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Protective role of broccoli extract on estradiol valerate-induced polycystic ovary syndrome in female rats

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SUMMARY

Polycystic ovary syndrome (PCOS) is one of the most common causes of infertility. The aim of this study was to investigate the protective role of broccoli extract on estradiol valerate (EV)-induced PCOS in female rats. Forty adult female rats were divided into four main groups; control, broccoli-treated, EV, single intramuscular injection of 16mg/kg)-treated, EV+broccoli (1 g/kg/day)-treated groups. The protected rats were treated orally by gastric tube daily for 4 weeks. At the end of the experiment, blood samples were collected and the ovary were subjected to histological and immunohistochemical analyses. EV treated group exhibited the characteristic features of PCOS. Disturbed ovarian cyclicity in addition to histopathological alterations, including decreased number of healthy follicles and corpora lutea, increased degenerated, cystic follicles and increased collagen fiber deposition were detected by light microscopic studies. Moreover, increased immune-reactivity for iNOS and altered proliferation index were observed by immunohistochemical assessments. Co-administration of broccoli extract improved EV-induced PCOS in rat model. In conclusion, broccoli may be an effective therapeutic candidate for the treatment of PCOS

Key words: Anti-oxidant – Brassica oleracea – Inos – Ki-67 – Picro-sirius – Polycystic ovary

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathy in women of reproductive age (Atis et al., 2012). The risk of endometrial hyperplasia, cancer, type 2 diabetes and cardiovascular diseases increase with long-term PCOS (Sadrefozalayi and Farokhi, 2014). In addition to ovulatory dysfunction, infertility, increased anxiety, depression and several other complications are associated with this disorder (Ouladsahebmadarek and Khaki, 2014). Imbalance between pro-oxidant molecules and antioxidant defensive system may result in PCOS; hence, antioxidants can play an important role in reducing infertility associated with this disease (Agarwal et al., 2014). Moreover, higher exhibition of pro-inflammatory agents, such as nitric oxide (NO), participates in endocrinal and pathophysiological events of PCOS (Lakzaei et al., 2013). A variety of mammalian species have been employed as animal models of PCOS, ranging from rodents to non-human primates. Rats are preferred over other animal species due to their stable genotype, ease of handling, shorter reproductive lifespan and short estrous cycles (Aitman et al., 2008; Walters et al., 2012), so rats are chosen for developing several experimental models for PCOS by different methods, such as subcutaneous injection or implantation of estrogen, androgens, antiprogesterone, letrozole, prenatal exposure to excess androgens and exposure to constant light (Danni and Donna, 2012). Estradiol valerate (EV) is a long-acting estrogen and on administration causes hypothalamic –pituitary dysregulation of gonadotrophin releasing hormone (GnRH), resulting in improper release and storage of luteinizing hormone (LH) (Danni

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and Donna, 2012) and causes a rapid appearance of PCOS due to disturbances in metabolic and physiologic processes (Atis et al., 2012). Broccoli (Brassica oleracea) has multiple pharmacological activities; including phytoestrogenic, anti-inflammatory, antimicrobial, antidiabetic and hypolipidemic effects in addition to its anti-cancer properties (Amnah and Alsuhaibani, 2013; Calder´on-Monta˜no et al., 2011; Farahmandi et al., 2013). It also contains major antioxidants as vitamin C, vitamin E, carotenoids and polyphenols, especially flavonoids, which all provide protection against free radicals (Monero et al., 2010). We sought to evaluate, for the first time, the protective role of broccoli extract in PCOS rat model.

MATERIALS AND METHODS

Animals and experiment design

Forty adult female Sprague-Dawley rats weighed 200±30 g were used in this study. They were obtained and housed at Theodor Bilharz Research Institute Animal House, Cairo, Egypt. The rats were kept at 12:12 h daylight/darkness with access to chow and water. The procedure was approved by the ethics committee for animal experimentation of the Faculty of Medicine, Menoufia University, in accordance with the international regulation on care and use of laboratory animals. All rats were checked daily throughout the whole experimental period for ovarian cycle using vaginal smear cytology for the determination of the estrous cycle phases. The rat estrous cycle usually lasts about 4 days, and only those with regular 4-5 days estrous cycles were used for the study. Abnormal estrous cycles and persistent vaginal cornification (PVC) were considered to be signs of the presence of ovarian cysts and early confirmation of PCOS induction (Jashni et al., 2016). The animals were randomly divided into four main groups; control, broccoli-treated, estradiol valerate (EV)-treated, EV+broccoli-treated groups (n = 10 per group). Polycystic ovary syndrome was induced in EV-treated and EV+broccoli-treated groups by single intramuscular injection of EV at a dose of (16 mg/kg) (Ouladsahebmadarek and Khaki, 2014). Broccoli-treated group received 1g/kg/day broccoli extract dissolved in distilled water by gavage (Hashem et al., 2013). The rats were anaesthetized lightly by diethyl ether inhalation, and then sacrificed after 4 weeks of the beginning of the experiment in the proestrus phase (Salvetti et al., 2009). There was no statistically significant difference between control and broccoli-treated group in all evaluated parameters; therefore, these groups were pooled into one group (control group).

