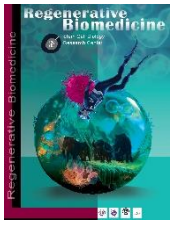

Regenerative Biomedicine

Production and Hosting: Shahid Sadoughi University of Medical Sciences

Journal homepage: jrb.ssu.ac.ir

Original Article

Exosomes Extracted from Human Embryonic Stem Cells-Derived Mesenchymal Stem/Stromal Cells Support Treatment of Polycystic Ovary Syndrome in Animal Model

Mahdieh Karimi[1], Morteza Anvari[2], Mahnaz Mohammadi[3], Fatemeh Hajizadeh-Tafti[1], Behrouz Aflatoonian[1,3,4*]

1. Stem Cell Biology Research Center, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
2. Department of Biology and Anatomical Sciences, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
3. Research and Clinical Center for Infertility, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
4. Department of Reproductive Biology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

***Corresponding Author:**
Aflatoonian, Behrouz
Email:
b.aflatoonian@ssu.ac.ir

Received:
2025-01-06
Revised:
2025-01-26
Accepted:
2025-02-17

Volume:1
Issue no.2

Editor-in-Chief:
Behrouz Aflatoonian Ph.D.

Abstract

Polycystic ovary syndrome (PCOS) is the most common medically treatable cause of female infertility. Several treatment methods have been proposed to treat this syndrome but nowadays researchers used cell therapy as the best treatment method, looking for a better way to treat it. Cell therapy is a treatment based on stem cells and mesenchymal stem/stromal cells (MSCs) are known as promising candidates for it. The secretome of these cells contains growth factors, cytokines and extracellular vesicles (exosomes (EXOs), microvesicles and apoptotic bodies), which are effective in the treatment of PCOS. The present study aimed to investigate the effect of EXOs extracted from human embryonic stem cells (hESCs) derived MSCs on PCOS in an animal model.

16 adult female Wistar rats were randomly divided into 3 groups of control (C), polycystic ovary syndrome (PCOS), test (T). After induction of PCOS in PCOS and T groups using letrozole (LTZ) (1mg/kg) dissolved in normal saline (2ml/kg), T group was treated with intra ovarian injection of EXOs. At the end of the treatment period, the amounts of Testosterone in blood serum were measured and a histomorphometric examination of the ovary was done using a caliper and Hematoxylin and Eosin (H&E) staining.

The weight and size of ovaries significantly increased in the PCOS group compared to C group and significantly decreased in T group. Also, Testosterone level in T group was significantly lower than those of the PCOS group. Moreover, the improvement of the histopathological status of the ovaries in T group along with the decrease in the number of cystic follicles and the increase in the number of corpora lutea were evident in PCOS group.

Our data indicated that EXOs can be considered as a medicinal product to improve PCOS.

Keywords: Exosomes (EXOs), Human embryonic stem cells (hESCs), Letrozole (LTZ), Mesenchymal stem/ stromal cells (MSCs), Polycystic ovary syndrome (PCOS)

How to cite this article:
Karimi, M., Anvari, M., Mohammadi, M., Hajizadeh-Tafti, F., Aflatoonian, B. Exosomes Extracted from Human Embryonic Stem Cells-Derived Mesenchymal Stem/Stromal Cells Support Treatment of Polycystic Ovary Syndrome in Animal Model. *Regenerative Biomedicine*, 2025; 1(2): 127-134.

Introduction

PCOS is one of the most common endocrine disorders in women and the most common cause of infertility due to lack of anovulation. Women with PCOS exhibit higher levels of Circulating Free Testosterone (CFT), postprandial glucose, fasting insulin and triglycerides, along with lower levels of sex hormone-binding globulin (SHBG) (1).

The use of animal models to evaluate various aspects of PCOS is one of the methods to research this disorder. The experimental PCOS has been induced and investigated in different rodent studies in very different ways and using various compounds have been used such as dihydrotestosterone (DHT), testosterone propionate (TP), estradiol valerate and aromatase inhibitors such as LTZ (2).

