TNFR1 is Upregulated and Mediates Apoptosis in Cumulus Cells of Women With Polycystic Ovarian Syndrome

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Research

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Abstract

Purpose

To investigate the changes of human granulosa cell, TNFR1, TNFR2 and their downstream molecules in patients with polycystic ovary syndrome (PCOS) and the control group.

Methods

We recruited infertile women with polycystic ovary syndrome (n = 30) and compared them with infertility due to fallopian tube obstruction(n = 30, control group). The ovaries were stimulated with GnRH agonists and gonadotropins. Follicular fluid from large follicles ([14 mm]) was pooled and granulosa cells (GCs) were separated by a cellular filter. The TNF-α level of follicular fluid was measured by ELISA. TUNEL assay were used to detect the apoptosis of purified GCs. Real-time PCR and Western blotting were used to detect the expression of TNF-related signaling molecules in GCs.

Results

The rate of high quality embryos in the PCOS group was lower than that in the control group. There were higher percentages of apoptosis in GCs of PCOS patients than in the control group. TNF-α is upregulated in follicular fluid of PCOS patients. TNFR1 and caspase-3 mRNA level were signifificantly higher in PCOS group than in the control group. TNF-α-mediated apoptosis of PCOS granulosa cells was mainly dependent on TNFR1.The TNF-α/TNFR1 signaling pathway mediates apoptosis rather than survival in cumulus cells of PCOS patients.

Conclusions

TNF-α expression was upregulated in follicular fluid of PCOS patients, and TNFR1 overexpression in female granulosa cells of PCOS was associated with higher levels of apoptosis in these cells, suggesting that the TNF-α/TNFR1 signaling pathway may be a candidate for higher apoptosis in female granulosa cells of PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is a common reproductive disorder, affecting 5–20% of the reproductive-age female population worldwide[1, 2]. In addition, PCOS is associated with ovulatory dysfunction, abdominal adiposity, insulin resistance, obesity, excessive androgen production and cardiovascular risk factors. However, the genetic mechanisms of PCOS remain largely unknown, since the etiology of the disease is very complex and affected both by genomic and environmental factors.

Therefore, an improved understanding of the genetic mechanisms of PCOS may provide novel insights into the treatment and diagnosis of PCOS[3].

Polycystic ovary syndrome (PCOS) is one of the most prevailing endocrine disorders with an incidence of 4–21% in women at reproductive age[3].

It is one of the main causes of infertility in women and accounts for almost 75% of anovulatory subfertilities [5]. It has been revealed that both inherited and environmental factors are involved in PCOS [3, 4]. Despite the numerous studies, the exact underlying cellular/molecular mechanisms of PCOS have not yet been understood.

Polycystic ovary syndrome (PCOS), a highly prevalent gynecological disease, is often characterized by amenorrhea, hirsutism, polycystic ovaries on ultrasound scan, elevated serum luteinizing hormone (LH), low serum follicle-stimulating hormone (FSH), anovulatory infertility, pregnancy and neonatal complications[6].Although assisted reproduction techniques (ARTs) are commonly used for PCOS associated infertility, there are still some questions regarding the paucity of high quality oocyte, low fertilization, and fnal pregnancy rate in women with PCOS following ART.In antral follicles, cumulus cells encircle the oocyte to form the cumulus -oocyte-complex (COC). Cumulus cells-oocyte cross-talk plays a very important role in the development,maturity, and fertilization ability of oocyte. In the literature, it has been shown that apoptosis of cumulus cells correlates with poor oocyte outcomes, embryo fragmentation, and impaired blastocyst development[7, 8]. Moreover, it

has been demonstrated that the percentage of apoptotic cells in women who achieved pregnancy was signifcantly lower than that of women who did not become pregnant[9]. Apoptosis is an active process of cellular deconstruction triggered by changes in the levels of specifc stimuli. An apoptotic cell is marked by shrinkage, nuclear chromatin condensation, fragmentation, and budding of the plasma membrane. Finally, the cell turns into apoptotic bodies which contain cell organelles and/or genetic material [10].

Apoptosis of granulosa cells seems to have a negative effect on IVF outcomes. A higher incidence of apoptotic granulosa cells has been related to poor embryo development, and low fertilization and pregnancy rates[11]. PCOS patients undergoing IVF procedure produce a high number of oocytes but approximately, all of them have poor quality leading to poor outcomes[12, 13]. Thus, investigation of the mechanisms of high apoptosis in granulosa cells of PCOS patients is of great clinical importance.

