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Inhibitory effect of bushen huoxue formula against dehydroepiandrosterone-induced inflammation in granulosa cells through TLR4/NF-κB signaling pathway

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Abstract: Androgen exposure may be an important factor in promoting the development of polycystic ovary syndrome (PCOS) and disease progression. Bushen Huoxue Formula (BHF), a traditional Chinese medicine, is prescribed in clinical settings as a PCOS remedy, albeit with unclear pharmacological effects on granulosa cells. The present research explores potentially advantageous BHF impacts and whereby BHF alleviates dehydroepiandrosterone (DHEA)-induced inflammation and endocrine disruption. Six chemical components in BHF were identified and fingerprint analysis showed good reproducibility. Using a human granulosa cell line (KGN), BHF effects on cell viability, secretion of steroidogenic and inflammatory factors were evaluated and TLR4/NF-κB pathway expression was examined. Our results demonstrate that BHF treatment of KGN cells in a DHEA-induced inflammatory state led to increased cell viability, decreased testosterone and estradiol production, and decreased CYP19A1 and HSD3B2 mRNA expression. Further experiments revealed that BHF inhibited the expression of pro-inflammatory cytokines and considerably hindered up-regulation in protein levels of TLR4, MyD88, and TRAF6, while inhibiting the activation of NF-κB and phosphorylation of IκBα. Collectively, BHF administration protected granulosa cells from DHEA-induced injuries through down-regulating pro-inflammatory cytokines and blocking the pathway of TLR4/NF-κB. Therefore, BHF hold promise as a therapeutic formulation for preventing androgen induced PCOS.

Keywords: Bushen Huoxue Formula, steroidogenic, inflammation, TLR4/NF-κB

INTRODUCTION

Polycystic ovary syndrome (PCOS) has been well-established as one of the most pervasive reasons for endocrine and metabolic disease based infertility in childbearing-aged women. Its cardinal features include menstrual disturbances, excess androgen-associated clinical and biochemical findings, with detection of polycystic ovaries revealed upon ultrasound examination (Stener-Victorin and Deng, 2021). Patients suffering from PCOS frequently represent dysregulation of reproductive hormones, such as greater than normal levels of luteinizing hormone and circulating testosterone that have been closely tied to increased miscarriage frequency and decreased fertility (Malini and Roy George, 2018). Nevertheless, despite decades of research into the occurrence and development of PCOS (Witchel *et al*., 2020), its pathophysiology and underlying molecular mechanisms are still unclear.

Recent evidence suggests that inflammation may play a crucial role in PCOS pathogenesis (Patel, 2018), whereby inflammation may trigger a series of events leading to insulin resistance, increased dyslipidemia, increased ovarian androgen production and eventually to PCOS-associated reproductive dysfunction (Shorakae *et al*., 2018). Notably, in numerous studies of women with PCOS, higher levels of inflammatory markers, such as pro-inflammatory cytokines and chemokines were observed in patients versus healthy controls (Piltonen *et al*., 2013, Samy *et al*., 2009). Thus, researchers have considered PCOS to be a pro-inflammatory state or chronic low-grade inflammatory state (Shimada *et al*., 2006). Meanwhile, Toll-like receptors (TLRs) have been detected on cell surfaces of a variety of ovarian tissue cells. Intriguingly, TLRs on granulosa cell surfaces apparently participate in ovulation, as support for the concept that ovulation is a controlled inflammatory response process (Woods *et al*., 2009). Additionally, results of an ovarian cancer study have shown that activated TLR4 triggers the myeloid differentiation initial response gene 88 (MyD88) pathway, which leads to phosphorylation and proteasomal degradation of IκB, and activation and nuclear localization of nuclear factor-kappa B (NF-κB) (Kelly *et al*., 2006). The aforesaid cascade of signaling has been illustrated to enhance pro-inflammatory cytokines production in addition to promoting tumor cell survival and proliferation (Woods *et*

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al., 2011). Taken together, these studies suggest that strategies geared toward reducing inflammation may have value for the prevention and/or treatment of early PCOS.

Recently, Traditional Chinese Medicine (TCM) therapeutic properties have attracted much attention worldwide, prompting researchers to focus on TCM compounds as potential treatments for PCOS. One such agent, Bushen Huoxue Formula (BHF) containing six types of herbs, was formulated based on TCM theory. Currently, BHF is an extensively applied TCM prescription for improving kidney function and enhancing circulation of blood in clinical experience, due to known effects of its herbal ingredients *Salvia miltiorrhiza* Bge., *Leonurus japonicus* Houtt and *Eupatorium japonicum Thunb*. to promote blood circulation, relieve blood stasis and adjust menstruation (Cheng *et al*., 2020, Jia *et al*., 2019, Yao *et al*., 2013). Meanwhile, *Rehmannia radix* Praeparata has traditionally been used in China to tonify kidney essence (Liu *et al*., 2017, Xia *et al*., 2019). Although the body of evidence demonstrating BHF beneficial effects has grown and prompted exploration of new uses for BHF, the underlying mechanisms for BHF's beneficial effects remain unclear. Therefore, in this report, we investigated BHF effect on dehydroepiandrosterone (DHEA)-induced KGN cell inflammation and possible mechanisms involved in observed BHF effects.