Chemicals

Estradiol valerate tablets (Bayer Weimar GmbHb und Co. KG, Germany) were obtained from a pharmacy in Egypt. The tablets were crushed and dissolved in distilled water to be given by intramuscular injection. Broccoli was purchased from a local supermarket. The plant material was authenticated in the Botany Department, National Research Center, El-Doki, Cairo, Egypt. Broccoli was then frozen at -40°C for 12 h, freeze dried for four days at 22° C (i.e. drying started at -40°C and ended at 22°C) and 200 Pascal. The dried broccoli was powdered by an electric grinder (Moulinex AR1043-UK0, Moulinex, Lyon, France), yielding 15.7 g powder per 100 g of fresh broccoli. The extract was extracted exhaustively with 80% ethanol (total ethanol extract) in a Soxhlet apparatus, Germany. The extract was dried at -40°C under vacuum.

Biochemical, histological and immunohistochemical studies

At the end of the experimental period, all rats were anaesthetized lightly by diethyl ether inhalation, and blood samples were collected for biochemical analysis. The rats were then sacrificed. The ovaries were extracted and preserved in 10% neutral buffered formaldehyde, and paraffin sections were prepared for histological study. Serial sections of 5 µm in thickness were stained with haematoxylin and eosin (H&E) for routine histological examination and with picro-sirius to detect collagen fibers. Paraffin sections (5 µm thick) were deparaffinized in xylene and then subjected to immunohistochemical stains using inducible nitric oxide synthase (iNOS), 1:500 (ThermoScientific) and Ki-67, 1:500 (ThermoScientific) (Mitchell et al., 2004). The serum samples were separated for estimation of serum estradiol, progesterone, testosterone, follicular stimulating hormone (FSH) and LH levels using special kits by Microparticle Enzyme Immunoassay (MEIA).

Quantitative assessments

For histological and immunohistochemical quantitative assessment, five non-overlapping fields (“200x” and “400x only for primordial follicles number”) per section were randomly captured by a Leica Microscope DML B2/11888111 equipped with a Leica camera DFC450. The number of follicles, percentage of collagen fiber deposition and immunoreactivity-positive cells in the fields taken from at least five sections / animal was counted using image J software (Maryland, USA) and averaged per field for each animal. The numbers calculated for at least five animals per experimental group and the biochemical results were considered statistical analyses.

Statistical analysis

Results were expressed as mean±SD. Kolmogorov-Smirnov test was used to assess the normality of data. One way-ANOVA followed by a post hoc Bonferroni test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA. The level of significance of $P \leq 0.05$ with 95% confidence interval was considered to be statistically significant.

RESULTS

Vaginal smear results

Control rats had 4-5 days regular estrous cycles (Fig. 1A), comprising proestrous, estrous,