Tumor necrosis factor-alpha (TNF-α) mediates a variety of biological activities by binding to TNF receptor (TNFR) 1 and TNFR2, including cell apoptosis and proliferation[14].TNFR1 is a member of the family of death receptors. The intracellular segment of TNFR1 includes an important and structured domains, namely the death domain (DD)[15, 16], which are required for the induction of apoptosis by TNFR1 ligands.The death domain of TNFR1 is well characterized and mediates homotypic interactions with other death domain-containing proteins. TNFR1 and TRADD interact through their DDs in response to sTNF-α. This interaction is the basis for the formation of two alternative protein complexes (complex I and complex II). Complex I is formed at the cell membrane by recruiting RIP1, TRAF2 and cIAP1/2. This

complex leads to activation of NF-κB and promotes cell survival and anti-apoptotic activities. TNFR1 can be also internalized into the cytoplasm, which is dependent on the TRID domain. Subsequently, a second cytosolic complex (complex II) can be formed by the recruitment of FADD and procaspase 8 to the internalized TNFR1-TRADD complex. This complex is called death-inducing signaling complex (DISC) as it is crucial for the induction of apoptosis[14, 17, 18]. In general, the default signaling pathway of sTNF-α is NF-κB pathway activated by complex I at the cell surface. Furthermore, NF-κB activation renders tumor cells resistant to sTNF-α-induced cytotoxicity. However, in case of impaired NF-κB activation the cytosolic complex II is formed, thus switching the pro-survival signaling to pro-death signaling.

The importance of TNF-α and its receptors has been demonstrated in ovarian function[19]. It has been reported that binding of sTNF-α and TNFR1 in granulosa cells can mediate ubiquitin-mediated phosphorylation and degradation IκB induces NF-κB migration into the nucleus, and activation of NF-κB is associated with XIAP synthesis, thereby promoting granulosa cell survival[20]. The binding of sTNF-α to TNFR1 also promoted the proliferation of granulosa cells by increasing the expression level of c-Jun[21]. However, little is known on the function of TNF-α in human ovary in PCOS condition. Reports indicate that TNF-α level was signifificantly higher in PCOS group than control[22], but the molecular mechanisms of TNF-α signaling pathway in this condition is still unclear. For this reason, we investigated the changes in TNFR1 and TNFR2 expressions in human granulosa cells. We hypothesized that the TNF-α signaling pathway in human granulosa cells play a crucial role in higher rate of apoptosis in PCOS patients.

Results

Characteristics and clinical outcomes of the study population

The characteristics and clinical outcomes of patients with PCOS and control group are presented in Table 1. The average age of women taking part in this study was 30.2 years (29.73 ± 2.46 years in control group and 28.1 ± 3.07 years in PCOS group). No statistically significant differences were found between PCOS or control group regarding age of women, infertility duration, duration of stimulation, number of oocytes, number of MII oocytes, number of 2PN oocytes, fertilized oocytes and high quality embryos. Moreover, the data revealed significantly higher BMI (p=0.0005), lower FSH (p=0.0089) and lower high quality embryos rate (p=0.0431) in the patient group in comparison with the control group.

Apoptosis of cumulus cells was increased in PCOS patients

As the rate of high quality embryos in the patient group was lower than that in the control group. Next, we collected cumulus cells from healthy and PCOS individuals to detect apoptosis by TUNEL assay. Apoptosis assay showed that there was a high rate of apoptosis among cumulus cells from PCOS patients compared to the cells obtained from healthy individuals (Fig 1).

TNF-α is upregulated in follicular fluid of PCOS patients

In order to explore the role of TNF-α in cumulus cells of PCOS patients, we collected follicular fluid from healthy and PCOS patients. The TNF-α level was measured by ELISA in 30 PCOS patients and 30 controls. Significantly increased follicular fluid TNF-α levels were found in PCOS patients (16.61±8.54) as compared to controls (4.61±3.71;$p < 0.0001$) (Fig 2).

TNF-α-mediated apoptosis of PCOS granulosa cells was mainly dependent on TNFR1

As shown in Fig. 3, we found that the mRNA expression level of TNFR2 has no differences in the cumulus cells of PCOS patients compared to the control group ($p>0.05$). In contrast, the mRNA expression levels of TNF-α, TNFR1 and caspase-3 were higher in the patients than the control group ($p<0.05$). Fluorescence staining(Fig. 4) and western blot(Fig. 5) results showed that TNFR1 expression was up-regulated, but TNFR2 expression was not changed.

The TNF-α/TNFR1 signaling pathway mediates apoptosis rather than survival in cumulus cells of PCOS patients

As we know, the TNF-α/TNFR1 signaling pathway mediates three different major cellular responses: cell survival through NF-κB activation, apoptosis, and necrosis[34]. Cumulus cells from healthy and PCOS patients were collected to extract total cell proteins, and related signal molecules were detected by Western blot. As shown in Fig. 5, TNFR1 activation by TNF-α did not cause IκB degradation. Nevertheless, the activation of caspase-3 was detected in cumulus cells of PCOS. These results suggest that TNF-mediated signal transduction in PCOS cumulus cells leads to apoptosis rather than survival.

