



Extracellular Vesicle-Derived Cord Blood Plasma and Photobiomodulation Therapy Down-Regulated Caspase 3, LC3 and Beclin 1 Markers in the PCOS Oocyte: An *In Vitro* Study

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Abstract

Introduction: Polycystic ovary syndrome (PCOS) is the communal endocrine illness in women and the most common cause of infertility due to lack of ovulation. The exact cause of PCOS is still unknown. Affected women may have difficulty getting pregnant due to ovulation problems. Various methods have not been effective in the treatment of PCOS due to the positive role of photobiomodulation therapy (PBMT) and extracellular vesicles (ECV) obtained from cord blood plasma in the treatment of various diseases. The aim of this study was to study the role of ECV and PBMT in maturation and improvement of infertility in women with PCOS.

Methods: In this research, a number of oocytes were obtained after ovarian stimulation from women who had been referred to the hospital for infertility treatment after obtaining personal consent, and they were divided into three groups: control, ECV and PBMT. Subsequently, *in vitro* maturation (IVM) was assessed, then some oocytes were cultured with a routine medium and others were treated with ECV and PBMT. Real-time PCR was used to evaluate BCL-2, BAX, caspase-3, and autophagy gene (ATG5, LC3, Beclin 1). Oocyte glutathione (GSH), oxidised glutathione (GSSG), and reactive oxygen species (ROS) were measured.

Results: The metaphase II (MII) oocyte ratio formation significantly increased in the ECV and PBMT groups ($P < 0.05$). The expression of the BCL-2 gene was significantly up-regulated in the ECV and PBMT groups, but the expression of BAX and caspase-3 significantly decreased ($P < 0.05$). The expression of the ATG5, LC3, BECLIN-2 genes significantly decreased in the ECV and PBMT groups ($P < 0.05$). ROS, GSSG decreased in ECV and PBMT groups but GSH increased ($P < 0.05$).

Conclusion: The use of ECV and PBMT can increase the rate of fertilization and maturation of an oocyte and cause a decrease in apoptosis, autophagy, and ROS in a PCOS oocyte.

Keywords: Caspase 3; Polycystic ovary syndrome; Extracellular vesicle; Cord blood plasma.



Introduction

Polycystic ovary syndrome (PCOS) is communal endocrine complaint in women of the reproductive period.^{1,2} The prevalence of PCOS is between 2.2% and 26%.³ More than 75% of infertility caused by lack of ovulation in women is due to PCOS, so the possibility of pregnancy decreases with the disease.⁴ In PCOS, the ovaries are stimulated to overproduce androgens, especially testosterone, so about three quarters of PCOS cases are hyper-androgenetic. Finally, the result of PCOS increases the LH/FSH ratio.^{5,6} PCOS patients experience irregular menstrual (oligomenorrhea) or

heavy (hypermenorrhea) or absence (amenorrhea) periods. Among the long-term complications of PCOS are an increased risk of endometrial and breast cancer, hypertension, cardiovascular diseases, and diabetes.⁷ PCOS is a heterogeneous disorder of an uncertain cause, but environmental and genetic factors such as overweight, insufficient exercise and family history are involved in the development of this syndrome. PCOS morphology is explained based on the number of follicles in each ovary or the volume of each ovary so that the number of follicles greater than 12 or the volume of more than 10 cc is called PCOS.^{8,9} In PCOS, there is an imbalance of hormones that

can lead to the development of multiple small cysts on the ovaries. Studies have shown that women with PCOS have decreased the expression of apoptotic genes (caspase, Bax, Bcl2) in their ovarian tissue, which may contribute to the formation and persistence of ovarian cysts. Additionally, some studies suggest that alterations in apoptotic gene expression may also be involved in insulin resistance and metabolic dysfunction commonly seen in PCOS. Nevertheless, more investigation is required to know the role of apoptotic genes in PCOS and how they may contribute to its pathogenesis.¹⁰ The treatment for PCOS depends on the symptoms and severity of the condition. Some new methods include the use of stem cells and their secretions. ECV are membranous vesicles derived from endosomes, which usually have a diameter of 30 to 150 nm. Also, ECV can be used in diagnostic work; for example, it is a marker for cancer diagnosis.¹¹ Basically, ECV are secreted from all cells in physiological and pathological conditions and are found in various body fluids such as breast milk, plasma, amniotic fluid, semen, cervical and vaginal mucus, and show an essential role in cellular communication and epigenetic regulation.¹²⁻¹⁴ The advantage of using ECV therapy is that it is non-toxic and non-immunogenic, so it is safe and has few complications.¹⁵

