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Mitochondria-targeted antioxidant MitoQ10 ameliorates p-ASK1 and TRAF2/6 in PCOS mouse model

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ABSTRACT

Mitochondrial dysfunction and the resulting oxidative stress have been increasingly recognized as playing a significant role in the development and progression of polycystic ovary syndrome (PCOS), which is the most prevalent endocrine disorder affecting women during their reproductive years. This study was designed to explore the potential therapeutic effects of MitoQ10, a novel antioxidant specifically targeted to the mitochondria, on mitochondrial function and oxidative stress markers in a mouse model of PCOS.We focused on examining the expression levels of phosphorylated apoptosis signal-regulating kinase 1 (p-ASK1) and tumor necrosis factor receptor-associated factors 2 and 6 (TRAF2/6) in granulosa cells (GCs) obtained from a dehydroepiandrosterone (DHEA)-induced PCOS mouse model by Real-Time PCR and Western blot. Female BALB/c mice were randomly assigned to one of three experimental groups: a Control group, a PCOS group induced by DHEA, and a PCOS + MitoQ10 (500 µmol/L) group. All groups were treated for a duration of 21 days.The results of our study revealed that the expression levels of p-ASK1, TRAF2, and TRAF6 were significantly elevated in the PCOS group compared to the control group, indicating increased oxidative stress and inflammatory signaling in the PCOS model. Importantly, treatment with MitoQ10 resulted in a downregulation of these elevated expression levels ($p < 0.05$), suggesting a mitigating effect of MitoQ10 on oxidative stress. However, it was noted that TRAF6 gene expression did not show a statistically significant difference between the MitoQ10-treated group and the PCOS group, indicating a potentially more complex regulatory mechanism for TRAF6.In conclusion, these preliminary findings provide evidence suggesting that MitoQ10, a readily available dietary supplement, holds promise as a potential effective adjunct in the treatment of PCOS. The observed beneficial effects warrant further investigation through properly designed clinical trials.

Introduction

Polycystic Ovary Syndrome (PCOS), the most common endocrinopathy of reproductive-aged women, has a prevalence of approximately 18% (1). PCOS is one of the leading causes of poor fertility and is associated with abdominal obesity, metabolic syndrome, and hyperandrogenism (2).

Oxidative Stress (OS) mediated by Reactive Oxygen Species (ROS) as free radicals is a

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common state affecting almost all living organisms. There is a balance between the generation of ROS and the cellular antioxidant system to scavenging ROS in any homeostatic condition (3). However, increased production of ROS more than cellular antioxidant capability of the cells leads to OS which was found to have a deleterious role in female reproduction, especially on ovarian functionality (4). Also, studies indicated evidence of OS in a range of reproductive disorders including PCOS, unexplained infertility, and endometriosis (5-7). Within the follicle, Granulosa Cells (GCs), the major cellular constituent, surround the oocyte and interact with the oocyte bi-directionally via gap junctions for the optimum follicular growth and ovulation (8). Furthermore, studies showed mitochondrial function is crucial for the ATP production in GCs which is the main source of energy for oocyte development (9, 10).

Mitochondrial respiratory chain is the major source of ROS production which they can cause cellular malfunction that can initiate pathological processes (1). Besides, functional GCs are related to the pro-oxidant state in the growing follicles (1).

The delivery of antioxidants to mitochondria is an ongoing field of active research (11, 12). Previously, we demonstrated that administration of the mitochondria-targeted antioxidant MitoQ10 has modulating effects on the ovarian folliculogenesis of PCOS mouse model. It also modulated the redox signaling pathway components, TXNIP, TRX, and ASK1 in GCs of this model which suggests it may have potential to attenuate OS and consequence damages (13).

ASK1 is a MAPKKK that regulates the JNK and p38 signaling cascades and plays pivotal roles in cellular responses to various types of stress. ASK1 detects environmental stress including pro-oxidants (14). During ASK1 signalosome formation, Regulatory proteins, Trx, TXNIP and TRAF2/6 that bind to ASK1 control its activity. Once reduced, binding of Trx suppressed ASK1 activation. However, in the presence of any types of stress like ROS, Trx is oxidized and dissociates from the signalosome, and a disulfide is formed between TXNIP and Trx. Phosphorylation of ASK1 and the recruitment of TRAF2/6 proteins activate ASK1(15).