Materials And Methods

Study population

Thirty control women and thirty patients with PCOS were recruited in this study from those who were referred to the reproductive Medicine Center, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology for in-vitro fertilization (IVF) treatment. The control group aged 29.73±2.46 years and had a regular menstrual cycle. These women selected from couples with only fallopian tube factors. The PCOS patients had a mean age of 28.1±3.07 years and were recruited if they had at least two of the Rotterdam-PCOS criteria (oligo/amenorrhea, clinical or biochemical hyper -androgenism, and polycystic ovaries on ultrasonography). We excluded women with endometriosis, immune and inflammatory diseases, endocrine disorders, and history of smoking. All the procedures were conducted according to the Declaration of Helsinki and approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. Signed informed consent was also obtained from all participants.

Isolation of the cumulus cells

Following follicular puncture, cumulus oocyte complexes (COCs) were collected and cumulus cells around the oocyte were removed using a syringe needle and transferred into GMOPS-PLUS (vitrolife

Sweden AB,V,Frolunda,Sweden). The cells were washed three times with sterile phosphate buffer brine (PBS) centrifugation (1000 RPM for 5 min). Oocytes were further inseminated and embryos were cultured in sequential media of G1 (vitrolife Sweden AB,V,Frolunda,Sweden) covered by mineral oil. Embryos were transferred or vitrified on Day 3 and the other embryos were cultured to blastula stage on Day 5–6.

Detection of TNF-α by ELISA

Follicular fluid was collected from healthy and PCOS patients after follicular puncture. The concentration of TNF-α in follicular fluid was determined by ELISA (Invitrogen, Austria) according to the instructions. After adding 100 μL/well of Stop Solution, the OD value at 450 nm was measured on a microplate reader (Tecan, Melbourne, Victoria, Australia).

Quantitative real-time PCR

Total RNA was extracted using the TRIZOL reagent(Invitrogen) and reverse-transcribed to cDNA usingHiFiScript cDNA Synthesis Kit (CoWinBiotech, Beijing,China) according to the manufacturer's instructions. Theprimers were synthesized by Tsingke (Wuhan, China) and their sequences were listed in Table 2. Realtime-PCR amplification of cDNA was conducted in20μl UltraSYBR Mixture (with ROX) (Beijing CoWinBiotech, Beijing, China) using the CFX Connect Real-Time PCR Detection System (Bio-Rad). The reactions were performed in triplicate as follows: 95 °C for 10 min,followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.Results were analyzed using the 2−□□Ctmethod andnormalized to the corresponding level of GAPDH.

DAPI and TUNEL staining

To evaluate apoptosis status of cumulus cells isolated from PCOS and control individuals, the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was performed with a commercial kit (Boster, China) according to the manufacturer's protocol. Nuclei were counterstained with DAPI. The cells were evaluated under a fluorescent microscope (Olympus system, Japan). This experiment was performed in triplicate.

Western blot analysis

50 mg of total protein was loaded and separated on 12% SDS-polyacrylamide gels by electrophoresis. The separated protein was then transferred to PVDF membranes (Millipore) using a semi-dry transfer system (Integrated Separation Systems). These membranes were blocked for 2 h at RT with 5% nonfat dry milk in TBS-Tween 20 (0.05%), and then probed overnight at 4°C with primary antibodies, including anti-TNF-α, anti-TRAF2, anti-caspase 3 (Cell Signaling Technology) ,anti-TNFR1, anti-TNFR2(Santa Cruz Biotechnology, Inc), anti-GAPDH, anti-IkB (ABclonal Technology). After washes, the membranes were incubated for 1 h with corresponding horseradish peroxidase-conjugated secondary antibodies, and then visualized using SuperSignal West Pico Chemiluminescence Substrate (Thermo).

Statistical analysis

Statistical analysis was performed with GraphPad Prism V6 software using one or two-way (where appropriate) ANOVA followed by Bonferroni post-test. Data are represented as mean ± SD. Statistical significance in the figures is indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Discussion

Our results have shown the high quality embryos rate was significantly lower in PCOS patients rather than non-PCOS. However, we could not find any statistically

significant differences in number of oocytes, number of MII oocytes and fertilized oocytes(%) between two groups. In line with our findings, Ludwig et al.[24]and Nikbakht et al. [25]concluded that a lower number of 'high quality oocytes' in PCOS compared to non-PCOS patients. One of the major limiting factors for improving the ART success rates of patients with PCOS is oocyte quality. Oocyte developmental competence or quality is achieved during folliculogenesis as the oocyte grows[26]. It has been observed that oocyte competence is altered in PCOS patients. Metabolic dysfunctions, endocrine/paracrine abnormalities and abnormal follicle microenvironment in PCOS are related to impaired oocyte competence [27].

