.* 2015). Fig. 4*b* shows the different distribution types. The mitochondrial even distribution ratio showed a decline in the model group, and the mitochondrial mean distribution rate showed an increase after NAC administration ($P < 0.01$) (Fig. 4*c*). These results indicated that NAC can alleviate abnormalities in the mitochondrial ultrastructure of oocytes after repeated COH and alter the mitochondrial distribution pattern in oocytes.

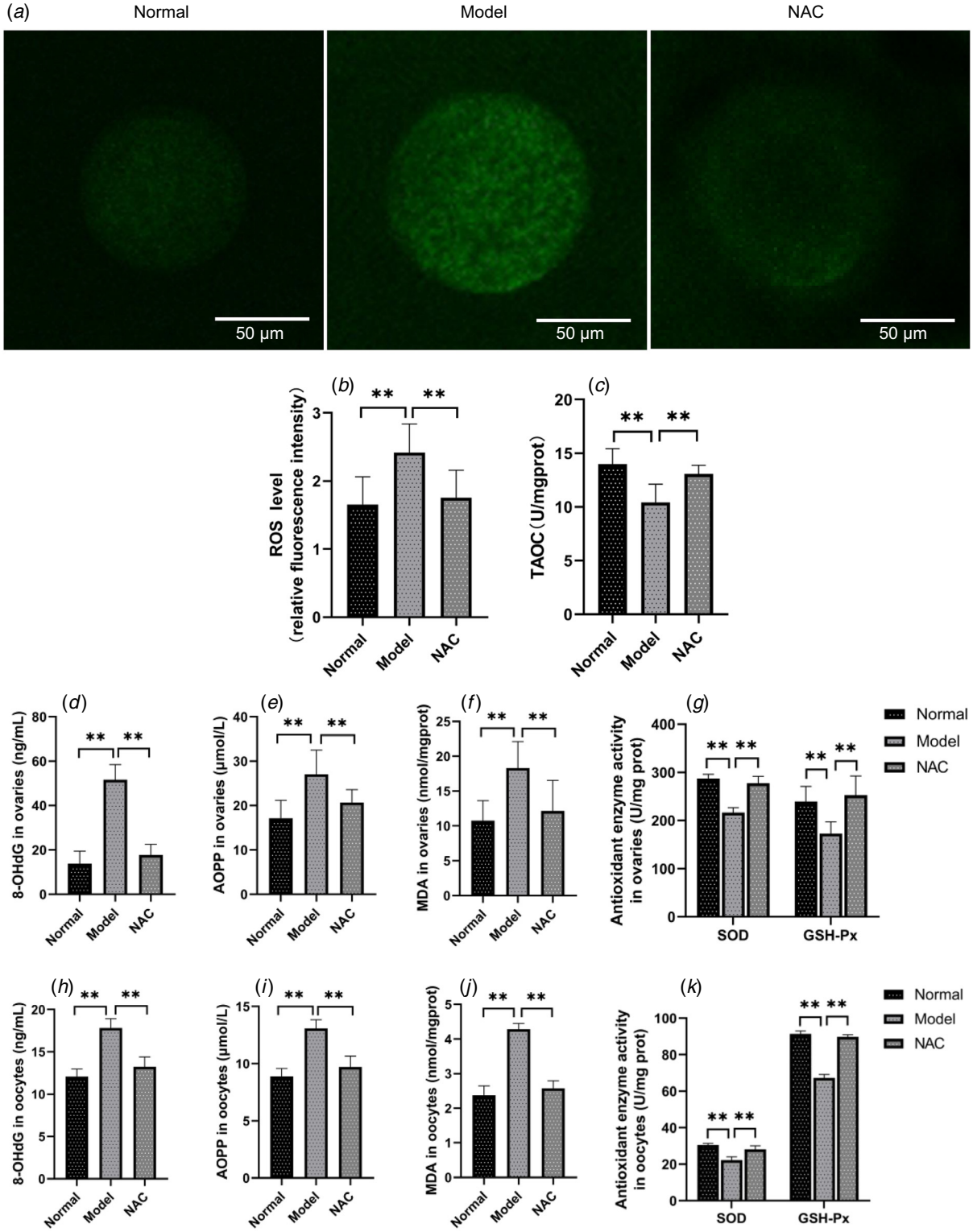

Fig. 3. NAC inhibits the repeated COH-promoted oxidative stress responses in ovary and oocytes. Female ICR mice were randomly assigned into three groups: normal (saline, $n = 10$), model (repeated COH, $n = 10$), NAC (repeated COH + NAC, $n = 8$). (*a*) DCFH-DA staining of oocytes (×40). (*b*) Comparison of ROS levels in ovaries. (*c*) Comparison of TAOC levels in ovaries. (*d*) 8-OHdG level in ovaries. (*e*) AOPP levels in ovaries. (*f*) MDA levels in ovaries. (*g*) SOD and GSH-Px activities in ovaries. (*h*) 8-OHdG levels in oocytes. (*i*) AOPP levels in oocytes. (*j*) MDA levels in oocytes. (*k*) SOD and GSH-Px activities in oocytes. $^{**}P < 0.01$. Data are shown as mean ± s.d.