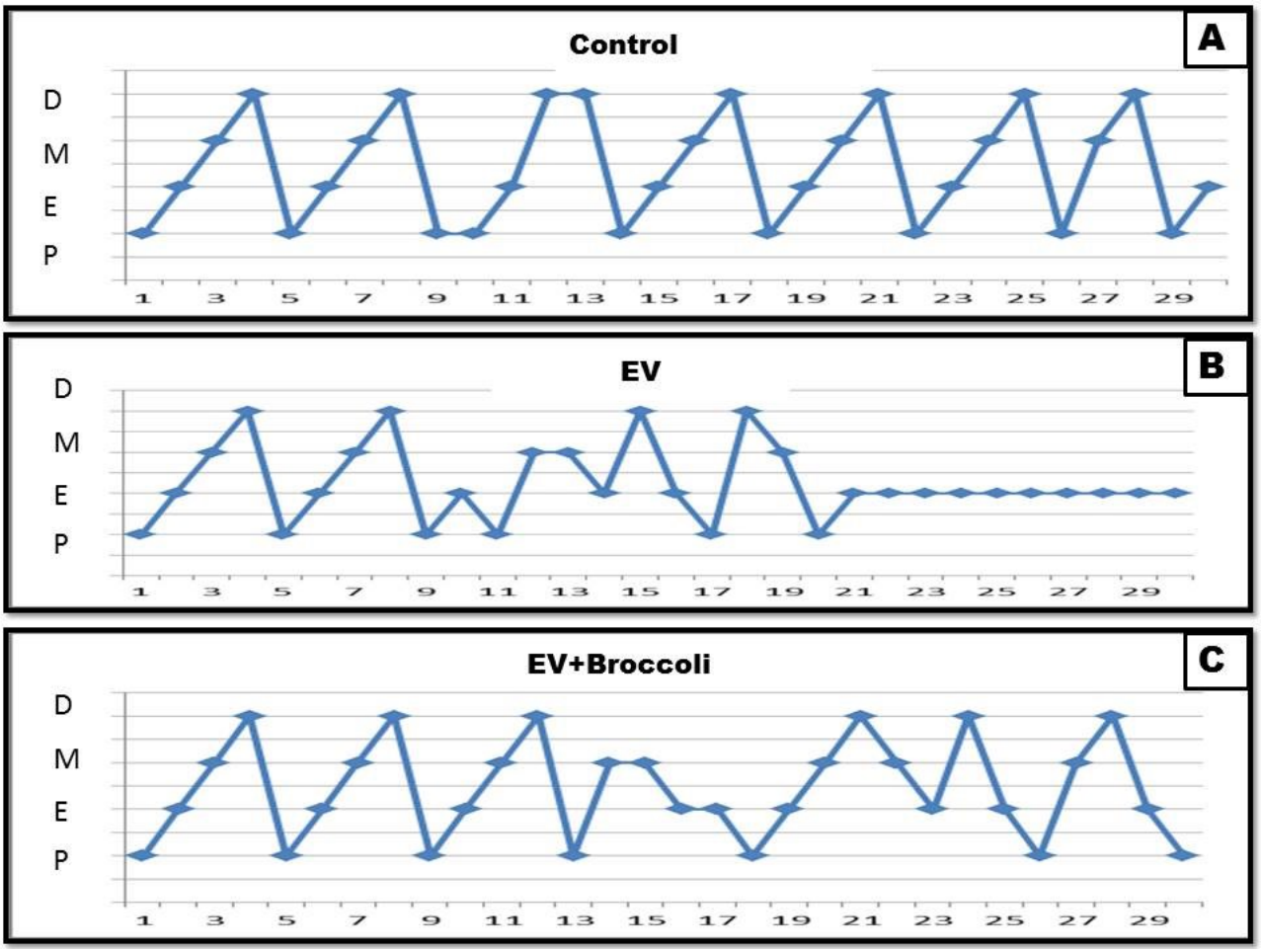

Fig 1. The estrous cycle in different experimental groups. Regular estrous cycles are observed in the control group (**A**). In EV-group, irregularity starts at the 10th day then turned to persistent estrous at the 20th day is detected (**B**). In EV+broccoli group, rats show irregular cycles at the 14th day without persistent estrous phase (**C**). P = proestrous, E = estrius, M = metaestrous, and D = diestrous.

metaestrous and diestrous, while EV group showed irregular cycles after 10 days of EV injection, then became constantly in estrous phase (Persistent Vaginal Cornification, PVC) after 20 days of EV injection till the time of sacrifice (Fig. 1B). Rats of the EV+broccoli group showed irregular estrous cycles after 2 weeks of EV injection till the time of sacrifice and did not exhibit PVC stage (Fig. 1C).

Biochemical results

Estrogen, LH and testosterone levels in the EV group had a highly significant increase compared to the control group. However, in the EV+broccoli these levels were significantly reduced compared to EV group. FSH and progesterone levels in the EV group were significantly reduced compared to the control group. In the EV+brocolli group, these levels were significantly increased compared to EV group (Fig. 2).

Histological results

Light microscopy H&E and picro-sirius studies

In H&E-stained sections, the control group showed large number of healthy follicles at different stages of differentiation and multiple large corpora lutea (Fig. 3A, B, C). Compared to control group, EV group showed decreased number of healthy follicles, increased number of atretic and cystic follicles (Fig. 3J), decreased number and size of corpora lutea and increased number of interstitial cells (Fig. 3D). Cysts had thin granulosa layer, thick theca layer (Fig. 3K) and desquamation of granulosa cells within the cyst fluid (Fig. 2E). Atretic follicles had degenerated oocytes (Fig. 3F). On the other hand, in EV+broccoli group a larger number of follicles was observed compared to EV group; most of them were healthy, few were degenerated or cystic. Corpora lutea increased in number and size indicating restoration of ovulatory function of the ovary (Fig. 3G, H, I). In control group picro-sirius-stained revealed that collagen was deposited in the tunica albuginea and the theca layer around the follicles (Fig. 4A). Compared to the control group, a significant increase (51.49±0.98 vs. 31.15±0.49; $P < 0.001$) in the surface area of collagen deposition in EV group, especially in the theca layer surrounding the cysts, was detected (Fig. 4 B, C). A significant decrease in the surface area of collagen deposition in the

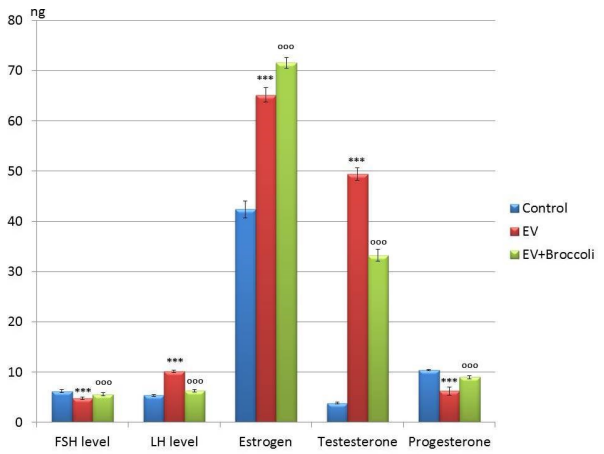

Fig 2. Biochemical results in different groups. *** P < 0.001 compared to the control group; and ^000 P < 0.001 compared to the EV-treated group.

EV+broccoli group was observed (82.52±2.16 vs. 51.49±0.98; P < 0.001) compared to EV group (Fig. 4D, E).