MATERIALS AND METHODS

Materials

DHEA, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM) and Fetal bovine serum (FBS) were provided from Gibco (Grand Island, NY, USA). Antibodies against p65 (#8242), phospho-p65 (#3033), phospho-IκBα (#2859), IκBα (#4814), and GAPDH (#5174) were provided from Cell Signaling Technology (Danvers, MA, USA). Antibodies specific for TLR4 (ab22048), MyD88 (ab2064), TRAF6 (ab62488) were supplied from Abcam (Cambridge, MA, USA). For all experiments, the analytical grade of reagents and chemicals was utilized.

Preparation of BHF and HPLC fingerprint analysis

The herbs of BHF were purchased from Tongrentang Chinese Medicine (Jilin, China). The herbal drugs and the voucher numbers of BHF are presented in table 1. The BHF herb formula extraction process was conducted as follows: the soaking of appropriate herbs was carried out by utilizing 8 volumes of water for 30 minutes, subsequently decocted for 2 h; the process was repeated one more time by using 6 volumes of water. For both aqueous extracts, pooling, filtration, and condensation were executed under vacuum at 60^{o}C. The extract yield of BHF was found to be 28.4% (w/w). The concentrated extract was filtered and powdered by implementing a method of spray drying succeeded by sifting mixing, and extrusion of the powder as dry formula to maintain potency (Huang *et al*., 2017).

According to the Chinese Pharmacopeia, we searched possible chemical compounds and purchased six reference standards from China Food and Drug Research Institute: Ursolic acid, Caffeic acid, Sodium Danshensu, Salvianolic acid B, Acteoside, Stachydrine hydrochloride. According to the previous method with minor modifications Ten different batches of BHF were separated implementing a TSKgel Amide-80 column (4.6×250 mm, 5μm, TSKgel, JP) and explored for specific components and chemical fingerprints utilizing a high-performance liquid chromatography (HPLC) system (SHIMADZU) with an ELSD detector (Alltech-2000ES). The mobile phase was comprised of acetonitrile (A) and 1% (V/V) glacial acetic acid solution（B）with the linear gradient elution: 10-25% B (0-25min), 25% B (25-40min); the temperature of the column was 40^{o}C and the rate of flow was 0.8mL/min. The temperature of drift tube is 90^{o}C and the volume flow rate of carrier gas is 2.5L / min. By comparing the retention time between standards and samples, six main components were identified in BHF (fig. 1A and 1B). The six main ingredients of BHF included Salvianolic acid B (from Salviae Miltiorrhizae Radix et Rhizoma), Sodium Danshensu (from Salviae Miltiorrhizae Radix et Rhizoma), Stachydrine hydrochloride (from Leonuri herba), Acteoside (from Rehmanniae Radix Praeparata), Caffeic acid (from Lycopi herba and Rehmanniae Radix Praeparata), Ursolic acid (from Lycopi herba and Salviae Miltiorrhizae Radix et Rhizoma). As shown in fig. 1C, the overall similarity of the 10 batches analyzed was more than 95%, suggesting that overall quality and composition of BHF exhibited good batch-to-batch reproducibility.

Cell culture and treatment

The human granulosa-like tumor cell line KGN was obtained from the Cell Resource Center of the Shanghai Institute for Biological Sciences (Shanghai, China). KGN cells were kept in DMEM/F12 with 10% fetal bovine serum, 100μg/mL streptomycin and 100U/mL penicillin at 37°C in a moisturized ambient of 5% CO_2. The medium of culture was altered every 2 to 3 days. KGN cells were seeded onto culture plates containing 96 or 6 wells and incubated with DHEA and/or 50, 25, or 12.5μg/mL BHF for 24 h.

Cell viability assay

The viability of DHEA-induced KGN cells was assessed through the assay of MTT, as previously described (Wang *et al*., 2020). The cells of KGN were seeded into a plate containing 96 wells (2×10^4 cells/well) and subjected to a number of DHEA processing for 24 h in the absence or presence of BHF. After incubation with MTT (0.5mg/mL)

for 4 h, the formazan was solubilized in 150 µL DMSO then the absorbance was measured at 490 nm employing a microplate reader (TECAN A-5082, Magellan, Austria). Achieved outcomes are given as the percentage of viable cells relative to the vehicle control.

