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ORIGINAL ARTICLE

Dual targeting of TNF-α and free radical toxic stress as a promising strategy to manage experimental polycystic ovary

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Abstract

Context: It is now clear that oxidative stress (OS) and chronic low-grade inflammation are two main pathways involved in polycystic ovary syndrome (PCOS) pathogenesis. Therefore, simultaneous targeting of these pathways by means of carvedilol and Semelil (ANGIPARS™), as established medicines with dual anti-cytokine and anti-oxidant potential may be a therapeutic alternative approach to the current treatments.

Objective: The objective of this study is to study the protective effects of carvedilol and ANGIPARS™ on inflammatory and oxidative response in hyperandrogenism-induced polycystic ovary (PCO).

Materials and methods: The murine model of PCO was induced by letrozole (1 mg/kg/d; orally) and effective doses of carvedilol (10 mg/kg/d; orally) and ANGIPARS™ (2.1 mg/kg/d; orally) were administrated for 21 d in PCO and non-PCO healthy rats. Ovarian folliculogenesis, sex hormones concentrations, OS, inflammatory, and metabolic biomarkers were assessed in serum and ovaries.

Results: PCO rats exhibited ovarian cystogenesis which was preserved by the application of carvedilol and ANGIPARS™. In comparison with controls, decreased level of the total antioxidant power (TAP) and higher levels of reactive oxygen species (ROS) and lipid peroxidation (LPO) in serum and ovaries (2.41 ± 0.67 versus 0.72 ± 0.11; and 0.17 ± 0.04 versus 0.05 ± 0.01; 5.48 ± 1.30 versus 10.56 ± 0.77; and 7.06 ± 1.94 versus 17.98 ± 0.98; $p < 0.05$, respectively) were detected in PCO rats. Moreover, the PCO rats exhibited hyperandrogenism due to a 3.7-fold increase in serum testosterone concentration (35.04 ± 3.17 versus 131.09 ± 13.24; $p < 0.05$) along with a 2.98-fold decrease in serum progesterone (6.19 ± 0.40 versus 18.50 ± 1.03; $p < 0.05$) and 5.2-fold decrease in serum estradiol (9.30 ± 0.61 versus 48.3 ± 2.10; $p < 0.05$) when compared with those of the control group. However, similar to the control group, normal levels of OS markers and sex hormones were detected in ANGIPARS™ and carvedilol co-treated PCO rats. Besides, when compared with controls, increased levels of TNF-α (770.75 ± 42.06 versus 477.14 ± 28.77; $p < 0.05$) and insulin (1.27 ± 0.10 versus 0.36 ± 0.05; $p < 0.05$) in PCO rats were significantly inhibited by carvedilol and ANGIPARS™ co-treatment.

Discussion and conclusion: We evidenced the beneficial effects of carvedilol and ANGIPARS™ in PCO, which underpin the new alternative approach in using these kinds of medicines in female reproductive disorders.

Keywords

Beta-blockers, herbal medicine, hyperandrogenism, oxidative stress

History

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Introduction

One-third of couples attending infertility clinics suffer from anovulation. Polycystic ovary syndrome (PCOS) is the cause in 90% of cases (Balen & Rutherford, 2007). The name of the syndrome comes from the wide spectrum lifetime complex multi-generic collection of signs and symptoms with a mild presentation in some to severe reproductive, endocrine, and metabolic disturbances (Mukherjee & Maitra, 2010). The endocrine abnormalities in women with PCOS include raised androgen secretion, menstrual irregularity, oligo-ovulation or anovulation, polycystic ovaries (PCO), infertility, and pregnancy complications (Goodarzi et al., 2011; Pasquali et al., 2011; Welt & Carmina, 2013). While PCOS is the core pathology, its contributory complications such as insulin resistance (IR), abdominal obesity, hyperinsulinemia, glucose intolerance, hypertension, and metabolic syndrome are associated with increased risk of type 2 diabetes and cardiovascular disease in later life (Moran et al., 2010).

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Although the etiology of PCOS is still unknown, there is ample evidence suggesting that uncontrolled steroidogenesis might be the primary abnormality in this disorder. Furthermore, elevated oxidative stress (OS) and inflammatory cytokines in the blood and histological samples of women with PCOS directed investigators to surmise that oxidative and nitrosative stress and inflammatory responses (overproduction of cytokines) might play pivotal roles in the pathogenesis of PCOS (Escobar-Morreale et al., 2011; Murri et al., 2013; Showell et al., 2013). In this regard, ovarian cystogenesis and follicular atresia leading to ovarian dysfunction in PCO rats might correlate with higher concentration of the serum TNF-α and the interplay between OS and pro-inflammatory cytokines, particularly TNF-α (Rezvanfar et al., 2012a,b, 2014, 2015). Therefore, their specific involvement in the pathogenesis of PCOS provides invaluable opportunity to block the disease progression using pharmacological agents with dual antioxidant and anti-TNF-α activities (Rezvanfar et al., 2012a,b).

Although several pharmacotherapeutic options are currently available for this multifactor disease, a complete cure has not been found yet and there is an uncertainty about potential adverse effects of these medicines (Johansson & Stener-Victorin, 2013).

Hence, in the light of these findings, to overcome hyperandrogenism-associated pathologic effects in ovary, we have examined in a series of studies, the beneficial effects of several medicines with both anti-inflammatory and anti-OS characteristics such as pioglitazon (Rezvanfar et al., 2012a), IMOD™ (Rezvanfar et al., 2012b), and pentoxifylline (Rezvanfar et al., 2015). In this study, for the first time, we applied carvedilol and ANGIPARS™ as medicines with both anti-OS and anti-inflammatory properties in letrozole-induced PCO rats and their protective properties were observed, which might be exerted by preventing TNF-α over-activity, free radical formation, and androgen ultra-concentration, resulting in betterment of antioxidant status.

Carvedilol, a third-generation, vasodilating, non-cardioselective β1, β2, and α1-adrenergic antagonist, is more effective than traditional β-blockers for treating hypertension and angina without the hemodynamic and metabolic actions of traditional β-blockers therapy (Fonarow, 2009). Further, several studies have shown that carvedilol has beneficial effects on oxidative biomarkers (it is approximately 10 times more potent than α-tocopherol) and metabolic parameters (glycemic control, insulin sensitivity, and lipid metabolism) (Dandona et al., 2007; Larijani et al., 2006; Saeidnia & Abdollahi, 2013). Such a distinctive characteristic has not been reported for other medicines of the same pharmacological class yet (Stafylas & Sarafidis, 2008). *Melilotus officinalis* (Feabaceae), yellow sweet clover, is known for its dual anti-inflammatory and antioxidant activities (Pleşca-Manea et al., 2002). This plant contains coumarin, flavonoides (kampeferol and quercetin glycosides), triterpene, and saponins. Semelil (ANGIPARS™) is a newly registered medicine derived from *M. officinalis* under the electromagnetic process. Preclinical safety studies of ANGIPARS™ in laboratory animals (Mousavi-Jazi et al., 2010), and phase II trials on diabetic foot ulcer healing were successful and indicated promising results (Larijani et al., 2008). Moreover, ANGIPARS™ has been found to be effective in some immunoinflammatory- and OS-based diseases, including periodontitis (Mousavi-Jazi et al., 2010) and aging (Ghanbari et al., 2012).