Our results indicated that TNF-α levels were elevated in cumulus cells of women with PCOS in comparison with the control group. Previous studies have also revealed that women with PCOS have increased serum levels of TNF-α[23, 28]. Elevated levels of TNF-α in the cumulus cells of PCOS patients could be a result of excessive inflammation in these patients. Moreover, Raei Sadigh et al. Concluded that expression levels of TNF-α in the cumulus cells were negatively associated with the fertilization rate of oocytes which is in good accordance with previous fndings regarding the negative correlation between TNF-α levels in follicular or embryo culture fuids with fertilization rate [29, 30].

In the present study, we found that the gene expression level of TNFR1 and TNF-α was higher in cumulus cells of women with PCOS while caspase-3 showed higher mRNA levels in PCOS patients compared to those of the control group. In addition, our results indicated that the altered expression of these genes in cumulus cells of PCOS patients may be related to poor quality of the corresponding embryos.

TNF-a is a pleiotropic cytokine with important functions in homeostasis and disease pathogenesis. TNF-a exerts a wide range of biological effects through two known transmembrane receptors[31]: the 55 kDa TNF receptor 1 (TNFR1), which is expressed on almost all cell types; and the larger 75 kDa TNF receptor 2 (TNFR2), which under normal physiological conditions is expressed typically at low levels in cells of the immune system[32]. In the present study, we found that both TNFR1 and TNFR2 were expressed in granulosa cells, while the gene expression level of TNFR1 was higher in cumulus cells of women with PCOS compared to those of the control group. Indicate that, TNFR1 serves as the major mediator of TNF-induced signaling pathways in cumulus cells of PCOS patients.

As we known, TNF-α induced three major cellular responses through different signal-transduction pathways: cell survival through NF-kB activation, apoptosis, and necrosis[33]. TNFR1 signaling has

pleiotropic functions, such as activation of nuclear factor kappaB (NF-kB) and inducing apoptosis, which depends on the cellular environment. It has been reported that binding of sTNF-α and TNFR1 in granulosa cells can mediate ubiquitin-mediated phosphorylation and degradation IκB induces NF-κB migration into the nucleus, and activation of NF-κB is associated with XIAP synthesis, thereby promoting granulosa cell survival[20].The binding of sTNF-α to TNFR1 also promoted the proliferation of granulosa cells by increasing the expression level of c-Jun[21]. However,our results indicate that granulosa cells in PCOS patients experience apoptosis rather than survival signaling despite increased TNF-α in follicular fluid.

The mechanism of TNF-α signaling from survival to apoptosis is still unclear, and we suggest further studies at the molecular and cellular level. Recent studies have shown that the ubiquitination of RIP1 determines whether it will function as a pro-survival or pro-cell death molecule[33, 34].Several proteins intercept TNF-induced NF-kB activation at the level of ubiquitinated RIP1. Studies published in the past 5 years have revealed the roles of Sharpin, A20 and ubiquitin carboxyl-terminal hydrolase CYLD in the decision to undergo cell death[35–39].In addition, PCOS is a common endocrine and metabolic disorder which is characterized by increased circulating androgen levels, anovulatory infertility, and, frequently, insulin resistance and hyperinsulinemia[40]. All of these factors may be related to changes in the direction of the TNF-α/TNFR1 signaling pathway.

Declarations

Acknowledgments

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Authors' contributions

Lei Jin contributed to the conception of the study; Yaping Jiang, Hongping Ba performed the experiment; Rui Jiang, Peng Zhang contributed significantly to analysis and manuscript preparation; Yaping Jiang, Rui Jiang performed the data analyses and wrote the manuscript; Qiong Yu helped perform the analysis with constructive discussions.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology.

Consent for publication

Not required.

Competing interests

The authors declare that they have no confict of interest.

