Effects of chitooligosaccharide-zinc on the ovarian function of mice with premature ovarian failure via the SESN2/NRF2 signaling pathway

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ABSTRACT: Chitooligosaccharide-zinc (COS·Zn) is a powerful anti-oxidant and anti-aging scavenger, whose anti-oxidative ability immensely exceeds vitamin C. Therefore, this study was aimed to investigate the protective effects of COS·Zn against premature ovarian failure (POF) and potential mechanisms. Female KM adult mice were divided into the following groups: a treatment group (150 mg·kg⁻¹·d⁻¹ COS·Zn), a treatment group (300 mg·kg⁻¹·d⁻¹ COS·Zn), a prevention group, two control groups and two CY/BUS groups. COS·Zn (150, 300 mg·kg⁻¹·d⁻¹) and COS·Zn (300 mg·kg⁻¹·d⁻¹) were therapeutically and preventatively administered to POF mice in the treatment and prevention studies, respectively. All the groups were administered for 21 days. Fewer primary and secondary follicles were observed in the COS·Zn-treated groups (including the treatment and prevention groups) than those of the control groups. Meanwhile, the ovarian index and the levels of FSH and LH notably increased in the treatment and prevention groups compared with those in the CY/BUS group. The levels of MVH, OCT4 and PCNA in the treatment group (300·kg⁻¹·d⁻¹ COS·Zn) and MVH in the prevention group remarkably increased compared with those in the CY/BUS groups. Meanwhile, the levels of P53 and P16 protein were down-regulated in the treatment and prevention groups compared with those in the CY/BUS groups. Additionally, the amounts of Sestrin2 (SESN2) and SOD2 protein were obviously higher in the treatment group (150 mg·kg⁻¹·d⁻¹ COS·Zn) than those in the CY/BUS groups. Similarly, the amounts of NRF2 and SESN2 protein were up-regulated in the prevention group. Besides, an increased GSH level was observed in the two treatment groups, compared with that in the CY/BUS groups, and the same trend was also present in the prevention group. Taken together, COS·Zn improves the ovarian and follicular development through regulating the SESN2/NRF2 signaling pathway. These results suggest the role of COS·Zn as a novel agent for the treatment and prevention of POF.

KEY WORDS: Premature ovarian failure; Chitooligosaccharide-zinc; Sestrin2-nrf2; Ovarian function; Aging

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Introduction

Premature ovarian failure (POF) refers to the development of amenorrhea due to cessation of ovarian function before the age of 40 and is the main cause of ovarian infertility [1-3]. This condition is associated with a wide spectrum of pathogenetic mechanisms, including chromosomal, genetic, auto-immune, metabolic (galactosemia), infectious (mumps) and iatrogenic (anti-cancer treatments) causes [4-6]. During the degenerative process, mitochondrial dysfunction leads to
overproduction of reactive oxygen species (ROS), resulting in oxidative damage. Oxidative stress may play a role in the degenerative process of POF, which becomes more noticeable in POF population, and impairs anti-oxidant defenses and increases susceptibility to oxidative stress and damage \(^1, 5, 8\).

Therefore, oxidative damage is considered inevitable in POF, and the underlying mechanism concerning oxidative-stress-related ovarian damage has attracted increasing attention.

Nowadays, the possible causes of POF are various, and there are no effective therapies available. Chitosan oligosaccharide (COS) molecules contain a large number of active amino and hydroxyl groups \(^9\), and can be chelated with inorganic zinc to produce an organic form of chitosoligosaccharide-zinc (COS·Zn) under controlled reaction conditions \(^10, 11\). As a basic amino oligosaccharide, COS·Zn is also a powerful anti-oxidant and anti-aging scavenger \(^12\), whose anti-oxidative ability immensely exceeds vitamin C. Cumulative evidence suggests that COS·Zn possesses biological properties against oxidative stress in different organs and tissues \(^13-15\). For example, COS·Zn effectively reduced the content of MDA in D-galactose-treated mice and significantly increased the activities of SOD, T-AOC, GSH-Px and CAT in the serum, kidneys and liver of mice; in particular, COS·Zn treatment also produced stronger effects of serum protective enzymes than COS, ZnSO\(_4\), and COS + ZnSO\(_4\) mixture \(^15\).