Immunohistochemical studies

To evaluate oxidative damage in the ovaries of different experimental groups, immunohistochemistry was used to assess the expression of iNOS. The expression of iNOS significantly up-regulated in the granulosa cells (28.2±0.77 vs. 3.4±0.32, P < 0.001) and the theca cells (55.2±0.95 vs. 28.7 ± 0.39; P < 0.001) of the EV group (Fig. 5B) compared with the control rats (Fig. 5A). Treatment with broccoli markedly down-regulated the expression of iNOS in the granulosa cells (16.4±0.4 vs. 28.2±0.77; P < 0.001) and the theca cells (32.2±0.92 vs. 55.2±0.95; P < 0.001) (Fig. 5C) compared with the EV group. There was no significant change in iNOS expression in interstitial cells of the control, EV and EV+broccoli groups.

Ki-67 expression, used to evaluate cell proliferation, was dramatically up-regulated in the theca cells (32.2±2.57 vs. 3.8±0.32; P < 0.001) and the interstitial cells (42.8±2.05 vs. 26.1±3.14; P < 0.001) in the EV-treated rats (Fig. 6B) compared to control group (Fig. 6A). This up-regulation was significantly down-regulated in the theca cells (8.0±0.797 vs. 32.2±2.57; P < 0.01) and in the interstitial cells (30.6±2.05 vs. 42.8±2.05; P < 0.001) of the EV+broccoli group (Fig. 6C), while its expression was down-regulated in the granulosa cells (8.8±2.4 vs. 70.47±8.34; P < 0.001) in EV-treated rats. This down-regulation was significantly increased in the granulosa cells (53.2±1.99 vs. 8.8±2.4; P < 0.001) of the EV+broccoli group.

DISCUSSION

Natural products can often provide more safety than synthetic medications with fewer side effects. Several clinical studies have revealed the beneficial effect of broccoli as antioxidant in different diseases such as diabetes (Farahmandi et al., 2013; Patel and Sharma, 2014) and hepatic diseases (Hashem et al., 2013; Ahmed et al., 2012). This study was the first to describe the protective effect of broccoli extract on the PCOS rat model via its anti-oxidative effect.

Estradiol-valerate (EV) rat model was used in the current study because it resembles human PCOS in many aspects, including hormonal abnormalities in the hypothalamic-pituitary-ovarian and anovulation, changes in the ovarian histology and systemic inflammation (Zare et al., 2015). This model is also easier, faster, and more accessible to perform than other models of this disease (Daneasa at al., 2016). Through short time of hormone exposure, it causes a rapid appearance of continuous estrous, which was reported to appear at 16-20 days by Brawer et al. (1986). Cystic and atretic follicles, decrease or absent of corpus luteum, and other morphologic characteristics resembling those observed in women can also be found in this model (Paixão et al., 2017) due to disturbances in metabolic and physiologic processes (Atis et al., 2012).

In the current study, LH, testosterone and estrogen levels were significantly increased in EV-treated group; however, there were significant decrease of progesterone and FSH levels compared to the control group. Increased androgen levels in the blood in this model was in agreement with several studies (Zangeneh et al., 2010; Karimzadeh et al., 2012; Zangeneh et al., 2012; Al-Moziel et al., 2013; Linares et al., 2013; Ugwah-Oguejiofor et al., 2014; Ghafurniyan et al., 2015; Nabiuni et al., 2015). Therefore, hyperandrogenic state in EV-rat model of PCOS is well-documented. Furthermore, the increase in androgen level in EV model was explained by Jashni et al. (2016), who reported that when the ratio of LH to FSH levels increases, the ovaries increase preferentially the synthesis of androgens. Another study reported that insulin resistance associated with EV model might play a role in upregulation of androgen levels. High insulin levels can increase the androgen levels through affecting the insulin receptor, which increases androgen response of theca cells to LH, reducing production of sex hormone-binding globulin (SHBG) in the liver and lowering production of the proteins that bind to insulin-like growth factor (IGF) (Ghafurniyan et al., 2015).

Highly significant increase in the estrogen level of EV group was observed in the current study compared to the control group, and this result was consistent with previous studies utilizing EV model (Karimzadeh et al., 2012; Zangeneh et al., 2012; Linares et al., 2013; Nabiuni et al., 2015; Jashni et al., 2016). High levels of estrogen reported in EV model are a consequence of anovulation (Mirabolghasemi and Kamyab, 2017). Co-treatment with broccoli remarkably ameliorated the biochemical changes with significant decrease of LH, testosterone and estrogen levels and significant increase of progesterone and FSH levels. In EV-treated group, many large cysts with very thin granulosa layer and thick theca layer and scanty corpora lutea were detected. These findings might be due to a sudden increase in LH levels (LH