Enzyme-linked immunosorbent assay

The supernatant from DHEA-stimulated KGN cells after 24 h exposure to BHF and/or DEHP were collected. Hormone levels of testosterone (T), estradiol (E2) and inflammatory cytokines TNF-α, IL-1β, IL-18, and IL-6 were quantified employing the kits of enzyme-linked immunosorbent (ELISA) (IBL International GmbH, Hamburg, Germany) conforming to the instructions of the manufacturer. The absorbances were observed at 410 nm implementing a microplate reader (TECAN).

Western blot analysis

Western blot analysis was performed based on the previous method (Wang *et al*., 2022). Cells were lysed with RIPA lysis buffer (Beyotime Biotechnology, Jiangsu, China) for 30 min on ice, than the concentration of protein was measured by utilizing the reagent of BCA (Beyotime Biotechnology). Samples of protein (30µg) were divided on 10% or 12% SDS-PAGE gels, and subsequently transferred to PVDF membrane. The membranes were blocked in 5% non-fat milk for 1 h then incubation was fulfilled with primary antibodies overnight at 4°C. After washing followed by incubation with proper secondary antibodies for 1 h, the specific bands were visualized by implementing an imaging system of chemiluminescent (FluorChem H2, ProteinSimple, San Jose, CA, United States).

Real-time quantitative PCR

The extraction of total RNA from cultured cells was accomplished by taking advantage of TRIzol (Invitrogen, Carlsbad, CA, USA). The purity and integrity of RNA were appraised spectroscopically by employing a NanoDrop 2000/c spectrophotometer (ThermoScientific, Waltham, MA, USA). 2µg of total RNA was reverse transcribed into cDNA utilizing a kit of PrimeScript RT reagent (TaKaRa, Dalian, China). qPCR was executed by applying SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA) and a Bio-Rad CFX96 System. The program is as following: 95°C 5 min, 95°C 15 s, 60°C 30 s, 72°C 30 s for 40 cycles. Primer sequences were as follows: FSHR, forward: 5′-TTC CTT ACT GCC AAC TCT CC-3′, reverse: 5′-TCA TAC ACC AGA CCG TCT GA-3′; LHR, forward: 5′-TCA ATT CTT GTG CCA ATC CA-3′, reverse: 5′-CCA TTT TTG CAG TTG GAG GT-3′; CYP19A1, forward: 5′-TTG GGC TGC AGT GCA TCG GT-3′, reverse: 5′-CCG GGG CCT GAC AGA GCT TTC ATA-3′; HSD3B2, forward: 5′-GCG GCT AAT GGG TGG AAT CTA-3′, reverse: 5′-CAT TGT TGT TCA GGG CCT CAT-3′; β-actin: forward, 5’-TTC CAG CCT TCC TTC CTG G-3’, reverse, 5’-TTG CGC TCA GGA GGA GCA AT-3’. The outcomes of various groups were analyzed through the approach of $2^{-\wedge\wedge CT}$ (Jin *et al*., 2020).

STATISTICAL ANALYSIS

The obtained outcomes are illustrated as the mean ± standard deviation calculated from data of three independent assessments. One-way analysis of variance (ANOVA) was exerted to appraise the significance of the difference by employing Graph Pad Prism 6.0 (San Diego, CA, USA). $p < 0.05$ was regarded as significant in all evaluations.

RESULTS

BHF inhibits DHEA-induced decrease in KGN cell viability

To explore the protective influence of BHF against androgen induced cytotoxicity for KGN cells, various concentration of DHEA were tested for KGN cell cytotoxicity. As shown in fig. 2A, 24 h exposure of KGN cells to DHEA at concentrations greater than 1µM led to significant cytotoxicity in a dose-dependent manner, with 10µM DHEA exposure reducing cell viability to 66.4% ($p<0.001$). Notably, 24h treatment with 25, 50 or 100µg/mL BHF with 10µM DHEA resulted in markedly increased cell viability (fig. 2B). These results thus demonstrate that BHF protects KGN cells from DHEA-induced cell death in a dose-dependent manner.

