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Original Research

Hyperandrogenism stimulates inflammation and promote apoptosis of cumulus cells

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Abstract: To explore roles of androgen and cytokines such as IL-1, IL-6 and TNF-α in PCOS. Cumulus cells were isolated from rat COCs and divided into two sets of experiments. Set 1 includes 4 groups: Control group, IL-1 group (final concentration at 5 nM), IL-6 group (final concentration at 5nM) and TNF-α group (final concentration at 5nM). Set 2 also include 4 groups: Control group, Androgen group 1 (final concentration at 1 nM), Androgen group 2 (final concentration at 10 nM) and Androgen group 3 (final concentration at 100 nM). Androgen, at final concentration of 100 nM, can induce secretion of cytokines include IL-1, IL-6 and TNF-α in cumulus cells. This in turn leads to cell apoptosis probably through ROS production as well as promotes oocyte maturation. Androgen and cytokines that developed during PCOS contribute to ROS production, apoptosis and oocyte maturation in that disease.

Key words: PCOS; Androgen; Cytokine; ROS; Apoptosis; Oocyte maturation.

Introduction

PCOS is a heterogeneous hormone-imbalance disorder with prevalence of approximately 4% to 18% in reproductive-aged women worldwide (1-2). PCOS is associated with hyper-androgenism, chronic oligo/anovulation, polycystic ovaries, insulin resistance and type 2 diabetes mellitus (3). Despite all these disturbance led by PCOS, its etiology is still to be elucidated.

PCOS is a pro-inflammatory state, and emerging data suggests that chronic low-grade inflammation underpins the development of metabolic aberration and ovarian dysfunction in the disorder (4-5). IL-1 (Interleukin-1), IL-6 (Interleukin-6) and TNF-α (Tumor necrosis factor-α) are all correlated with inflammation in PCOS (6-7). CRP is the most reliable circulating marker of chronic low-grade inflammation in PCOS. However, CRP was shown to be more related with obese that is the complication of PCOS, rather than with PCOS itself (8-10).

Circulating androgens can raise the inflammatory load in lean healthy reproductive-age women who were normally not inflamed at baseline (10). On the other hand, in PCOS women, inflammation may promote hyper-androgenism in the disorder (11). However, hyper androgenism per se in PCOS exerts an anti-inflammatory effect rather than pro-inflammatory effect when obesity is present in the disorder (7).

Women with PCOS frequently present with reproductive dysfunction, and PCOS is the main cause of infertility due to dysfunctional follicular maturation and ovulation, distinctive multicystic ovaries and dysregulation of reproductive hormones including luteinizing hormone (LH) hypersecretion and hyperandrogenism in this disorder (12). Human follicle is formed by oocytes combining with its surrounding supported cells include granulosa cells and theca cells (both are somatic cells) (13). Follicle development is an ordered process. Primordial follicles are first recruited into a cohort of growing follicles, then only one growing follicle is selected to ovulate to a mature oocyte (14). The primordial follicle comprises an oocyte arrested in meiotic prophase I which is surrounded by squamous granulosa cells. When the diameter of oocyte reached 140 μm, antral follicle formed. At this point, extracellular fluid accumulated and granulosa cell layers differentiated into mural and cumulus cell subpopulations(15-16). Cumulus cells (CCs) are specialized somatic cells that surround and nourish oocytes by providing a number of specific proteins and enzymes which are required for oocyte development and fertilization (17). PCOS can perturb the intrafollicular environment, through cumulus cell-oocyte signaling directly or indirectly (18).

Suppressing inflammation of PCOS promoted apoptosis of theca-interstitial, another supporting cells of oocytes other than granulosa cells, so inflammation might play a vital role in PCOS (19). In this study, we revealed that inflammation itself without other PCOS background can induce apoptosis and oxidative stress while inhibit proliferation of primary cultured cumulus cells from SD rats. What's more, hyperandrogenism per se can induce apoptosis in cumulus cells.

Materials and Methods

Rat Husbandary

Female Sprague-Dawley rats of 1 months of age were kept in a 12 h light: 12 h darkness schedule (lights from 08.00 to 20.00 hours) and an ordinary laboratory diet was available ad libitum. The animals were injec-

ted with a superovulatory dose (20 IU) of PMSG (pregnant mare serum gonadotrophin), followed by a single injection of 10 IU hCG (Human chorionic gonadotropin) 48 h later to induce ovulation (which occurred 14-16 h later). During various periovulatory periods, the animals were killed by cervical dislocation and ovaries were removed. , large follicles (0.6-1.0 mm in diameter) were punctured under microscope and the follicle content was expressed into the medium. Cumulus-oocyte complexes were collected into fresh medium and washed three times by repeated transfer.

