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Reproductive Sciences

ISSN 1933-7191

Reprod. Sci.
DOI 10.1007/s43032-020-00168-9

Springer

Reproductive Sciences
https://doi.org/10.1007/s43032-020-00168-9

REVIEW

Potential of Auraptene in Improvement of Oocyte Maturation, Fertilization Rate, and Inflammation in Polycystic Ovary Syndrome Mouse Model

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Received: 2 November 2019 / Accepted: 13 January 2020

Abstract

Polycystic ovary with poor-quality oocytes has remained problematic in polycystic ovary syndrome (PCOS) patients. It is well documented that the inflammation and production of reactive oxygen species (ROS) in PCOS ovaries are significantly higher than normal voluntaries. In this study, we hypothesized that auraptene (AUR), as a coumarin derivative with anti-inflammatory properties, may be effective in improvement of oocyte maturation and fertilization rate in PCOS patients. For this purpose, PCOS model was induced in NMRI mice and confirmed by ovarian histopathology observations and hormonal assays. PCOS-induced mice were administrated with AUR (PCOS-AUR) and metformin (PCOS-MET), and their effects on inflammation, apoptosis rate, oocyte maturation, and in vitro fertilization capacity were determined and compared with those normal and PCOS animals treated with sesame oil (PCOS-sesame oil) and no treatment (PCOS). Treatment with AUR and MET decreased the inflammation and apoptosis rates in PCOS mice compared with PCOS animals with no treatment. PCOS-AUR and PCOS-MET oocytes also showed higher intracellular glutathione and lower ROS concentrations compared with PCOS mice, indicating improved oocyte maturation rate. PCOS-AUR and PCOS-MET groups showed higher percentages of expansion rate and MII stage oocytes, and lower rate of abnormal oocytes compared with PCOS with no treatment. The rate of fertilization in the oocytes isolated from PCOS-AUR and PCOS-MET groups was higher than PCOS-sesame oil and PCOS groups. Our findings suggest that AUR can be considered as a potential candidate for improvement of oocyte maturation and fertilization capacity in PCOS patients, comparable to MET.

Keywords Polycystic ovary syndrome · In vitro fertilization · Oocyte maturation · Auraptene · Metformin

Introduction

Polycystic ovary syndrome (PCOS) is recognized as one of the most common endocrine-metabolic disorders, affecting 5–10% of women of reproductive age and 50% of women with subfertility [1–3]. Developmental pathophysiology of PCOS is still not well understood, but it is characterized by anovulation, increased production of

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Published online: 02 March 2020

ovarian androgen, and polycystic ovaries [4–7]. Infertile patients with PCOS show abnormal folliculogenesis and hyperinsulinemic insulin resistance, which negatively affect the maturation, development, and fertilization of their oocytes. Therefore, PCOS patients finally show small cysts on one or both ovaries and poor-quality oocytes [8, 9]. Anti-estrogenic drugs, such as clomiphene citrate, are considered as the first-line treatment for induction of ovulation in infertile PCOS women. However, resistance to clomiphene citrate remains a big problem [10, 11]. Gonadotropin therapy is an alternative treatment for induction of ovulation in clomiphene citrate–resistant PCOS patients. However, gonadotropin administration increases the risk of multiple-pregnancy and ovarian hyperstimulation syndrome (OHSS) [12, 13]. Metformin (MET) is a frequently prescribed orally available drug for the management of PCOS. Nevertheless, digestive disorders (diarrhea, vomiting), nausea or gastrointestinal discomfort, and weight loss are the most common side effects associated with MET [14–17]. Therefore, during the past years, the efforts have been made to develop an efficient treatment strategy with minimal adverse effects for PCOS women [18–20].

Auraptene (AUR), a coumarin derivative, is mostly found in citrus fruits. There is growing evidence indicating anti-tumor and anti-inflammatory properties of AUR [21–24]. Since it is well documented that inflammatory mediators such as TNFα increase in PCOS patients, and subsequently induce apoptosis in antral follicles granulosa cells [25–27], we hypothesized that administration of AUR can be considered as a potential candidate for the treatment of PCOS patients via its anti-inflammatory effects. For this purpose, PCOS-induced mice were administrated with AUR and MET, and the apoptosis rate, inflammatory responses, oocyte maturation (OM), and in vitro fertilization (IVF) were evaluated and compared with those in normal animals, and PCOS animals with no treatment.

Materials and Methods

Animals

Prepubertal Naval Medical Research Institute (NMRI) female mice ($n = 54$) were purchased from Royan Institute, Tehran, Iran, and kept in the medical sciences animal house of Shahid Beheshti University, under standard conditions of ventilation and humidity, required by the Ethical Committee. All the animal surgeries and procedures were carried out in accordance with the 8th edition of the "Guide for the Care and Use of Laboratory Animals" [28], and approved by the Ethical Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1397.334).

PCOS Mouse Model Generation

PCOS mouse model was generated by a procedure described previously by Aragno et al. In brief, prepubertal female NMRI mice (21-days old) were subcutaneously injected with 60 mg/kg dehydroepiandrosterone (DHEA, dissolved in 1/9 mixture of 95% ethanol and sesame oil), for twenty consecutive days [29]. Animals with no treatment were served as normal (control) group.