In this study, we hypothesized that application of carvedilol and ANGIPARS™ can lead to a simultaneous blockade of OS and pro-inflammatory condition in the ovaries of the hyperandrogenism-induced PCO in a rat model, restraining local, systemic inflammatory and oxidative responses, and maintaining normal folliculogenesis.

Materials and methods

Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (Munich, Germany). Krebs-ringer-bicarbonate (KRB), ethyl acetate, from Fluka (Steinheim, Germany), rat TNF-α ELISA kit from Bender Med System (Vienna, Austria); steroid hormone radioimmunoassay kits from Neogen (Shanghai, China); letrozole (>99% purity) from SOHA Pharmaceutical Co. (Tehran, Iran); carvedilol (99.96% purity) from Dr. Abidi Pharmaceutical Co. (Tehran, Iran); and ANGIPARS™ (>99% purity) from RosePharmed Co. (Tehran, Iran), were used in this study.

Animals

Sixty adult female albino Wistar rats, weighing 180–200 g with the normal estrous cycle were kept under standard conditions of temperature (23 °C ± 1 °C), relative humidity (55% ± 10%), and 1 h dark and 12 h light cycle, with standard pellet feeding diet and water *ad libitum*. The estrous cyclicity was monitored by vaginal smears, and the rats with at least three consecutive 4–5 d regular estrous cycles were used in this experiment and injections were started on the same day of the estrous cycle for all rats. A vaginal smear was taken daily to determine the phase of the estrous cycle throughout the entire treatment up to the day of autopsy. Ethical rules of the investigation on animals were considered carefully, and the experimental protocol was approved by the Institutional Review Board and ethical committee with the code number 91-04-33-20198.

Experimental design

Rats were randomly divided into six experimental groups: healthy control, letrozole-induced PCO, carvedilol-treated non-PCO, ANGIPARS™-treated non-PCO, carvedilol-treated PCO, and ANGIPARS™-treated PCO. As described previously (Rezvanfar et al., 2012a,b, 2014, 2015), induction of PCO was performed by letrozole administration once daily at the concentration of 1 mg/kg orally dissolved in 0.9% NaCl. The control group received only vehicle (0.9% NaCl solution) orally, once daily. The carvedilol non-PCO and ANGIPARS™ non-PCO groups received 10 mg/kg of carvedilol and 2.1 mg/kg of ANGIPARS™, respectively, by gavage (OP). Animals in carvedilol-treated PCO and ANGIPARS™-treated PCO groups were given letrozole orally (1 mg/kg dissolved in 0.9% NaCl), 30 min before carvedilol or ANGIPARS™ treatment. The treatment period was 21 d and the effective doses of letrozole, carvedilol, and ANGIPARS™

were selected according to pilot studies and the previous experiments (Boshra & El Wakeel, 2013; Rezvanfar et al., 2012a,b).

Sampling

On the 22th day, the animals were weighed and anesthetized, and the blood samples were directly taken from the heart and were centrifuged at $1000 \times g$ for 15 min. The collected serum was stored at $-70\,^{\circ}C$ until evaluation of sex hormones, insulin, TNF-α, and OS markers. After that, all rats were sacrificed by an overdose of ether inhalation. The abdomen was then rapidly dissected, and ovaries were removed, cleansed gently with normal saline, weighed, and divided as follows: five ovaries of each group were immediately fixed in 4% (w/v) paraformaldehyde for histopathological assessment, whereas the remaining ovaries were homogenized in 10-volume ice cold potassium phosphate buffer (50 mM, pH 7.4), sonicated, and centrifuged for 30 min at $3500 \times g$. The supernatants were then transformed into several micro-tubes for separate biochemical assays and all were kept at $-80\,^{\circ}C$ until analyses (Rezvanfar et al., 2012a,b).

Histopathological assessment of ovarian dynamics

The morphological and histopathological studies were performed as described previously (Rezvanfar et al., 2012a,b). Briefly, the ovaries from each group were removed, cleansed of adherent connective fat tissue, and fixed in 4% (w/v) paraformaldehyde for at least 24 h. Thereafter the samples were dehydrated and imbedded in paraffin and finally sectioned longitudinally at 4 μm and every tenth section was placed on the glass slide and stained with hematoxylin and eosin (H & E). The presence of healthy and atretic follicles, follicular cysts, and corpora lutea was assessed microscopically by two persons blind to the origin of the sections. Quantifying ovarian folliculogenesis, all healthy and atretic follicles containing an oocyte with a distinct nucleus were counted and characteristically divided to primordial follicles (an oocyte with a single layer of granulosa cells, and a diameter <100 μm), growing follicles (an oocyte enclosed by several layers of granulosa cells, without an antrum formation, and diameter of 100–300 μm), or mature graafian follicles (a peripherally located oocyte enclosed by cumulus cells and a large fluid filled antrum and diameter >300 μm). Follicles considered atretic when one of the following features was seen: scattered pyknotic nuclei in the granulosa cell layer, detachment of the granulosa cell layers, loss of oocyte-granulosa cell communication, fragmentation and malformation of the oocyte, disruption of the zona pellucida (ZP), and presence of cellular debris in the antrum of the follicle. Histopathological changes of the ovarian specimens were also semi-quantitatively scored from 0 (normal) to 4 (severe changes).

Evaluation of OS biomarkers

To investigate OS biomarkers, the cellular LPO index, ROS activity, and the level of TAP were analyzed in serum and ovaries.

Evaluation of cellular LPO

The level of cellular LPO within collected serum samples and ovaries was assessed through thiobarbituric acid reactive substance (TBARS) measurement as described previously (Rezvanfar et al., 2012a,b) and expressed as the extent of malondialdehyde (MDA) production during an acid-heating reaction and data were reported as μmol/ml.

Evaluation of ROS

As described previously (Rezvanfar et al., 2014), to determine ROS production, a portion of the homogenized ovaries and the blood serum were evaluated using fluorescence DCFH with some modifications. The oxidation rate from DCFH to dichlorofluorescein (DCF) indicates oxidant production and is followed by an excitation wavelength of 488 nm and an emission wavelength of 525 nm which were measured every 6 min for 60 min using an ELISA F-2000 fluorescence spectrometer (Hitachi, Tokyo, Japan). The rate was linear for at least 60 min at various concentrations of protein present and corrected for the auto-oxidation rate of DCFH.