Moreover, some studies reported that COS·Zn regulates the growth and development of the reproductive system in mice, and plays an essential role in improvement of the tissue structure of reproductive organs/fertility in female and male mice \(^13-15\). Another study indicated the intimate relationship between COS·Zn and mammalian ovarian function \(^16\). COS·Zn treatment can obviously improve female fertility rate, resulting in increases in the number of ovarian follicles and endometrial thickness, an abundance in the uterine glands, and increased egg vitality and fertilization \(^9\). However, during the process of POF, the oxidative damage to ovaries can not be avoided, so we included COS·Zn in our histological study to determine the effect of oxidative damage on POF, in consideration of the potential of COS·Zn in therapies for POF.

In mammals, the Sestrin family comprises three members (Sestrin 1−3), which can be induced by oxidative stress and DNA damage in a p53-dependent manner \(^17-20\). SESN2, an essential member of the Sestrin family, is a highly conserved protein expressed in endothelial and macrophage cells and displays a protective role in the body \(^21, 22\). According to our previous research, sestrin is strongly expressed in ovarian tissue, but the exact mechanism underlying ovary-protective effects have not been clearly elucidated. To date, the effect of SESN2 on ovarian function has not been fully determined.

NF-E2-related factor 2 (NRF2) is an important endogenous anti-oxidant factor, and acts as an essential part in oxidative stress responses \(^23-25\). When oxidative stress occurs, NRF2 is separated from Kelch-like ECH-associated protein 1 (Keap1) and enters into the nucleus \(^26-30\). Recent studies indicated that NRF2 is mainly regulated by Keap1 \(^31\), and plays an important role in regulating ovarian function and treating ovarian cancer \(^32-35\). Furthermore, SESN2 can directly or indirectly participate in the regulation of NRF2 during the process of metabolism \(^36, 38\).

Therefore, this study was designed to investigate the protective effects of COS·Zn against oxidative damage in POF and evaluate the role of COS·Zn in relieving ovarian dysfunction. The findings will provide theoretical evidence for daily COS·Zn treatment against oxidative damage.

**Material and Methods**

**Ethical approval**

All animal procedures were approved by the Institutional Animal Care and Use Committee of Nanchang, and conducted in accordance with the Guide for the Care and Use of Laboratory Animals Eighth Edition.

**Preparation of COS·Zn**

COS·Zn was prepared under optimal reaction conditions \(^39\). COS and zinc sulfate were weighed in a mass ratio of 2 : 1 and separately dissolved in two beakers with an equal amount of distilled water. Then, the two solutions were mixed in equal volumes and the pH value was slowly adjusted to 7 with 1% ammonium hydroxide solution. The resultant mixture was then chelated at 40 °C for about 30 min, to which anhydrous ethanol (three times in volume) was slowly added. Then, the product was dried at 60 °C, obtaining COS·Zn as yellow powder, with a zinc percentage of 10%.

**Mice and treatment**

Female KM mice aged 6 weeks (weighing 20−25 g) were purchased from the Laboratory Animal Center of Nanchang University, and acclimatized for seven days prior to experiments. The mice were housed on a 12-hour light/dark cycle at (22 ± 1) °C, with free access to food and water. They were then randomly divided into the following groups (n = 6): a treatment group (150 mg·kg\(^{-1}\)·d\(^{-1}\) COS·Zn), a treatment group (300 mg·kg\(^{-1}\)·d\(^{-1}\) COS·Zn), a prevention group, two control groups (for treatment and prevention studies, respectively) and two CY/BUS groups (for treatment and prevention studies, respectively).

All the mice, except those in the control groups and prevention group, were administered with CY/BUS for 21 days to establish a model of POF. The treatment group (300 mg·kg\(^{-1}\)·d\(^{-1}\) COS·Zn) showed significantly stronger effects on reversing the process of POF, so mice in the prevention group were preventative treated with COS·Zn (300 mg·kg\(^{-1}\)·d\(^{-1}\)) for 21 days. No special procedures were provided for the control groups.