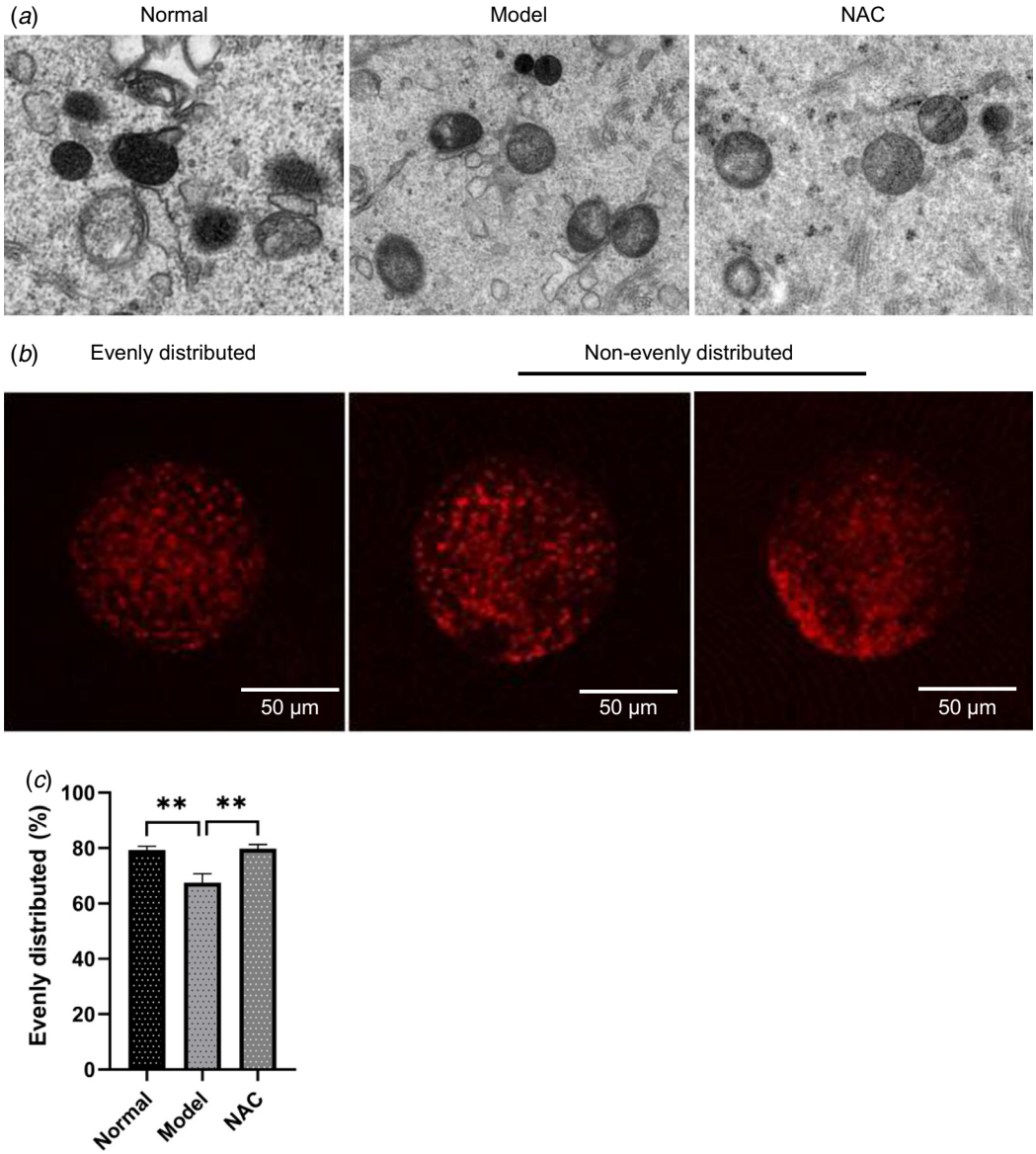

Fig. 4. The ultrastructure and distribution of mitochondrial. Transmission electron microscopy (TEM) was used to observe mitochondrial ultrastructure and $n = 10$ in each group. (*a*) Mitochondrial ultrastructure in oocytes (×5000). And mitochondrial distribution revealed by Mitochondrial Staining Kit-Red. (*b*) Different distribution types of mitochondrial in oocytes (×200). (*c*) Evenly distribution rates in oocytes. $^{**}P < 0.01$. Data are shown as mean ± s.d.