Evaluation of TAP

Ferric ion-reducing antioxidant power (FRAP assay) was used to determine the level of TAP in the ovaries and serum through their ability to reduce the TPTZ–Fe^{3+} complex to TPTZ–Fe^{2+} complex. The interaction of tripyridyl triazine (TPTZ) with Fe^{2+} results in the formation of a blue color, with a maximum absorbance at 593 nm. Data were reported as mmol/L ferric ions reduced to ferrous per mg of protein (Rezvanfar et al., 2014, 2015).

Evaluation of inflammatory and metabolic parameters

Evaluation of TNF-α concentration

TNF-α concentration was determined in serum using rat sandwich ELISA kit as a formed color product in proportion to the amount of cytokine present in the sample and expressed as pg/mg protein. After adding stop solution to terminate the reaction, the absorbance was measured at 450 nm as the primary wavelength and the absorbance was measured at 620 nm as the reference wavelength (Hosseini-Tabatabaei et al., 2009). To determine the concentrations of TNF-α per unit of protein, the Bradford method was used to measure the protein content using concentrated Comassie blue as reagent and BSA as the standard.

Evaluation of insulin concentration

As described previously (Pakzad et al., 2013), rat ELISA Kit was used to determine the serum insulin concentrations as a metabolic biomarker. According to the manufacturer's recommendation, after 8 h fasting for all the studied rats, the serum insulin level was measured and reported as ng/ml.

Evaluation of sex steroids

The levels of steroid hormones produced by ovaries were measured in all examined groups by competitive radioimmunoassay, using commercial RIA kits (Rezvanfar et al., 2012a,b). Assay sensitivity was 0.03 ng/mL for estradiol,

0.35 ng/mL for progesterone, and 0.006 ng/mL for testosterone. For each hormone, intra- and inter-assay CVs were $\leq$10%.

Statistical analysis

Data were analyzed by StatsDirect 3.0.107 software (StataCorp, College Station, TX). Kruskal–Wallis test was used to test non-parametric data. One-way ANOVA followed by the Tukey multiple comparison *post-hoc* tests for multiple comparisons were used. Differences between groups were considered significant when p values <0.05 and results were presented as means $\pm$ SD.

Results

The body and ovary weights (g)

In comparison with the control group, the hyperandrogenized PCO rats gained significantly ($p<0.05$) more body and ovarian weights, whereas treatment of PCO rats with ANGIPARS™ and carvedilol caused similar body and ovarian weights as the control (Table 1). Furthermore, administration of the ANGIPARS™ and carvedilol in the non-PCO rats did not affect the body and ovarian weights and exhibited similar results as the control group.

Sexual cycle

With respect to the sexual cycle, all rats in control, ANGIPARS™, and carvedilol-treated non-PCO groups exhibited regular estrous cycles with an expected time of 4–5 d. However, all rats in the hyperandrogenized PCO group were completely acyclic and exhibited constant estrous state. Our data indicated that when the PCO rats were concurrently treated with ANGIPARS™ and carvedilol, they exhibited a normal sexual cycle in a large extent as seen in the control group.

Morphological and histopathological findings

The ovaries in the animals of control, ANGIPARS™, and carvedilol-treated non-PCO groups exhibited normal follculogenesis confirmed by the appearance of follicles in various stages of development and several intact corpora lutea, indicating normal ovulation (Table 1 and Figure 1A–C). Significantly higher scores were found for histological parameters in ovaries of PCO rats compared with those of controls ($p<0.05$). The ovaries in the hyperandrogenized rats displayed typical PCO-like changes including multiple fluid-filled sacs on the ovarian surface (follicular cysts) along with a clear absence of both large secondary and tertiary follicles as well as a clear CL absence (Figure 1D and E). There was the large number of atretic pre-antral and antral follicles as well as hyperplastic ovarian theca-interstitial tissues compared with ovaries in control animals (Figure 1E and F). Moreover, the majority of oocytes in the atretic follicles were found malformed and showed signs of the zona pellucida (ZP) breakdown and fragmentation (Figure 1G). In carvedilol- and ANGIPARS™-treated PCO rats, scores for histological parameters were detected similar to the control. Concurrent ANGIPARS™ and carvedilol-therapy in PCO-rats exhibited normal folliculogenesis

Table 1. Comparative assessment of histopathological findings of ovary in experimental and control groups and the protective effects of ANP and CLV.

	Control	PCO	ANP	PCO + ANP	CVL	PCO + CVL
Body weight (g)	236.13 ± 3.18	256.38 ± 3.46[a]	234.38 ± 4.50	236.50 ± 2.45[b]	235.88 ± 3.14	235.13 ± 1.25[b]
Ovary weight (g)	0.043 ± 0.004	0.059 ± 0.002[a]	0.041 ± 0.001	0.045 ± 0.001[b]	0.042 ± 0.002	0.043 ± 0.002[b]
Mean no. of atretic primordial follicles	26.82 ± 2.64	48.54 ± 2.84[a]	27.27 ± 3.85	34.18 ± 2.89[a,b]	25.18 ± 2.96	32.27 ± 3.58[a,b]
Mean no. of atretic growing follicles	43.45 ± 4.27	77.73 ± 4.38[a]	42.45 ± 6.64	48.82 ± 3.03[b]	39.82 ± 4.28	45.27 ± 3.50[b]
Mean no. of atretic graafian follicles	6.78 ± 1.19	21.54 ± 2.80[a]	5.82 ± 1.33	10.73 ± 1.79[a,b]	4.82 ± 1.17	8.73 ± 2.19[b]
Mean no. of cystic primordial follicles	0	0	0	0	0	0
Mean no. of cystic growing follicles	0	6.82 ± 1.17[a]	0.27 ± 0.47	1.54 ± 0.52[a,b]	0	1.36 ± 0.50[a,b]
Mean no. of cystic graafian follicles	0.64 ± 0.50	12.36 ± 1.43[a]	0.54 ± 0.15	2.18 ± 0.60[a,b]	0.45 ± 0.16	1.36 ± 0.50[b]
Mean no. of corpus luteum (CL)	4.64 ± 1.03	0.09 ± 0.30[a]	4.73 ± 1.00	3.18 ± 0.75[a,b]	6.18 ± 1.17[a]	4.18 ± 0.75[b]
Luteinization of follicular wall and granulosa cells median (min–max)	0 (0–0)	4 (3–4)[a]	0 (0–0)	2 (2–2)[b]	0 (0–0)	0 (0–1)[b]
Vascularization within follicular wall median (min–max)	0 (0–0)	3 (2–4)[a]	0 (0–0)	1 (1–2)[b]	0 (0–0)	1 (1–2)[b]
Oocyte degeneration median (min–max)	0 (0–0)	4 (3–4)[a]	0 (0–0)	2 (1–2)[b]	0 (0–0)	0 (0–1)[b]
Pyknosis of granulosa cells median (min–max)	0 (0–0)	4 (2–4)[a]	0 (0–0)	1 (1–2)[b]	0 (0–0)	1 (1–2)[b]
Granulosa cells Dissociation median (min–max)	0 (0–0)	4 (3–4)[a]	0 (0–0)	2 (2–2)[b]	0 (0–0)	1 (1–2)[b]
Hyperplasia of theca cells median (min–max)	0 (0–0)	4 (3–4)[a]	0 (0–0)	1 (1–1)[b]	0 (0–0)	0 (0–1)[b]

