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Protective effects ROS up-regulation on premature ovarian failure by suppressing ROS-TERT signal pathway

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Abstract. – OBJECTIVE: Premature ovarian failure (POF) refers to the condition of pre-onset ovarian function failure, and is one commonly occurred disease in gynecology. Its pathogenic mechanism, however, is still unclear. Early study found decreased activity of telomerase reverse transcriptase (TERT). As an important factor to suppress TERT, oxidative stress has not been studied in POF. We, thus, investigated the role of reactive oxygen species (ROS)-TERT in POF.

MATERIALS AND METHODS: Rat POF model was induced by a single intraperitoneal injection of cyclophosphamide plus 12 mg/kg busulfan. Level of follicle stimulating hormone (FSH) and inhibin B was measured by enzyme-linked immunosorbent assay (ELISA), along with hematoxylin and eosin (HE) staining to confirm successful generation of models. Western blot was applied to measure TERT expression, and N-acetyl-cysteine (NAC) or TERT small interfere RNA (siRNA) was injected to suppress ROS or TERT level, followed by HE staining to observe POF condition.

RESULTS: In POF model, ovary tissues showed atrophy, less follicles, and more follicular atresia, plus mesenchymal hyperplasia. FSH and inhibin B level were significantly up-regulated and down-regulated, respectively ($p<0.05$). In POF rat, ROS level was elevated ($p<0.05$) whilst TERT level was decreased. NAC inhibited ROS level and enhanced TERT expression. In contrast, TERT siRNA further aggravated POF condition.

CONCLUSIONS: ROS up-regulation inhibits TERT expression, suppresses TERT activity and facilitates POF. The ROS-TERT pathway may work as the target for treating POF.

Key Words:
Oxidative stress, Reactive oxygen species, Premature ovarian failure, Telomerase.

Introduction

Premature ovarian failure (POF) refers to the condition of menopause before 40 years old. It is mainly manifested as hypoestrinemia and hyperprolanemia in clinics, and severely affects fertility of women[1,2]. The female infertility has attracted increasing attentions as life style transition. Currently hormone replacement is still the major approach for POF, but its application is largely limited due to unclear pathogenic mechanism, thus requiring further investigations regarding epidemiology[3]. Telomere is the cap-like structure at the end of eukaryotic chromosome. Telomerase reverse transcriptase (TERT) is critical enzyme maintaining telomere length[4,5]. Early study showed decreasing TERT activity in normal ovarian tissues with aging[6], as ovarian TERT activity was significantly lower in females over 38 years. This is probably related with follicle deposit inside ovary[7]. We thus proposed critical regulatory role of TERT in POF. Oxidative stress refers to the over-production of hyperactive molecules such as reactive oxygen species (ROS) to surplus clearance potency of cells under harsh stimuli. Such imbalance of oxidation/anti-oxidation system can lead to tissues damage[8]. Persistent up-regulation of ROS is thus the major feature of oxidative stress in cells, and is important factor causing cell aging. It is also a key upstream index regulating TERT activity[9]. Previous study showed that persistently high level ROS stimuli in colon cancer cells can lead to cell aging caused by TERT down-regulation[10], suggesting the close correlation between POF and cell aging. So whether ROS-TERT pathway is involved in POF course has not been reported. In recent years, with increased incidence of tumor and immune disease, chemotherapy has become the major reason causing POF. We thus utilized a chemotherapy-induced POF animal model, on which the role of oxidative stress and telomere function in POF were investigated,

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in order to reveal effective target of molecular pharmacology.

Materials and Methods

Major Materials and Reagents

Beta-actin reference antibody was purchased from Kangcheng Bio. (Lianyugang, China).
Gene of phosphate and tension homology deleted on chromsome ten (PTEN), protein kinases B (AKT) and phosphorylated AKT (p-AKT) antibody was purchased from Abcam Biotech (Cambridge, MA, USA). Rabbit anti-mouse IgG (H+L), rabbit anti-mouse IgG (H+L) were purchased from Yantai Qriental Protein Tech. Co. Ltd. (Zhaoyuan, China). Enzyme linked immunosorbent assay (ELISA) kits for rat ROS, follicle-stimulating hormone (FSH) and inhibin B were purchased from Huamei Biotech (Beijing, China). HE staining kit was purchased from ZSJQ Bio. Tech. (Beijing, China). Lentiviral vector for TERT siRNA was purchased from Gimma (Shanghai, China). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Major Equipment

Gel imaging system UVP Multispectral Imaging System UVP (Sacramento, CA, USA) and PS-9 semi-dry transferring electrophoresis were purchased from Jingmai Biotech. (Jiangsu, China). Thermo-354 microplate reader was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Experimental Animals

Healthy female Wistar rats (6-8 weeks age) were purchased from Laboratory Animal Center, Xinjiang Medical University with body weight 180-220 g. Rats were kept in clean facility which was maintained at 24°C and 60% relative humidity, plus 12 h daily light and food and water *ad libitum*. Bedding was changed every day to avoid infection. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of The Fifth Affiliated Hospital of Xinjiang Medical University.