Confirmation of PCOS Model

Ovarian Histopathology

PCOS model was identified by staining of ovarian tissue with hematoxylin and eosin (H&E) and ovarian histopathology observations [30]. Ovaries were collected, washed with normal saline and then fixed in 10% formalin for 48 h at 37 °C. The tissues were dehydrated through a graded series of ethanol and cleared in xylene using a tissue processor. The samples were then paraffin-embedded and serially sectioned at 10 μm thickness. The slides were stained with hematoxylin and eosin (H&E). The presence of ovarian cysts with multiple cystic follicles, as indicators of polycystic ovary and PCOS model, was identified under light microscope (Nikon; ECLIPSE E200, Tokyo, Japan).

Hormonal Assays by ELISA

The serum level of progesterone, testosterone, and 17β-oestradiol was quantified by immunoassay technique using enzyme-linked immunosorbent assay (ELISA) kits (CUSABIO.com; Cat No: CSB-E05104m, CSB-E05101m, and CSB-E05109m for progesterone, testosterone, and 17β-oestradiol ELISA kits, respectively), according to the manufacturer's protocols, to further confirm PCOS model.

Administration of AUR and MET

Animals were randomly divided into six experimental groups ($n = 9$) as follows: normal mice with no treatment (Con); normal mice with subcutaneous administration of 10 mg/kg/day AUR (495-02-3, Golexir Pars CO., Mashhad-Iran) (Con-AUR); PCOS mice with subcutaneous administration of AUR (10 mg/kg/day) (PCOS-AUR); PCOS mice with oral gavage administration of 500 mg/kg/day MET (1115-70-4, Kharazmi Pharmaceutical CO., Tehran-Iran) (PCOS-MET); PCOS mice with subcutaneous administration of sesame oil (PCOS-sesame oil); and PCOS with no treatment (PCOS); for 20 consecutive days. It is noted that the PCOS-sesame oil group was considered as a sham group, to ensure that sesame oil, as solvent of AUR, has no negative/positive effects in this study. On day 20, animals were sacrificed by neck dislocation.

Ovary, fallopian tube, and uterine horns were removed and subjected to the following evaluations.

Evaluations

TNFα Assay by ELISA

The concentration of tumor necrosis factor-alpha (TNFα), as a pro-inflammatory cytokine, in ovary was measured by a commercially available ELISA kit (Tumor Necrosis Factor Alpha, Cat No: MBS764227), according to the manufacturer's protocols. The optical density of each reaction was measured at a wavelength of 450 nm using an automated ELISA reader.

Apoptosis

The concentration of caspase-3, as an indicator of apoptosis, in cumulus cells was quantified by a commercially available ELISA kit (Human Caspase-3 ELISA Kit, Mybiosource, Cat No. MBS260710), according to the manufacturer's protocols. An automated ELISA Reader was used to measure the optical density of each reaction at a wavelength of 450 nm.

Cumulus-Oocyte Complexes Collection

Cumulus-oocyte complexes (COCs) were obtained from superovulated mice and collected for evaluations of oocyte maturation (OM) and in vitro fertilization (IVF) capacity. At first, animals were received an intraperitoneal injection of 10 IU pregnant mare serum gonadotrophin (PMSG, Gestyl, Organon, Oss, The Netherlands). Forty-eight hours later, mice were intraperitoneally injected with human chorionic gonadotrophin (hCG; Pregnyl, Organon, Oss, The Netherlands) to induce ovulation. Fourteen to sixteen hours after hCG injection, animals were sacrificed by neck dislocation. Oviducts were removed and transferred into 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered tissue cell culture medium (TCM199) containing 10% FBS. Antral follicles were punctured with a 28-G sterile needle, and cumulus-oocyte complexes (COCs) were carefully collected under stereomicroscope (Olympus; SZ1145, Tokyo, Japan). The ovulated COCs were treated with 0.1% hyaluronidase (Cooper surgical, Denmark). The detached oocytes were isolated and transferred into HEPES-buffered α-MEM supplemented with 3 mg/mL bovine serum albumin (BSA; Sigma-Aldrich, USA), then subjected to further examinations.

Oocyte Maturation Assay

For OM assay, the isolated oocytes were observed under a stereomicroscope (Olympus; SZ1145, Tokyo, Japan). The complete expansion of cumulus cells and presence of the first polar body were recognized as expansion rate and MII stage oocytes, respectively. Observation of a round and clear zona pellucida (ZP) with small perivitelline space and pale moderately granular cytoplasm without inclusions were considered as criteria for matured oocytes [31–33]. OM was further determined by intracellular glutathione (GSH) and reactive oxygen species (ROS) assays in the collected oocytes. Oocytes cytoplasmic maturation was evaluated by detection of intracellular GSH with 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (Cell Tracker™ Blue; Molecular Probes, Invitrogen, Carlsbad, CA). Intracellular ROS in oocytes was identified by 2-7-dichlorodihydrofluorescein diacetate (H2DCFDA). For this purpose, the oocytes from each group were incubated for 30–45 min in the dark at 37 °C in PBS comprising 1 mg/ml PVA and 10 μM Cell Tracker™ Blue or 10 μM H2DCFDA. After incubation, oocytes were washed with PBS/PVA for 5 min and then observed under fluorescence microscope (Nikon; ECLIPSE TS 100, Tokyo, Japan) with UV filters of 370 nm for GSH (blue fluorescent) and 460 nm for ROS (green fluorescent) [19]. The fluorescence intensities of the stained samples were analyzed using ImageJ software (version 1.40, National Institutes of Health, Bethesda, MD, USA) and compared between the experimental groups.