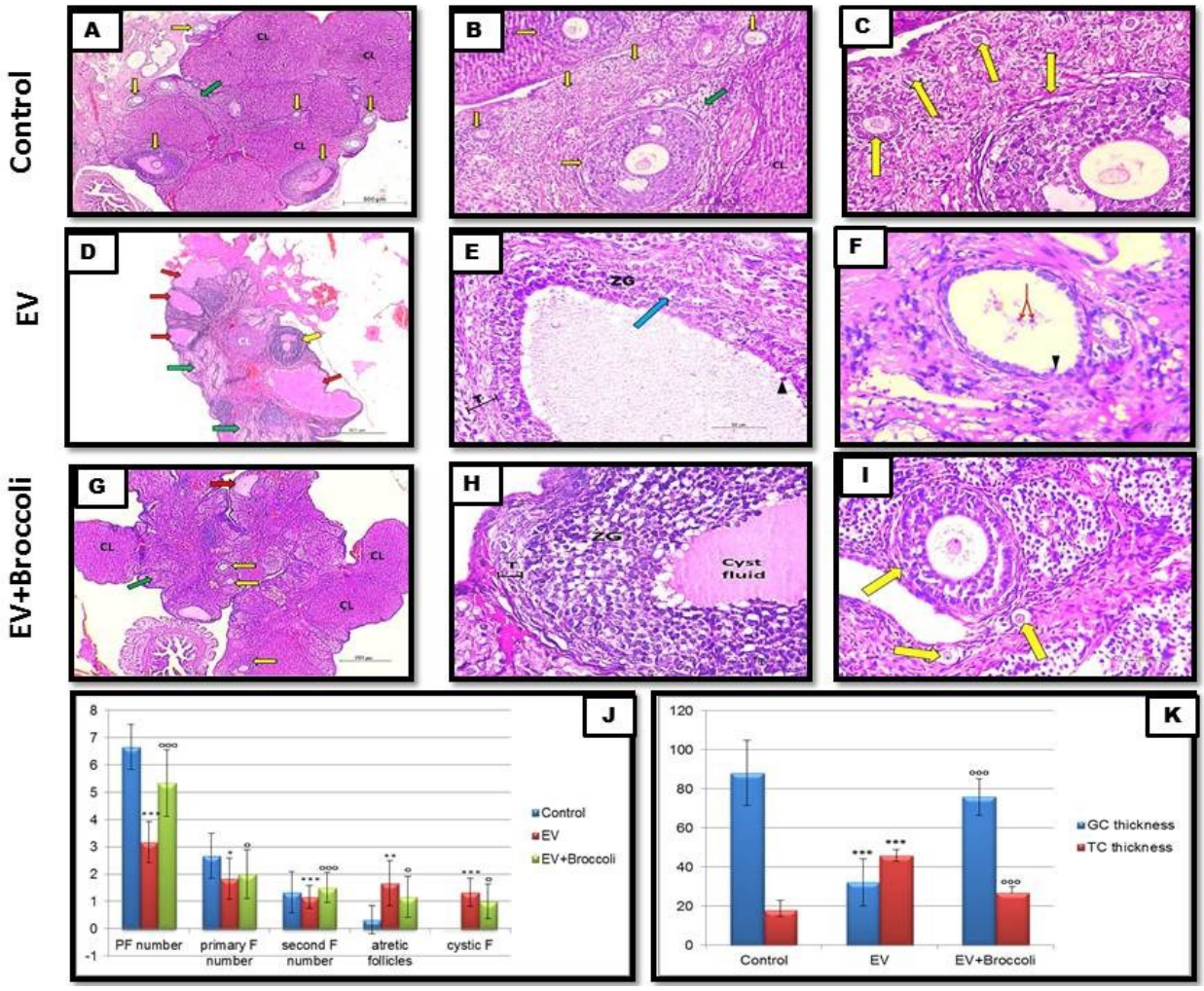

Fig 3. Representative images of H and E-stained sections from rats of different groups. In the control group, cortex with large number of corpora lutea (CL) and healthy follicles (yellow arrows) with interstitial cells in between (green arrow) are seen (**A, B** and **C**). In EV-group, large number of cysts (red arrows) and interstitial cells (green arrows, **D**) are detected. Cysts have thick theca layer (T), thin granulosa layer (ZG) with pyknotic nuclei (blue arrow), desquamated cells (arrow head, **E**) and Atretic follicle with divided micro nucleus (double-head arrow) and desquamated granulosa cells (arrow head, **F**) are detected. In EV+broccoli group, many normal follicles (yellow arrows), corpora lutea (CL) and few cysts (red arrows, **G, H** and **I**) are seen. * P > 0.05, ** P < 0.05 and *** P < 0.001, compared to the control group; and 0 P > 0.05 and 000 P < 0.001, compared to the EV-treated group. Scale bars = 500 µm (A, D and G), 100 µm (B), 50 µm (C, E, F, H and I).

surge), which was considered an essential process for ovulation. The steady rise of this hormone in PCOS model caused the formation of antral follicles without ovulation, and thus produced cystic follicles (Jashni et al., 2016). In the current work, hyperplasia of stromal interstitial cells was detected. As the cell proliferation rate is higher in the theca interna in the PCOS group, this might result in the accumulation of interstitial cells in ovarian stroma leading to the high hormone levels (Lombardi et al., 2014). Similar findings have been reported in other studies using EV animal models (Atis et al., 2012; Ghafurniyan et al., 2015; Jashni et al., 2016). In the present study, Picro-Sirius stained sections of the EV group showed significant increase in collagen fibers deposition as reported by previous studies (Zhang et al., 2013). The theca cell layer was thickened and an increased amount of collagen accumulated around follicles, which might mechanically inhibit follicle rupture and ovulation (Zhang et al., 2013). Fibrosis might be due to chronic persistent inflammation like fibrotic conditions. Chronic inflammation had been demonstrated to participate in the pathophysiological process of PCOS (Boulman et al., 2004; Krishna et al., 2017; Xiong et al., 2011). Treatment with broccoli significantly decreased collagen fibers deposition in the ovary; this might be attributed to the anti-inflammatory capability of broccoli.