The mass ratio of COS to Zn in the COS·Zn synthesized in the current study was 9 : 1, and all of the concentrations of COS·Zn mentioned in this article were measured by COS (Fig. 1). Based on literature review and our previous data, the
daily food intake of the experimental mice is about 6 g, which means that every mouse in the COS·Zn (150 mg·kg\(^{-1}·d\(^{-1}\)) group requires 1 mg COS·Zn per day and 2 mg COS·Zn per day is suggested for each mouse in the COS·Zn (300 mg·kg\(^{-1}·d\(^{-1}\)) group. Accordingly, 0.04 g COS·Zn powder was taken and dissolved in 8 mL normal saline, to which 2 mL sodium carboxymethyl cellulose was added to prevent the deposition of COS·Zn powder. The resultant solution was then shaken and mixed, obtaining a solution of COS·Zn at 4 mg·mL\(^{-1}\). After POF modeling, the mice underwent the following procedures: (1) the COS·Zn (150 mg·kg\(^{-1}·d\(^{-1}\)) group was intragastrically administered with 0.125 mg·mL\(^{-1}\) COS·Zn solution per day for 21 days; (2) the COS·Zn (300 mg·kg\(^{-1}·d\(^{-1}\)) group was intragastrically administered with 0.25 mg·mL\(^{-1}\) COS·Zn solution per day for 21 days; (3) the prevention group was intraperitoneally with CY/BUS on day 1 and kept a normal life without any operations in the following 20 days; (4) the control and CY/BUS groups were intragastrically administered with 0.25 mL normal saline per day for 21 days.

**Histological analysis of ovarian tissue and measurement of ovarian follicle count**

Mice were sacrificed by cervical dislocation. The ovaries were collected, and fixed in 4% paraformaldehyde followed by paraffin embedding, as previously described [37]. The paraffin-embedded ovaries were cut into serial sections and stained with hematoxylin and eosin (HE). The ovarian follicles were counted as previously described [37]. Then, immunostaining was performed using ovary specimens for treatment and prevention studies, according to previously published procedures [38].

The primary antibodies used were listed as follows: anti-MVH (1 : 100, ab27591), anti-OCT4 (1 : 100, ab18976), anti-PCNA (1 : 100, ab56701), anti-P53 (1 : 100, ab54073), anti-P16 (1 : 100, ab54073), anti-IL2 (1 : 100, ab54073), anti-IL4, anti-TNF\(\alpha\), anti-NRF2, anti-SESN2 and anti-SOD2 (1 : 100, ab51134). The secondary antibodies used were goat anti-mouse and goat anti-rabbit IgG conjugated with fluorescein isothiocyanate at a dilution of 1 : 200 (Proteintech, China). All the images were taken under a NIKON Eclipse 80i microscope.

**Western blot**

Total protein from different ovary tissues was extracted with RIPA lysis solution (Beyotime, P0013C). The proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membrane (Millipore Corp., Bedford, MA).The results were analyzed by the gel imaging analysis system and converted to semi-quantitative data by the GelScan software. Meanwhile, the level of \(\beta\)-actin was quantified, as an internal quantitative control. Each experiment was repeated at least three times.

**Tunnel assay**

The apoptotic rates of ovarian sections were detected by terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL) method using an apoptosis detection kit (Solarbio Biotech Co., Ltd., Beijing, China), according to the manufacturer’s instructions. After exposure to TUNEL reaction mixture for 1 h, the sections were washed twice with PBS for 10 min and counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 5 min. After being washed with ddH\(_2\)O for three times, the sections were photographed under a confocal laser scanning microscope (Leica, TSP8), and the apoptotic rate was analyzed using IPP 6.0 Software.

**SOD and GSH detection**

Superoxide dismutase (SOD): The activity of SOD was assessed using the WST-1 method. Blank wells, standard wells, determination wells and control wells were prepared according to the experimental requirements. The serum from mice was incubated at 37 °C for 20 min. The absorbance value of each well was detected at 450 nm by a microplate.