ANP, ANGIPARS™; CVL, carvedilol; PCO, polycystic ovary; min, minimum; max, maximum.
[a]Represents a significant difference between control and other treated groups at $p<0.05$ level.
[b]Represents a significant difference between PCO and PCO + ANP and PCO + CVL treated groups at $p<0.05$ level.
(0–4): Semi-quantitative microscopic scores in each treated group in terms of ovarian histopathological changes in comparison with the control group ($p<0.05$).

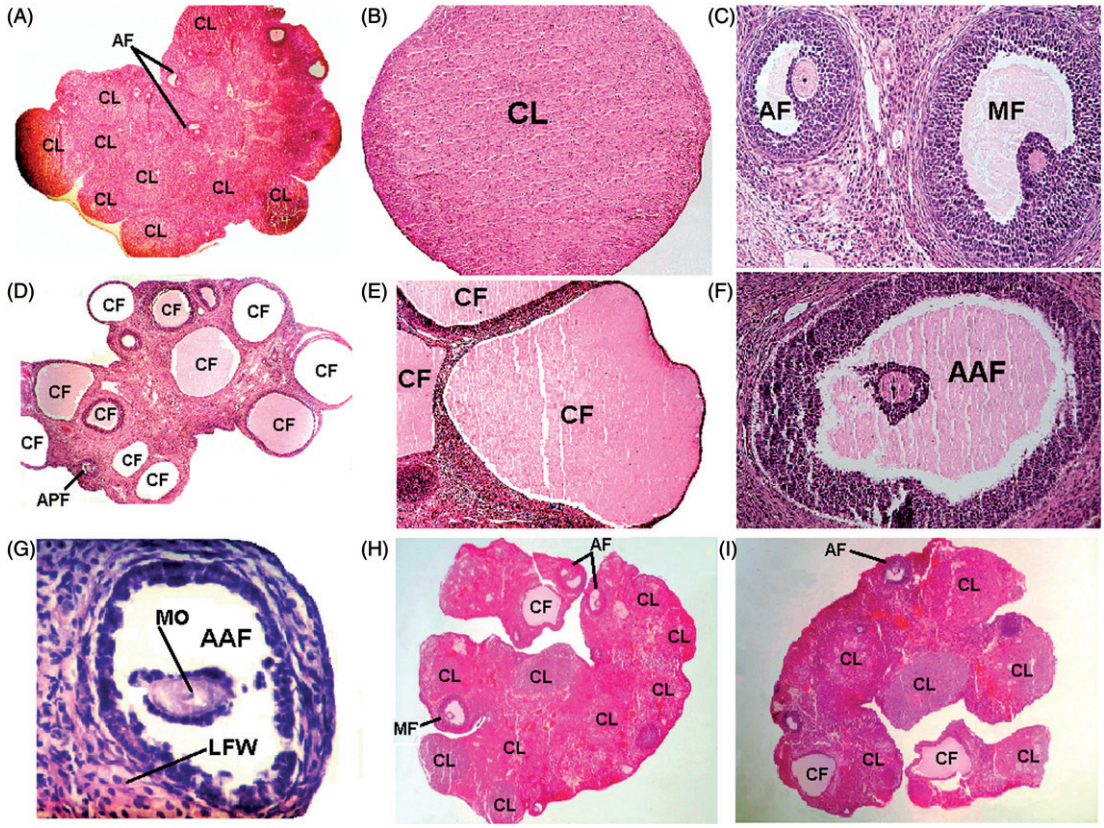

Figure 1. Ovarian sections of control and experimental groups. (A) Lower magnification (40×) of ovarian section showing normal folliculogenesis as well as several CL from the control rat. (B) Higher magnification (400×) of control ovary showing fresh CL. (C) Higher magnification (400×) of two intact antral follicles with a clear oocyte surrounded by cumulus cells and several layers of granulosa cells (×400). (D) Section of ovary from PCO rats showing multiple fluid-filled sub-capsular cysts (40×). (E) Higher magnification (400×) of a fluid-filled large cystic follicle of PCO rats with a degenerated thin layer of granulosa cells and hyperplasia of theca cells (×400). (F) Higher magnification (400×) of one antral degenerating atretic follicle showing detached floating oocyte. (G) An antral atretic follicle with a malformed oocyte and luteinized theca cells (400×). (H) Section of the ovary from the PCO + CVL group showing normal developing follicles and several CL (×40). (I) Section of the ovary from the PCO + ANP group showing normal developing follicles and several CL (×40). AF, antral follicle; AAF, atretic antral follicle; CF, cystic follicle; CL, corpora lutea; LGW, luteinized follicular wall; MF, mature follicle; MO, malformed oocyte.

confirmed by the absence of cystogenesis along with a significant decrease in total populations of ovarian atretic follicles and significant increase in the number of fresh CL (Table 1 and Figure 1H and I). Although, histopathologically, the maintenance of normal folliculogenesis in PCO-rats was in favor of carvedilol compared with the ANGIPARS™, the difference was not significant (Table 1).