Many studies reported oxidative stress as one of the pathological factors for PCOS (Reddy et al., 2016). The high levels of androgen and advanced glycation end products (AGEs) in PCOS women could induce oxidative stress and inflammation (Krishna et al., 2017). To determine the degree of oxidative damage in the ovaries of different experi-

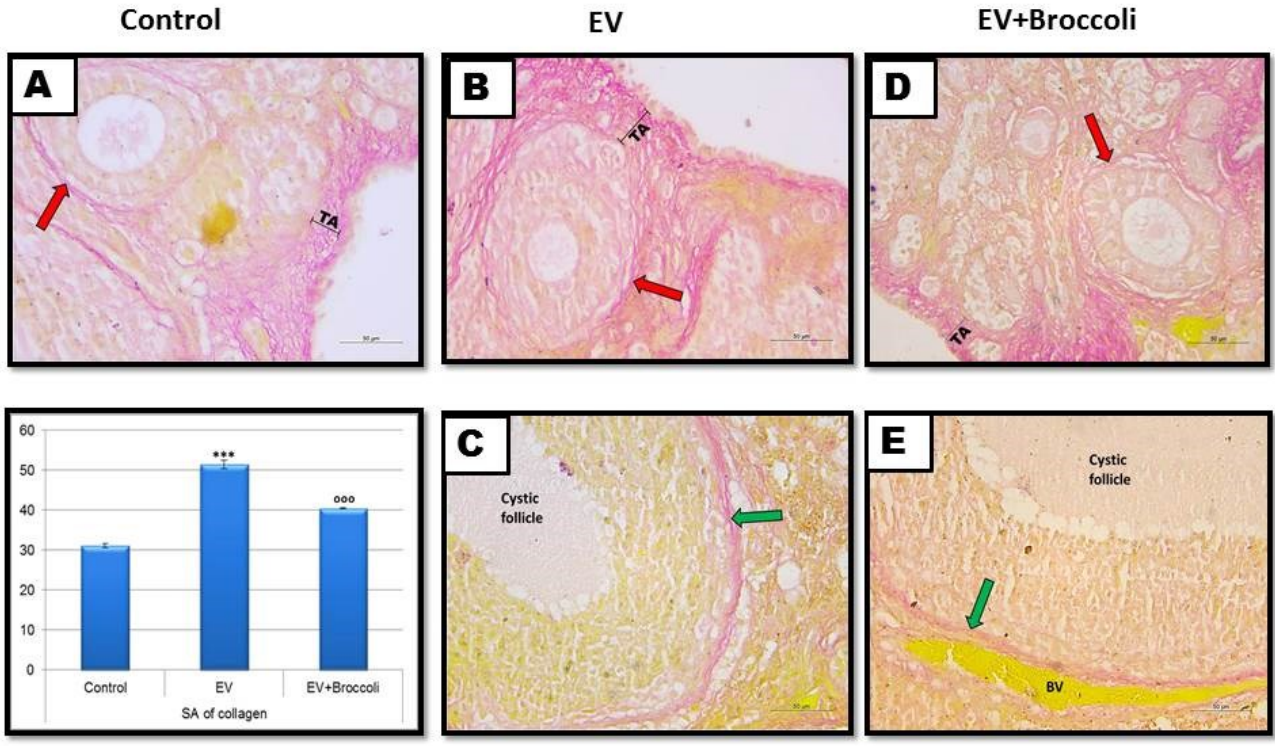

Fig 4. Representative images of picro-sirius-staining in the ovaries of the control (**A**), EV-treated (**B** and **C**), and EV+broccoli (**D** and **E**) groups. In the control group, collagen fibers are noticed in tunica albuginea (TA) and around follicles (red arrow, B). EV-group shows significant increase in collagen fibers deposition in tunica albuginea (TA), around follicles (red arrow) and cysts (green arrow) compared with control ($P < 0.001$). These increases are significantly reduced in EV+broccoli group. *** $P < 0.001$, compared to the control group; and 000 $P < 0.001$, compared to the EV-treated group. N= 10 for each group. Scale bars = 50 µm.

mental groups, iNOS expression was assessed using an immunohistochemical technique. The expression of iNOS was up-regulated in EV group in the granulosa and theca cells confirming the presence of an imbalance of oxidative/ antioxidative status in PCOS with no significant change in the interstitial cells. These findings were consistent with that reported by previous studies (Karabulut et al., 2012). Broccoli co-treatment dramatically down-regulated the iNOS expression in the granulosa and theca cells. These findings indicated that the anti-oxidative abilities of broccoli by played an important role in treating PCOS. In the current study, the percentage of immunopositive cells of Ki-67 was significantly higher in granulosa cells of the antral follicles in the control group compared to the granulosa cells of ovarian cysts present in EV group. This could be explained by high level of oxidative stress markers, which might enhance apoptosis and decrease proliferation (Patel and Sharma, 2014).