In Vitro Fertilization Assay

In vitro fertilization (IVF) assay was performed by a method described elsewhere with some modifications [19]. Briefly, sperms were isolated from caudal epididymis of male NMRI mice (10–12 weeks old) and capacitated using HamsF10 medium. The morphologically normal and healthy oocytes (normal oocytes at MII stage) were incubated with capacitated sperm in fertilization media (KSOM; Merck Millipore, Burlington, MA) containing 15 mg/mL BSA (Sigma-Aldrich, USA) for 4 h at 37 °C in a humidified atmosphere of 5% CO_2. For evaluation of IVF capacity, zygotes were washed three times with washing media, transferred into cleavage media containing 4 mg/mL BSA (Sigma-Aldrich, USA). The presence of two pronuclei (2PN), two-cell embryos, blastocyst rate of fertilization, and subsequent on-time development (from 24 to 96 h) were evaluated in droplets (25 μL containing zygotes, KSOM, and 4 mg/mL BSA) using stereomicroscope (Olympus; SZ1145, Tokyo, Japan).

Statistical Analysis

Distribution of data was determined by the Shapiro-Wilk test. The data were analyzed by parametric (ANOVA) and non-parametric (Kruskal Wallis) statistical tests where appropriate using Prism software (Prism, Version 6.0). P value < 0.05 was considered as a level of significance. The graphs were plotted using SigmaPlot 1n 2.0 (Systat, San Jose, CA) and Prism

software (Prism, Version 6.0). Each assay was repeated three times. Data was reported as mean ± SD for each experiment.

Results

Confirmation of PCOS Model

Ovarian Histopathology

Histopathology of animals at day 20 post-DHEA injection confirmed PCOS model (Fig. 1). Ovaries from normal mice showed normal follicle development with antral follicles (Fig. 1a), while PCOS-like ovarian histology with multiple cystic follicles, abnormal ovarian morphology, and granulosa cells detachment were clearly seen in the ovary of DHEA-injected mice (Fig. 1b), confirming polycystic ovary morphology and PCOS model.

Hormonal Assays by ELISA

Elevation in serum levels of steroid hormones such as progesterone, testosterone, and 17β-oestradiol is of the most reliable indicators confirming PCOS model [4, 7]. For this purpose, the PCOS model was further confirmed by measuring the serum levels of these hormones. As shown in Fig. 2, the concentration of progesterone ($p < 0.01$), testosterone ($p < 0.001$), and 17β-oestradiol ($p < 0.001$) were significantly elevated in serum of animals after DHEA administration when compared with normal mice.

Evaluations After Administration of AUR and MET

TNFα Assay by ELISA

The concentration of TNFα in ovaries of PCOS animals treated with AUR and MET was measured and compared with their control groups, and the results are shown in Fig. 3. The PCOS animals treated with AUR (PCOS-AUR) and MET (PCOS-MET) showed a significant decrease in TNFα concentration when compared with those PCOS animals without treatment (PCOS) and treated with sesame oil (PCOS-sesame oil) ($p < 0.0001$). As can be seen in Fig. 3, the level of TNFα in the animals administrated with AUR and MET was very close to those in Con and Con/AUR groups, although the difference was still significant ($p < 0.0001$).

Apoptosis

Apoptosis in cumulus cells of COCs in the animals (normal and PCOS model) treated with AUR, MET, and sesame oil and without treatment (PCOS) was determined by evaluation of caspase-3 concentration by a commercially available Caspase-3 ELISA kit. As can be seen in Fig. 4, caspase-3 concentration in cumulus cells of PCOS-AUR and PCOS-MET showed a significant increase compared with Con and

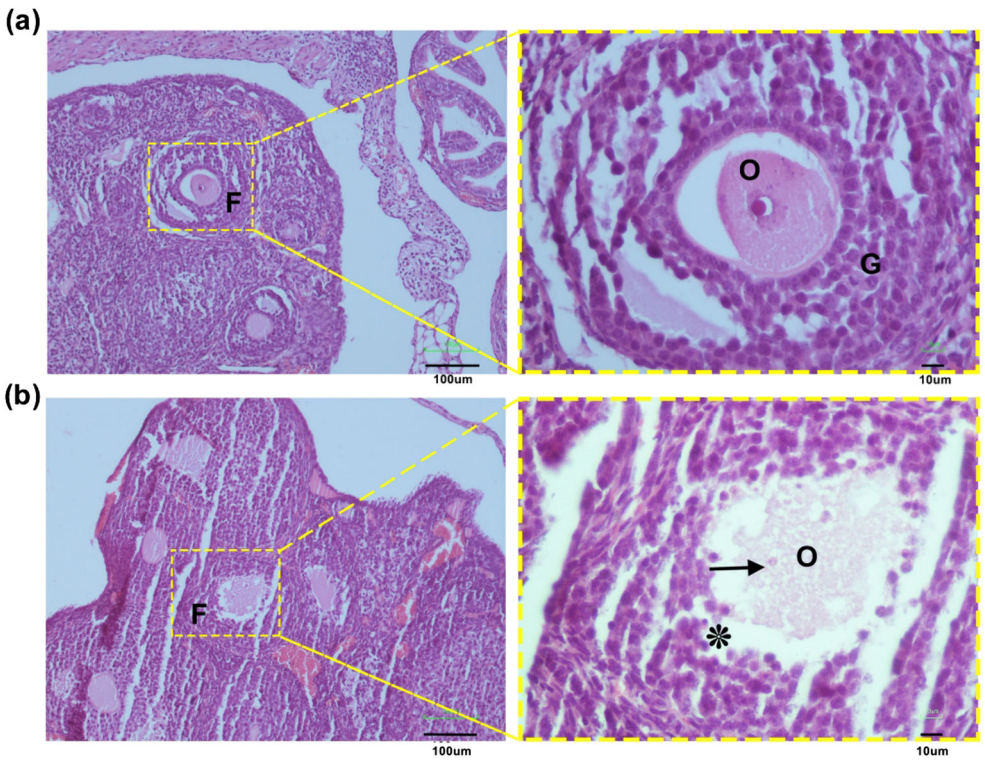

Fig. 1 Histopathology of ovaries in normal (**a**) and DHEA-injected (**b**) mice. Antral follicle (F); granulosa cells (G); and oocyte (O). Black arrow and black star indicate follicular cyst and granulosa cells detachment, respectively