The OS biomarkers

Compared with controls, higher levels of LPO in serum (5.48 ± 1.30 versus 10.56 ± 0.77; $p < 0.05$, respectively) and ovaries (7.06 ± 1.94 versus 17.98 ± 0.98; $p < 0.05$, respectively) of PCO rats (Figures 2 and 3) were found. Moreover, as shown in Figures 4 and 5, higher levels of ROS in serum (2.41 ± 0.67 versus 0.72 ± 0.11; $p < 0.05$, respectively) and ovaries (0.17 ± 0.04 versus 0.05 ± 0.01; $p < 0.05$, respectively) were detected in PCO rats in comparison with those in controls. In contrast, in carvedilol- and ANGIPARS™-treated PCO rats, the levels of LPO and ROS were detected similar to the control. In addition, the measured ovarian and serum TAP, which reflect local and systemic total antioxidants levels,

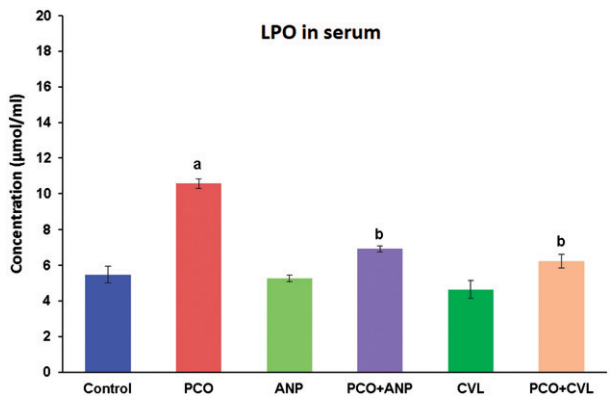

Figure 2. Comparative assessment of serum LPO in control and experimental groups of rats. [a]A significant difference between control and other treated groups at $p < 0.05$ level. [b]A significant difference among PCO–, PCO + ANP–, and PCO + CVL-treated groups at $p < 0.05$ level. CVL, carvedilol; ANP, ANGIPARS™; PCO, polycystic ovary; LPO, lipid peroxidation.

were significantly decreased in PCO animals ($p < 0.05$) compared with the controls. ANGIPARS™ and carvedilol cotreatments in PCO rats retained all OS-related parameters close to those of the controls (Figures 6 and 7).

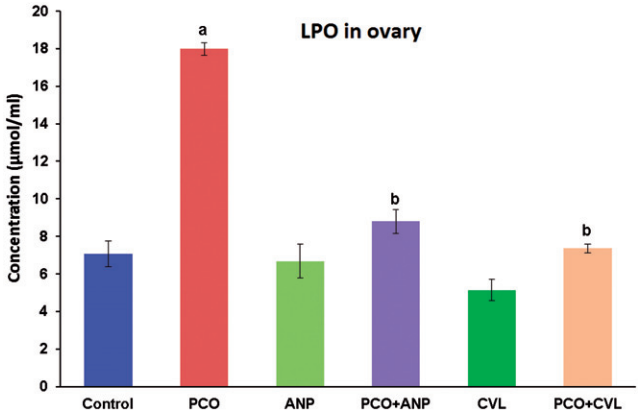

Figure 3. Comparative assessment of ovarian LPO in control and experimental groups of rats. [a]A significant difference between control and other treated groups at $p<0.05$ level. [b]A significant difference among PCO–, PCO + ANP–, and PCO + CVL-treated groups at $p<0.05$ level. CVL, carvedilol; ANP, ANGIPARS™; PCO, polycystic ovary; LPO, lipid peroxidation.

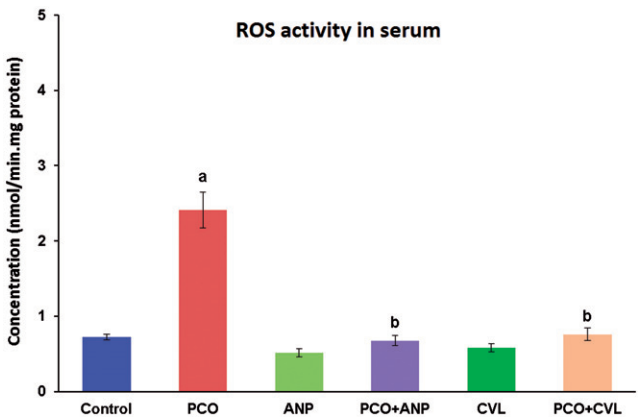

Figure 4. Comparative assessment of ROS in serum in control and experimental groups of rats. [a]A significant difference between control and other treated groups at $p<0.05$ level. [b]A significant difference among PCO–, PCO + ANP–, and PCO + CVL-treated groups at $p<0.05$ level. CVL, carvedilol; ANP, ANGIPARS™; PCO, polycystic ovary; ROS, reactive oxygen species.

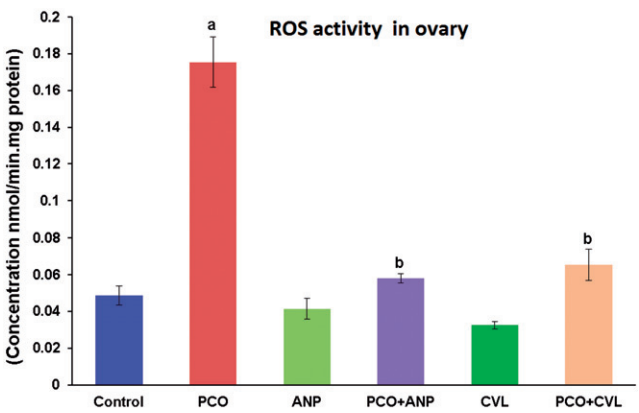

Figure 5. Comparative assessment of ROS in ovary in control and experimental groups of rats. [a]A significant difference between control and other treated groups at $p<0.05$ level. [b]A significant difference among PCO–, PCO + ANP–, and PCO + CVL-treated groups at $p<0.05$ level. CVL, carvedilol; ANP, ANGIPARS™; PCO, polycystic ovary; ROS, reactive oxygen species.

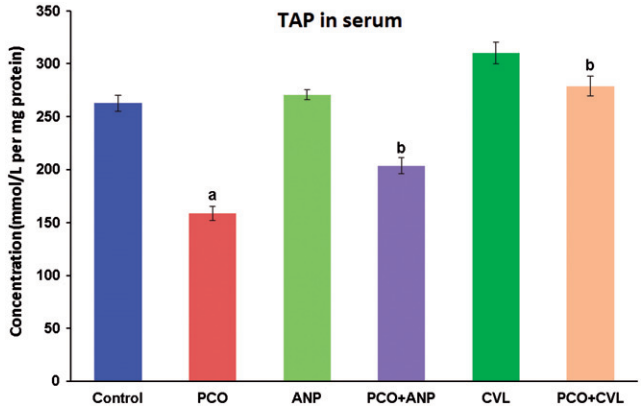

Figure 6. Comparative assessment of TAP in serum in control and experimental groups of rats. [a]A significant difference between control and other treated groups at $p<0.05$ level. [b]A significant difference among PCO–, PCO + ANP–, and PCO + CVL-treated groups at $p<0.05$ level. CVL, carvedilol; ANP, ANGIPARS™; PCO, polycystic ovary; TAP, total antioxidant power.