BHF effect on steroid synthesis pathway-associated in DHEA-induced KGN cells

Exposure to excessive androgen levels induces a granulosa cell hormone imbalance that is also a hallmark of PCOS. To investigate the influence of BHF on steroid hormones production, levels of T and E2 in cells processed with various concentrations of BHF were examined. The results showed significantly greater T ($p<0.001$) and E2 ($p<0.01$) production in DHEA-induced cells than observed in the control group, with production gradually decreasing with were increasing BHF concentration (fig. 3A and 3B). 100µg/mL concentration of BHF significantly ($p<0.01$) counteracted DHEA-induced reduction of follicle stimulating hormone receptor (FSHR) mRNA expression (fig. 3C). On the contrary, the observed DHEA-induced enhancement in luteinizing hormone receptor (LHR) mRNA expression was counteracted by cell exposure to high BHF concentration (fig. 3D). Moreover, after monitoring the expression of key steroid synthesis pathway enzymes, we found that BHF treatment effectively inhibited increases of DHEA-stimulated CYP19A1 and HSD3B2 mRNA (fig. 3E and 3F). These results indicated that BHF regulates key factors in the steroid synthesis pathway and effectively corrects DHEA-induced steroid hormone secretion imbalances.

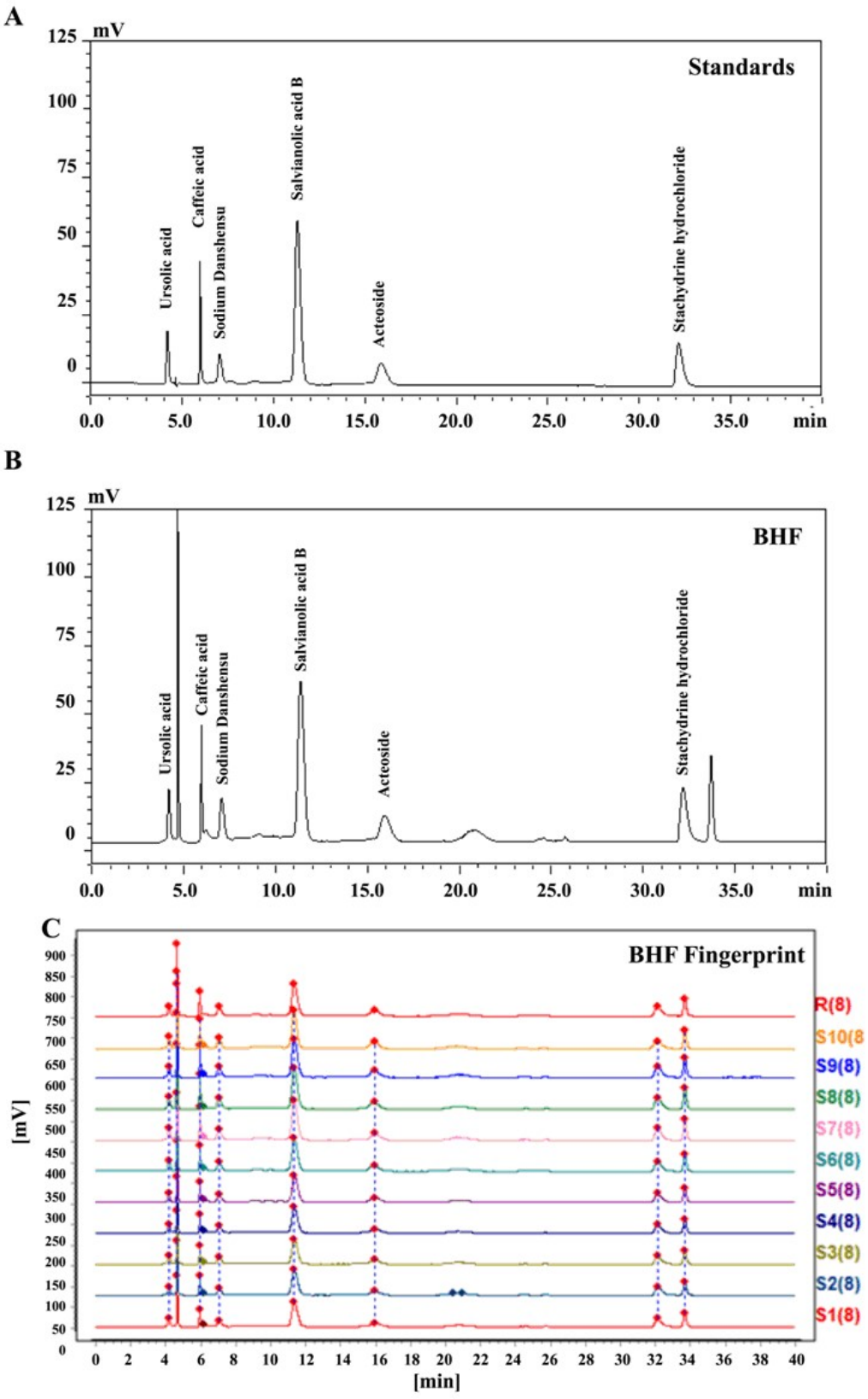

Fig. 1: HPLC chromatogram of BHF. (A) HPLC chromatograms of Ursolic acid, Caffeic acid, Sodium Danshensu, Salvianolic acid B, Acteoside and Stachydrine hydrochloride are shown. (B) HPLC chromatogram of BHF is shown. (C) HPLC fingerprints of 10 batches of BHF (S1-10) are shown.