NAC improves the quantity and quality of mitochondria in oocytes after repeated COH

To verify the effects of NAC on the mitochondrial function of oocytes, we measured the MMP by calculating the red/green fluorescence intensity ratio in oocytes. As shown in Fig. 5*a*, *b*, in the model group, the MMP was lower than in the normal group ($P < 0.05$). NAC significantly elevated the MMP ($P < 0.01$). Then, we performed fluorometry analysis to measure the ATP content. The ATP content was significantly lower in the model group than in the normal group ($P < 0.05$) and significantly restored in the NAC group ($P < 0.05$) (Fig. 5*c*). During oocyte maturation, the mtDNA copy number dramatically increases (Combelles *et al.* 2002). Then the mtDNA copy number was measured. We found that the mtDNA copy number was significantly decreased in the model group ($P < 0.05$) and elevated after NAC administration ($P < 0.01$) (Fig. 5*d*). These results demonstrated that NAC can increase the quantity and quality of mitochondria after repeated COH.

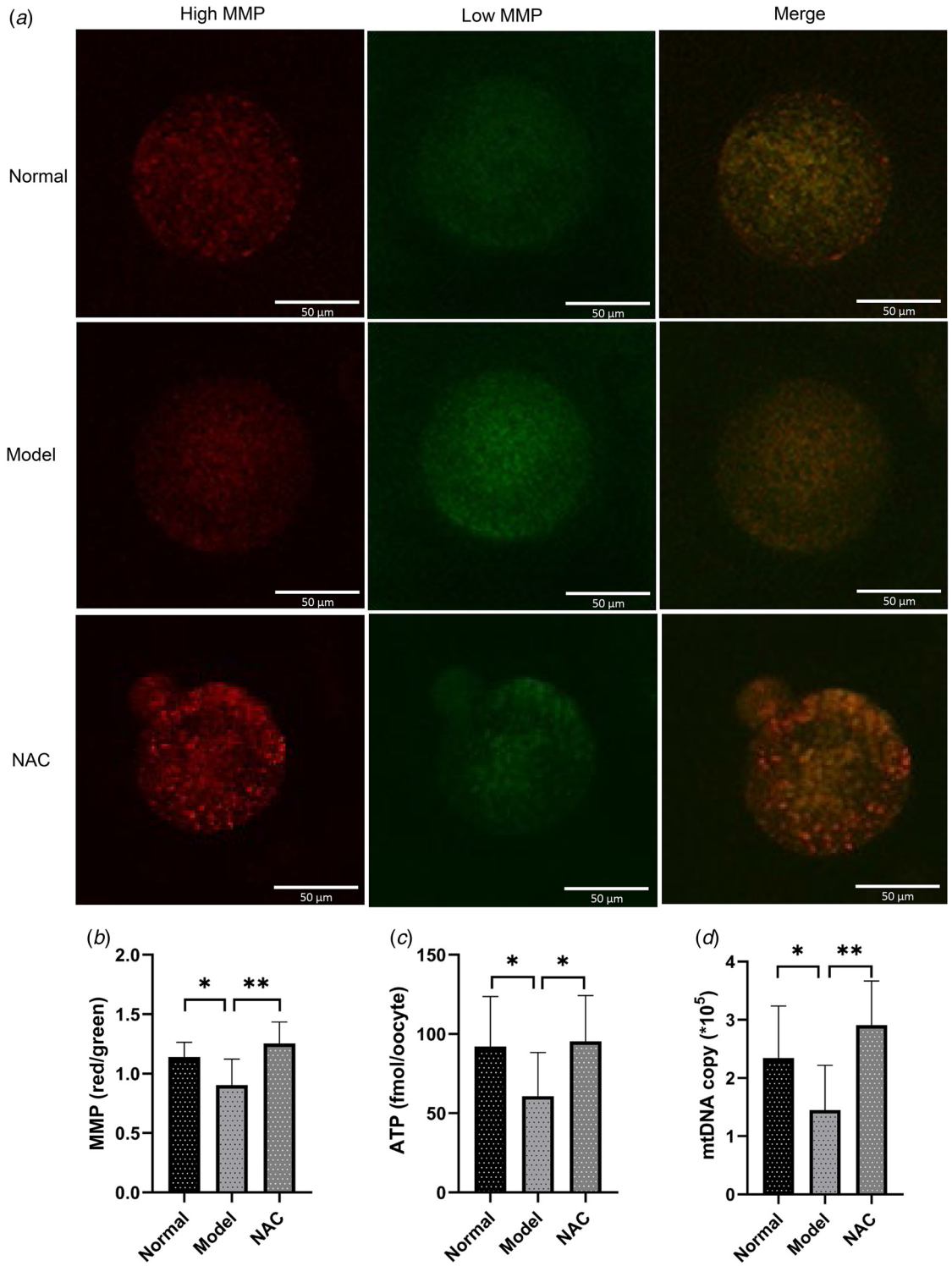