It has been proven in various studies that ECV have a high ability to treat various diseases due to their characteristics such as capacity to irritate the blood-brain barrier (BBB).^{16,17} Thus, ECV have shown the dual effects of suppressing and spreading cancer.¹⁸ It has been proven that the ECV taken from HUC-MSCs play a role in reducing and controlling inflammation through the mechanisms of reducing the apoptosis of ovarian pGCs, increasing anti-inflammatory cytokines (IL-10), and also inhibiting the making of inflammatory cytokines TNF- α , TNF- γ .¹⁹ This increases apoptosis and controls the proliferation of granulosa cells.²⁰ The biological functions of ECV are related to the activation of follicular growth of ovarian follicles of PCOS patients using mRNA and lncRNA profiles.²¹ The findings show that miR-19 and miR-199, which regulate processes in a positive direction, may be the cause of PCOS. This idea originated from the fact that the expression of ECV miR-19 and miR-199 is amplified in follicular fluid (FF) samples of PCOS patients.²² According to the studies, miRNA-ECV markers taken from PCOS FF affect the guideline of the aging process of granulosa cells and lead to a decrease in cell proliferation, so they can be a solution for early detection of PCOS.^{23,24} Despite many studies on the therapeutic effect of ECV on PCOS, the exact mechanism of ECV action is still not clear. The fact that chronic inflammation is related to the pathogenesis of PCOS and the genes present in ECV can be effective in the process of inflammation causes the idea of possible treatment of PCOS with the help of

ECV to be strengthened.^{19,25} Another treatment method is the use of photobiomodulation therapy (PBMT) in PCOS treatment. PBMT involves the use of low-level lasers to stimulate cellular processes, and it works by targeting the cysts on the ovaries and reducing their size. This can help to restore normal hormone levels and improve fertility. The procedure involves using PBMT to treat the cysts, causing them to shrink or disappear and helping to reduce inflammation in the ovaries, which can improve such symptoms as acne, hirsutism, and alopecia. In addition to improving fertility, PBMT has been shown to reduce symptoms associated with PCOS such as irregular menstrual cycles, acne, hirsutism, and alopecia. On the other hand, PBMT is used to stimulate the growth and maturation of immature oocytes by increasing mitochondrial activity and promoting DNA repair mechanisms.²⁶⁻²⁹ This can lead to improved quality and quantity of matured oocytes.

PBMT is applied directly to the follicles containing the immature oocytes during the culture period. The laser energy penetrates into the follicle and stimulates cellular activity without causing damage or harm to the cells.

Studies have shown that PBMT can improve the maturation rate and quality of oocytes obtained through in vitro maturation (IVM). It has also been shown to increase fertilization rates and embryo development *in vitro*.^{28,29} Overall, PBMT is an effective treatment option for women with PCOS can improve the fertility or reduce the symptoms associated with this condition. In this study, we investigated the effect of ECV-derived cord blood plasma and PBMT on maturation, fertilization and apoptosis genes in the PCOS oocyte in vitro.

Materials and Methods

The current study included GV oocytes from 36 intracytoplasmic sperm injection (ICSI) procedures. Contributors were all women whose ages ranged from 24 to 40 years. We included cycles identified as having polycystic ovarian syndrome and excluded the ones with endometriosis, male factor, tubal factor, uterine, unexplained infertility, or genetic disorders.

Ovarian Stimulation Protocol

After ovarian stimulation using hormones gonadotropin GnRH agonist (Superfact, Aventis Pharma, Germany) and rFSH (Gonal-F, Merck Serono, Germany) for five days, the diameter of the follicles was determined using ultrasound. While one of the follicles reached >18 mm, 10000 IU of human chorionic gonadotropin (hCG) (Ovitrelle, Merck Serono Europe; Pregnyl, Organon) was administered intramuscularly. Next, the oocyte was collected from the patient 36-40 hours after hCG administration via transvaginal ultrasound-guided oocyte retrieval procedure.