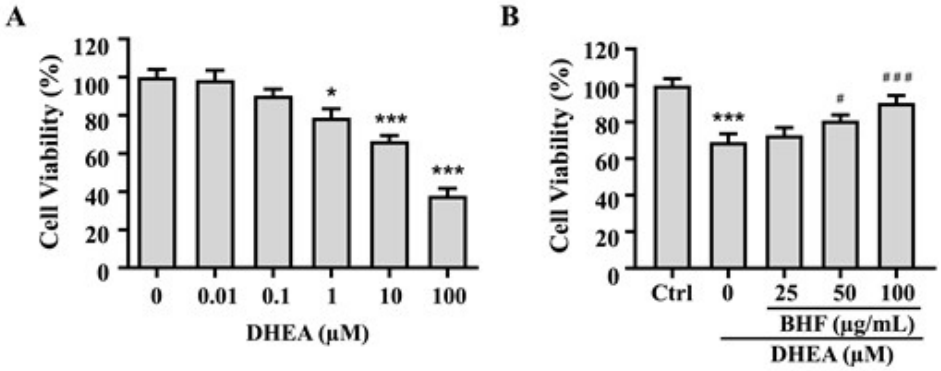

Fig. 2: Effect of BHF on cell viability in KGN cells stimulated with dehydroepiandrosterone (DHEA). (A) KGN cells were treated with various concentrations of DHEA for 24 h then cell viability was measured. (B) Cell viability after 24-h exposure of cells to DHEA with or without different concentrations of BHF. Viability of KGN cells was assessed using the MTT assay. *p<0.05, ***p<0.001 vs. Ctrl; #p< 0.05, ###p<0.001 vs. DHEA.

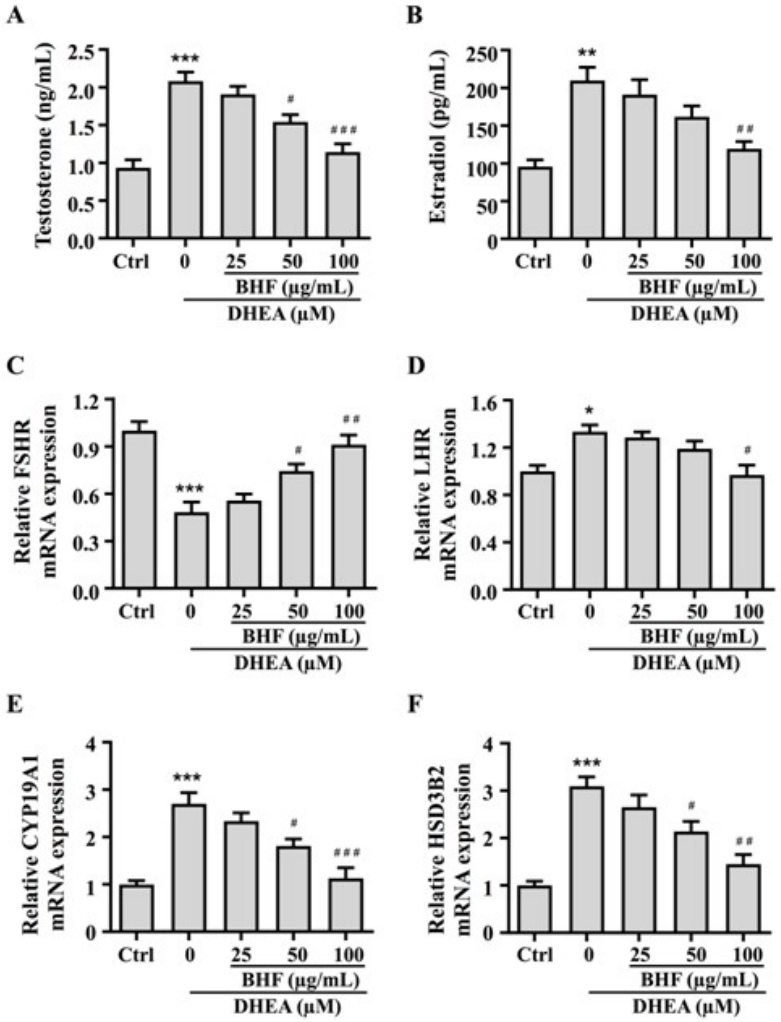

Fig. 3: Effects of BHF on the expression of hormones and steroid synthesis factors in KGN cells stimulated with DHEA. BHF effects after 24 h exposure of cells to DHEA with or without various concentrations of BHF. (A, B) The supernatants from DHEA-stimulated KGN cells treated with different concentrations of BHF were collected and assayed for testosterone (T) and estradiol (E2) production using an ELISA kit. Cells were collected for analysis of FSHR (C), LHR (D), CYP19A1 (E) and HSD3B2 (F) mRNA levels by qPCR. *p<0.05, ***p<0.001 vs. Ctrl; #p< 0.05, ##p<0.01, ###p<0.001 vs. DHEA.

