

Effect of *Allium Cepa* seeds Ethanolic Extract on Experimental Polycystic Ovary Syndrome (PCOS) Apoptosis induced by Estradiol-Valerate

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Abstract: Polycystic ovary syndrome (PCOS) is the most frequent cause of female infertility, affecting about 5–10% of women in age of procreation. Apoptotic effects of *Allium cepa* seeds ethanol extract on experimental PCO induced by estradiol-valerate (PPA) in rats. Wistar female rat (n=60) were allocated into three groups, control (n=30); C1, an equal volume of (0.9% NaCl); C2, extract (0.3cc/rat/orally/daily); C3, Sesame oil(0.3cc/rat/orally/daily) and test groups (n=30), that subdivided into groups of 3, one group received extract supplement (0.3cc Sesame oil+ 0.3cc *Allium Cepa* /rat/orally/daily), second and third groups were induced PCO by single injection of estradiol-valerate (4mg/rat/IM), third group received extract supplement, for 60 consequence days. Animals were kept in standard conditions. In last day of study the blood samples and ovarian tissue of rats in whole groups were removed and prepared to biochemical and pathology analysis. Means of hyperaemia, number of cyst and granulosa apoptotic cells were significantly increased in PCO groups (p<0.05), and these were significantly decreased in group receiving extract in comparison to experimental PCO and control groups. Level of TAC in all extract groups were significantly increased as compared to control and PCO groups (p<0.05). Means of Large antral follicle was significantly decreased in PCO groups (p<0.05). Results revealed that administration of *Allium Cepa* ethanol extract significantly compensation blood antioxidants level in PCO- induced rats that led to modulating the apoptosis.

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Key words: *Allium cepa*, Apoptosis, Estradiol-Valerate, sesame oil, TAC.

1. Introduction

Polycystic ovary syndrome (PCOS) is the most frequent female endocrinopathy during reproductive age with a prevalence of 7-10% worldwide. Clinical manifestations of this disease include health disorders such as diabetes, coronary heart diseases, cancer, subfertility etc. PCOS is a hormonal disorder characterized by excess amount of androgens, so androgenic disorders including acne, hirsutism, menstrual disorders, and metabolic abnormalities are common in PCOS. All of disorders are the results of multiple genetic and environmental factors. The PCOS characterizations include small antral follicles arrested in their development. Disorders in growth factors (e.g. EGF/TGF alpha) expression are responsible for blocking of apoptosis and atresia leading to multiple small antral follicles. The hormonal levels in PCOS reach to abnormal levels; estrogen level is lower, but the progesterone level is more than normal level and LH/FSH is three times of the normal level (Dasgupta and Reddy, 2008; Homburg and Amsterdam, 1998; Franks, 1995; Lewandowski et al., 2011). Reactive oxygen species (ROS) produced by mitochondria are considered as by-products of normal oxidative metabolism (Raha and Robinson, 2001). Free radicals are produced in physiological processes like cellular metabolism; they also control apoptosis. Two major types of free radicals species include Reactive oxygen species

(ROS) and reactive nitrogen species (RNS), in a healthy body ROS and antioxidants are in balance, but in antioxidant disorders that this balance disrupted would lead to increasing in ROS production, causing cell damage and oxidative stress. Oxidative stress involves in physiological processes including oocyte maturation, fertilization, embryo development and pregnancy. Antioxidants are involved in suppression of apoptosis by modulating of free radicals levels. Apoptosis means physiologic cell death occurs primarily through an evolutionarily conserved form of cell suicide (Wood and Youle, 1994; Agarwal and Allamaneni, 2004; Thompson, 1995). Herbs have been used for medicinal purposes since centuries ago; the herbs include hypolipidemic, antiplatelet, antitumor, or immune-stimulating properties that cause to decrease the cardiovascular and cancer risk. Many herbs include antioxidant content. *Alliums* are from herbs group with properties of anti-bacteria, anti-fungi and antioxidant power. *Allium cepa* (Linn) one of the *Alliums* with beneficial impact upon disease includes antioxidative properties too (Craig, 1999; Khaki et al., 2008a; Benkeblia, 2005). The purpose of our study is assessment effect of *Allium cepa* ethanolic extract on modulation of apoptosis in the experimental induced PCO rats.