Following oocyte retrieval, the oocytes were stripped by

hyaluronidase (LifeGlobal) and regular pipetting.

Extracellular Vesicle Extraction

To collect umbilical cord blood, after cutting and tying the umbilical cord, a special bag with a specific barcode is selected for collection. The needle connected to the bag is inserted into the umbilical vein under sterile conditions, and the bag is placed at a lower level so that the blood inside the umbilical vein can quickly and completely enter the sterile collection bag with CPDA. After drawing blood into the CPDA bag, we mix it slowly for about 3-5 minutes and centrifuge it for a maximum of 10 minutes at 3000-3500 rpm. In the next step, we use a multi-stage centrifuge to separate the ECV, and the ECV are stored in a -20 degree freezer until use.

PBMT Procedure

Afterward, the oocytes of the PBMT group were placed in the droplet of the culture medium, then they were covered with mineral oil and placed under the class 2 laminar hood. Next, the laser device was moved under the hood, and laser radiation was performed under this parameter (the continuous He-Ne laser irradiance of 0.032 W/cm², Wavelength of 640 nm, laser beam area of 0.79 cm², average power output of 9.5 mW, 1.85 J/cm²).^{29,30}

In Vitro Maturation

After oocyte retrieval, the whole GV oocytes were achieved from women with sufficient MII oocytes. Separated oocytes were moved to 40 µL drops in a culture medium (Global R, Life Global) for 60 minutes before IVM. The GV oocytes in the control group were cultured in a global medium. Oocytes from the ECV group were transferred to droplets of culture medium+ECV suspended in mineral oil (LifeGlobal) and incubated at 37 °C in a 6% CO₂. Oocytes in the PBMT group were affected by the laser. Oocyte maturation was assessed after 24 hours.

Real-Time RT-PCR Analysis

In order to examine the expression of ATG5, LC3,

BECLIN, BCL2, BAX, caspase-3, and GAPDH genes in different groups, real-time PCR was used. After obtaining the sample from the oocytes, the oocytes were washed in phosphate-buffered saline (PBS, Invitrogen Corp.) + 1% polyvinyl alcohol (PVA) and pooled into six Eppendorf tubes (7 oocytes in each microtube) with 1.5 L of lysis buffer (kept at -80 °C). Next, lyse the sample to release the cellular contents. Finally, we used a phenol-chloroform extraction to separate the oocyte RNA from other cellular components such as proteins and DNA and to purify the RNA using spin column chromatography. After Complementary DNA (cDNA) synthesis, we added Master Mix (Amplicon, Denmark), and forward and reverse primers (Table 1) to the PCR Eppendorf tubes and ran them for 5 minutes at 94 °C, 30 seconds at 60 °C, 45 seconds at 72 °C, for 40 extension cycles. Next, we calculated the relative expression levels of specific genes by the standard curve generated from serial dilutions of identified concentrations of target DNA molecules or using a software program such as qPCR Express.^{31,32}

ROS, GSSG and Glutathione Assay

The level of reduced glutathione (GSH) was measured by means of Ellman's method. Based on this method, 25 microliters of the culture medium with 140 microliters of 2 M trisethylenediaminetetraacetic acid with pH=8 and 30 microliters of 1 M dithio-bis-2-nitrobenzoic acid mixture and dithio-nitrobenzoic acid-group complex sulfhydryl yellow reduction were measured at a wavelength of 412 nm by spectrophotometry. Reactive Oxygen Species (ROS) Detection Assay Kit (ab287839) was used to measure the ROS level and GSH+GSSG / GSH Assay Kit (Colorimetric) (ab239709) for GSSG level.

Statistical Analysis

The *t* test and the chi-square test from SPSS (SPSS, Chicago, IL, USA) software (version 16.0) were used to compare the data between the groups. The records were analyzed using the mean, standard deviation (SD), and percentages.