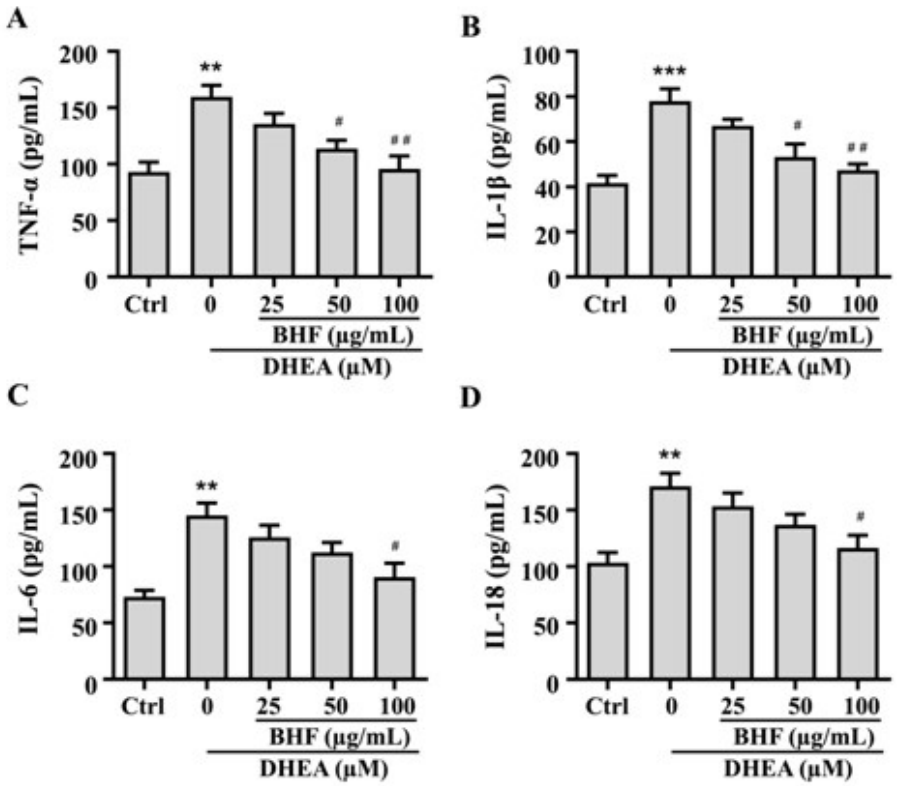

Fig. 4: Effect of BHF on levels of inflammatory factors in KGN cells stimulated with DHEA. Cells were treated with 25, 50, or 100μg/mL BHF and 10μM DHEA for 48 h. The concentrations of TNF-α (A), IL-1β (B), IL-6 (C), IL-18 (D) were measured with an ELISA kit. ***p*<0.01, ****p*<0.001 vs. Ctrl; #*p*<0.05, ##*p*<0.01 vs. DHEA.