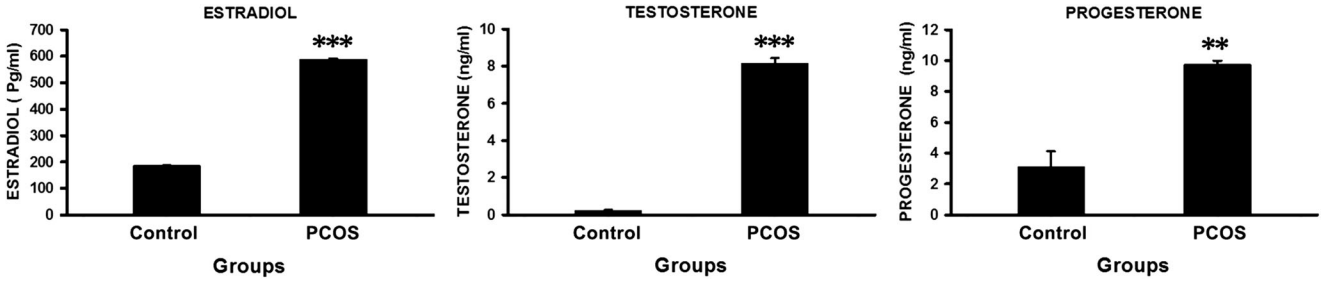

Fig. 2 The concentration of progesterone, testosterone, and 17β-oestradiol hormones in the serum of normal (control) and PCOS-induced animals. ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively

Con-AUR, and significant decrease compared to PCOS-sesame oil and PCOS experimental groups ($p < 0.0001$).

Oocyte Maturation Assay

Intracellular GSH in oocytes of different experimental groups was stained with Cell Tracker™ Blue, as GSH is stained in blue (Fig. 5a). PCOS animals administrated with AUR and MET showed the oocytes with a higher level of intracellular GSH when compared with the PCOS and PCOS-sesame oil groups (Fig. 5b). Additionally, ROS (green dots) were detected by staining of oocytes with H2DCFDA, as shown in Fig. 6a. A significant decrease in ROS level was observed in the oocytes derived from PCOS animals treated with AUR and MET compared with PCOS and PCOS-sesame oil groups (Fig. 6b).

Maturation of oocytes isolated from normal and PCOS animals after treatment was also evaluated under a stereomicroscope (Olympus; SZ1145, Tokyo, Japan) and the results are shown in Table 1. A significant decrease in abnormality rate was observed in PCOS-MET oocytes compared with the oocytes isolated from PCOS and PCOS-sesame oil. Although the rate of abnormal oocytes in PCOS-AUR was lower than PCOS and PCOS-sesame oil groups, the difference was not significant. PCOS animals administrated with AUR and MET showed a significant decrease in germinal vesicle rates in comparison with PCOS and PCOS-sesame oil groups. PCOS-AUR and PCOS-MET groups had higher percentages of expansion rate and MII stage oocytes compared with PCOS and PCOS-sesame oil groups. Oocytes derived from control and control-AUR animals had significantly lowest abnormality, germinal vesicle rates and MI stage percentage, and highest MII stage percentage and expansion rate compared with other experimental groups.

In Vitro Fertilization

In vitro fertilization (IVF) assay was carried out to determine the fertilization capacity and subsequent oocyte developmental competence in the oocytes derived from normal and PCOS animals treated with AUR and MET. The fertilized oocytes were observed under a microscope and the representative images were analyzed for the rates of fertilization, 2-Cell embryo, and blastocyst formation (Fig. 7a). The IVF results were

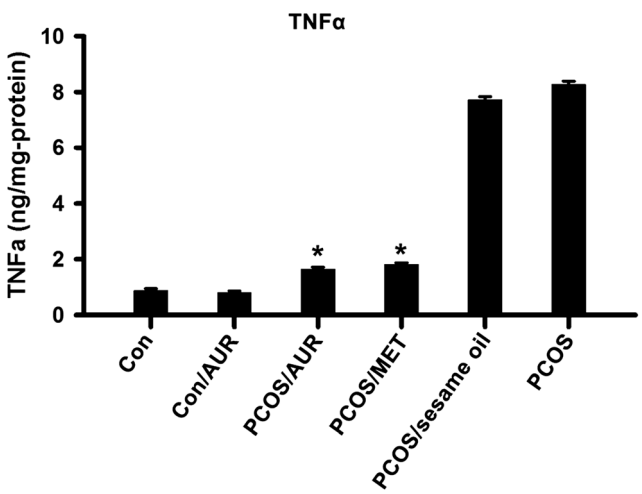

Fig. 3 Concentration of TNFα in ovaries of normal (Con) and PCOS animals treated with auraptene (PCOS-AUR), metformin (PCOS-MET), sesame oil (PCOS-sesame oil), and no treatment (PCOS). * indicates significant difference with Con, Con/AUR, PCOS-sesame oil, and PCOS groups ($p < 0.0001$)