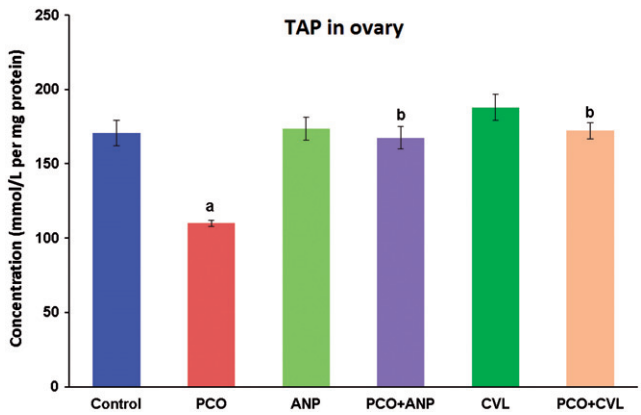

Figure 7. Comparative assessment of TAP in ovary in control and experimental groups of rats. [a]A significant difference between control and other treated groups at $p<0.05$ level. [b]A significant difference among PCO–, PCO + ANP–, and PCO + CVL-treated groups at $p<0.05$ level. CVL, carvedilol; ANP, ANGIPARS™; PCO, polycystic ovary; TAP, total antioxidant power.

Inflammatory and metabolic biomarkers

As shown in Figure 8, the ANGIPARS™- and carvedilol-treated non-PCO rats had a similar level of serum TNF-α in comparison with control rats. The serum TNF-α concentration was significantly higher in the letrozole-induced PCO rats when compared with controls (770.75 ± 42.06 versus 477.14 ± 28.77; $p<0.05$). However, cotreatment with ANGIPARS™ and carvedilol significantly prevented the elevation of TNF-α concentration in PCO rats and kept it close to that of controls. Furthermore, serum insulin concentration was detected remarkably higher in hyperandrogenized PCO rats when compared with the control (1.27 ± 0.10 versus 0.36 ± 0.05; $p<0.05$; Figure 9). In contrast, treatment of PCO rats with ANGIPARS™ and carvedilol prevented the elevation of insulin and retained it close to that of controls. No significant change was observed in serum insulin concentration in the control and non-PCO rats treated with ANGIPARS™ and carvedilol.

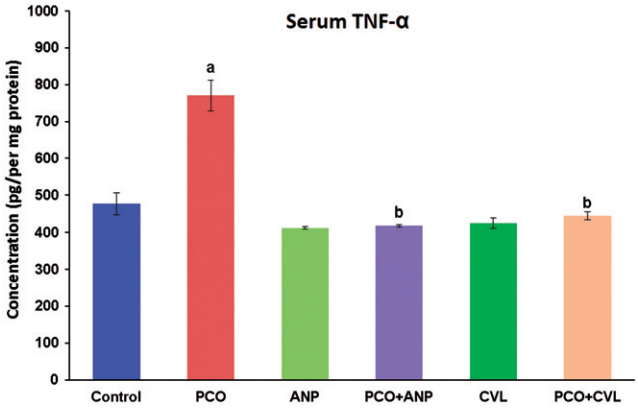

Figure 8. Comparative assessment of serum TNF-α concentration in control and experimental groups of rats. [a]A significant difference between control and other treated groups at $p<0.05$ level. [b]A significant difference among PCO−, PCO + ANP−, and PCO + CVL-treated groups at $p<0.05$ level. CVL, carvedilol; ANP, ANGIPARS™; PCO, polycystic ovary; TNF-α, tumor necrosis factor alpha.

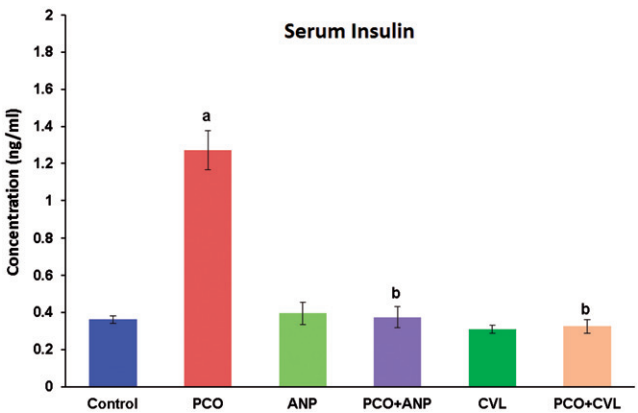

Figure 9. Comparative assessment of serum insulin concentration in control and experimental groups of rats. [a]A significant difference between control and other treated groups at $p<0.05$ level. [b]A significant difference among PCO−, PCO + ANP−, and PCO + CVL-treated groups at $p<0.05$ level. CVL, carvedilol; ANP, ANGIPARS™; PCO, polycystic ovary.

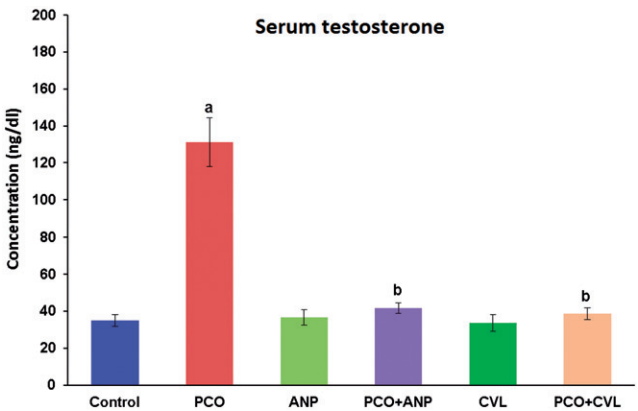

Figure 10. Comparative assessment of serum testosterone concentrations in control and experimental groups of rats. [a]A significant difference between control and other treated groups at $p<0.05$ level. [b]Significant difference among PCO−, PCO + ANP−, and PCO + CVL-treated groups at $p<0.05$ level. CVL, carvedilol; ANP, ANGIPARS™; PCO, polycystic ovary.

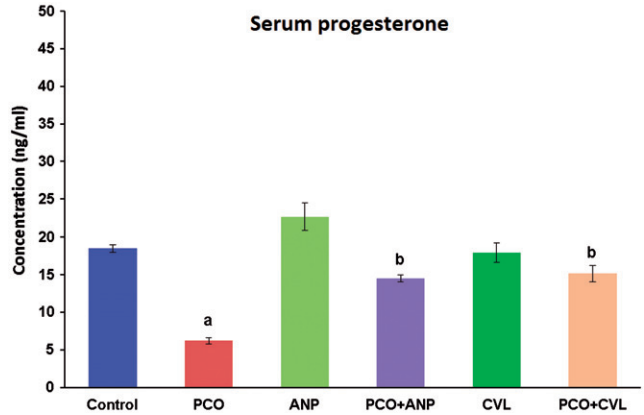

Figure 11. Comparative assessment of serum progesterone concentrations in control and experimental groups of rats. [a]A significant difference between control and other treated groups at $p<0.05$ level. [b]A significant difference among PCO−, PCO + ANP−, and PCO + CVL-treated groups at $p<0.05$ level. CVL, carvedilol; ANP, ANGIPARS™; PCO, polycystic ovary.