2. Material and methods

2.1. Animals

Sixty adult 8 weeks old Wistar albino female rats of 250 ± 10 grams were obtained from Animal Facility of Pasture Institute of Iran. Rats were housed in temperature controlled rooms (25°C) with constant humidity (40-70%) and 12h/12h light/ dark cycle prior to use in experimental protocols. All animals were treated in accordance to the Principles of Laboratory Animal Care [NIH]. The experimental protocol was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals prepared by Tabriz medical University. All rats were fed a standard diet and water. The daily intake of animal water was monitored at least one week prior to start of treatments in order to determine the amount of water needed per experimental animal. Wistar female rat were allocated into three groups, control (n=30), C1: an equal volume of (0.9% NaCl); C2: extract (1cc/rat/orally/daily); C3: Sesame oil (0/3cc/rat/orally/daily) and test groups (n=30), that subdivided into groups of 3, one group received extract supplement (0.5cc Sesame oil+ 0.5cc *Allium Cepa* /rat/orally/daily), second and third groups were induced PCO by single injection of estradiol-valerate (4mg/rat/IM), third group, was received extract supplement, for 60 consequence day.in 60th day of study, 5cc blood samples of Rats in whole groups were removed and prepared to biochemical pathological analysis. Animals were kept in standard conditions.

2.2. Measurement of Serum Total Antioxidant capacity (TAC)

TAC was measured in serum by means of a commercial kit (Randox Co-England). The assay is based on the incubation of 2, 2'-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS) with a peroxidase (methmyoglobin) and hydrogen peroxide to produce the radical cation ABTS⁺, which has a relatively stable blue-green color, measured at 600 nm. The suppression of the color is compared with that of the Trolox, which is widely used as a traditional standard for TAS measurement assays, and the assay results are expressed as Trolox equivalent (mmol/L).

2.3. TUNEL analysis of Granulosa apoptotic cell

The in-situ DNA fragmentation was visualized by TUNEL method (Khaki et al., 2008b). Briefly, dewaxed tissue sections were predigested with 20 mg/ml proteinase K for 20 min and incubated in phosphate buffered saline solution (PBS) containing 3 % H₂O₂ for 10 min to block the endogenous peroxidase activity. The ovarian sections were incubated with the TUNEL reaction mixture, fluorescein-dUTP (in situ Cell Death

Detection, POD kit, Roche, Germany), for 60 min at 37°C. The slides were then rinsed three times with PBS and incubated with secondary anti-fluorescein-POD-conjugate for 30 min. After washing three times in PBS, diaminobenzidine- H₂O₂ (DAB, Roche, Germany) chromogenic reaction was added on sections and counterstained with hematoxylin. As a control for method specificity, the step using the TUNEL reaction mixture was omitted in negative control serial sections, and nucleotide mixture in reaction buffer was used instead. Apoptotic cells were quantified by counting the number of TUNEL stained nuclei per ovarian cross section. 100 cross sections of per specimen were assessed and the mean number of TUNEL positive dark brown Granulosa cell per each cross- section was calculated.

2.4. Light microscopic study of Ovary

The ovarian tissues were fixed in 10% buffer formalin and embedded in paraffin wax. Five micron thick sections were obtained and prepared than stained with haematoxylin and eosin (H&E). The 100 cross sections of per specimen were assessed under Olympus 3H light microscope for reveal hyperaemia, percentage of cysts and large antral follicle.

2.5. Statistical analysis

Statistical analysis was done by using the ANOVA and test for comparison of data in the control group with the experimental groups. The results were expressed as mean \pm S.E.M (standard Error of means). P-value less than 0.05 were considered significant and were written in the Parentheses.

3. Results:

3.1. Total Antioxidant capacity Results

Level of TAC in all extract groups were significantly increased as compared to control and PCO groups (P<0.05).