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Fig. 1  Infrared spectroscopic analysis of COS·Zn. (A) Preparation of COS·Zn and COS. (B) Percentage transmittance of COS·Zn and COS at a wavenumber of 1600 cm\(^{-1}\). * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\) vs COS·Zn, \(n = 6\)
reader.
Malondialdehyde (GSH): The content of GSH was determined using the thiobarbituric acid method. According to the manufacturer’s instructions, blank wells, standard wells, determination wells and control wells were prepared. The absorbance value of each well was detected at 450 nm by a microplate reader (set zero by distilled water).

### Measurement of hormone level
Blood samples were collected from the retro-orbital plexus and the serum was collected at 4 °C for 20 min, 4000 r min⁻¹. The levels of E2 and FSH were measured by NanJing JianCheng Bioengineering Institute.

### Statistical methods
All analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). The statistical comparisons between different groups were analyzed by the Student’s paired t-test. The threshold of \( P < 0.05 \) was considered significant; \( P < 0.01 \) and \( P < 0.001 \) were considered extremely significant.

### Results
**COS·Zn is identified by infrared spectroscopy**
The bimodal C=O bond disappeared at about 1600 cm⁻¹, and the absorption peak obviously weakened, which indicated that the C-O double bond chelated with the zinc ion in the COS molecule and “C.O-Zn” was formed. According to infrared spectroscopy, COS chelated with the zinc ion and formed a stable chelate as “COS·Zn” (Fig. 1A, B).

**COS·Zn improves ovarian and follicular development in POF mice**
The ovarian morphological changes of the control, CY/BUS and treatment groups were determined by HE staining (Fig. 2A). The number of primary follicles, secondary follicles, corpus lutea, and atretic follicles was counted (Fig. 2A). For the CY/BUS group, the number of atretic follicles dramatically rose compared with the 300 mg kg⁻¹·d⁻¹ COS·Zn-treated group and the control group (\( P < 0.05 \) or \( P < 0.01 \)). However, the number of atretic follicles in the 150 mg kg⁻¹·d⁻¹ COS·Zn-treated group was still higher than that of the control group (\( P < 0.05 \)). Besides, the number of atretic follicles was not significant between the 150 and 300 mg kg⁻¹·d⁻¹ COS·Zn-treated groups.

The number of primary follicles was significantly higher in the 300 mg kg⁻¹·d⁻¹ COS·Zn-treated group than that in the CY/BUS group (\( P < 0.05 \)). However, no significant difference was found in the number of secondary follicles between the treatment groups (150 and 300 mg kg⁻¹·d⁻¹ COS·Zn) and the CY/BUS group. Furthermore, neither level of the secondary follicles in the treatment groups (150 and 300 mg kg⁻¹·d⁻¹ COS·Zn) had reached that of the control group. Nevertheless, we found that both the ovarian index (ovarian weight/body weight ratio) and the levels of FSH and LH remarkably increased in the COS·Zn-treated groups in a dose-dependent manner, compared with that in the CY/BUS group (\( P < 0.01 \) or \( P < 0.001 \), Figs. 2B, C).

To further clarify the changes in the number of follicles and their proportion among different groups, we analyzed the prevention group (300 mg kg⁻¹·d⁻¹ COS·Zn) and its matched control and CY/BUS groups by HE staining. The results showed decreases in the number of atretic follicles in the prevention group compared with that in the CY/BUS group (Fig. 3A, \( P < 0.01 \)). We also found that the number of primary and secondary follicles increased in the prevention group and control group compared with that in the CY/BUS group (Fig. 3A, \( P < 0.01 \)). Furthermore, the prevention-ovary index and the levels of FSH and LH dramatically increased in the prevention group compared with those in the CY/BUS group (Figs. 3B, C, \( P < 0.01 \) or \( P < 0.001 \)). All data suggest that COS·Zn (300 mg kg⁻¹·d⁻¹) improves ovarian and follicle development in POF mice.