Model Preparation and Drug Delivery

Experimental rats received single intraperitoneal injection of 120 mg/kg cyclophosphamide plus intra-cutaneous injection of 12 mg/kg busulfan to generate PFO model. Based on the grouping, 5 mg/kg N-acetyl-cysteine (NAC) or 50 ng/kg lentiviral vector containing TERT siRNA were injected into the tail vein 3 days before model preparation.

HE Staining

Rats were sacrificed and ovarian tissues were fixed in 4% paraformaldehyde for 24 h, and were dehydrated in 30% sucrose solution until sedimentation. The tissue block was embedded in optimal cutting temperature (OCT), and was prepared into consecutive slices on a cryotome. After drying, tissue slices were stained by HE approach, and were rinsed in distilled water, followed by dehydration in gradient ethanol and xylene treatment. The coverslip was mounted and images were taken under a microscope.

FSH and Inhibin B Assay

Rat whole-blood samples were centrifuged to collect the serum. Assay for FSH and inhibin B was performed following the manual instruction of ELISA kits, and results were presented as mean ± standard deviation (SD). In specific, test serum was added into 96-well plate following the manual instruction, followed by the addition of horseradish peroxidase labeled secondary antibody in 37°C incubation for 1 h. The plate was rinsed for 5 times, and TMB substrate was added for 20 min dark reaction. The quenching buffer was added and absorbance value at 490 nm was measured by a micro-plate reader.

Whole-Protein Extraction

Rat ovarian tissues samples stored at -80°C were diluted in 1 ml radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotech., Shanghai, China) for 15 min incubation. The supernatant was saved after 4°C and 12000 ×g centrifugation to obtain total protein solution. The concentration of protein was measured by bicinchoninic acid (BCA) approach, and was adjusted to equal level with saline. Adding with 2× loading buffer, proteins were boiled in 5 min for denature.

Western Blot

Total protein solution was loaded into 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for electrophoresis until the separation of target protein and adjacent proteins. All samples were transferred to polyvinylidene difluoride (PVDF) membrane under 300 mA current for 1 h. The membrane was incubated overnight using Akt or p-Akt antibody

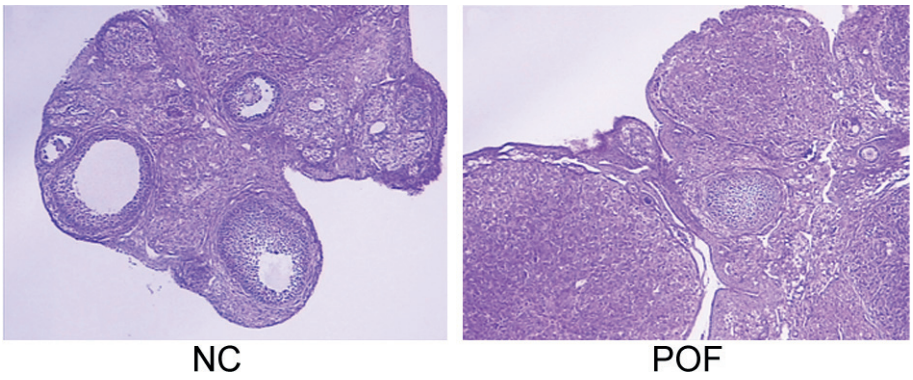

Figure 1. HE staining of all rat ovarian tissues. NC, normal control. POF, premature ovarian failure. (Magnification: 40×).

(1:1000 dilution). After three times of Tris buffer saline Tween-20 (TTBS) rinsing, secondary antibody (1:1000) was added for 37°C overnight incubation for 2 h. Chromogenic substrate was added to develop the target band.

ROS Level Assay

Rat ROS level was measured by ELISA following the manual instruction. In specific, test serum was added into 96-well plate following the manual instruction, secondary antibody with horseradish peroxidase (HRP) labeling was added for 37°C 1 h incubation. The plate was rinsed for 5 times using washing buffer, and 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added for 20 min dark incubation. After adding quenching buffer, a microplate reader was used to measure absorbance value at 490 nm.