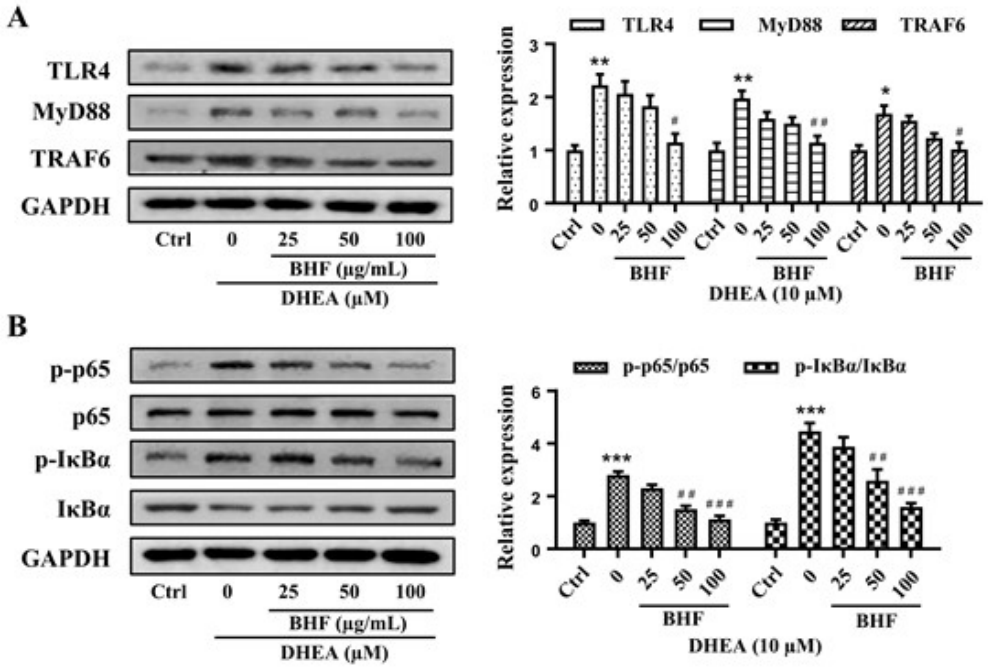

Fig. 5: Effect of BHF on expression of TLR4/NF-κB pathway in KGN cells stimulated with DHEA. Cells treated with 25, 50, or 100μg/mL BHF and 10μM DHEA were lysed by lysis buffer, the levels of TLR4, MyD88, TRAF6 (A) and p-p65, p65, p-IκB and IκB (B) were analyzed by Western blot. Quantification of bands relative to GAPDH using Image J software. **p*<0.05, ***p*<0.01, ****p*<0.001 vs. Ctrl; #*p*<0.05, ##*p*<0.01, ###*p*<0.001 vs. DHEA.

Table 1: The Chinese herb drugs contained in Bushen Huoxue Formula (BHF)

Latin name	Chinese name	English name in Chinese pharmacopoeia (Ch.P.)	Plant part (s), processing	Weight (g)	Voucher numbers
Salvia miltiorrhiza Bge.	Dan Shen	Salviae Miltiorrhizae Radix et Rhizoma	Root and rhizome	20	CM0274
Leonurus japonicus Houtt.	Yi Mu Cao	Leonuri herba	Above-ground parts	20	CM0365
Atractylodes lancea (Thunb.) DC	Cang Zhu	Atractylodis Rhizoma	Root and rhizome	20	CM0386
Platycladus orientalis (L.) Franco	Bai Zi Ren	Platycladi Semen	Seed	20	CM0288
Rehmannia glutinosa Libosch	Shu Di Huang	Rehmanniae Radix Praeparata	Root tuber, steamed with yellow wine	15	CM0320
Eupatorium japonicum Thunb.	Ze Lan	Lycopi herba	Above-ground parts	15	CM0341

Influence of BHF on DHEA-induced inflammatory response in KGN cells

Inflammation is considered to perform an essential task in the pathophysiological process that leads to PCOS. We therefore investigated the inflammatory status of DHEA-induced cells by analyzing levels of pro-inflammatory cytokines. As expected, expression levels of TNFα ($p<0.01$, fig. 4A), IL-1β ($p<0.001$, fig. 4B), IL-6 ($p<0.01$, fig. 4C) and IL-18 ($p<0.01$, fig. 4D) were clearly elevated in the DHEA-induced group versus control group levels. Thus, BHF treatment inhibited pro-inflammatory cytokine level increases, indicating that inhibition of granulosa cell inflammation might be a benefit of BHF treatment.

Effects of BHF on TLR4/NF-κB signaling pathways in KGN cells with DHEA-induced inflammation

To further explore the potential molecular mechanism by which BHF attenuates DHEA-induced granulosa cell injury, we determined whether or not BHF regulates inflammation through TLR4 and p65 signaling pathways. As shown in fig. 5A, high TLR4 protein level expression observed ($p<0.01$) in DHEA treated cells subsequently decreased ($p<0.05$) after BHF treatment. Moreover, expression of downstream signaling molecules MyD88 and TRAF6 responded as expected in response to altered TLR4 expression. More specifically, our results show that DHEA treatment led to degradation of IκB-α protein and increased p65 phosphorylation, conversely, BHF treatment prevented DHEA-induced IκB-α degradation and p65 phosphorylation in a dose-dependent manner without affecting total p65 protein content (fig. 5B). Collectively, these findings indicated that BHF treatment significantly reversed DHEA-induced activation of the signaling pathway of TLR4/NF-κB, thereby regulating the production of pro-inflammatory mediators.