Fig. 5. Mitochondrial membrane potential (MMP), ATP levels and mitochondrial DNA copy number in oocytes. (*a*) JC-1 staining in oocytes (×200), red indicated mitochondria with high MMP, green indicated mitochondria with low MMP. (*b*) The comparison of MMP in each group. (*c*) ATP levels in oocytes. (*d*) mtDNA copy number in oocytes. $^{*}P < 0.05$, $^{**}P < 0.01$. $n = 25$ in each group. Data are shown as mean ± s.d.

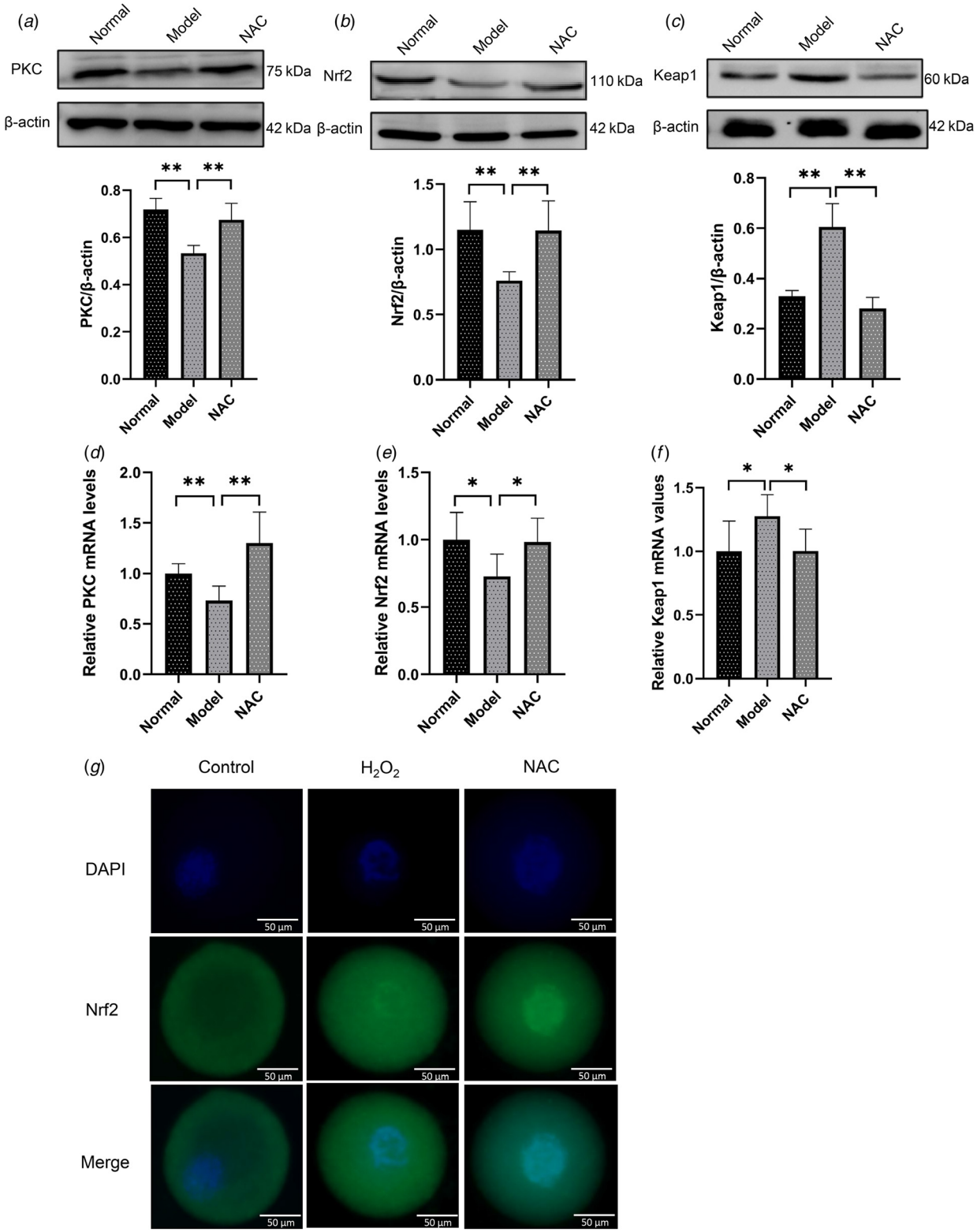

Fig. 6. NAC activates PKC-Nrf2 signaling pathway in oocytes. *In vivo* MII oocytes were used to detect the expression of PKC-Nrf2 signaling pathway after repeated COH. (*a*) PKC protein expression of oocytes in the normal-, repeated COH-, and repeated COH + NAC-treated mice. (*b*) The protein expression of Nrf2 in oocytes of mice. (*c*) The protein expression of Keap1 in oocytes of mice. (*d*) PKC mRNA levels in oocytes of mice. (*e*) Nrf2 mRNA levels in oocytes of mice. (*f*) Keap1 mRNA levels in oocytes of mice. (*g*) The nuclear translocation of Nrf2 in oocytes. $^{*}P < 0.05$, $^{**}P < 0.01$. $n = 30$ in each group. Data are shown as mean ± s.d.