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Figures

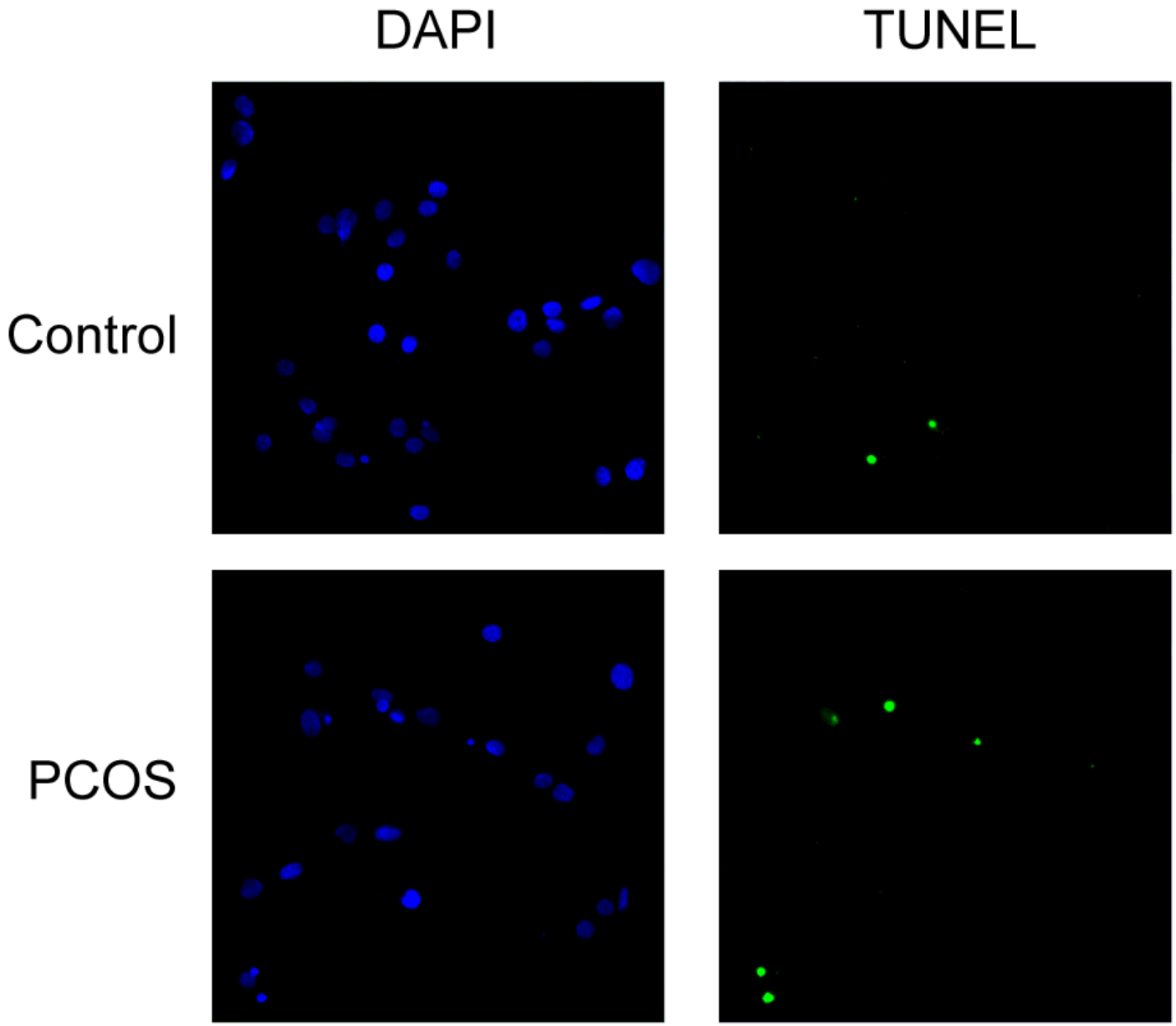

Figure 1

Apoptosis of cumulus cells was increased in PCOS patients. GCs obtained from control and polycystic ovarian syndrome (PCOS) patients, Evaluating apoptosis rate of granulosa cells (GCs) using TUNEL and DAPI staining.