Grouping

Cumulus cells were isolated from COC which itself is harvested from SD rats according to published protocols (20). Cells were then divided into two sets of experiments. Set 1 includes 4 groups: "Control group", "IL-1 group (addition of IL-1 at final concentration of 5 nM)", "IL-6 group (addition of IL-6 at final concentration of 5nM)" and "TNF-α group (addition of TNF-α at final concentration of 5nM)". Set 2 also includes 4 groups: "Control group", "Androgen group 1 (addition of Androgen at final concentration of 1 nM)", "Androgen group 2 (addition of Androgen at final concentration of 10 nM)" and "Androgen group 3 (addition of Androgen at final concentration of 100 nM)".

Cell Count

Granulosa cells were cultured to reach 80% confluence and grouped as cumulus cells did. Cells in each group were inoculated into a 24- well plate.

Quantitative PCR

mRNA were extracted from cultured cumulus cells then q-PCR were performed using commercial available kits (Thermo 11746-100). Relative quantitative expression level was calculated byΔΔCT Method.

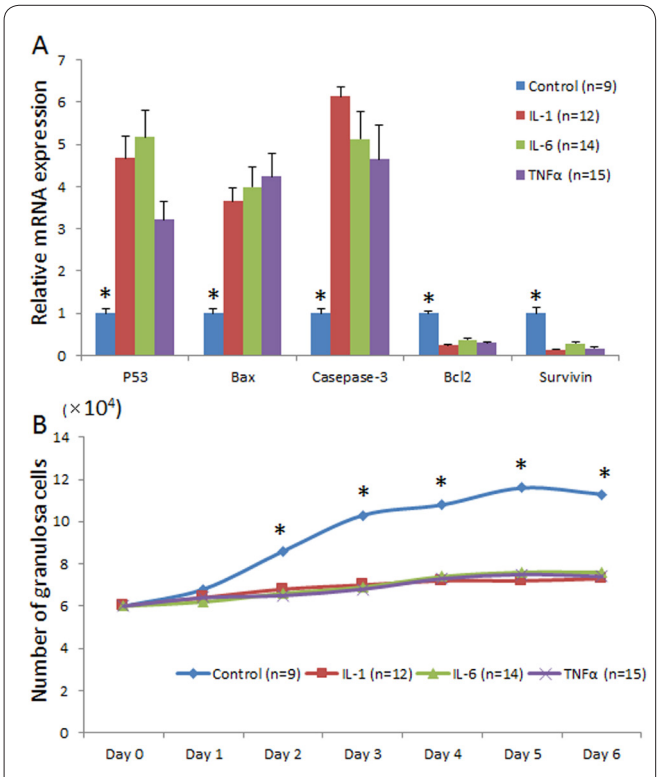

Figure 1. (A) All these three cytokines up-regulated pro apoptotic factors include p53, Bax, Caspase-3 but down-regulated anti-apoptotic factors: Bcl-2 and Survivin. (B) All these three cytokines suppress granulosa cell proliferation from day 2 until day 6.

mtDNA Copy

The number of mtDNA (mitochondria DNA) Copy was determined as previously reported (21). In brief, DNA was extracted from cumulus cells using phenol – chloroform methods. Primers were designed using software and synthesized by Sangon biotech (Shang hai). Then the target fragments were amplified using PCR, and the number of mtDNA was determined by RT-PCR.

ROS level

The level of ROS was assessed with 2,7-dichlorofluorescein diacetate (DCFH-DA, Beyotime) as previously described (22).

Statistical Analysis

All statistical analysis was calculated using SPSS17.0 software. Statistic significance was defined when $P < 0.05$.

Results

To set up the chronic inflammation environment that mimics that in PCOS, cytokines include IL-1, IL-6 and TNF-α were added into cumulus culture respectively, which were noted as IL-6 group, IL-1 group and TNF-α group as described in material and methods. All these cytokines can up-regulated pro-apoptotic proteins include p53, Caspase-3 and Bax but down-regulated anti-apoptotic proteins Bcl-2 and Survivin (Figure 1A). Consistent with this, these three cytokines all reduce the number of cultured granulosa cells (Figure 1B) which confirmed the role of inflammation in suppressing cell proliferation through inducing apoptosis. Interestingly,