NAC activates the PKC–Nrf2 signaling pathway in oocytes

In response to oxidative stress, Nrf2 is dissociated from Keap1 and translocates into the nucleus, initiating transcription of antioxidant enzyme genes (Shaw and Chattopadhyay 2020). To further investigate whether NAC activates the PKC–Nrf2 pathway to suppress oxidative stress in oocytes, we observed the effect of NAC on the expression of PKC, Nrf2, and Keap1 in oocytes treated *in vivo* with repeated COH and the nuclear translocation of Nrf2 in oocytes cultured *in vitro* treated with H_2O_2. As shown in Fig. 6*a*, *b*, the protein expression of PKC and Nrf2 was significantly reduced in the model group ($P < 0.01$) but significantly elevated in the NAC group ($P < 0.01$). By contrast, the Keap1 protein level was dramatically increased in the model group ($P < 0.01$) and decreased in the NAC group ($P < 0.01$) (Fig. 6*c*). The mRNA expression levels of PKC, Nrf2, and Keap1 were consistent with the protein expression levels (Fig. 6*d, e, f*). Then we examined the nuclear translocation of Nrf2 in oocytes *in vitro*. After incubation in IVM culture medium for 2 h, the GV oocytes were treated with or without NAC for 6 h. In the control group, Nrf2 was only expressed in the cytoplasm and not in the nucleus. H_2O_2 slightly promoted Nrf2 translocation from the cytoplasm to the nucleus and NAC treatment significantly increased Nrf2 expression in the nucleus (Fig. 6*g*).