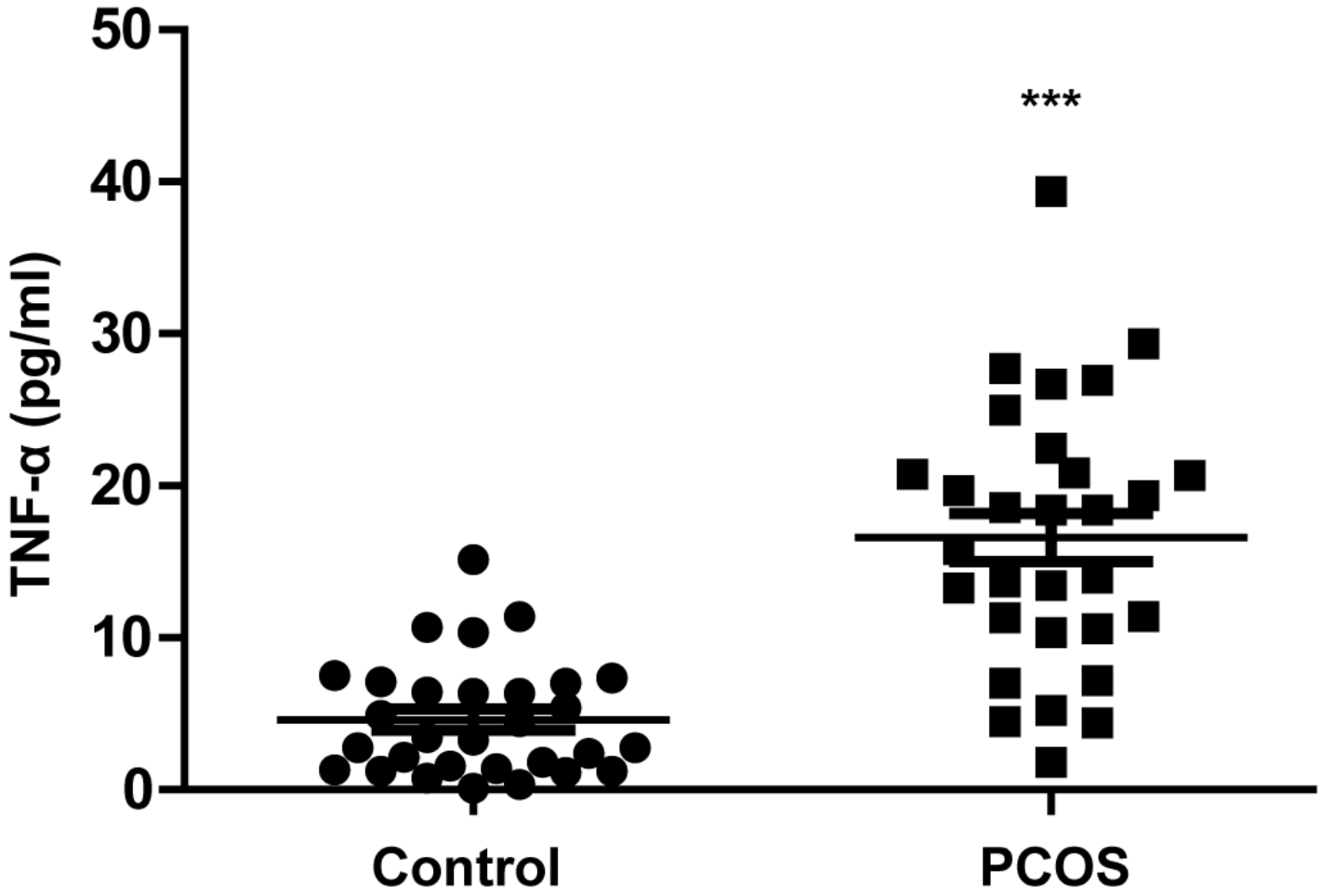

Figure 2

TNF-α is upregulated in follicular fluid of PCOS patients The TNF-a level was measured in 30 PCOS patients and 30 controls. Significantly increased follicular fluid TNF-a levels were found in PCOS patients (16.61±8.54) as compared to controls (4.61±3.71;$p < 0.0001$).