**COS·Zn improves ovarian function in mice with POF**
To evaluate the changes in the ovarian function of COS·Zn-treated mice, the expression of MVH and OCT4 was detected. The results revealed increased MVH and OCT4 levels in the 300 mg kg⁻¹·d⁻¹ COS·Zn-treated group compared with those in the CY/BUS group (Figs. 4A, C, \( P < 0.05 \) or \( P < 0.001 \)). Significant increases were observed in MVH for the prevention group (Figs. 4B, E, \( P < 0.01 \)). In addition, PCNA levels also increased in the 150 and 300 mg kg⁻¹·d⁻¹ COS·Zn-treated groups compared with that in the CY/BUS group (Figs. 4A, C, E, \( P < 0.05 \); however, this trend was not obvious in the prevention group (Figs. 4B, F). Through TUNEL assay, we found that the apoptotic signal was negative in granulosa cells and oocytes (Figs. 4D, F). The number of apoptotic cells per section in the 300 mg·kg⁻¹·d⁻¹ COS·Zn-treated group was significantly lower than that in the control and CY/BUS groups.

Meanwhile, the levels of P53 and P16 protein were markedly down-regulated in the treatment and prevention groups compared with those in the CY/BUS groups, except for the expression of P53 in the prevention group (Figs. 5A, B). **COS·Zn causes oxidative stress and enhances anti-aging capacity through regulating the SESN2/NRF2 signaling pathway in POF mice**
To investigate the mechanisms of changes in ovarian function, we detected oxidative stress and anti-aging capacity in the ovaries of COS·Zn-treated mice. The levels of NRF2 protein in the 150 and 300 mg kg⁻¹·d⁻¹ COS·Zn-treated groups markedly increased (Fig. 6A, \( P < 0.05 \)). Meanwhile, the amounts of SESN2 and SOD2 protein were significantly higher in the 150 mg kg⁻¹·d⁻¹ COS·Zn-treated group than those in the CY/BUS groups (Fig. 6A, \( P < 0.01 \) or \( P < 0.001 \), but the 300 mg kg⁻¹·d⁻¹ COS·Zn-treated group and CY/BUS groups showed no significant difference in the level of SESN2. Similarly, the amounts of NRF2 and SESN2 protein were up-regulated in the prevention group (Fig. 6C, \( P < 0.01 \)).
without statistical changes in SOD2. Then we analyzed the changes in the levels of GSH and SOD2 in different groups. The results revealed increased GSH and SOD2 levels in the 150 and 300 mg·kg$^{-1}$·d$^{-1}$ COS·Zn-treated groups compared with those in the CY/BUS group (Fig. 6B), and the same trend was shown in the prevention group (Fig. 6D).

**Discussion**

COS·Zn produces good anti-aging effects and enhances immune function in recent studies, as documented by Guijuan et al. [13], who found that COS·Zn complexes possess significantly stronger scavenging effects than COS, ZnSO$_4$, or COS + ZnSO$_4$ [13]. Meanwhile, with an increase in the concentrations of COS·Zn, the scavenging ability of DPPH free radicals gradually increases [39]. There are several studies associated with the role of COS·Zn in inhibiting the activities of microorganisms, and improving the indexes of organs such as the liver, thymus, spleen, and kidneys. Notably, the ovary is the major target organ for COS·Zn, but few studies have been reported to investigate the function of COS·Zn in the ovary. Thus, the aim of this study is to analyze the anti-oxid-
The number of primary and second-order follicles significantly increased in the 300 mg·kg⁻¹·d⁻¹ COS·Zn-treated group, and the number of atretic follicles also decreased in the 300 mg·kg⁻¹·d⁻¹ COS·Zn-treated group. Furthermore, the ovarian index results suggested that COS·Zn decelerates apoptosis in the ovary and stimulates ovarian follicular development, which is consistent with previous studies [16]. All the data above suggest that COS·Zn (300 mg·kg⁻¹·d⁻¹) improves ovarian and follicle damage in POF mice. Thus, the prevention group was treated with 300 mg·kg⁻¹·d⁻¹ COS·Zn. The decrease in the number of atretic follicles in the prevention group was consistent with those in the treatment groups. We also found that the number of primary and secondary follicles increased in the prevention group compared with those in the control and CY/BUS groups. Therefore, the development of the ovary is promoted by COS·Zn treatment in POF mice. Follicular development-related genes might play certain roles during this process [40]. Surprisingly, significant increases were observed in the levels of MVH, PCNA and OCT4 in the COS·Zn-administered groups, compared with those in the CY/BUS groups.