Table 1. Primer Sequences

ID	Gene Name	Sequence (5'-3')	Temperature	RT-PCR Primer length (bp)
NM_004346	Caspase-3	TCAGAGGAGACCGATGCAAGAC AAGTCGGCCTCCACTGGAATAC	58	180
NM_000633	BCL-2	GAAGACACGGCACTCCTTGC AGGAGAAATCAAACAGAGGCC	62	178
NM_001286106	ATG5	TGCAGATGGACAGTTGCACACG GCTCAGATGTTCACTCAGCCAC	65	195
NM_001291428	BAX	TCAGGATGCGTCCACCAAGACC TGTGTCCACGGCGCAATCACTA	55	185
NM_003766.4	Beclin1	CAAGATCCTGGACCGTGTC GGCACTTCTGTGGACATCATC	59	190
NM_181509.2	LC3	TACAGCAGATACGCGACCAG TTCACCAGCAGGAAGAAGGC	65	193
NM_001256799.2	GAPDH	ACACCCACTCCTCCACTTTG TCCACCACCCTGTTGCTGTAG	60	112

Results

Effects of ECV and PBMT Treatment on Oocyte Maturation

Overall, 36 cycles contributed in this study. Out of 454 GV oocytes, 290 oocytes reached the MII phase and 79 arrested in the MI phase. There were 58 oocytes that arrested in the GV phase and 27 oocytes were degenerated. Result showed that the MII oocyte level in PBMT and ECV groups was higher compared to the control group (Table 2, Figures 1 and 2).

Effects of ECV and PBMT Treatment on Apoptosis and Autophagy-Related Gene Expression

The expression of ATG5, LC3, Beclin 1, *BCL-2*, *BAX*, and *Caspase-3* was analyzed by real-time PCR. The result showed that the expression of *BCL-2* was significantly up-regulated by ECV and PBMT, but the expression of *BAX* and *Caspase-3* significantly decreased ($P<0.05$) (Figure 3). Moreover, the expression of *ATG5* and *LC3*, *Beclin 1* genes significantly decreased in PCOS oocytes in the PBMT and ECV groups compared to the control group ($P<0.05$) (Figure 4).

Effects of ECV and PBMT Treatment on Glutathione and Oxidative Stress

Results showed that PBMT and ECV treatment caused a significant increase in GSH ($P<0.05$), but ROS and GSSG content of PCOS oocytes significantly decreased in

PBMT and ECV groups ($P<0.05$) (Figure 5).

Discussion

The results of this in vitro clinical study displayed that the use of PBMT and ECV can cause a decrease in apoptosis and autophagy in PCOS oocytes, and it can also reduce the amount of ROS and GSSG in oocytes. Patients with PCOS using clomiphene reported improved ovarian and menstrual cycle function and glucose metabolism.^{10,11} The treatment methods used in clinics are the injection of human chorionic gonadotropin along with clomiphene citrate, one of the most popular treatments.⁹ It is possible that clomiphene has molecular similarities to estrogenic compounds and produces negative effects on the thickness of the endometrium.^{12,13} The effects of the long-term use of chemical drugs, such as clomiphene citrate, metformin, and other contraceptives, are destructive.^{14,15} Unfortunately, various treatments have not been able to cure this disease so far, and it is necessary to use new treatments. ECV and PBMT are two emerging treatments for PCOS. PBMT is a non-invasive procedure that uses

Table 2. Baseline Characteristics of the Study Population

	Control	PBMT	ECV
Number of cycles	12	12	12
Number of total oocytes	159	155	140
GV oocytes	32	11	15
MI oocytes	34	25	20
MI oocytes	78	111	101
Degenerated oocytes	15	8	4

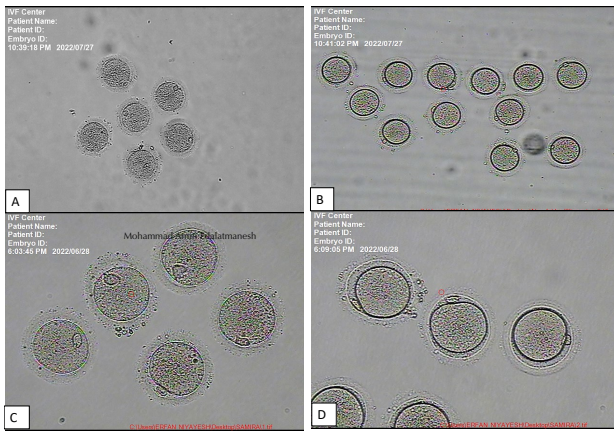


Figure 1. The Results of the Treatment of PCOS Oocyte With PBMT. The results showed that PBMT can improve the maturation rate in the PCOS oocyte in the in vitro model