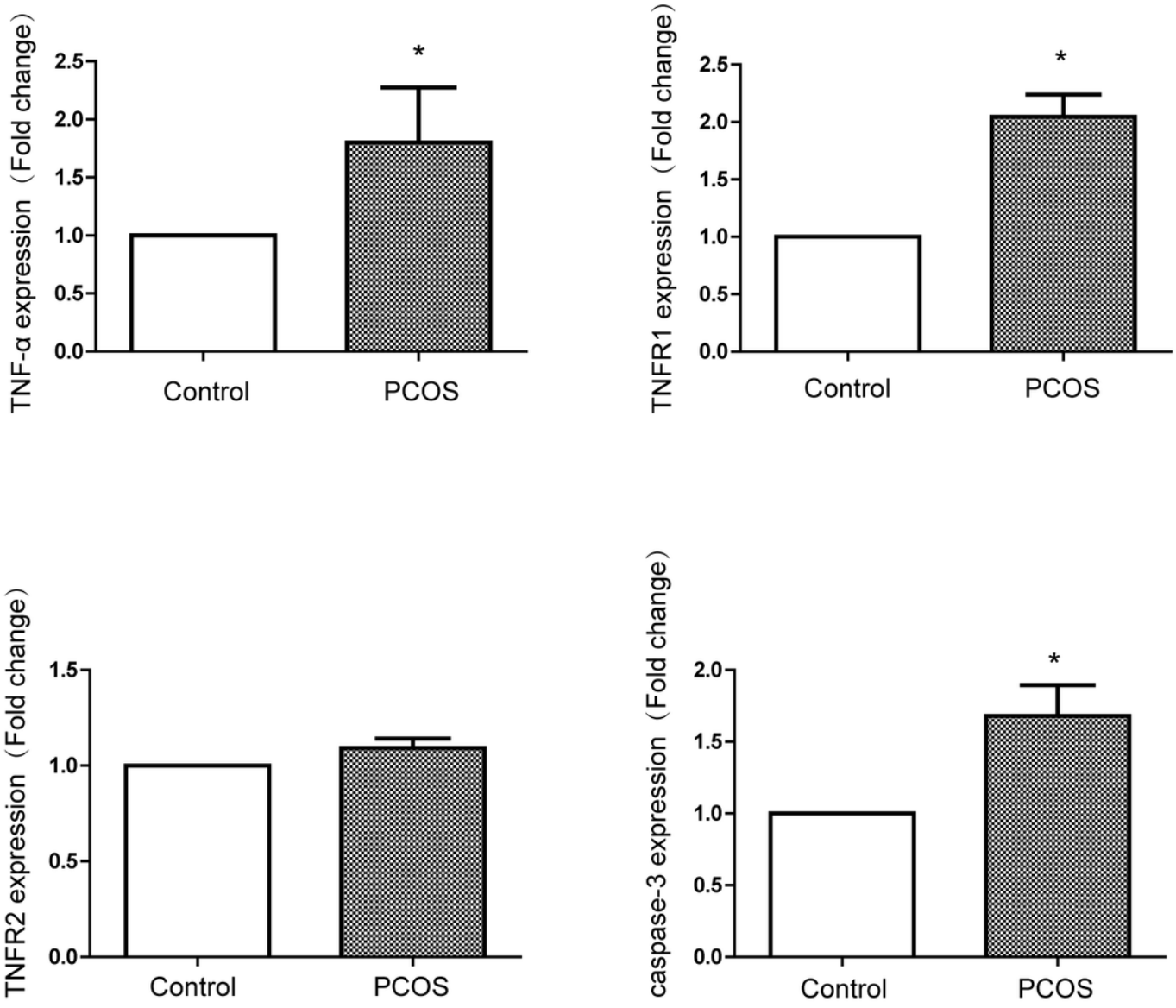

Figure 3

TNF-α,TNFR1,TNFR2, caspase-3 genes expression in PCOS and control groups. TNF-α tumor necrosis factor-alpha,TNFR1 Tumor Necrosis Factor Receptor 1,TNFR2 Tumor Necrosis Factor Receptor 2. *Represents a signifcant diference between the groups ($p<0.05$)

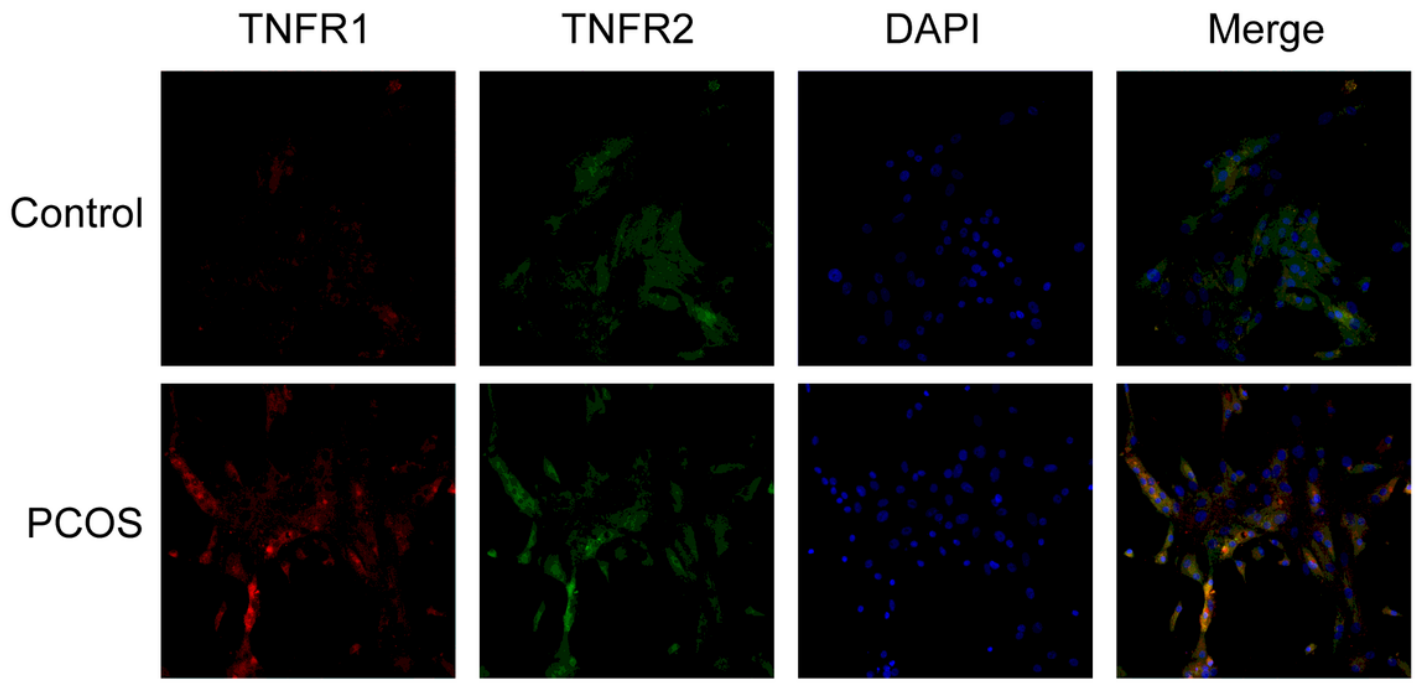

Figure 4

TNFR1 and TNFR2 expression in PCOS and control groups. TNFR1(red) and TNFR2(green) protein levels in human GCs in PCOS and control groups. Fixed cells in culture slides were incubated with anti-TNFR1/TNFR2 antibody. Nuclei were counterstained with DAPI (blue).