![Fig. 3](image-url)
To further explore the mechanism of COS·Zn on the ovary, we performed ovarian proteomic analysis. SESN2/NRF2 is probably the most important master of the expression of molecules that exert anti-oxidant functions within organs and cells. In the ovaries of POF mice, COS·Zn up-regulated the levels of SESN2, NRF2 and SOD2, which can be attributed to antioxidant effects. Taken together, these results indicate that COS·Zn improves ovarian and follicular development through regulating the SESN2-NRF2 signaling pathway, which suggest the role of COS·Zn as a novel agent that is effective for POF prevention and treatment.

**Conclusion**

Currently, there are few effective treatments for POF in the field of reproductive medicine. COS·Zn is the basic and primary requirement for the growth and development of the reproductive system in mice, and plays a major role in improving the tissue structure of reproductive organs/fertility in female and male mice. Increasing the level of COS·Zn is an effective way to improve ovarian and follicular development through regulating the SESN2-NRF2 signaling pathway, which suggest the role of COS·Zn as a potential new agent for POF prevention and treatment.
**Fig. 4** The expression of MVH/OCT4 genes in the COS·Zn-treated mice in the treatment and prevention studies. (A−B) Western blotting shows the levels of MVH, OCT4 and PCNA in the treatment and prevention studies, respectively. The relative protein levels of MVH, OCT4 and PCNA were measured by densitometry analysis, and calculated relative to the intensity of β-actin. Results are presented as the mean ± SEM of determinations in five mice of each group. *P < 0.05, **P < 0.01, ***P < 0.001 vs control group. ¹P < 0.05, ²P < 0.01 vs CY/BUS or NaCl + CY/BUS group. ³P < 0.05 vs COS·Zn (150 mg·kg⁻¹·d⁻¹) group. (C−D) Representative micrographs of immunohistochemically stained ovarian sections for MVH, OCT4 and PCNA. Scale bar: 100 μm. (E−F) Apoptosis was detected by Tunnel assay. Scale bar: 100 μm.

**Fig. 5** The expression of P53/P16 genes in the COS·Zn-treated mice in the treatment and prevention studies. (A−B) The relative protein levels of P53/P16 were assessed by densitometry analysis, and calculated relative to the intensity of β-actin. Results are presented as the mean ± SEM of determinations in five mice of each group. *P < 0.05, **P < 0.01, ***P < 0.001 vs control group. ¹P < 0.05, ²P < 0.01 vs CY/BUS or NaCl + CY/BUS group. ³P < 0.05 vs COS·Zn (150 mg·kg⁻¹·d⁻¹) group.
Fig. 6 The expression of NRF2/SESN2/SOD2 genes in the COS·Zn-treated mice in the treatment and prevention studies. (A) Western blotting shows the levels of NRF2, SESN2 and SOD2 in the treatment study. (B) Effect of COS·Zn on SOD2 and GSH levels in the peripheral blood of each group. (C) Western blotting shows the levels of NRF2, SESN2 and SOD2 in the prevention study. (D) Effect of COS·Zn on the levels of follicle SOD2 and GSH in the peripheral blood of each group. (A, C): *P < 0.05, **P < 0.01, ***P < 0.001 vs CY/BUS or NaCl + CY/BUS group. #P < 0.05, ##P < 0.01 vs control. +P < 0.05 vs COS·Zn (150 mg·kg$^{-1}$·d$^{-1}$) group. (B, D) *P < 0.05, **P < 0.01 vs control. *P < 0.05 vs COS·Zn (150 mg·kg$^{-1}$·d$^{-1}$) group.
Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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