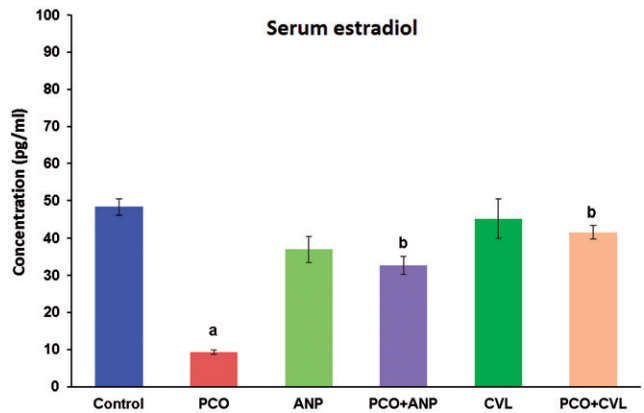

Figure 12. Comparative assessment of serum estradiol concentrations in control and experimental groups of rats. [a]S significant difference between control and other treated groups at $p<0.05$ level. [b]A significant difference among PCO−, PCO + ANP−, and PCO + CVL-treated groups at $p<0.05$ level. CVL, carvedilol; ANP, ANGIPARS™; PCO, polycystic ovary.

Sex steroid concentrations

The serum concentrations of testosterone, progesterone, and estradiol are shown in Figures 10–12. In comparison with controls, the rats in the PCO group exhibited hyperandrogenization due to a 3.7-fold increase in serum testosterone concentration (35.04 ± 3.17 versus 131.09 ± 13.24; $p<0.05$). In contrast, letrozole-induced PCO rats showed a 2.98-fold decrease in serum progesterone (6.19 ± 0.40 versus 18.50 ± 1.03; $p<0.05$, respectively) and a 5.2-fold decrease in serum estradiol (9.30 ± 0.61 versus 48.3 ± 2.10; $p<0.05$, respectively) when compared with those of the control group. However, similar to the control group, normal serum concentrations of testosterone (41.56 ± 2.88 and 38.70 ± 2.17), progesterone (14.48 ± 0.47 and 15.13 ± 1.12), and estradiol (32.67 ± 2.43 and 41.60 ± 1.86) were detected in PCO rats co-treated with ANGIPARS™ and carvedilol, respectively.

Discussion

In this study, we demonstrated protective effects of the carvedilol and the ANGIPARS™ in hyperandrogenism-induced PCO rats by presenting ovarian morphology, endocrine profiles, steroidogenesis, and alteration of selected markers involved in OS and inflammation. Our data showed that carvedilol and ANGIPARS™ significantly decrease inflammatory biomarkers, especially TNF-α and OS biomarkers, in addition to preserved antioxidant potential in letrozole-induced PCO rats. Interestingly, these protective effects were consistent with the maintenance of physiological ovarian steroidogenesis and significant improvement in histological findings evident by the appearance of intact follicles, normal sexual cycling activity, and presence of corpus lutea when compared with the PCO group.

The biochemical and histological characteristics of letrozole-induced PCO in rats have been extensively discussed in previous studies (Rezvanfar et al., 2012a,b, 2014, 2015). Similar to the present results, it was already found that cystogenesis in the letrozole-induced hyperandrogenemia was associated with several alterations in biochemical factors. Those measured alterations included: an increase in the cellular LPO and peroxynitrite production, over-activity of inflammatory cytokines like TNF-α and prostaglandin-E (PGE), and reduced activity of enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Rezvanfar et al., 2012a,b).

Letrozole, a non-steroidal aromatase inhibitor, reduces conversion of androgens to estrogens in the ovary and results in a condition termed hyperandrogenemia, in which serum estradiol concentration significantly decreases and consequently non-aromatizable androgens such as testosterone increase. Hyperandrogenism as a key regulator in the pathogenesis of a majority of PCOS cases developed cystogenesis by impairing maturation of developing follicles in the ovaries (Goodarzi et al., 2011). Emerging data from experimental models and from clinical studies suggest that hyperandrogenism is progenitor of chronic low-grade inflammation that in turn directly stimulates excess ovarian androgen production (González, 2012). It can arrest follicular development via apoptosis in granulosa cells resulting to poor oocyte quality and ultimately progressive follicular atresia (Jonard & Dewailly, 2004; Sasson et al., 2002) through higher serum TNF-α concentrations (Escobar-Morreale et al., 2011; Murri et al., 2013) and OS (Belgorosky et al., 2010).

Moreover, decreased rate of normal ovulations or anovulation and lack of developing CL have been shown to lead to decreased serum progesterone concentrations in the present model, as also seen in PCOS women (Meenakumari et al., 2004). Such a hormonal disturbance was expected, which in turn led to constant estrus manifestation in PCO rats.

Besides, the association of high concentrations of insulin (hyperinsulinemia) with hyperandrogenemia and the formation of follicular cysts have already been reported (Rezvanfar et al., 2014, 2015). Hyperinsulinemia is believed to be a stimulator of hyperandrogenism and chronic oligo- or anovulation (González, 2012). Insulin excess can cause fulliculogenesis disruption through stimulating androgen production by theca cells and elevating serum-free testosterone levels (Dumesic & Richards, 2013).

Carvedilol has been reported to be effective in various ROS-related tissue injury models (Huang et al., 2007; Rodrigues et al., 2011; Saeidnia & Abdollahi; 2013; Singh et al., 2004) such as alcoholic fatty liver disease, hepatic IR, insulin sensitivity, and sympathetic hyperactivity in rats, which can be of great interest as there is a close association between PCOS and above-mentioned disorders (Bhatt et al., 2007; Hakucho et al., 2014; Kveiborg et al., 2006). Supporting our results, administration of carvedilol also had a beneficial effect on the bone quality in ovariectomized postmenopausal rat model via estradiol enhancement, antioxidants promotion, and diminishing in resorbing TNF-α and IL-6 (Boshra & El Wakeel, 2013).