Statistical Analysis

SPSS 19.0 software package was used to process all experimental data (IBM, Armonk, NY, USA). Results were presented as mean ± standard deviation (SD). Student's *t*-test was used to compare the differences between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. Further paired comparison was performed using SNK-Q test. $p<0.05$ was considered as statistical significance.

Results

Generation of Rat POF Model

HE staining of ovarian tissues in normal and POF rats was show in Figure 1. Normal rat ovary showed normal follicle number without arrestia or major mesenchymal hyperplasia. In contrast, model rat ovarian tissues presented atrophy morphology, reduced follicle number and enhanced follicle arrestia, accompanied with significant mesenchymal hyperplasia.

Rat FSH and Inhibin B Assay

Elevated FSH and suppressed inhibin B levels are critical markers for POF[11]. In our model rats, FSH and inhibin B levels were measured as

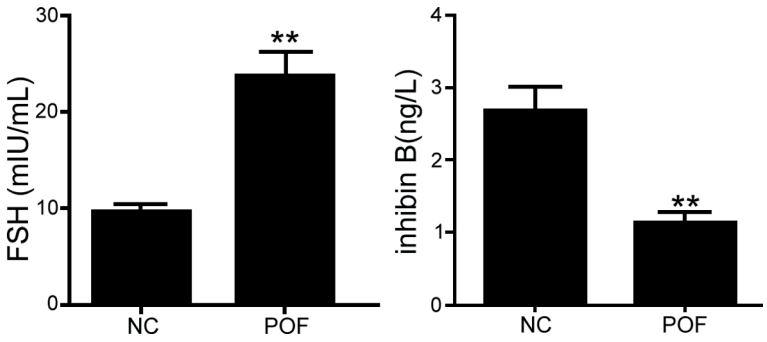

Figure 2. FSH and inhibin B level in all groups of rats. *$p<0.05$ comparing to normal control group.

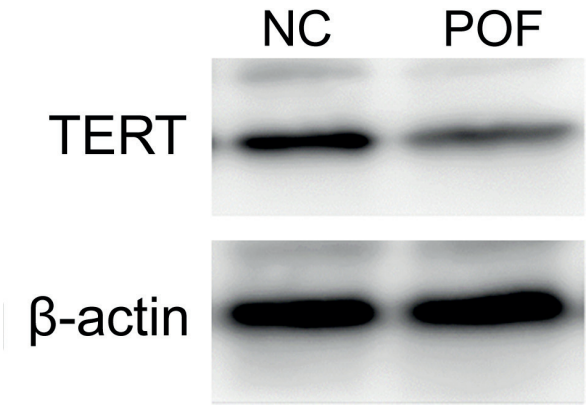

Figure 3. Expression of TERT in POF model. NC, normal control. POF, premature ovarian failure.

shown in Figure 2. In POF model, comparing to normal control group, FSH level was significantly elevated ($p<0.05$) whilst inhibin B expression was remarkably suppressed ($p<0.05$). These results indicated successful generation of POF model.

Expression of TERT in Rat POF Model

Telomere has critical roles in cell aging process, and TERT is the critical enzyme maintaining telomere length[12]. In POF model, we measured TERT expression level (Figure 3). Comparing to normal control group, POF model showed significantly depressed TERT protein level, indicating the potential involvement of TERT in POF pathogenesis.

ROS Level in POF Model

Persistently high ROS is the major feature of aging cells[13]. By measuring ROS level in POF rats, we found significantly elevated serum ROS level in model rats, with significant difference against control group ($p<0.05$). These results suggested that ROS up-regulation might be the important reason causing POF (Figure 4).

Functional Role of ROS and TERT in POF Model

To investigate the role of ROS and TERT in POF model, we suppressed ROS level or specifically inhibit TERT expression by using siRNA at animal level. As shown in Figure 5A, comparing to normal control group, POF model rats showed significantly elevated ROS level ($p<0.05$). The application of NAC via tail vein remarkably decreased ROS level ($p<0.05$ comparing to model group). Similarly, in Figure 5B, we observed remarkably decreased TERT expression in POF rats ($p<0.05$ comparing to normal control group). The application of TERT siRNA further depressed TERT expression in addition to lower TERT in model rats. To further investigate the correlation between ROS level and TERT expression, we measured TERT expression after NAC treatment. As shown in Figure 5, NAC application remarkably elevated TERT protein expression ($p<0.05$), suggesting that inhibition of ROS level effectively up-regulated TERT. Furthermore, we utilized HE staining to observe follicle morphology in Figure 5D. NAC treatment remarkably improved atrophy status of rats, increased follicle number, decreased follicle atresia, without major mesenchymal hyperplasia. In siTERT treatment group, follicle number was further decreased comparing to POF model group, accompanied with aggravated follicle atresia and significant mesenchymal hyperplasia, indicating severer POF condition comparing to model rats. These results suggested that ROS-induced TRET down-regulation was the major reason causing POF, and inhibition of ROS over-production improved ovarian function.