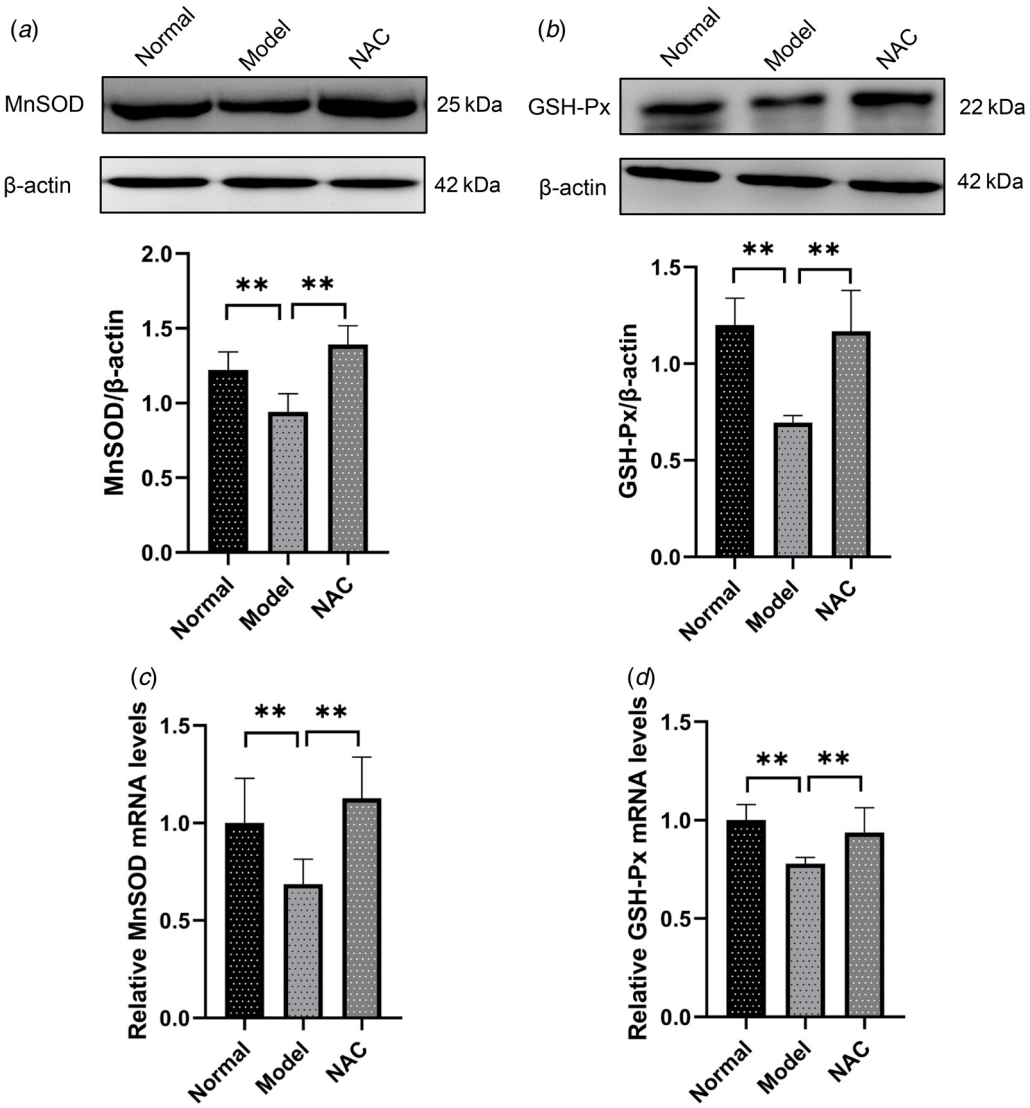

Fig. 7. NAC promotes the expression of SOD, GSH-Px in oocytes. *In vivo* MII oocytes were used to validate the transcription of downstream enzymes SOD and GSH-Px of the Nrf2 signaling pathway. (*a*) The protein expression of MnSOD in oocytes of mice. (*b*) The protein expression of GSH-Px in oocytes of mice. (*c*) MnSOD mRNA levels in oocytes of mice. (*d*) GSH-Px mRNA levels in oocytes of mice. $^{**}P < 0.01$. $n = 25$ in each group. Data are shown as mean ± s.d.

NAC promotes the expression of SOD and GSH-Px in oocytes

The PKC–Nrf2 signaling pathway could regulate the expression of its downstream antioxidant enzymes such as SOD and GSH-Px (Shaw and Chattopadhyay 2020). Therefore, we evaluated the protein levels of SOD and GSH-Px in normal, model and NAC mice. The protein levels of SOD and GSH-Px were significantly decreased in the model group ($P < 0.01$). NAC enhanced the protein expression of SOD and GSH-Px ($P < 0.01$) (Fig. 7*a*, *b*). Simultaneously, we checked the mRNA levels of SOD and GSH-Px. The results were consistent with the protein levels ($P < 0.01$) (Fig. 7*c*, *d*). These results suggested that NAC regulates the expression of antioxidant enzymes through modulation of the Nrf2 signaling pathway, thereby exerting antioxidant effects and improving oocyte quality after repeated COH.

Discussion

In this study, we investigated the protective effects of NAC on oocyte quality in terms of antioxidative stress and mitochondrial function. We found that NAC increased the expression of Nrf2 and promoted the nuclear translocation of Nrf2, which activated the expression of the antioxidant enzymes SOD and GSH-Px, leading to a reduction in the levels of oxidative stress products upon repeated COH (Fig. 8). These results indicate that NAC could be used to improve oocyte quality in humans by protecting mitochondrial function.

According to the WHO, infertility is considered to be the failure to conceive after 12 months of unprotected intercourse. IVF-ET is an effective treatment for infertility. COH is an important step in IVF-ET that regulates the development and maturation of multiple follicles through exogenous gonadotropins. However, the pregnancy rate of a single IVF cycle is only about 30% (Smith *et al.* 2015). Therefore, repeated COH is very common in clinical settings. The quality of the oocyte is crucial to fertilisation and the formation and development of the embryo. Repeated COH can cause oxidative stress, which leads to a reduction of oocyte quality and abnormal mitochondrial structure and function (Miyamoto *et al.* 2010). Shapiro *et al.* (2001) observed that pregnancy and embryo implantation rates decreased significantly after repeated COH. Our previous clinical studies indicated that repeated COH results in oxidative stress and changes the follicle microenvironment, possibly causing poor IVF outcomes in patients (Ma *et al.* 2021). In the present study, we also found that oxidative stress affects ovarian and follicular morphology, the PB1 discharge rate, and the pregnancy rate in mice (Fig. 2).