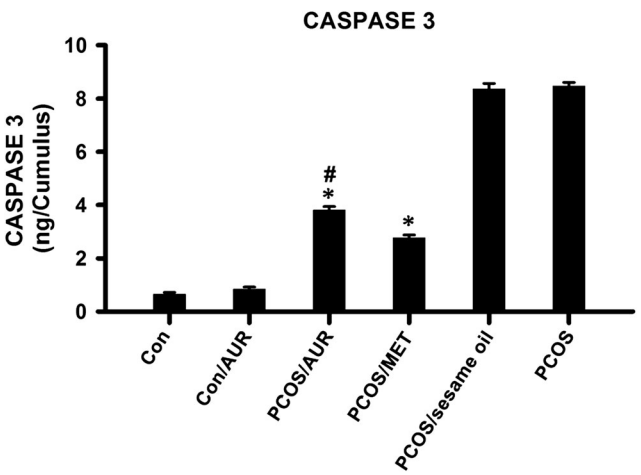

Fig. 4 The concentration of caspase-3 in cumulus cells of normal (Con) and PCOS animals treated with auraptene (PCOS-AUR), metformin (PCOS-MET), sesame oil (PCOS-sesame oil), and no treatment (PCOS). * indicates significant difference with Con, Con/AUR, PCOS-sesame oil, and PCOS groups ($p < 0.0001$). # indicates significant difference with PCOS/MET group ($p < 0.001$)

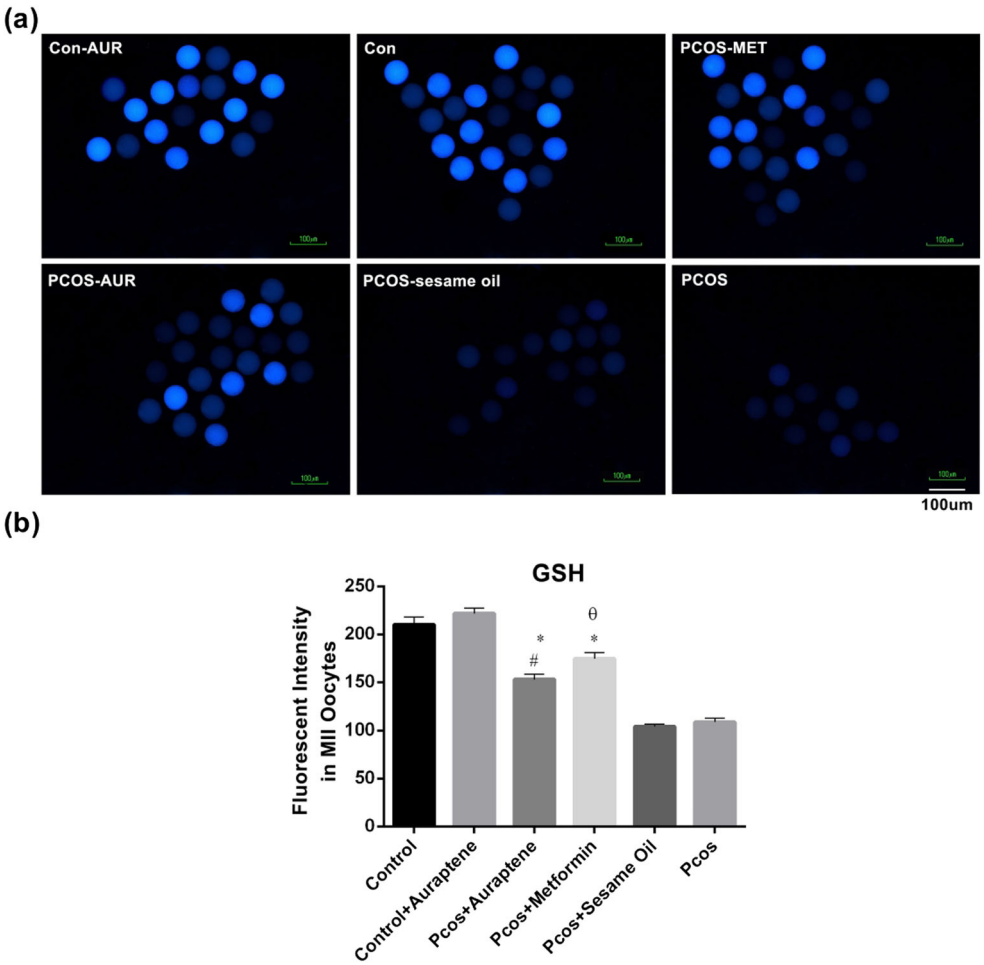

Fig. 5 **a** Cell Tracker™ Blue staining for detection of intracellular glutathione (GSH) in oocytes of six experimental groups. Blue dots indicate GSH. **b** PCOS animals treated with auraptene (PCOS-AUR) and metformin (PCOS-MET) showed higher levels of GSH compared with those PCOS animals with no treatment (PCOS) and treated with sesame oil (PCOS-sesame oil), and decreased GSH concentration in comparison with normal animals treated with AUR (Con-AUR) and with no treatment (Con). The data were analyzed using ImageJ software version 1.40. * $P < 0.05$ indicates statistically significant difference with PCOS-sesame oil; # $P < 0.01$ indicates statistically significant difference with PCOS; $P < 0.001$ indicates statistically significant difference with PCOS

quantified and compared between the experimental groups (Fig. 7b). PCOS-AUR and PCOS-MET groups showed significantly higher rates of fertilization (~ 70% and 71%, respectively) compared with those in PCOS-sesame oil and PCOS groups (~ 42% and 42%, respectively) ($p < 0.05$). Although the rate of fertilization showed a slight decrease in PCOS-AUR and PCOS-MET groups when compared with control and con-AUR groups (~ 80% and 81%, respectively), the difference was not significant. The same differences were found in 2-Cell embryo and blastocyst formation rates (Fig. 7b). As control, con-AUR, PCOS-AUR, PCOS-MET, PCOS-sesame oil, and PCOS experimental groups showed ~ 71%, ~ 72%, ~ 63%, ~ 67%, ~ 39%, and ~ 39% rates of 2-Cell, respectively, and ~ 54%, ~ 54%, ~ 43%, ~ 47%, ~ 20%, and ~ 21% rates of blastocyst formation, respectively.