3.2. Pathology results

Light microscopic study with H&E staining methods in control group normal structure of follicle was seen and not any cyst and hyperemia seen, in group that poly cystic ovarian (PCO) was induced by estradiol-valerate (4mg/rat/IM), hemorrhagic, hyperemia and many of cyst were seen in tissue structural area, the rate of these pathological change was decreased in poly cystic ovarian group that received supplement (0.3cc Sesame oil+ 0.3cc *Allium Cepa* /rat/orally/daily),(Fig:A,B,C). Means of hyperaemia, number of cyst were significantly increased in PCO groups (P<0.05), and these were significantly decreased in group receiving extract in

comparison to experimental PCO and control groups. Means of large antral follicle was significantly decreased in PCO groups ($P < 0.05$), (Table-1).

3.3. Apoptosis results

Light microscopic study with TUNEL methods in control group showed dark brown cells

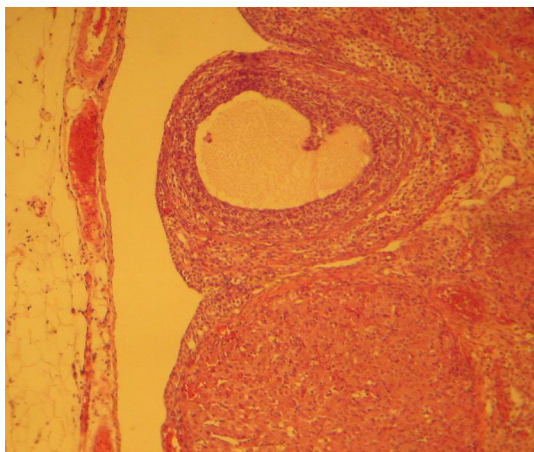
indicated Granulosa apoptotic cell this cells were symbols of apoptotic body, the number of this cells were increased in PCO group and number of this dark brown cells were significantly decreased in PCO group that received supplement (0.3cc Sesame oil+ 0.3cc Allium Ceba /rat/orally/daily),(Table-1).

Table 1: effects of Allium Ceba seeds ethanol Extract, on TAC, Granulosa apoptotic cell, large antral Follicle, Ovary weight's cyst and Artery hyperaemia in PCO rats.

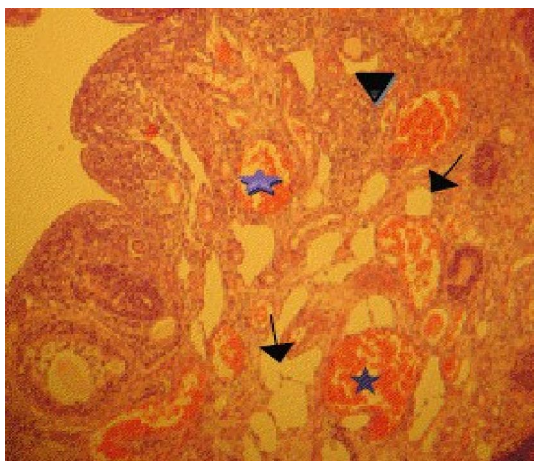
PCO+ (0.3cc Sesame oil+ 0.3cc Allium Ceba /rat/orally/daily)	PCO estradiol- valerate (4mg/rat/IM)	Sesame oil(0.3cc/rat/orall y/daily)+ Allium Ceba seeds ethanol Extract	Sesame oil(0.3cc/rat/orall y/daily)	Allium Ceba 0.3cc/rat/orally/d aily)	Control 0.9% NaCl	Groups
1.0 ±0.05*	0.6 ±0.05*	1.9 ±0.05	1.6 ±0.05	3.6 ±0.05*	1.8 ±0.05	TAC(mmol/ml)
7.05 ±0.05*	10.05 ±0.05*	3.05 ±0.05	3.05 ±0.01	3.03 ±0.05	4.01 ±0.03	Granulosa apoptotic cell (%)
6.05 ±0.05*	4.05 ±0.05*	9.05 ±0.05	11.15 ±0.05	11.01 ±0.05	10.05 ±0.05	Large antral Follicle (%)
12.01±0.0*1	15.00±0.01*	1.54±0.371	1.57±0.73	1.50±0.01	1.50±0.05	Ovary weight's cyst (%)
3.15 ±0.01*	4.35 ±0.01*	0.05 ±0.01	0.04 ±0.03	0.05±0.05	0.05 ±0.01	Artery hyperaemia (%)

Data are presented as mean ± SE.