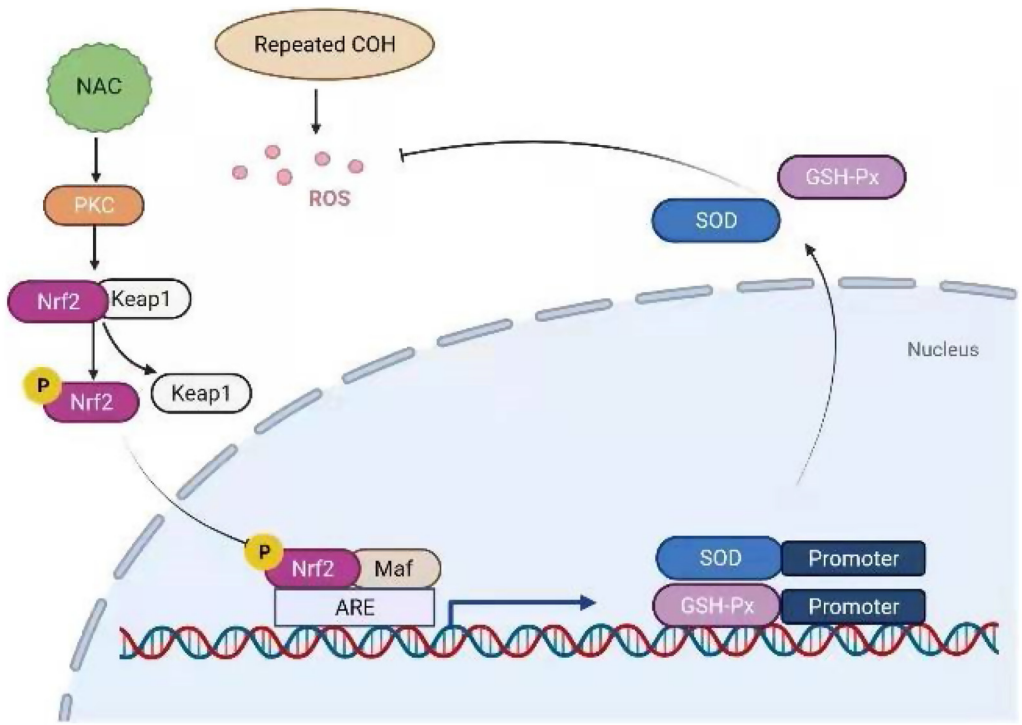

Fig. 8. Proposed model for the mechanism by which NAC reduces oxidative stress after repeated COH through the PKC-Nrf2-Keap1 signaling pathway. NAC could promote the uncoupling of Nrf2 from Keap1, induce Nrf2 nuclear translocation and then promote transcription and expression of antioxidant enzymes, thus preserving the quality of oocytes by protecting against oxidative stress.

These results suggest that oxidative stress affects ovulation and estrogen levels in the ovaries of mice with repeated COH.

NAC has many pharmacological properties, including antioxidant, anti-inflammatory, and anti-apoptotic activities (Firuzi *et al.* 2011; Mikolka *et al.* 2016; Dash *et al.* 2020). NAC, as a ROS scavenger, has already been widely used in medicine for several years, especially as a potent drug for acetaminophen poisoning. Previous studies showed that NAC, which is known to replenish stores of the antioxidant GSH, can provide sulfhydryl groups to cells (Barrozo *et al.* 2021). In recent years, NAC was shown to elicit a variety of responses to improve female fertilisation and treat endometriosis, polycystic ovary syndrome and other conditions. (Cheraghi *et al.* 2016; Adeoye *et al.* 2018). NAC can preserve the follicles in the ovary and improve the pregnancy rate through inhibiting apoptosis (Salehpour *et al.* 2012). Cheraghi *et al.* (2016) found that NAC improves oocyte and embryo quality in polycystic ovary syndrome patients undergoing intracytoplasmic sperm injection. In addition, NAC has functions in the preservation of morphological characteristics, proliferation, and DNA integrity in human ovarian tissue after cryopreservation (Fabbri *et al.* 2015). However, we still do not know whether NAC plays a protective role in oocytes during repeated COH. In the present study, we established a repeated COH mouse model and showed that NAC improves follicle and ovarian morphology, promotes oocyte maturation, and increases the pregnancy rate (Fig. 2). Thus, NAC is a promising antioxidant substance that has the potential to improve the efficacy of assisted reproductive technologies.

In homeostatic conditions, ROS are important in physiological processes, functioning as second messengers and are involved in the processes of ovulation (Sohel *et al.* 2019). *In vivo*, owing to its antioxidant potential, the addition of NAC to the maturation medium of COCs from mice that were treated with bisphenol A (BPA) led to a reduction in ROS levels and an increase in GSH content (Li *et al.* 2019). The addition of NAC can promote cumulus cell expansion and cause a reduction in ROS levels (Lai *et al.* 2015). Ambruosi *et al.* (2011) found that treatment with NAC led to damage minimisation induced by di(2-ethylhexyl) phthalate (DEHP) in matured oocytes of horses by effectively abrogating excess ROS in DEHP-treated cumulus cells. *In vitro*, NAC can protect germinal vesicle breakdown (GVBD) oocytes from impairment induced by heat stress through reversing disorganised spindle assembly and reducing the accumulation of ROS (Hu *et al.* 2020). Our study showed that NAC could reduce the levels of ROS, 8-OHdG, AOPP, and MDA and increase the expression of T-AOC, SOD, and GSH-Px in ovaries and oocytes (Fig. 3). We speculate that the simultaneous development of multiple follicles may cause an abnormal increase in the production of ROS, resulting in oxidative stress in ovaries and oocytes and that NAC can reduce oxidative stress. The underlying mechanisms deserve further investigation.