Discussion

Patients with PCOS experience repeated IVF failures and poor pregnancy outcomes [34]. It is well documented that the inflammation and production of ROS in PCOS ovaries is significantly higher than normal voluntaries [18, 35]. Although PCOS is a multifactorial disease, it was reported that reduction of inflammatory responses and apoptosis rate in ovaries of PCOS can improve in vitro maturation and IVF potential of their oocytes [20, 25, 36]. Therefore, in this study, we hypothesized that the administration of an anti-inflammatory and anti-oxidant agent such as AUR might be a potential candidate for minimizing the complications associated with PCOS. The present study provides evidence that administration of AUR improves the maturation and IVF potential of PCOS oocytes via its anti-inflammatory property, an increase in intracellular GSH level, and protection against ROS activity. Here, we induced a PCOS model in mouse by subcutaneous injection of 60 mg/kg DHEA to NMRI mice [29, 37]. The formation of polycystic ovary with abnormal ovarian morphology and degenerated granulosa cells in induced PCOS mice was confirmed by histopathological observations. PCOS model was further identified by elevated concentrations of steroid hormones such as progesterone, testosterone, and 17β-oestradiol in serum of PCOS mice [4, 7, 18, 38]. PCOS-induced mice were administrated with AUR and MET, and their effects on inflammation, apoptosis rate, OM, and IVF capacity were determined.

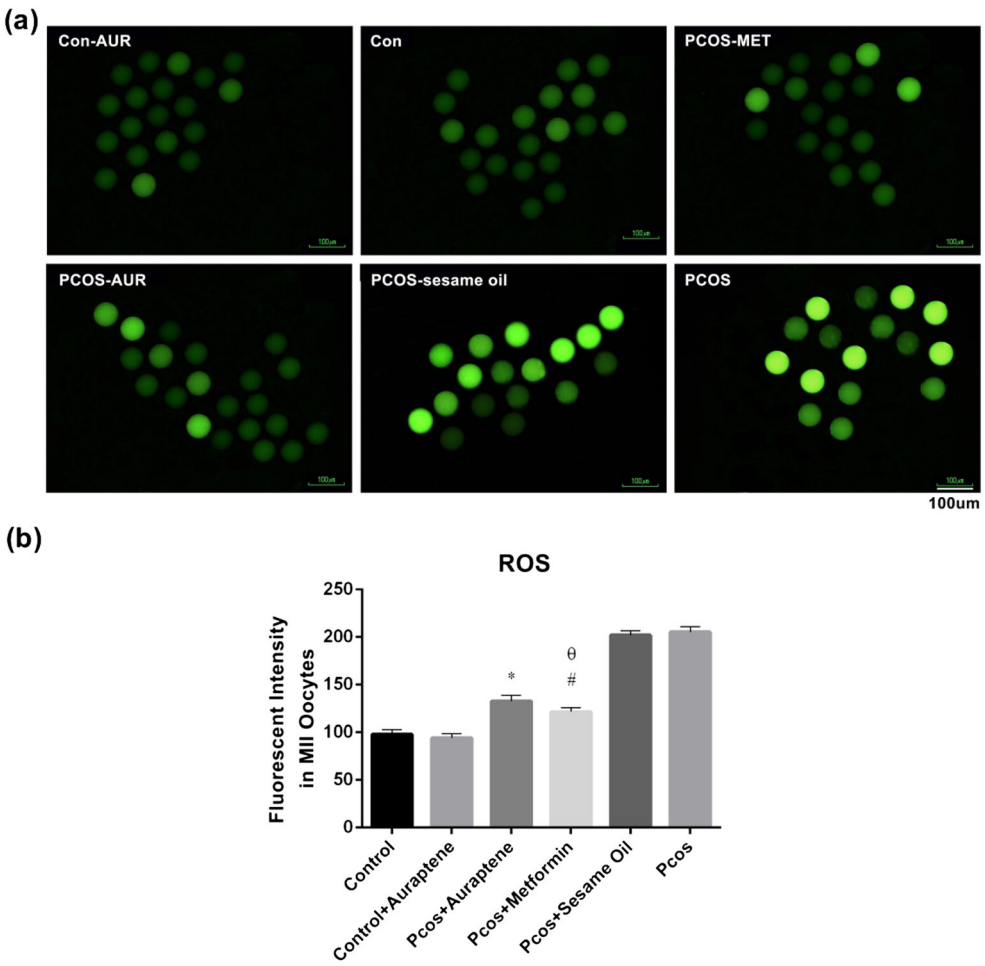

Fig. 6 **a** H2DCFDA staining for detection of reactive oxygen species (ROS) level in oocytes of six experimental groups. Green dots indicate ROS. **b** PCOS animals treated with auraptene (PCOS-AUR) and metformin (PCOS-MET) showed a significant decrease in ROS levels compared with those PCOS animals with no treatment (PCOS) and treated with sesame oil (PCOS-sesame oil), and increased ROS concentration compared with normal animals treated with AUR (Con-AUR) and with no treatment (Con). The data were analyzed using ImageJ software version 1.40. * $P < 0.05$ indicates statistically significant difference with PCOS and PCOS-sesame oil; $P < 0.01$ indicates statistically significant difference with PCOS. # $P < 0.0001$ indicates statistically significant difference with PCOS-sesame oil