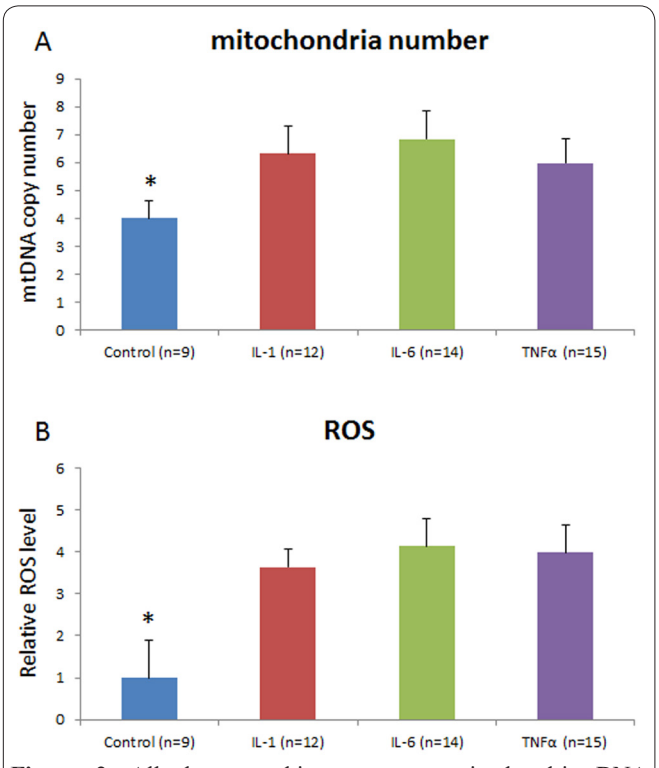

Figure 2. All three cytokines promote mitochondria DNA expanding (A) while increase ROS levels of cumulus cells (B).

these cytokines also increase ROS levels in cumulus cells (Figure 2B). ROS was known to induce apoptosis in granulosa cells, so it might be one of the mechanisms that these cytokines induce apoptosis(23). What's more, these three cytokines boost mitochondria DNA expansion therefore revealed its role in oocyte maturation (24-25).

As shown in Figure 3, androgen enhanced release of IL-1, IL-6 and TNF-α by cumulus cells at final concentration of 100 nM but not at 1 nM and 10 nM. As a result, androgen, at final concentration of 100 nM, also induced effects as those by these three cytokines. It promoted expressions of pro-apoptotic factors p53, Bax and Caspase-3 while restrained expressions of anti-apoptotic factors Bcl-2 and surviving (Figure 4A). It hindered proliferation of granulosa cells (Figure 4B). It also favored oocyte maturation as shown by increased number of mitochondria DNA (Figure 4C). Finally, androgen at all three concentrations (1 nM, 10 nM and 100 nM) all rise ROS levels in cumulus cells, and to a greater extend at final concentration of 100 nM (Figure 4D).

Discussion

Although many studies of PCOS, its exact etiology was still to be elucidated. Here, we induce inflammation in cumulus cells and tried to explore the isolated role of chronic inflammation in PCOS without other background.

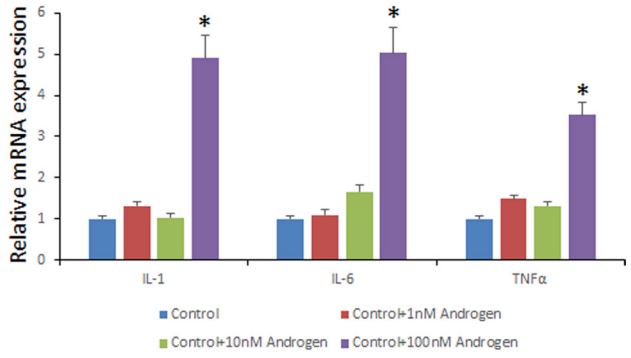

Figure 3. Androgen at final concentration of 100 nM restrained expression of cytokines include IL-1, IL-6 and TNF-α.

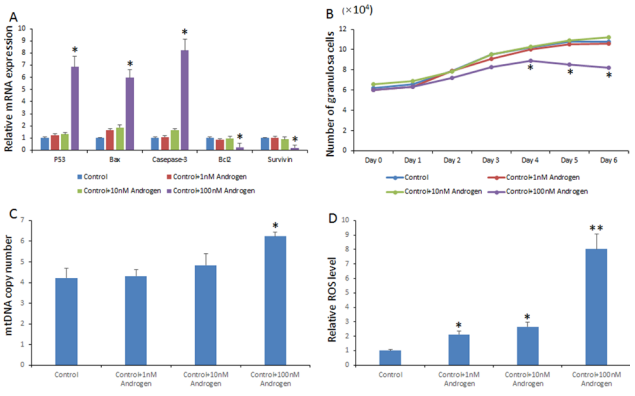

Figure 4. Androgen at final concentration of 100 nM enhanced expressions of pro-apoptotic factors such as p53, Bax and Caspase-3 but depressed anti-apoptotic factors include Bcl-2 and Survivin (A)therefore inhibited proliferation of granulosa cells with significant difference at day 4 to day 6 (B) and stimulated mitochondria DNA expansion (C). Androgen up-regulated ROS levels at 1 nM and 10 nM but in a greater extend at 100 nM (D).