Due to logistic and ethical limitations of experimentation on humans, several animal models that resemble many or all PCOS traits have been developed. Although animal models of chronic anovulation and PCO may not fully reproduce the reproductive events seen in the human syndrome, these models can improve our understanding of the pathophysiology of PCOS and have the potential to support development of innovative and curative treatments (Paixão et al., 2017).

A variety of mammalian species have been employed as animal models of PCOS, ranging from rodents to non-human primates. Rats are preferred over other animal species due to their stable genotype, ease of handling, shorter reproductive lifespan, and short estrous cycles (Aitman et al., 2008; Walters et al., 2012). Is spite of the fact that the rat is a polytocous rodent, the female has regular ovarian cyclicity of 4 or 5 days, with distinct proestrous, estrous, and diestrous phases; therefore, this species is a good model for studying the pathophysiology of PCOS (Mahajan, 1998). Indeed, utilization of several different animal models of PCOS may offer the best approach to improve our understanding and to investigate potential interventions (Danni and Donna, 2012).

EV rat model of PCOS does not share all features with human PCOS; however, the results obtained from the present study revealed that broccoli extract attenuated PCOS symptoms in this animal model. Broccoli extract modulated gonadotropin levels, hyperandrogenemia, improved the ovarian morphology, and downregulated oxidative stress. Indeed, further studies on PCOS women are needed to confirm the protective effects of broccoli extract before considering wider clinical application.

CONCLUSION

The results obtained from the present study re-

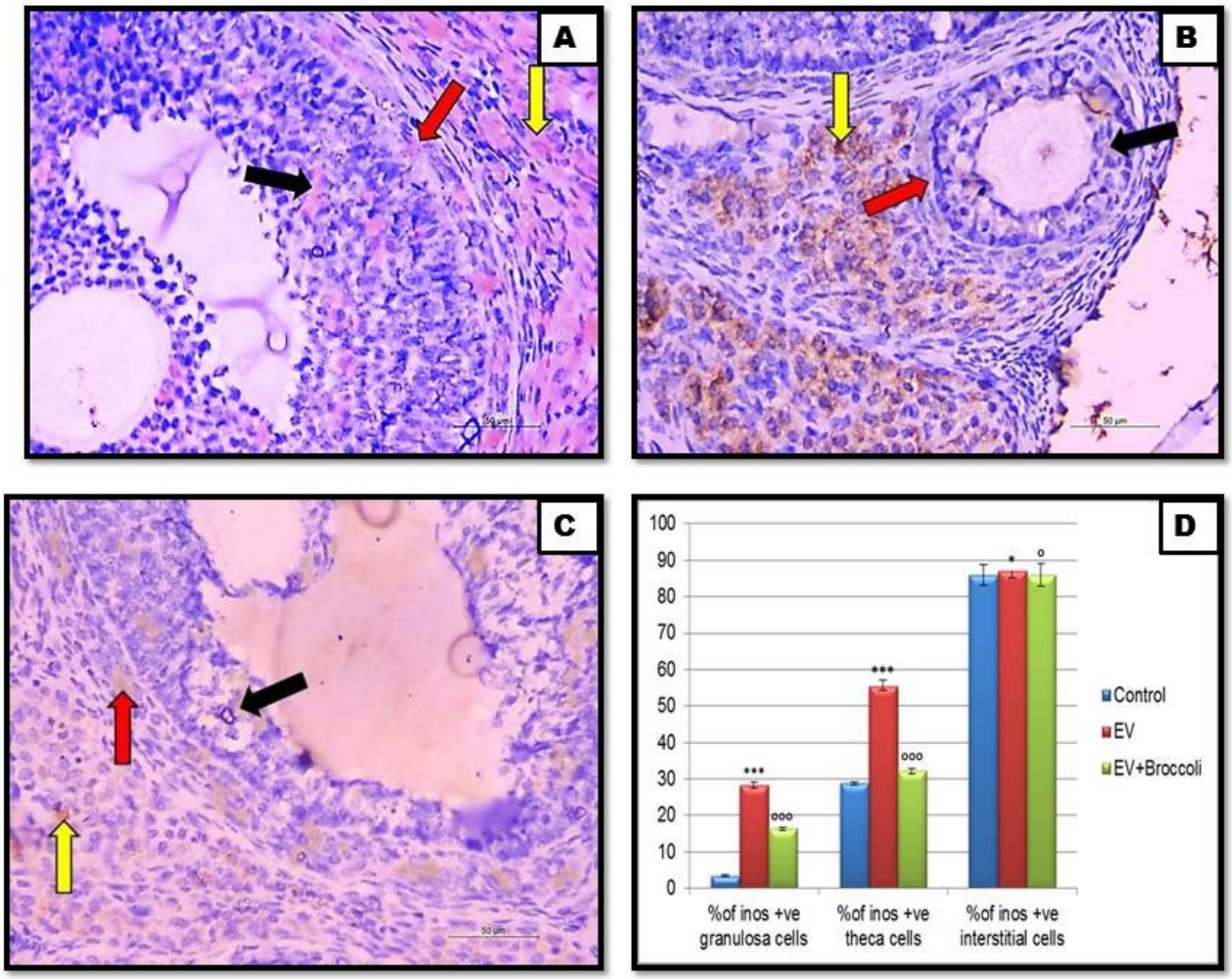

Fig 5. Expression of iNOS immunostaining in the ovaries of the control (**A**), EV-treated (**B**), and EV+broccoli (**C**) groups. Expression of iNOS in granulosa (black arrows), theca cells (red arrows) and interstitial cells (yellow arrows) is dramatically upregulated in EV-group compared with control group ($P < 0.001$). These increases are significantly downregulated in EV+broccoli group. N= 10 for each group. * $P > 0.05$ and *** $P < 0.001$ compared to the control group; and ^{0} $P > 0.05$ and ^{000} $P < 0.001$ compared to the EV-treated group. Scale bars = 50 µm.

vealed that co-treatment with broccoli extract provided a beneficial role against EV-induced PCOS rat model through its antioxidant property.