It is needed to clarify a regulatory mechanism between redox molecular modifications in signaling pathways and a pathological process of PCOS to find out a possible treatment. To address, we studied the effects of mitochondria-targeted antioxidant, MitoQ10 on the redox signaling pathway′s elements, phosphorylated ASK1, TRAF2, and TRAF6 in GCs of PCOS mouse model.

Materials and Methods

Antibodies and Primers

The antibodies were obtained from SANTA CRUZ BIOTECHNOLOGY, INC. and applied in the present study: p-ASK 1 (B-5): sc-166967, TRAF6 (D-10): sc-8409, TRAF2 (F-2): sc-136999, β-Actin: (C4): sc-47778, m-IgGκ BP-HRP: sc-516102, and mouse anti-rabbit IgG-HRP: sc-2357 (HRP).

The primer sequences were as follows: ASK1: forward, 5′- CCCTGGAGACCCTGCATTT -3′, reverse: 5′- CATCTCCACCACAGCAATATCTG -3′; TRAF2: forward: 5′- ATCGCTACTGCTCCTTCTGC -3′, reverse: 5′- AGCTGCTCTCCAGGATTGAG -3′; TRAF6: forward: 5′- TGCGGGTCCAGCCAGTCGT -3′, reverse: 5′- TTCCCGTAAAGCCATCAAGCAGA -3′.

Induction of PCOS and Treatment

BALB/c female prepuberal mice (25 days old and 13±1grams, n= 7-10) were randomly divided into three groups as follow: Control, PCOS, PCOS + MitoQ10 groups. PCOS induction was performed based on (13). Briefly, animals in the PCOS groups were injected Dehydroepiandrosterone (DHEA) (Sigma-Aldrich, USA) (6 mg/100 g bodyweight, dissolved in 0.05 ml sesame oil (Sigma-Aldrich) and 0.01 ml 95% ethanol (Merck, Germany) for 21 consecutive days. Animals in the control group received daily injection oil (0.05 ml) for 21 days. 500 µmol/L MitoQ10 (MitoQ Ltd. New Zealand) were dissolved in clean drinking water and given fresh every third day in PCOS + MitoQ10 group (16). Animals in control and PCOS groups were given tap water, were kept under the controlled temperature and lighting conditions, and had free access to food and water. During the whole treatment period, the animals were weighed every two days, and the vaginal smears were daily provided beginning 10 days after performing the first injection until the end of the experiments.

Animals were euthanized by cervical dislocation following treatment.

Quantitative Real-Time PCR Analysis

40 μm cell strainer (BD Falcon, MA, USA) was used to separate GCs from the follicle-derived cell suspension as descried before (13) and treated with TRIzol reagent (Invitrogen, ThermoFisher) according to the manufacturer's protocol for total RNA extraction to analysis of phosphorylated ASK1, TRAF2, and TRAF6 mRNA expression. Quantitative RT-PCR was carried out as described in (13). *GAPDH* was used as an internal control. Relative expressions of each messenger RNA (mRNA) were analyzed using a comparative CT (2ΔΔCT) method. The quality of all PCR products was monitored using a post-PCR melting curve analysis. The primer sequences of the genes used in these experiments showed in table 1 were designed via Oligo Primer Analysis Software v.7. The qRT-PCR reactions were set up in 15 μl using Buffer Solution (ThermoFisher) (10 × PCR), template DNA (50ng), dNTPs (ThermoFisher) (0.1 M), each primer (2 pmol), and Taq polymerase (ThermoFisher) 2U plus double-distilled water (ThermoFisher) to 15 μl. The thermal cycling conditions were 95 °C for 15 min, 45 cycles of denaturation at 95 °C for 20 s, annealing at 59 °C for 30 s, with a final extension step at 72 °C for 30s. The qRT-PCR was performed using Master Mix BioFACT™ 2X Real-Time PCR Master Mix (For SYBR Green I) (BioFACT, Korea) on a Light Cycler® 96 System (Roche, Life Science). All the experiments were conducted in triplicate.