As discussed earlier in this article, the ovary of women with PCOS experiences an inflammation with increased expression of some pro-inflammatory cytokines such as TNFα and abnormal apoptotic activities [39, 40]. TNFα has important roles in regulation of normal activity of ovary and follicles development. It is reported that the increase in expression of TNFα in PCOS patients induces apoptosis in antral follicles granulosa cells, and subsequently results in increase and decrease in thickness of theca and granulosa layers, respectively [25]. Actually, apoptosis is a mechanism by which abnormal embryos are rid and removed from ovary [41]. Therefore, a balance between pro-apoptotic and anti-apoptotic factors is crucial in healthy and successful fertilization and pregnancy [8]. Any threatening conditions such as increase in ROS in stress situations can negatively affect this balance and lead to unwanted apoptosis and embryo arrest [42]. But, the role of apoptosis in OM and IVF potential of oocytes derived from PCOS patients is still a controversial issue. It was reported that upregulation of heat shock protein 27 (Hsp27), Hsp27, as an apoptosis inhibitor factor, in PCOS patients inhibits OM while

Table 1 The effects of AUR and MET administration in the maturation of oocytes isolated from normal and PCOS model groups

Groups	No. of oocytes	Ab (%)	GV (%)	MI (%)	MII (%)	Exp (%)
Control	228	4.97 ± 0.38*#	3.65 ± 0.14*#	5.84 ± 0.14*	85.82 ± 0.63*#	96.49 ± 1.01*#
Control-AUR	224	4.61 ± 0.39*#	3.27 ± 0.39*#	5.50 ± 0.17*#	87.65 ± 0.39*#	97.47 ± 0.9*#
PCOS-AUR	218	9.32 ± 0.40	5.65 ± 0.15	6.88 ± 0.26	78.59 ± 0.66#	83.33 ± 0.80
PCOS-MET	220	7.42 ± 0.41	5.15 ± 0.17	6.51 ± 0.12	81.12 ± 0.4*	84.24 ± 0.92
PCOS-sesame oil	209	9.25 ± 0.44	8.77 ± 0.19*#	10.69 ± 0.17*#	72.09 ± 0.31*#	73.37 ± 1.24*#
PCOS	207	10.14 ± 55#	8.37 ± 0.16*#	10.79 ± 0.16*#	71.18 ± 0.42*#	73.59 ± 0.97*#

Data are presented as mean ± SEM. * and # indicate statistically significant ($P < 0.05$) difference with PCOS-AUR and PCOS-MET, respectively. *Abbreviations*: *Ab*, abnormal oocyte; *GV*, germinal vesicle; *MI*, metaphase I; *MII*, metaphase II; *EXP*, expansion rate