LTZ is a non-steroid aromatase that blocks the conversion of androgen to strogen. In adult rat, it causes cyclical irregularity, anovulation and the development of ovaries that have a large number of large follicular cysts and no corpus luteum or few corpus luteum for at least 21 consecutive days. LTZ can also increase gonadotropin and testosterone levels and decrease estrogen levels (2). Numerous studies have been conducted to minimize and prevent the complications of this disease but nowadays researchers use cell therapy as the best treatment method, looking for a better way to treat it. Cell therapy is a treatment based on stem cells and MSCs are known as promising candidates for it. The secretome from these cells includes growth factors, cytokines and Extracellular vesicles, which are effective in the treatment of PCOS. Extracellular vesicles can be classified in to exosomes (EXOs) (30–150 nm), microvesicles (MVs) (100–1000 nm) and apoptotic bodies (500–5000 nm) according to their characteristics and functions (3). EXOs are tiny extracellular vesicles, measuring 30 to 150 nm and protected by membrane packets, secreted by a variety of live cells under physiologically healthy or pathological circumstances (4).

Research indicates that EXOs derived from MSCs exhibit functions similar to those of MSCs, including the repairing tissue damage, suppressing inflammatory responses and modulating the immune system. However, the mechanisms are still not fully understood and the results remain controversial. In comparison to cells, EXOs are more stable and reservable, have no risk of aneuploidy, a lower possibility of immune rejection following in vivo allogeneic administration and may provide an alternative therapy for various diseases (5).

Also, since no research has been conducted on the effects of this EXOs on PCOS, we aimed to investigate the effects of the EXOs extracted from hESC derived MSCs on Wistar rats induced PCOS by LTZ.

Materials and Methods

Yazd2/MSCs culture

The cells (Yazd2/MSCs, passage number 14 (P14)) (Fig.1) (6) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) + 10% Fetal Bovine Serum (FBS) (Gibco) and incubated at 37℃ with 5% CO2. After the confluency reached 70%, culture media was replaced with a DMEM free FBS for 72h.

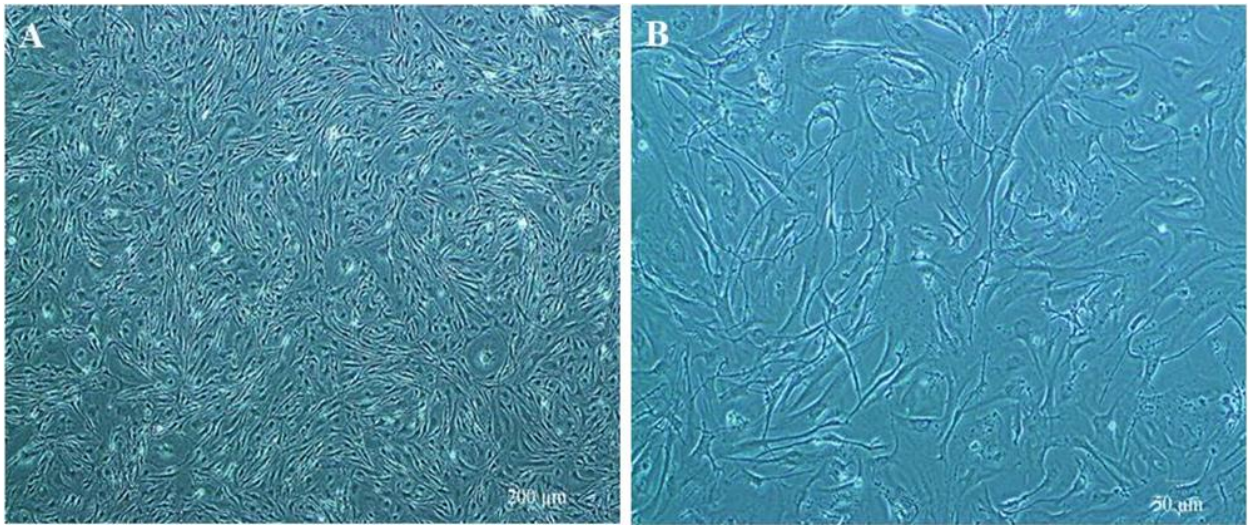

Figure 1. Cellular morphology of human embryonic stem cells (hESCs) derived mesenchymal stem/sromal cells (MSCs) culture in Passage 14 (P14) (A : x4 (scale bar : 200µm), B : x10 (scale bar : 50µm)

Animals, grouping and sample collection

This study was conducted experimentally on 18 adult female rats (Wistar breed) with a weight range of 160-200 grams and an age range of 8-9 weeks. The animals were kept in the Animal Breeding Department of Yazd Science and Reproduction Research Institute with standard conditions of 12 hours of light and 12 hours of darkness, temperature of 22 to 25℃ a 40-55% humidity and proper ventilation. During the research period, the rats had free access to water (purified) and food (They were fed pellets).