Western Blot and Quantitative Analysis

RIPA lysis buffer fattened with protease inhibitor cocktail (Sigma-Aldrich) was used to total protein extraction of the ovarian GCs. The concentration of the protein was measured by utilizing Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Afterward, 20 μg of protein was loaded into each lane, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was used for separating electrophoretically and finally moved to Polyvinylidene difluoride membrane (PVDF; Millipore, Billerica, MA, USA). The membrane was blocked with 2% non-fat dried milk (Sigma-Aldrich) for 75 min based on the room temperature to decrease the non-specific binding.

Next, the PVDF membrane was incubated with primary antibodies (final dilution, 1:1000) mouse monoclonal antibody p-ASK 1 (B-5), mouse monoclonal antibody TRAF2 (F-2), mouse monoclonal antibody TRAF6 (D-10), and mouse monoclonal antibody β-actin (C4) in TBST (Tris-buffered saline/Tween-20) containing 5% non-fat dried milk powder (16-18h). The membrane was washed with TBST three times within 15 min, and HRP-conjugated anti-rabbit secondary antibodies were incubated in TBST with 5% non-fat milk powder at the room temperature for 75 m. Subsequently, the TBST was implemented for washing incubated membrane three times with each 15 min. In addition, an enhanced chemiluminescence system (ECL-plus, Lumigen, Inc., Southfield, MI, USA) was used for monitoring Immunoreactivity and signals. Finally, the Image J software (NIH, Bethesda, MD, USA) was utilized for quantifying the protein bands. All the experiments were performed in triplicate.

Statistical Analysis

SPSS software (version 25 for Windows; SPSS Inc., USA) and GraphPad Prism (version 8 for windows, USA) were used for data analyses. One-way analysis of variance (ANOVA) followed by post-hoc Tukey test was used to assess the statistical significance of the differences between the groups. The P-values less than or equal to 0.05 were statistically significant**.**

Results

P-ASK1, TRAF2, and TRAF6 gene expression following MitoQ10 treatment

The present study evaluated the expression levels of the genes in redox signaling pathway, p-ASK1, TRAF2, TRAF6, and GAPDH (as a reference gene) in the GCs of the mouse ovary in control and treated groups. P-ASK1, TRAF2, and TRAF6 mRNA levels significantly rise in PCOS and downregulated after treatment by MitoQ10 which were meaningful except in TRAF6 expression among MitoQ10 and PCOS groups (Fig. 1a-c) ($p < 0.05$).

Western blot analysis of p-ASK1, TRAF2, and TRAF6 protein levels following MitoQ10 treatment

Protein assay results confirmed almost similar findings as gene expression levels showed a significant rise of p-ASK1, TRAF2, and TRAF6 protein expression levels in GCs of PCOS mouse

model which were downregulated after treatment by MitoQ10 in three groups of study (Fig. 2a-d) ($p < 0.05$).

Discussion

The results from the present study have clearly showed that MitoQ10, a mitochondria- targeted compound with profound free radical scavenger activity, has modulating effects on OS signaling pathways' components in PCOS, the most common endocrine and metabolic disorder occurring in females. Quantitative Real-Time PCR analysis revealed that phosphorylated ASK1 expression significantly increased in GCs from the PCOS mouse model as compared to the control group. Highly conserved ASK1 in an inactive state, forms a high molecular mass complex by thioredoxin (Trx-(SH)2) through the N-terminal domain. Oxidation of Trx (Trx-S2) under the oxidative stress leads to the dissociation of ASK1 from Trx. Meanwhile, the tumor necrosis factor-α receptor-associated factor (TRAF) 2 and 6 are reciprocally recruited to accelerate oligomerization of ASK1. This oligomerization induces auto-phosphorylation of ASK1 and its full activation (17). The biological activity of ASK1 depends on conditions and the cell type (18).