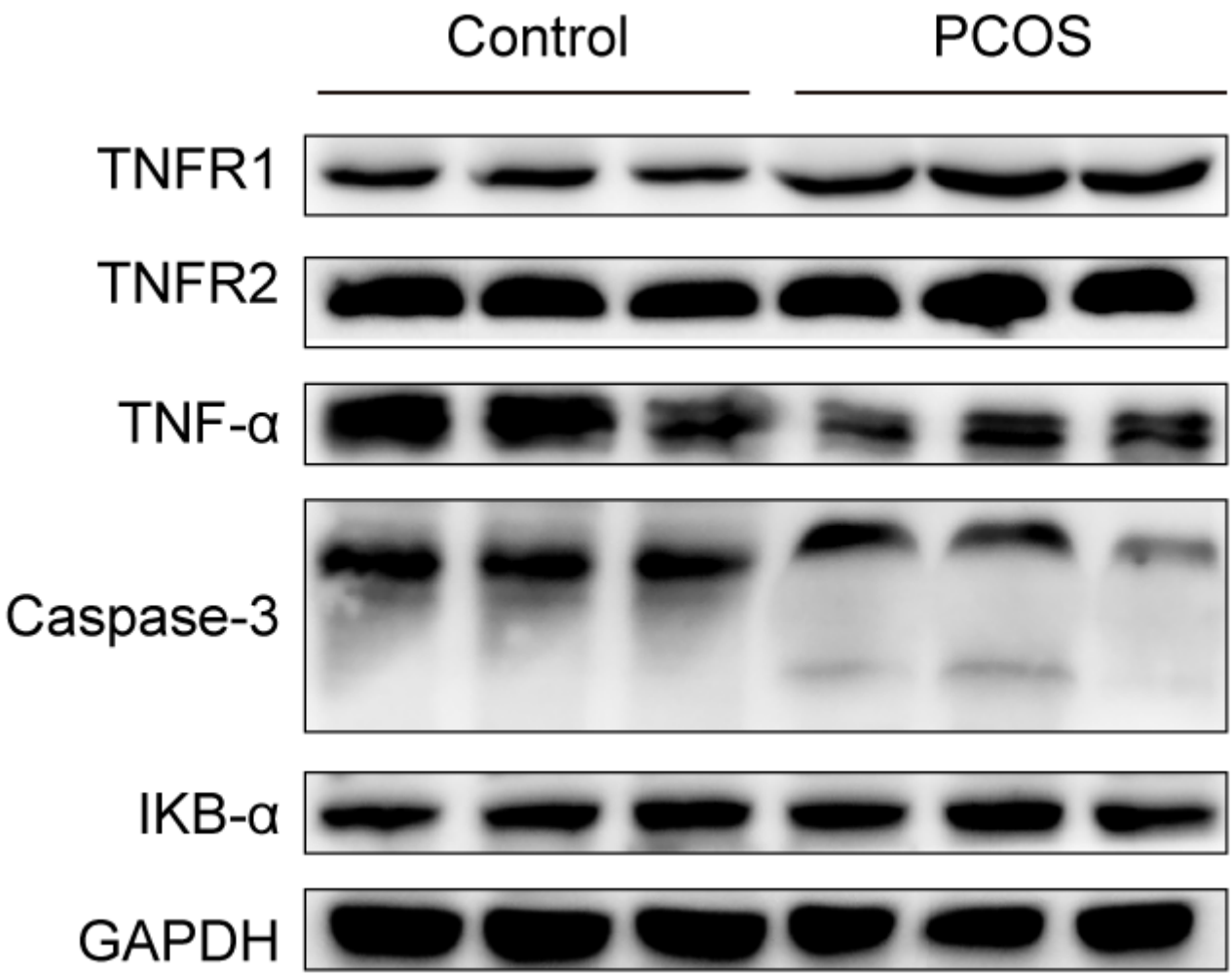

Figure 5

The TNF-α/TNFR1 signaling pathway mediates apoptosis rather than survival in cumulus cells of PCOS patients The total protein was analyzed by immunoblotting with the indicated antibodies.TNFR1, TNFR2 and TNF-α expression was assessed by western blotting for control group and PCOS group. Caspase 3 activation and degradation of Iκ-B was analyzed by western blotting that is representative of three independent experiments. The GAPDH was regarded as internal control in both groups.

Supplementary Files

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- OnlineTable2.png