We showed that inflammation in cumulus cells induced apoptosis and restrained cell proliferation. The roles of IL-1, IL-6 and TNF-α in apoptosis are inconsistent, probably in a tissue specific manner. For example, both IL-1 and IL-6 promoted apoptosis in human fetal membranes (26). But IL-1 failed to induce apoptosis in intestinal epithelial cells (27) and IL-6 improves proliferation rather than lead to apoptosis in head and neck squamous carcinoma cell (28). TNF-α induces apoptosis in the lower gastrointestinal tract (29) but exert anti-apoptotic role in chronic lymphocytic leukemia (30). p53 was known to be associated with cytokines include IL-1, IL-6 and TNF-α (31). And cytokines such as IL-1 are reported to induce apoptotic signaling, the reduction of Bax/Bcl-2 ratio and caspase-3 activity (32). Consistent with their role in a tissue specific manner, cytokine up-regulate survivin level in normal eosinophils (33) while down-regulate survivin in cumulus cells in this study. In many studies, cytokines such as IL-1, IL-6 and TNF-α stimulate cell proliferation (34-35) but they inhibit granulosa cells growth in this study which might through inducing cell apoptotic process. Up-regulating ROS might be one of the mechanism that cytokines inducing apoptosis as shown in Figure 2B. Although, IL-1, IL-6 and TNF-α were demonstrated to be up-regulated by ROS in a dose dependent manner (36), here we show these cytokines can also increase ROS levels.

Comply with the role of cytokines in oocyte maturation, IL-1 was shown to involved in equine oocyte in vitro maturation (37). Other cytokines such as IL-6, TNF-α and CSF-1 play important roles in the regulation of embryonic development (38).

Androgen was revealed to inhibit IL-1α or TNF-α induced pro-inflammatory cytokine production from synovial fibroblast-like cells (39) and deprivation of androgen increase those cytokines in human plasma (40). Different from results from these studies regarding relationship between cytokines and androgen, we report that administration of androgen at 100 nM induce high transcription levels of IL-1, IL-6 and TNF-α. Since hyperandrogenism exert anti-inflammation role in obese PCOS patient but rise inflammation load in lean women (7, 10), we speculate lipid metabolism also play a role in the regulation of cytokines by androgen. Indeed, cytokines like IL-1, IL-6 and TNF-α affect the phospholipid metabolism and subsequent production of ROS in brain tissue after stroke (41). As a result of induction of cytokines in cumulus cells, androgen (100 nM of final concentration) increase pro-apoptotic factors p53, Bax and Caspase-3 but decrease anti-apoptotic factors Bcl-2 and Survivin. This is contradictory to other reports that advocate the apoptosis inhibition role of androgen through suppressing cytokines (42-43). It can be explained by the different role of androgen in different tissues. What's more, androgen up-regulated suppressor of cytokines signaling (SOCS) which can also be induced by interleukins (44). Therefore, androgen administration might first rise cytokine levels and activate SOCS at the same time to down-regulate the increased cytokine levels. And cytokines themselves worked in a negative feedback loop to prevent further accumulation also through activating SOCS. The releasing of SOCS and

the negative feedback of cytokine might not be accomplished within one type of cells. Therefore, we observed the up-regulation of cytokine then apoptosis process after administration of androgen in cumulus cells and androgen suppress its proliferation.

Androgen, at final concentration of 100 nM, also favors oocyte maturation as shown by increasing the number of mitochondria DNA in cumulus cells. This is consistent with its role of promoting oocyte maturation in Xenopus laevis ovaries(45). Androgen was also demonstrated in this study to boost ROS levels in cumulus cells probably through increasing cytokine levels. Androgen was also known to induce oxidative stress both in transformed cells (46) and in normal cells such as hair follicle dermal papilla cells (47).

In summary, Androgen, at final concentration of 100 nM, can induce secretion of cytokines include IL-1, IL-6 and TNF-α in cumulus cells. This resulted in favoring apoptosis process then lead to proliferation inhibition. Increasing ROS levels might contribute to the apoptosis process. Cytokines, which could be induced by androgen, also promote oocyte maturation as shown by the number of mitochondria DNA.

Androgen and cytokines that developed during PCOS contribute to ROS production, apoptosis and oocyte maturation in that disease.

Declaration of Interest

There is no known conflict of interest and all procedures were complied with local ethic committee regulations.

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