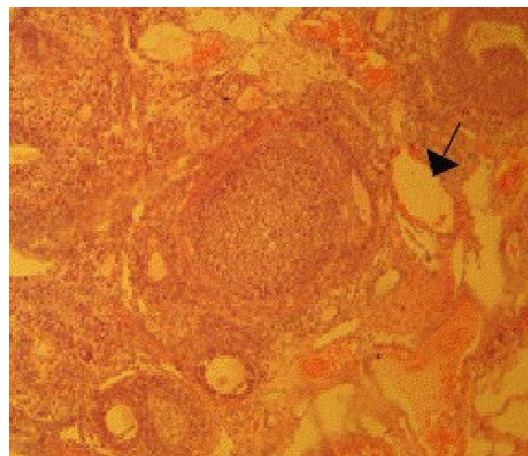
*Significant different at $P < 0.05$ level, (compared with the control group).



A



B



C

Figure A) Photomicrograph of ovary tissue in control group show normal structure of follicle, *H&E staining, X160*. **Figure B)** photomicrograph of ovary tissue in of PCO group show cyst (arrow), inflammation cells (triangle), and hemorrhage (star), *H&E staining, X160*. **Figure C)** Photomicrograph of ovary tissue in of PCO group that treated with ethanolic extract of Allium Ceba, show decreasing of cystic follicle and hemorrhage (arrow) and present of growing follicle (bold arrow) *H&E staining, X160*.

4. Discussion

Free radicals, Reactive oxygen species (ROS), and reactive nitrogen species (RNS), are products of normal cellular metabolism. ROS and RNS have deleterious and beneficial effects on the

body. Beneficial effects of ROS in low concentration include defence against infectious agents involving in cellular signalling systems and induction of a mitogenic response. The deleterious effects of free radicals are biological damage by oxidative stress and nitrosative stress (Valko et al., 2007). In the reproductive tissue, the elevated level of ROS is followed by active metabolism and steroidogenesis and may cause oocyte and DNA damage; besides, the ROS play a physiological role during ovulation that is similar in some responses to inflammation. ROS may play a role in the regulation of growth of ovarian mesenchyme for example in a pathological condition like PCOS, excessive oxidative stress may contribute to ovarian mesenchyme hyperplasia. One of protective mechanisms of the body against the ROS effects is anti-oxidative enzymes. ROS can activate scavenging system, a Redox system that can repair oxidized and damaged molecules by using NADPH as an original electron source (Agarwal et al., 2005; Fujii et al., 2005). The ROS cause lipid peroxidation, so it can damage DNA and/or change in cell signalling and cellular function. The oxidative stress (OS) lead to damage to DNA of ovarian epithelium or cell apoptosis; however, oxidative status of the cell modulates follicular growth, corpus luteum formation endometrial differentiation and embryonic growth. Oxidative stress may also be the cause of preeclampsia, abortion, endometriosis, PCOS, infertility, mole hidatyform, radical-induced birth defects; hence, evaluating and protecting against OS is very important in reproductive science. Recent findings illustrated the positive influence of oxygen radicals and ROS in many physiological states like development of germ cells, the uterine environment, oocyte maturation, ovulation, and corpus luteum function and regression. Oxidative stress (OS) may also play a role in the pathophysiology of PCOS. OS may affect Insulin resistance (IR) that is common in young non-obese PCOS women (Agarwal et al., 2005; Riley and Behrman, 1991). ROS and free radicals can be generated by environmental factors (e.g. cigarette smoke, exhaust, chemicals in food), immune responses and some diseases. ROS cause to damage to DNA, lipids and protein; cell protection against these damages is by using specific enzymes such as Superoxide dismutase, Catalase, Glutathione peroxidase, and the peroxiredoxins. Protection is also achieved with a number of chemical antioxidants including tocopherol (vitamin E), ascorbate, GSH and. The mechanisms by which oxidants induce apoptosis are unknown, but may involve the generation of some signalling factor in addition to more direct damage. Apoptosis involves events requiring active cell participation and is the basis for normal tissue remodelling and the result of certain toxic effects;