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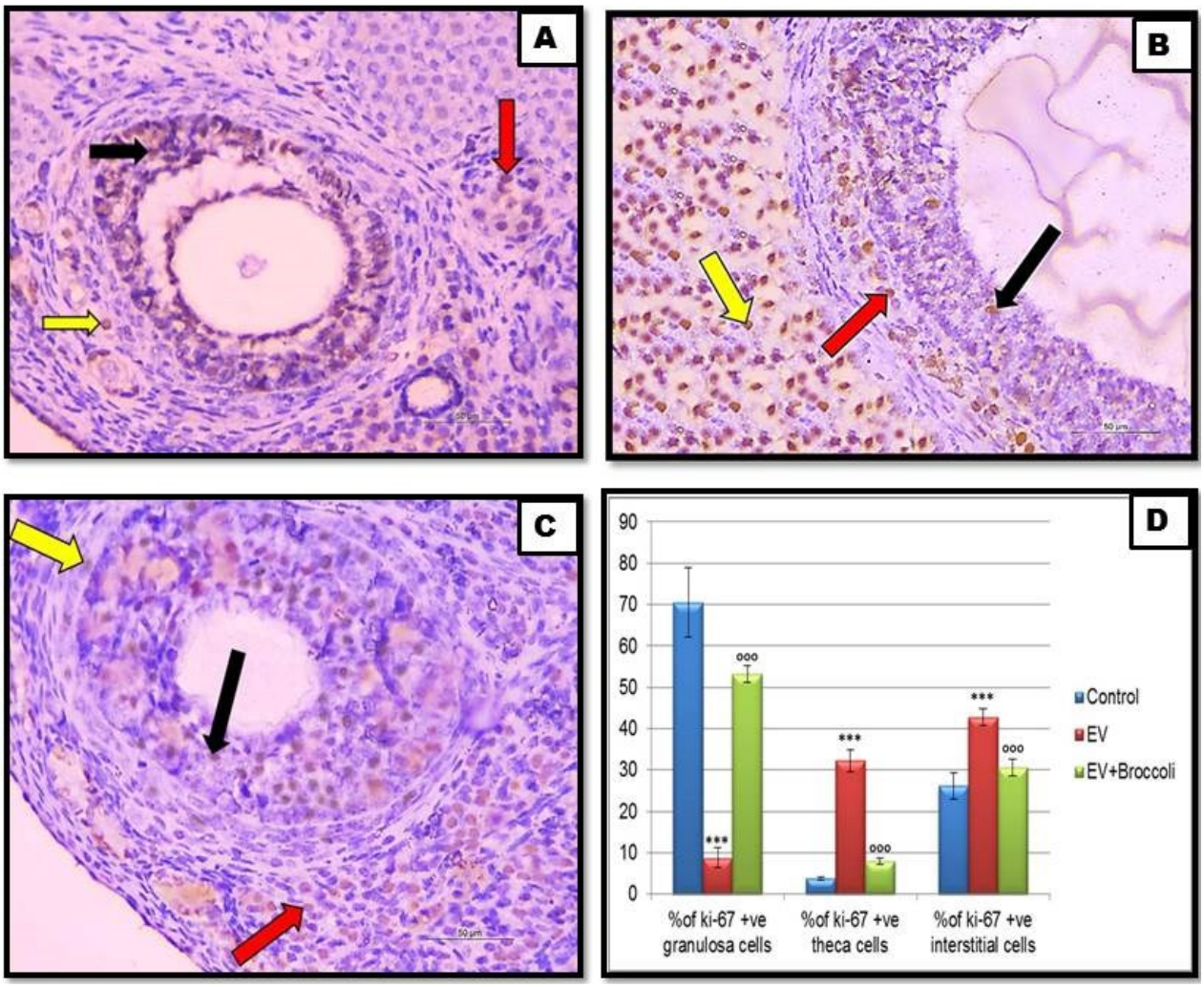

Fig 6. Expression of Ki-67 immunostaining in the ovaries of the control (**A**), EV-treated (**B**), and EV+broccoli (**C**) groups. Ki-67 expression is significantly increased in the theca cells (yellow arrow) and the interstitial cells (red arrow), while its expression is significantly decreased in the granulosa cells (black arrow) in EV-group compared with control group ($P < 0.001$). In EV+broccoli group, Ki-67 expression is decreased in the theca cells and the interstitial cells, while its expression is increased in the granulosa cells. *** $P < 0.001$, compared to the control group; and ^{000} $P < 0.001$, compared to the EV-treated group. N= 10 for each group. Scale bars = 50 μm.

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