PCOS was induced in female rats by LTZ with a dose of 1mg/kg dissolved in 2ml/kg normal saline orally once daily for 21 days. After induction of PCOS, the estrous cycle was determined by vaginal smear and the rats in diestrous phase were selected (7).

The rats were randomly divided in to 3 groups of 6 :

Group 1: C (healthy rats given 2 mg/kg per body weight of normal saline for 28 days).

Group 2: PCOS (rats with PCOS given 2mg/kg per body weight of normal saline for 28 days).

Group 3: T (rats with PCOS given EXOs).

For histological examination also, after dissecting, the ovaries were removed from the abdominal cavity, they were washed with physiological serum solution and their weight and diameter were measured and placed in a 10% formalin solution.

Exosome isolation by commercial kit

The isolation of EXOs with the Exocib kit (Cibzist) was performed according to the kit protocol. The conditioned media collected after 72h of the cultured cells. The particles and cell debris were eliminated by centrifuging the conditioned medium at 3000 rpm at 4℃ for 10 minutes (for better result samples were filtered through a 0.22µm filter). Then the collected supernatant was vortexed with reagent A (5:1 ratio) for 5 minutes and allowed to incubate overnight. Next, vortex the mixture for 1 minute to ensure thorough mixing, centrifuge it at 3000 rpm at 4℃ for 40 minutes and completely remove and discard the supernatant. Finally,

resuspend the EXOs plate with 50-200 µl of reagent B and keep it at -80°C for extended storage.

Dynamic Light Scattering (DLS)

The hydrodynamic diameter distribution of the EXOs was determined using DLS (Particles suspended in a solution are constantly in motion due to Brownian movement. Given that this Brownian motion relies on the particle size's velocity, the size can be determined by analyzing the fluctuations related to this velocity. By obtaining the autocorrelation function of the observed fluctuation, the particle size and distribution can be determined. DLS uses the above principle and provides size information of a wide range of particles easily and accurately) analysis. For this aim, EXOs were appropriate diluted using Phosphate buffered saline (PBS) (One hundred microliter suspensions of EXOs were diluted to 1000 µL in PBS, added to a cuvette, and the air bubbles were carefully removed) and analyzed on Nanopartica SZ-100V2, Horiba instrument (According to kit, the ideal size for EXOs are around 50 to 120 nm).

Enzyme Linked Immunosorbent (ELISA) assay

After the treatment period, the animals were anesthetized using ketamine and xylazine intraperitoneally and blood was collected directly from their hearts. To obtain the sera, blood samples were centrifuged at 6000 rpm for 5 minutes and the collected blood sera were kept at minus 20°C for hormone analysis. The serum level of Testosterone was detected according to the manufacturer's instructions using by ELISA kit

Histological Examinations

Preparation of Tissue Sections

After fixing the tissue samples in 10% formalin (for two weeks), the samples of the ovaries were placed in special baskets to be processed in Autotechnicon (Tissue Processor). After paraffin embedding, the tissues were sectioned in to 5µm thickness slices. The slices were stained with H&E to observe the ovarian morphology and follicle counts.

After preparing the section tissues and performing H&E staining, a histophotometric study including investigation of ovary cysts and corpora lutea in serial sections of the ovary was done using an optical microscope.

Ethical considerations

All experiment protocols were under supervision of the Ethics Committee of the Shahid Sadoughi University of Medical Sciences, Yazd (IR.SSU.AEC.1403.003).

Data Analysis

Data were analyzed using SPSS version 25 software, statistical differences between the groups were determined by one-way analysis of variance (ANOVA) and Tukey's test. Results were expressed as mean ± standard deviation (mean ± SD). $P<0.05$ was considered to be statistically significant.

Results

DLS

DLS results (Fig.2.) show that mean size of EXOs isolated are 112.9 nm (According to kit, the ideal size for EXOs are around 50 to 120 nm).