Appearance of apoptosis is characterized by cell shrinkage and localized, non-inflammatory death. Several genes and several antioxidative enzymes are involved in modulating of apoptosis. Apoptosis plays harmful and beneficial role in cells; beneficial role involves in controlling death during embryonic cell development, during normal cell turnover, the immune system responses to different stimuli and termination of critically damaged cells that have been exposed to toxicants; However, excess apoptosis can have adverse consequences that occurs in neurodegenerative diseases and autoimmune disorders. GSH involved in the reduction of lipid hydro peroxides (which induce apoptosis). There is a significant correlation in numerous cells between the levels of GSH and resistance or induction of apoptosis. GSH can scavenge ONOO⁻ with the formation of oxidized glutathione (GS-SG), which is converted back to GSH by the NADPH-dependent Glutathione reductase. GSH effectively scavenges ROS (e.g., lipid peroxyl radical, peroxynitrite, and H) directly and indirectly through enzymatic reactions; GSH can conjugate with NO, resulting in the formation of a S-nitrosoglutathione adduct, which is cleaved by the thioredoxin system to release GSH and NO; besides, GSH interacts with glutaredoxin and thioredoxin (thiol-proteins) which play important roles in the regulation of cellular redox homeostasis. Assessments of lipid peroxidation have included the analysis of lipid peroxides, isoprostanes, and breakdown products of lipids (e.g., malonaldehyde, ethane, pentane, and 4-hydroxynonenal). Among these products, malondialdehyde (MDA) is often used as a reliable marker of lipid peroxidation. The increased SOD activity decreases superoxide content in the cells and thus reduces the ROS-mediated stimulation of cell growth and it also can regulate cell growth and decrease expression of cancer cells. Superoxide promotes cell proliferation whereas hydrogen peroxide induces apoptosis and activates protein kinase C, suggesting a role for protein kinase C in ROS-mediated vascular disease. Catalase as an enzymatic antioxidant plays role in ethanol metabolism, inflammation, apoptosis, aging and cancer, it causes to break down hydrogen peroxide to water and oxygen. Free radicals can be defined as molecules containing one or more unpaired electrons from molecular oxygen. Considerable degree of reactivity to the free radical is due to unpaired electron(s); Free radicals include superoxide, hydroxyl, peroxyl (RO₂•), alkoxyl (RO•), and hydroperoxyl (HO) radicals, nitric oxide and nitrogen dioxide (•NO). Oxygen and nitrogen free radicals can be converted to other non-radical reactive species, such as hydrogen peroxide, hypochlorous acid (HOCl), hypobromous acid (HOBr), and peroxynitrite (ONOO₂). Mechanisms of harmful effect of free