Discussion

A prominent feature of POF is elevated level of gonadotropin plus lower estrogen level, accompanied with primary or secondary meno-

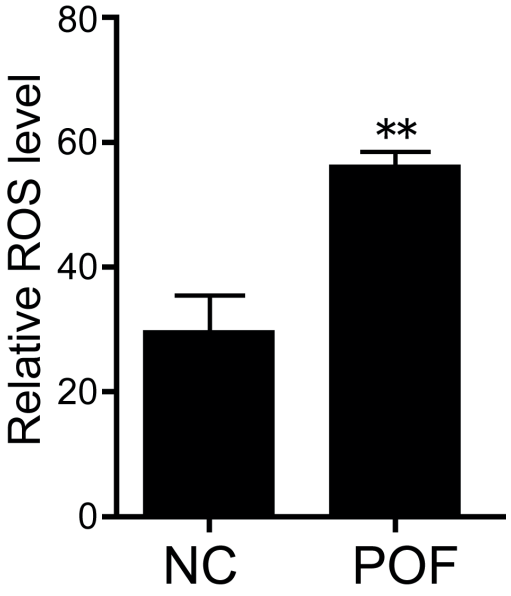

Figure 4. Rat ROS level. $^{**}p<0.01$ comparing to normal control group. NC, normal control. POF, premature ovarian failure.