DISCUSSION

Investigations have demonstrated that exposure to sex hormones, particularly androgen, may interfere with the functions of the axis of the hypothalamic-pituitary-ovarian (HPO) and lead to the manifestation of PCOS phenotypes (Li *et al*., 2019). Granulosa cells, which provide an essential environment for healthy ovarian follicle development and oocyte maturation, regulate gonadal functions by acting as the main source of ovarian estrogens and progesterone, in response to triggering of FSH and LH receptors on these cells (Liu *et al*., 2019). Here, we demonstrated that BHF reverses DHEA-induced granulosa cell decreases in FSHR expression and increases in LHR expression. Meanwhile, we also monitored cell-specific expression of a series of steroidogenic enzymes required for synthesis of various steroids. For example, HSD3B2 is an essential key enzyme involved in both testosterone biosynthesis and in estradiol production, whereby an absence of HSD3B2 leads to inhibition of estradiol production (Zhang *et al*., 2018). Another enzyme, CYP19, encodes an aromatase that catalyzes the irreversible conversion of androstenedione to estrone and of testosterone to estradiol (Goodarzi *et al*., 2015). These reactions comprise the final steps of estrogen biosynthesis (Schleutker, 2012). Therefore, regulating aromatase CYP19 expression can alter the rate of estrogen production and thus control levels of estrogens. Herein, our results indicate that BHF might protect granulosa cells against DHEA-induced damage by maintaining a normal hormonal environment and by inhibiting HSD3B2 and aromatase CYP19 expression.

The etiology of PCOS is complicated, as shown in numerous studies implicating increased pro-inflammatory cytokines and decreased anti-inflammatory factors in PCOS pathogenesis. A resulting heightened inflammatory state may therefore lead to the elevated the production of ovarian androgen and reproductive dysfunction observed in PCOS patients (Escobar-Morreale *et al*., 2011, Hu *et al*., 2020). In addition, such inflammatory conditions may also trigger cascading events leading to increased insulin resistance and PCOS progression, as well as increased

local inflammation within PCOS ovaries which is able to impair follicular growth and maturation (Bhatnager *et al*., 2019). The PCOS pathophysiological mechanism may also involve roles of IL-18, which is important for follicular growth and oocyte maturation (Lima *et al*., 2018), and IL-6, which is closely related to hyperandrogenism and insulin resistance (Marciniak *et al*., 2016). Notably, our results clearly indicated that BHF inhibits DHEA-induced enhances in inflammatory factors TNF-α, IL-1β, IL-18 and IL-6 in KGN cells.

The TLR4/NF-κB pathway plays an active role in the innate immunity of ovarian granulosa cells. Of note, research studies investigating biological functions of TLRs during ovulation, a main research topic in reproductive immunology (Xie *et al*., 2020). TLR4 usually plays a key role in inflammatory response through MyD88-dependent pathway (Luchner *et al*., 2021). Upon ligand binding to TLR4, recruitment of IRAK4 and IRAK1 to the MyD88-signaling complex and induces IRAK4 to phosphorylate IRAK1. Subsequently, phosphorylated IRAK1 interacts with TRAF6, leading to phosphorylation and degradation of IκB-α, the inhibitor of NF-κB, leading to NF-κB initiation that ultimately promotes production of inflammatory factors (Mitchell and Carmody, 2018). This mechanism is supported by previous studies demonstrating that ligation of TLR4 with bacterial lipopolysaccharide resulted in degradation of IκB and activation of NF-κB in KGN cells (Woods *et al*., 2011). Notably, it has also been observed that ovarian surface epithelium cancer cells responded to TLR4 activation through acute activation of NF-κB that results in further up-regulation of pro-inflammatory cytokines (Kelly *et al*., 2006).

By exploring mechanisms of BHF action, here we found that BHF significantly reduced TLR4 signal transduction and activation of the MyD88-dependent pathway in cells with DHEA-induced injury. However, BHF is a compound prescription composed of Traditional Chinese Medicine and extensively implemented in clinics for treating PCOS, the absorbed bioactive compositions derived from BHF that execute the protective functions remain unknown. It is well known that determining the effective chemical composition of Traditional Chinese Medicine formula has been the focus and difficulty of the field because of its complicated ingredient compositions. Further investigation for illuminating the molecular mechanism of the safe and effective bioactive ingredients in BHF against inflammation in vivo model of PCOS will be our next direction. Collectively, these findings provide additional evidence warranting additional BHF studies toward the development of better therapies to prevent and/or treat PCOS.

CONCLUSION

Here we provide evidence of a protective effect of BHF against the development of PCOS-like disease in granulosa cells. Specifically, our findings indicate that BHF could ameliorate DHEA-induced hormonal disorders and inflammatory responses via a mechanism that potentially involves inhibition of TLR4/NF-κB pathway activation. Additional investigations are required to discover the molecular basis of the PCOS relationship with the inflammatory state in order to build a scientifically based foundation for understanding PCOS pathophysiology.

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