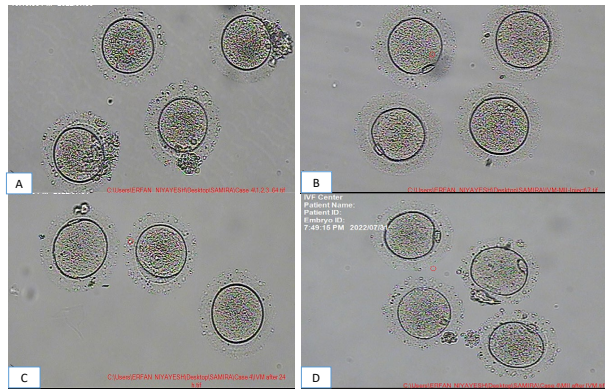


Figure 2. The Results of the Treatment of PCOS Oocyte With ECV. The results showed that ECV can improve the maturation rate in the PCOS oocyte in the in vitro model

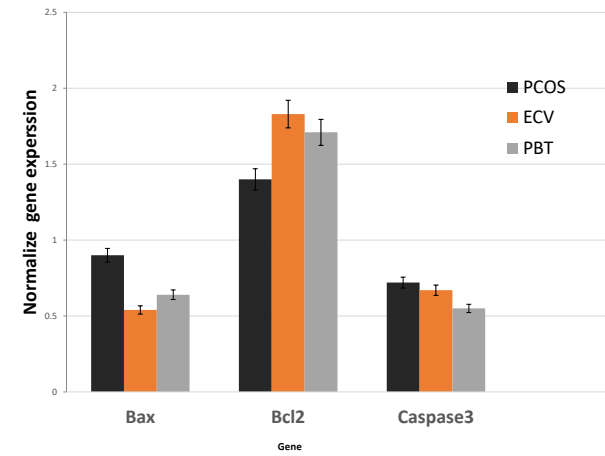


Figure 3. The Results of the Effect of ECV and PBMT on the Expression of Apoptotic Genes. The results showed that PBMT and ECV can have a positive effect on apoptotic gene expression, and these changes are significant ($*P<0.05$).

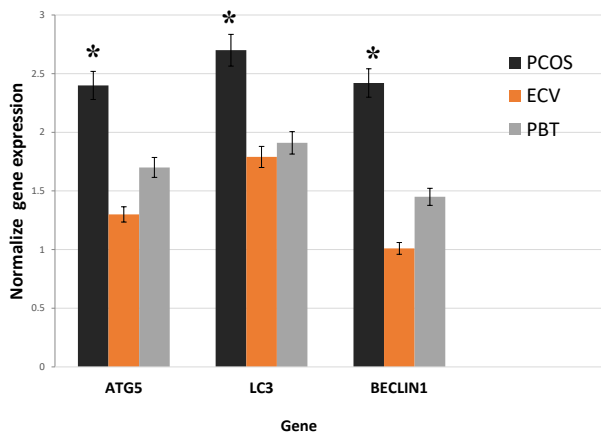


Figure 4. The Results of the Effect of ECV and PBMT on the Expression of Autophagy Genes. The results showed that PBMT and ECV can have a decreased the autophagy gene, and these changes are significant (* $P < 0.05$)