radicals impose by lipid peroxidation, mitochondrial damage and DNA damage; moreover, the pathological role of free radicals leads to various diseases like ageing, cancer, lung diseases, liver diseases, coronary artery disorders, inflammatory diseases, diabetes mellitus, neurological disorders, female and male infertility that the cause of female infertility is induction of inflammatory cytokines like TNF- α , IFN- γ and IL-1 involved in various aetiologies of infertility disorders like PCOS, endometriosis, tubal obstruction, pre-eclampsia, birth defects and recurrent abortions. (Chithra, 2010; Putnam et al., 2000). There is a balance of cell proliferation and apoptosis in a healthy body. Any imbalance of the two processes could lead to pathological changes. Massive apoptosis accounts for the demise of a majority of gonadal cells (ovarian granulosa cells and male germ cells) during reproductive life. Intraovarian survival factors in the ovary (oestrogens, insulin-like growth factor I, epidermal growth factor, basic fibroblast growth factor, interleukin-1 β , nitric oxide, etc.) and testis (androgens) have been shown to act along with the gonadotrophins as survival factors. In contrast, several apoptotic factors (androgens, gonadotrophin-releasing hormone-like peptide and interleukin-6) may be important in inducing the demise of ovarian follicles (Billig et al., 1996). The altered developmental capacity of follicles from PCO is due to effects of intrafollicular inhibitors and stimulators. It has previously been shown that EGF and TGF α have inhibitory actions on follicular development, aromatization and LH receptor formation. EGF/TGF α may have a causal relationship in the mechanisms of anovulatory infertility in women with PCOS. Ovarian folliculogenesis is regulated by balance between endocrine and intraovarian factors. Main systems implicated in polycystic ovary folliculogenesis are the growth hormone and insulin-like growth factor system, vascular endothelial growth factor, and the transforming growth factor- β family. The transforming growth factor- β family is composed of various molecules, which have different roles in cellular proliferation. Thus, a series of different factors seems to be involved in altered polycystic ovary follicular growth (Almahbobi and Trounson, 1996). The majority of ovarian follicles undergo atresia, a hormonally controlled apoptotic process. During follicle development, gonadotropins, together with local ovarian growth factors (IGF-I, EGF/TGF- α , basic FGF) and cytokine (interleukin-1 β), as well as estrogens, activate different intracellular pathways to rescue follicles from apoptotic demise (Kaipia and Hsueh, 1997). The various species of *Alliums* have wide antioxidative activity due to inclusion of antioxidative enzyme and non-enzymic antioxidants (Štajner et al., 2006). According to USDA nutrient

database the nutritional value of *Allium Cepa* seed includes antioxidants such as polyunsaturated fatty acids, monounsaturated fatty acid, vitamin C, vitamin E, vitamin A, zinc, folate, niacin. Since erythrocyte lipid peroxidation products (MDA), Glutathione (GSH), ascorbic acid, plasma vitamin E and activities of antioxidant enzymes Super oxide dismutase (SOD), Glutathione peroxidase (GPX), Catalase in erythrocyte, plasma Glutathione-S-transferase (GST) and serum homocysteine levels exchange to abnormal levels in polycystic ovary syndrome, we designed this study so as to assess the impact of *Allium Cepa* antioxidants on TAC related to apoptosis in rats with PCOS. We subdivided the rats into 3 experimental groups, and control groups. One experimental just group received *Allium Cepa* extract, second and third groups had been induced PCOS by estradiol valerate, third group received *Allium Cepa* extract in addition; after 60 days results indicated the higher level of antioxidants such as CAT, SOD, GPx in first experimental group than control groups and the improvement of antioxidant levels in The PCOS group that received *Allium Cepa* extract while these antioxidant levels in the PCO group that didn't receive *Allium Cepa* was lower significantly (Mohan and Priya, 2009). According to this study the polyunsaturated fatty acids may cause to increase the blood antioxidant levels that account for prevention of apoptosis responsible for pathophysiology of PCOS (Ouladsahebmadarek et al., 2013). This study is a confirmation of the other our research on the effect of polyunsaturated fatty acids (PUFAS) as antioxidant that had indicated reduction of lipid peroxidation products in rats with PCOS (Richard et al., 2008). Since PUFAS (polyunsaturated fatty acids) reduce lipid peroxidation products, the risk of atherosclerosis and cardiovascular would decrease after supplementation with PUFA. PUFA is necessary in many reproductive processes including prostaglandin production and steroid metabolism; they also play a significant role in fertilization of spermatozoa. Recently, the positive effect of these antioxidants in reproductive tract has demonstrated that PCOS also may be modulated (Wathes et al., 2007).

5. Conclusion

Results of group receiving *Allium Cepa* extract showed increased total antioxidant levels as well as lowered apoptosis in this group. According to this study and the other studies, it seems PUFAS is significant as an antioxidant to modify apoptosis in rats with PCOS.

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