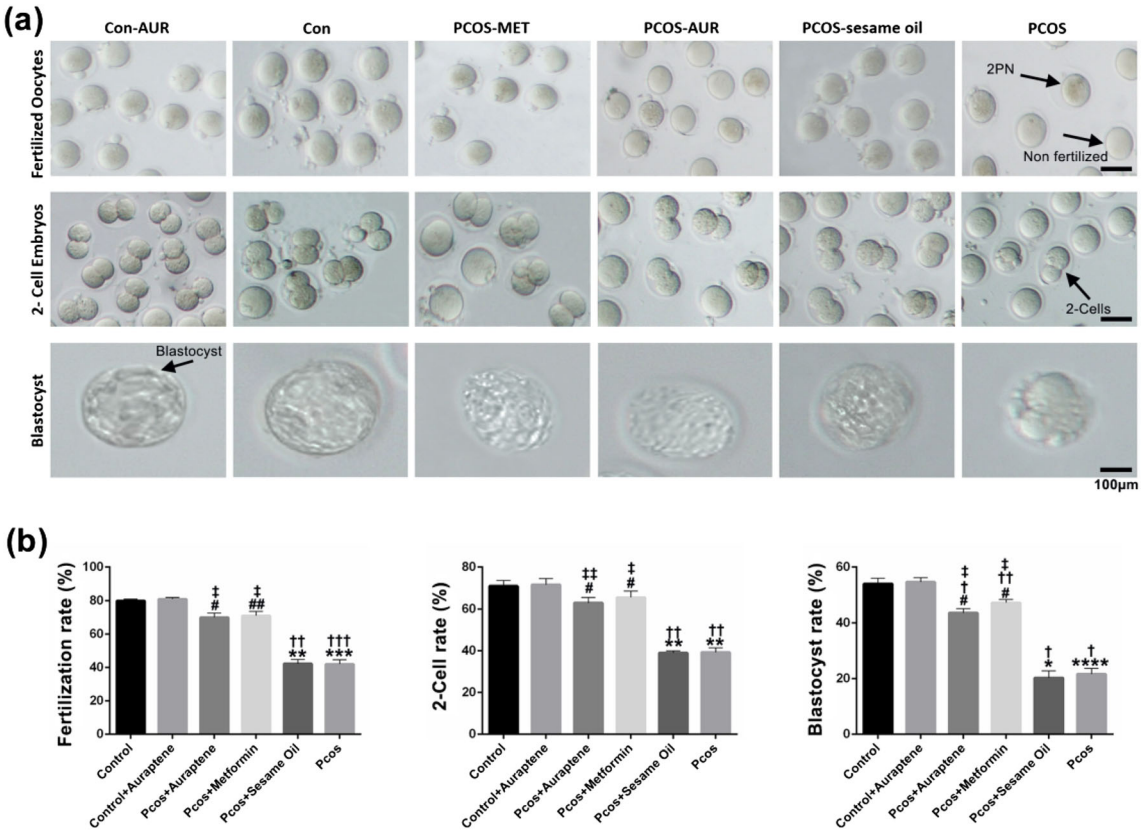

Fig. 7 **a** Evaluation of in vitro fertilization (IVF) in the oocytes isolated from normal and PCOS animals after AUR and MET administration under a stereomicroscope (Olympus; SZ1145, Tokyo, Japan). Two-cell embryos, blastocysts, and fertilization rates were determined and compared between the experimental groups. **b** Fertilization rate, 2-cell rate, and blastocyst rate were quantified. PCOS-AUR and PCOS-MET showed significantly higher rates of fertilization, 2-Cell, and blastocyst formation when compared with PCOS-sesame oil and PCOS with no treatment (PCOS). * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$), and **** ($P < 0.0001$) indicate significant difference with control. † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.001$) indicate significant difference with control-AUR. # ($P < 0.05$) and ## ($P < 0.01$) indicate significant difference with PCOS-sesame oil. ‡ ($P < 0.05$) and ‡‡ ($P < 0.01$) indicate significant difference with PCOS

increasing the potential of embryonic development [9]. Our results showed that AUR and MET significantly decreased the concentration of TNFα in ovary of PCOS mice compared with those PCOS animals without treatment and treated with sesame oil. As expected, the animals administrated with both AUR and MET showed a significant decrease in level of caspase-3 in their cumulus cells, indicating decreased apoptosis rate, in comparison with PCOS-sesame oil and PCOS groups. Anti-inflammatory effects of AUR have been demonstrated by some studies [43]. For example, Okuyama and coworkers indicated that AUR could suppress inflammatory responses and subsequently show neuroprotective effects in the ischemic brain [43]. In another study, it was revealed that thymoquinone, an active component of black seed oil (*Nigella sativa*), can improve OM and fertilization in PCOS-derived oocytes through minimizing oxidative stress and apoptosis rate [36]. It was shown that the number of oocytes in ovary of PCOS patients increases; however, the oocytes are immature with poor quality and low rate of IVF potential [44–47]. Low IVF and pregnancy rate and subsequently higher risk of abortion in PCOS patients was reported to be more likely due to the increased risk of aneuploidy in these patients compared with normal women [46]. The embryos derived from IVF of oocytes isolated from women with PCOS was shown to have suboptimal development with very low quality, which makes them more sensitive and not appropriate candidates for biopsy and extended culture [48–50]. Nuclear and cytoplasmic maturation of oocyte is a molecular process, which involves many events including synthesis of biochemical compounds, phosphorylation of proteins, and various signaling pathways. Synchronization of these events guarantees the quality of matured oocytes. It is well known that intracellular GSH, as a non-enzymatic antioxidant, can protect oocytes from ROS activity, and improve oocyte maturation and embryo development [51–54]. Therefore, intracellular GSH is considered as a reliable and valuable indicator of cytoplasmic maturation of oocytes [53]. In our study, we showed a significant increase in intracellular GSH level and subsequently decrease in ROS level in the PCOS animals treated with AUR and MET compared with those PCOS animals treated with sesame oil and no treatment. These findings provide evidences that AUR improves maturation of PCOS

oocytes through increase in intracellular GSH and protection against ROS activity.

The results obtained from IVF assay revealed that the positive effects of administration with AUR on fertilization rate and subsequent oocyte developmental competence in PCOS animals were comparable to those PCOS animals treated with MET. Administration with AUR and MET significantly increased the rates of fertilization, 2-Cell, and blastocyst formation in the oocytes derived from PCOS animals when compared with PCOS-sesame oil and PCOS groups. The results obtained from OM and IVF assays are consistent with other relevant studies showing the effects of resveratrol [55], thymoquinone [36], and trans-ε-viniferin [19] on OM and IVF by increasing intracellular GSH level and reducing ROS activity. Jeon et al. [56] showed that sufficient zinc supplementation (0.8 μg/mL) in in vitro maturation medium is required for in vitro oocyte maturation and embryo development. They showed that zinc increased intracellular GSH concentration and reduced ROS activity.

Conclusion

The current study revealed that the administration of AUR had positive effects on oocyte maturation, decreased inflammation and apoptosis, and subsequently improved fertilization rate and oocyte developmental competence in PCOS oocytes, comparable to those PCOS animals treated with MET. Taken together, AUR administration is suggested as a potential candidate for therapeutic management of PCOS patients, although further examinations are required to confirm the safety of AUR administration.

Funding Information This study is a part of the thesis written by Marzieh Abizadeh, PhD student of Reproductive Biology and financially supported by "Research Department of the School of Medicine Shahid Beheshti University of Medical Sciences" (Grant No. 16826).

Compliance with Ethical Standards All the animal surgeries and procedures were carried out in accordance with the 8th edition of the "Guide for the Care and Use of Laboratory Animals," and approved by the Ethical Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1397.334).

Conflict of Interest The authors declare that there is no conflict of interest.

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