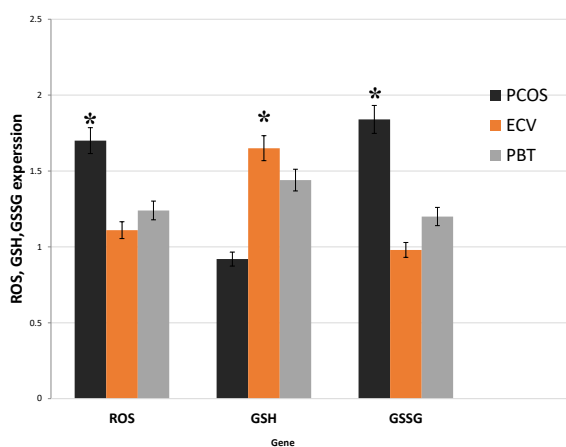


Figure 5. The Results of the Effect of ECV and PBMT on ROS, GSH and GSSG. The results showed that PBMT and ECV can cause a decrease in ROS, GSSG and an increase in GSH; these changes are significant (* $P < 0.05$)

light energy to reduce the size of ovarian cysts and improve hormone levels. Both treatments have been found to be safe and effective in reducing symptoms of PCOS, including hirsutism, acne, irregular menstrual cycles, and infertility.²⁸ ECV works by delivering therapeutic molecules directly to the cells of the ovaries. This helps to restore normal hormone levels and improve fertility in women with PCOS. In addition, ECV can help reduce inflammation and oxidative stress in the ovaries, which can lead to improved oocyte quality and increased chances of conception. ECV has been studied in both animal models and clinical trials for PCOS. In one study, mice with PCOS were treated with ECV derived from mesenchymal stem cells (MSCs). The results showed that the ECV were able to reduce inflammation in the ovaries and improve ovarian function.²²⁻²⁴ In another study, women with PCOS were treated with ECV for three months. The results showed that the treatment was associated with improved ovarian function as well as increased pregnancy rates compared to those who

did not receive treatment. Overall, ECV is a promising new treatment option for women with PCOS.²⁵ In the current study, we found that ECV and PBMT increased GSH and decreased ROS and GSSG levels. ECV therapy has been shown to be effective in decreasing reactive oxygen species (ROS) and increasing glutathione levels in women with PCOS. ECV can act as signaling molecules to regulate gene expression and cellular metabolism. Studies have shown that ECV can reduce ROS levels by upregulating antioxidant enzymes while increasing the levels of glutathione. This helps to protect the cells from oxidative stress and damage caused by ROS.³³

Additionally, ECV has been found to improve reproductive outcomes in PCOS patients by reducing inflammation and improving ovarian function. The results of this study displayed that ECV could increase anti-apoptotic gene while decreasing apoptotic gene expression in PCOS oocytes. ECV has been shown to reduce the expression of Bax and caspase 3, which are involved in the initiation of apoptosis. This decrease in expression can help to prevent cell death and promote cell survival.³⁴ Furthermore, ECV therapy has been shown to increase the expression of Bcl-2 and Bcl-xL, which can further protect cells from apoptosis. Thus, ECV therapy can be an effective tool for decreasing Bax and caspase 3 levels and promoting cell survival. BCL-2 inhibits the mitochondrial internal apoptotic path. Bcl2 has an antioxidant-like property linked to the rule of the concentration of GSH, in addition to its anti-apoptotic function.³⁵ Previous research has shown that increased BCL2 expression increases GSH content by increasing GSH synthesis and decreasing cellular GSH efflux.³⁶ These results were consistent with the observations of our research.

Conclusion

The use of PBMT and ECV therapy can reduce the amount of apoptosis and autophagy gene expression in PCOS oocytes, increase the rate of maturation and fertilization, and cause the reduction of ROS and GSSG, which can be used in the clinic as an effective method.

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Authors' Contribution

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Investigation: Samira Sahraeian, Hojjat Allah Abbaszadeh,

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Writing—original draft: Samira Sahraeian, Hojjat Allah Abbaszadeh.

Writing—review & editing: Hojjat Allah Abbaszadeh, Samira Sahraeian.

Competing Interests

The authors declare that there is no conflict of interest.

Ethical Approval

The Ethics Committee at Shahid Beheshti Medical University in Tehran, Iran, approved the in vitro clinical study (IR.SBMU.RETECH.REC.1401.223). Contributors agreed verbally and in writing to contribute to the study. Patients from Erfan Niyash hospital (Tehran, Iran) donated oocytes for the current study between 2021 and 2022.

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