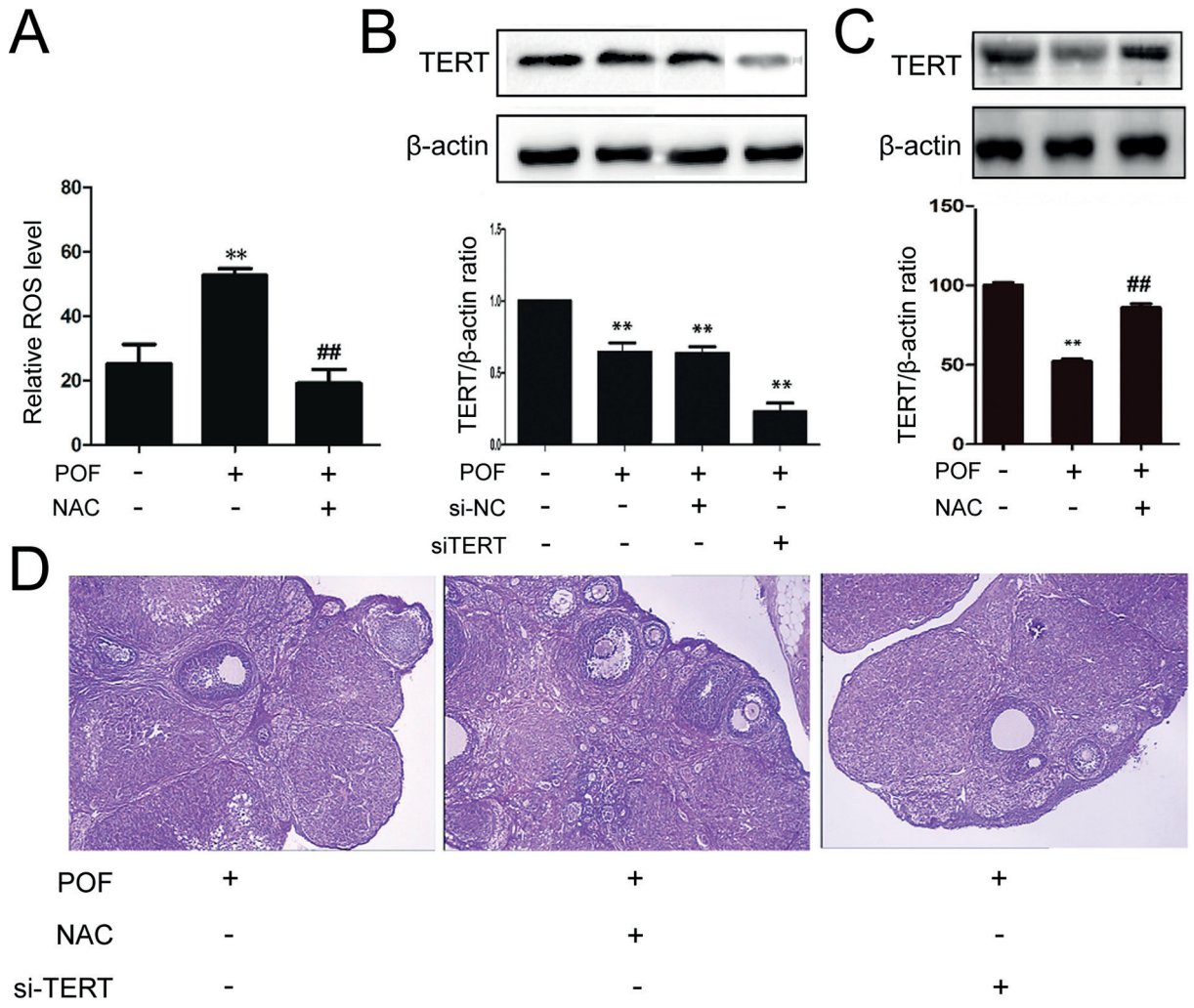

Figure 5. ROS over-production induced TERT inhibition to accelerate POF. ***(A)*** ROS level after NAC treatment. ***(B)*** TERT expression after si-TERT treatment. ***(C)*** TERT expression after NAC treatment. ***(D)*** HE staining by NAC or si-TERT treatment. $^{**}p<0.01$ comparing to control group. $^{\#\#}p<0.01$ comparing to POF group. (Magnification: 40×).

pause. Clinical features also include a series of hypo-estrogen related syndromes including depression, face reddish and sexual depression[14]. As one heterogeneous disease, POF pathogenic mechanism is still unclear yet. Elevated FSH level is the major marker for POF[15]. Under low FSH level, basal follicle starts in induce enlargement of oocyte within primordial follicle near puberty stage. With enhanced FSH receptor expression on cell surface, androgen may be secreted under gonadotrophin induction, and was later converted into estrogen under catalyze of aromatase. With enhanced synthesis of estrogen from follicles, blood FSH level was decreased, leading to atrophy and atresia of some follicular cells. Inhibin B is secreted by reproductive cells and is closely correlated with fertility, via autocrine, paracrine and endocrine regulatory roles[16]. Currently it has been confirmed that inhibin B was the major marker of ovarian depository function and testicular convoluted tubules, and can be used in the diagnosis of meal infertility caused by convoluted tubule dysfunction and female infertility related with ovarian factors[17]. In this study, we measured FSH up-regulation and inhibin B down-regulation, which were consistent with clinical diagnosis of POF. Oxidative stress refers to the over-production of ROS and reactive nitrogen species (RNS) inside body under harmful stimuli, with oxidative level surmounting clearance potency, leading to imbalance of oxidation/anti-oxidation system, causing tissue injury. In recent years, certain theory has related various factors with aging velocity, meta-

bolic control, gene regulation module and ROS over-production[18]. Increasing evidence showed that cell aging led to a series of age related dysfunction, including shortened telomere, accumulated DNA damages, abnormal oncogene activity and metabolic change[19]. With development of free radicals biology, ROS has been demonstrated to regulate apoptosis and proliferation of certain tumor cells[14, 20]. In POF women, ROS level was found to be up-regulated[21]. However, whether these mechanism are involved in POF pathogenesis is still unclear. In this study, we measured ROS up-regulation in POF rats, and found that NAC suppression of ROS level alleviated POF. As the critical enzyme maintaining telomere length, the role of TERT in POF has not been reported. However, in aged aorta smooth muscle cells, TERT up-regulation can extend telomere length and relieve DNA damage[22]. The activation of aorta telomerase can also prevent oxidative stress induced endothelial cell apoptosis[23]. In this study, we firstly identified TERT down-regulation in a rat POF model, and found that TERT suppression further aggravated POF condition via siRNA experiment. In our POF model, NAC treatment enhanced TERT expression to certain extents and relieve POF condition. These results confirmed that ROS induced TERT down-regulation might play important roles in POF pathogenesis.

Conclusions

We demonstrated the participation of ROS-TERT signal in POF pathogenesis in a rat POF model, and ROS level directly affected TERT expression. ROS-TERT thus might be important targets for treating POF. However, how does ROS regulate TERT still requires further *in vitro* assay for substantiation. ROS up-regulation can inhibit TERT expression, decrease telomerase activity, and facilitate POF. ROS-TERT pathway might be the important target treating POF.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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