In eukaryotes, oxidative metabolism takes place in mitochondria, which is the 'power station' of the cell and the energy released by the oxidation of respiratory substrates drives ATP synthesis and metabolite transport (Galluzzi *et al.* 2012). Oocyte mitochondria are essential for supporting oocyte maturation and early embryo development (Babayev and Seli 2015). The oxidative stress caused by ROS accumulation in oocytes can result in decreased oocyte MMP and a reduction in oocyte ATP and mtDNA content (Lord and Aitken 2013). On oocyte maturation, the mitochondria migrate toward the central region. Homogeneous localisation of mitochondria is considered to be a marker of cytoplasmic maturity, whereas peripheral localisation is more commonly observed in meiotically incompetent oocytes (Sanchez *et al.* 2015). We found that the mitochondrial ultrastructure of oocytes was disturbed upon repeated COH and improved by NAC (Fig. 4). The presence of a high MMP is a prerequisite for mitochondria to perform oxidative phosphorylation to produce ATP and is a hallmark of oocyte cytoplasmic maturation (Van Blerkom and Davis 2007). A distinct range of oocyte mtDNA copy numbers was shown in patients with diminished ovarian reserve (100 000 ± 99 000 copies) compared to women with a normal ovarian reserve (318 000 ± 184 000 copies) (May-Panloup *et al.* 2005). Low ATP levels in oocytes are associated with reduced embryo viability and implantation potential (Van Blerkom *et al.* 1995). Repeated COH leads to a decreased number of oocytes, increased cytoplasmic fragmentation, an abnormal mitochondrial distribution, spindle damage, increased intracellular oxidative stress and a decrease in oocyte numbers, resulting in poor developmental potential of the embryos (Kalthur *et al.* 2016). Our results showed that NAC treatment results in elevated mtDNA copy numbers, increased MMP and increased ATP content in oocytes, suggesting that repeated COH damages oocyte mitochondria and NAC protects them (Fig. 5).

The Keap1–Nrf2–ARE signaling pathway is one of the most important defense mechanisms against oxidative stress and this pathway can be activated by sulfhydryl-responsive small molecule drugs such as NAC (Wakabayashi *et al.* 2010). Under physiological conditions, Keap1 acts as a negative regulatory protein targeting Nrf2 for ubiquitin-dependent proteasomal degradation. Once oxidative stress occurs, Nrf2 dissociates from Keap1 and translocates into the nucleus, where it binds to AREs, resulting in the expression of several antioxidant enzymes, such as SOD and GSH-Px (Shaw and Chattopadhyay 2020). Kim *et al.* (2020) have shown that melatonin-activated Nrf2 signaling synergistically improves mammalian oocyte maturation and embryonic development. Ma *et al.* (2018) reported that the expression of Nrf2 is related to female age in ovarian granular cells, suggesting that the decreased expression of Nrf2 may be related to the decline in the reproductive

capacity of older women. Combining these results, we propose the hypothesis that the mechanism by which NAC improves oxidative stress-reduced oocyte quality is related to the activation of the Nrf2 signaling pathway. We observed decreased expression of PKC and Nrf2 in oocytes after repeated COH, while Keap1 was upregulated. The expression of the antioxidant enzymes SOD and GSH-Px, which are transactivated by the Nrf2 signaling pathway, was also downregulated. After NAC administration, PKC and Nrf2 upregulation was accompanied by Keap1 downregulation (Fig. 6). A prominent NAC-induced increase in (1) the nuclear translocation of Nrf2 and (2) the expression of antioxidant enzymes in oocytes was found (Figs 6 and 7). The mechanism by which NAC reduces oxidative stress in oocytes after repeated COH and improves oocyte treatment is related to the regulation of the Nrf2 signaling pathway, which in turn increases the expression of antioxidant enzymes.

Conclusions

In summary, this study demonstrates the potential effect of NAC on oocyte quality in mice with repeated COH. NAC could promote the nuclear translocation of Nrf2, which transcriptionally activate the expression of SOD and GSH-Px, which removed excessive ROS that cause mitochondria damage of oocyte and ovaries in mice during repeated COH. It appears that NAC addition promotes oocytes mitochondria function and improves oocyte quality through decreasing the oxidative stress in mice during repeated COH. This study provides a meaningful foundation for the future clinical application of NAC during repeated COH.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest. The authors declare that they have no conflicts of interest.

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