Study of Sirotkin et al. indicates healthy ovarian granulosa cell functions, including promoting apoptosis, inhibiting proliferation, and alter progesterone release were suppressed by ASK1 (19). It also decreases ovarian cancer cell survival and proliferation and promotes apoptosis in these cells (20). P-ASK1 gene expression downregulated following MitoQ10 treatment in GCs from the PCOS mouse model which was approved by western blot results.

There is a complex relationship between GCs and oxidative stress in reproductive system (4). Shortly before ovulation, overproduction of ROS occurred in the follicular microenvironment, following the recruitment of inflammatory cells which facilitate follicular rupture, indicating that GCs are also exposed to a higher concentration of ROS and the consequence oxidative stress during the ovulatory process (4). Studies indicated oxidative stress and inflammation have been related to the pathogenesis of PCOS (21, 22).

Mitochondria-targeted antioxidants such as MitoQ10 have been developed to decrease mitochondrial oxidative damage because of little efficacy of conventional antioxidants against oxidative stress pathologies in vivo (23). Mitochondrial membrane potential and lipophilic moiety of this class of compounds lead to accumulation of them several hundred folds within the mitochondria in vivo and makes it far more protective against oxidative damage and cell death than untargeted antioxidants (24).

Tumor necrosis factor (TNF) receptor associated factor-2 (TRAF2) is an intracellular adapter protein, which interacts with an abundance of other signaling proteins. TRAF2 is crucial for MAPK pathways stimulation, autophagy, the control of cell death programs, and endoplasmic reticulum (ER) stress signaling. In a context-dependent manner, TRAF2 can act as a tumor suppressor as well as tumor development promoter (25). TRAF2 promotes tumorigenesis in gastric and breast cancers (26). Furthermore, TRAF2 mediates JNK and p38 signaling which trigger inflammatory gene expression and promote cell survival. Studies indicated TRAF2-deficiency results in chronic inflammation and is lethal in mice (27). TRAF2 is involved in TNF-α signaling in human myometrium whereas TNF-α plays a central role in the processes of human labour and delivery (28). Other investigation suggested that ASK1 activation is responsible for the induction of inflammation that leads to preterm birth (29).

TRAF6 another cytoplasmic adaptor protein which was first discovered as a signal transduction molecule for IL1 and CD40, triggers multiple signaling pathways involved in cancer and regulates tumor cell proliferation, survival, apoptosis, and invasion (26).

To our knowledge, it was the first study that demonstrated the TRAF2 and TRAF6 expression alteration in PCOS. TRAF2, TRAF6 gene and protein levels increased significantly in PCOS condition whereas they were downregulated following MitoQ10. In this regard, our group has previously shown mitochondria-targeted antioxidant mitoQ10 may attenuate oxidative stress effects on the GCs of the PCOS mouse model, by downregulating TXNIP and ASK1 as well as upregulating the antioxidative element, Trx.

Conclusions

In conclusion, the possible role of oxidative stress in the pathophysiology of PCOS provides us with another point of view for the treatment of the disease. Evidence from the current study demonstrated that successful treatment of experimental models of PCOS can be accomplished by MitoQ10 through increased antioxidant capacity that could provide insights into the new PCOS treatment modalities, which is an area of ongoing research. This preliminary information suggests that MitoQ10 as a common and cost-saving product that is available as dietary supplements may be used as an effective supplement in the medical regimen of PCOS, following proper clinical trials.

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Data availability statement

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

Ethics approval and consent to participate

All treatments and animal care procedures were conducted based on the guidelines of the National Institute of Health on caring and using animals and approved by the Research Deputy and Ethics Committee of Tehran University of Medical Sciences.

Conflict of interests

The authors of the current paper declare no conflict of interest regarding to all time periods of working, neither financial support nor personal relationship.

Figure legends:

Fig 1. *P-ASK1*, *TRAF2*, and *TRAF6* gene expression following MitoQ10 treatment

Fig 2. P-ASK1, TRAF2, and TRAF6 protein expression following MitoQ10 treatment

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