Although the exact mechanism of carvedilol beneficial effects has not been fully understood, it seems that carvedilol exerts its effect through OS markers normalization (lipid peroxides, nitric oxide, and protein thiols) and lowers the release of inflammatory cytokines such as TNF-α, interleukin-6 (IL-6), and eicosanoids (Arab & El-Sawalhi, 2013), suggesting its beneficial effects in hyper-inflammation (Mizuochi et al., 2007) and reducing OS (Oettl et al., 2001; Ouyang et al., 2013; Zepeda et al., 2012). The hydroxylated compounds of SB 211475, BM 910228, and SB 209995 as carvedilol's main metabolites exert the greatest antioxidant activity, which may be responsible for carvedilol protective effect (Lysko et al., 2000).

As another possible mechanism, we also inferred that the beneficial effects of carvedilol on ovarian function are at least partly mediated through blockade of β-adrenergic activity in the ovary leading to the preservation of ovarian steroidogenesis, and therefore saving ovaries from follicular atresia and cystogenesis (Luna et al., 2012). An increased sympathetic input to the ovary seems to involve norepinephrine and β2-adrenergic receptors and causes development of the polycystic condition in animal models and probably in women. Evidently, our results are supported with those found by Luna et al. (2012), which reported that β-adrenergic receptor activation induces PCO condition in the rat which could be prevented by simultaneous administration of the β-antagonist propranolol. They concluded a causal relationship between adrenergic activation and ovarian cyst development *in vivo*. In another recently published report, blockade of the β-adrenergic receptors using propranolol has been found to partially improve ovarian function including increased number of healthy antral follicles and corpora lutea, and improved ovarian cycling activity leading to an increased rate of ovulation and a decrease in the number of cystic and precystic follicles (Fernandois et al., 2012). It is also well-documented that the activation of β2-adrenergic receptors in theca cells is involved in elevated androgen secretion from the ovary (Barria et al., 1993), thus, the normal testosterone concentration observed in our study in carvedilol-treated PCO rats represents a possible direct or indirect modulatory effects of carvedilol on ovarian cells.

Another hypothesis for the beneficial effect of carvedilol to protect follicules to be cystit is: carvedilol might have decreased the levels of ovarian matrix methaloproteinase-2 (MMP-2) and MMP-9, which are enzymes involved in the

pathogenesis of PCO (Lewandowski et al., 2006; Rezvanfar et al., 2014). In that regard, the blocking effect of carvedilol on β-adrenergic receptors has been indicated to decrease the concentrations of MMP-2 and MMP-9 (Ohtsuka et al., 2003; Romana-Souza et al., 2009). Altogether, considering the prominent role of β-adrenergic stimulation as an activator of the sympathetic nerves in women with PCO, carvedilol therapy could be put into practice to manage PCO(S) complications.

Although several conventional therapies have been effectively applied to induce ovulation in PCOS patients, there is no consensus yet in favor of their long-term application which can lead to negative side effects such as cardiovascular complications and multiple pregnancies (Johansson & Stener-Victorin, 2013). Accordingly, finding alternative safe and effective medicinal herbs is highly recommended for the management of such diseases (Hasani-Ranjbar et al., 2009). To explain the safety and cardiovascular effects, ANGIPARS™ has been indicated to maintain the heart contractility and hence blood pressure stability in myocardial injury conditions (Joukar et al., 2013). Moreover, it has been well-documented that selenium-based agents with antioxidant potential have protective or therapeutic effects on disorders related to the OS. More specifically selenium is an essential trace element, which can protect cells from OS (Abdollahi et al., 2001; Brozmanová et al., 2010; Ogunro et al., 2006; Steinbrenner & Sies, 2009) by modulating the expression of selenoprotein genes and anti-inflammatory mechanisms (Duntas, 2009). On the contrary, several studies have reported about the association of selenium deficiency and increased prevalence of ovarian cysts (Harrison et al., 1984), infertility (Paszkowski et al., 1995), and idiopathic miscarriage (Barrington et al., 1996). Supporting that, the beneficial effect of a selenium-based multi-herbal medicine in the letrozole-induced experimental PCO rats has been found to be mediated through the inhibition of synthesis or release of inflammatory mediators, reduction of OS, and maintaining the steroid status (Rezvanfar et al., 2012b).

In addition, further *in vitro* studies have demonstrated that selenium can improve the development of ovarian follicles through enhancement of proliferation in granulosa cells, decreasing ROS, and increasing follicular TAP and GPx (Abedelahi et al., 2010). Furthermore, FSH receptor expression and biosynthetic pathway of estradiol were predominantly influenced by selenium (Basini & Tamanini, 2000). Selenium can also stimulate luteal cell proliferation and decrease toxic lipid peroxides, and consequently elevate progesterone level affecting luteal cells (Kamada & Hodate, 1998; Kamada & Ikumo, 1997). Therefore, it can be concluded that the observed maintenance of estrous cyclicity in PCO rats, after treatment by ANGIPARS™, was mainly due to restoring steroid status and enabling ovaries to function normally.

In contrast, selenium can exert antioxidant effects by degrading the hydroperoxides and hydrogen peroxide as a component of GPx (Wichtel, 1998). In this study, our results indicated that ANGIPARS™ approximately maintains TNF-α level close to the control level. Therefore, it was inferred that induction of antioxidants (either direct or indirect) reduces free radicals, especially via inhibition of the secretion of cytokines, e.g., TNF-α is one of the main possible protective mechanisms of actions of ANGIPARS™. Several studies have demonstrated that ANGIPARS™ was effective in various OS-related disorders through reducing TNF-α, IL-β, IL-6, and nuclear factor-kappaB (NF-κb) as well as increasing TAP and LPO (Ghanbari et al., 2012; Mousavi-Jazi et al., 2010).

Conclusion

Taken collectively, the treatment with carvedilol appeared to be superior to the treatment with ANGIPARS™ in PCO rats, especially in the case of histopathological findings, which further reinforce occurrence of a dysfunction in adrenergic innervation in ovarian cystogenesis. This was an expected result because carvedilol inhibits the negative effects of an excessive β-adrenergic receptor sympathetic activity in PCO condition (Rizzo et al., 2011). We infer that carvedilol and ANGIPARS™ could protect ovarian function through the possible following mechanisms: (i) suppression of TNF-α over-activity leading to normalized inflammatory status, (ii) suppression of systemic and intra-ovarian OS inducers concomitant with TAP enhancement, and (iii) preserved ovarian steroidogenesis leading to decrease in follicular atresia and cystogenesis. It is now evident that there is a strong association between OS, inflammation, and hyperandrogenism in PCOS that has been the focus of ongoing research in female fertility. Therefore, from a clinical point of view and based on the promising current results as well as those from previous studies, it sounds that application of medicines with dual anti-OS and anti-inflammatory effects can be effective in the management of PCOS.

Declaration of interest

The authors report that they have no conflicts of interest. The authors acknowledge the partial support of INSF and the TUMS with the grant number 91-04-33-20198.

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