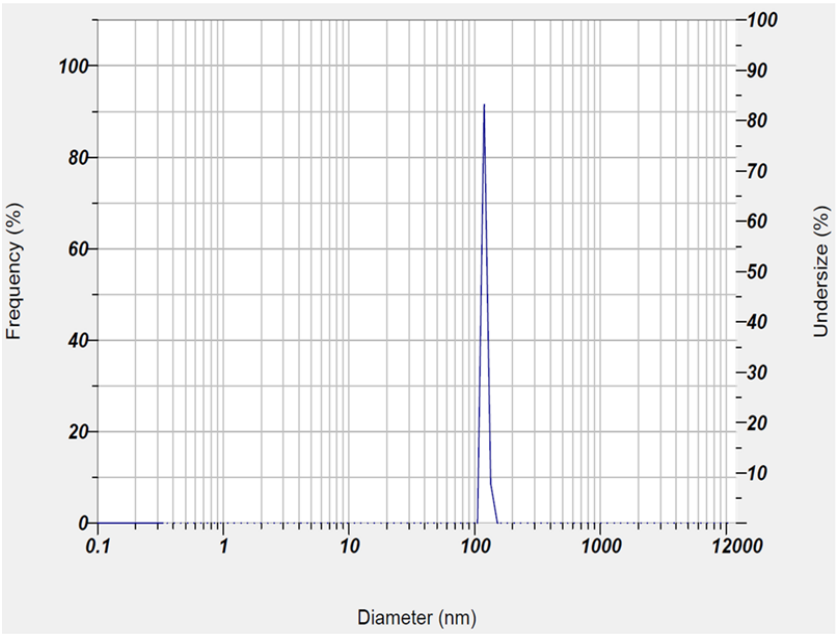

Figure 2. Dynamic Light Scattering (DLS) results : DLS analysis for identifying size distribution of isolated exosomes (EXOs). DLS results show that mean size of EXOs isolated are 112.9 nm

Body weight Changes (g)

On the first day of the treatment period, as seen in Table 1. there were no significant differences between the groups in terms of mean body weight. However, at the end of the third and fourth weeks, the mean body weight in the PCOS group was significantly increased compared to C group and the mean body weight in T group was significantly decreased compared to the PCOS group ($p<0.05$).

Ovaries Weight and Size Changes

Mean ovaries weights and Size are shown in Table 2. for different groups. The analysis results indicate that this parameter has significantly increased in the PCOS group compared to C group. T group showed an significantly decrease in this parameter compared to the PCOS group ($p<0.05$).

Testosterone Levels in Blood Serum (ng/ml)

Mean blood serum testosterone levels are shown in Table 3. The results are indicative of an increase in the PCOS group compared to C group. Also, T group show a decrease in blood serum testosterone levels compared to the PCOS group ($p<0.05$).

H&E Staining Results

Figure 3. Iillustrates the microscopic sections of ovaries in various groups. As seen here, the ovary size is increased in the PCOS group compared to C group. However, it is associated with a decrease in Corpus Luteum (CL). Also, an improvement in histopathologic conditions of the ovaries in T group alongside a decrease in Cystic Follicles (CF) count and an increase in CL count can be observed.

Table 1: Comparison of mean body weight between studied groups. (non-identical letters are indicative of a significant difference at p<0.05)(control (C), polycystic ovary syndrome (PCOS) and test (T))

Days	C	PCOS	T
0	176.2±6.776[a]	176.19±7.271[a]	176.21±7.17[a]
21	182.7±6.842[a]	197.69±7.304[b]	183.16±6.96[a]
28	185.5±6.776[a]	205.49±7.27[b]	194±7.04[a]

Table 2: Comparison of mean ovaries weight and size between studied groups. (non-identical letters are indicative of a significant difference at p<0.05)(control (C), polycystic ovary syndrome (PCOS) and test (T))

groups	Ovary weight(g)	Ovary size(mm)
C	0.0735±0.00672[a]	0.2248±0.01001[a]
PCOS	0.2415±0.05730[b]	0.5020±0.05813[b]
T	0.1017±0.00163[a]	0.1443±0.00383[c]

Table 3: Comparison of mean blood serum testosterone levels between studied groups. (non-identical letters are indicative of a significant difference at p<0.05)(control (C), polycystic ovary syndrome (PCOS) and test (T))

groups	Testosterone(ng/ml)
C	0.3250±0.01871[a]
PCOS	1.3850±0.01871[b]
T	0.5250±0.01871[d]

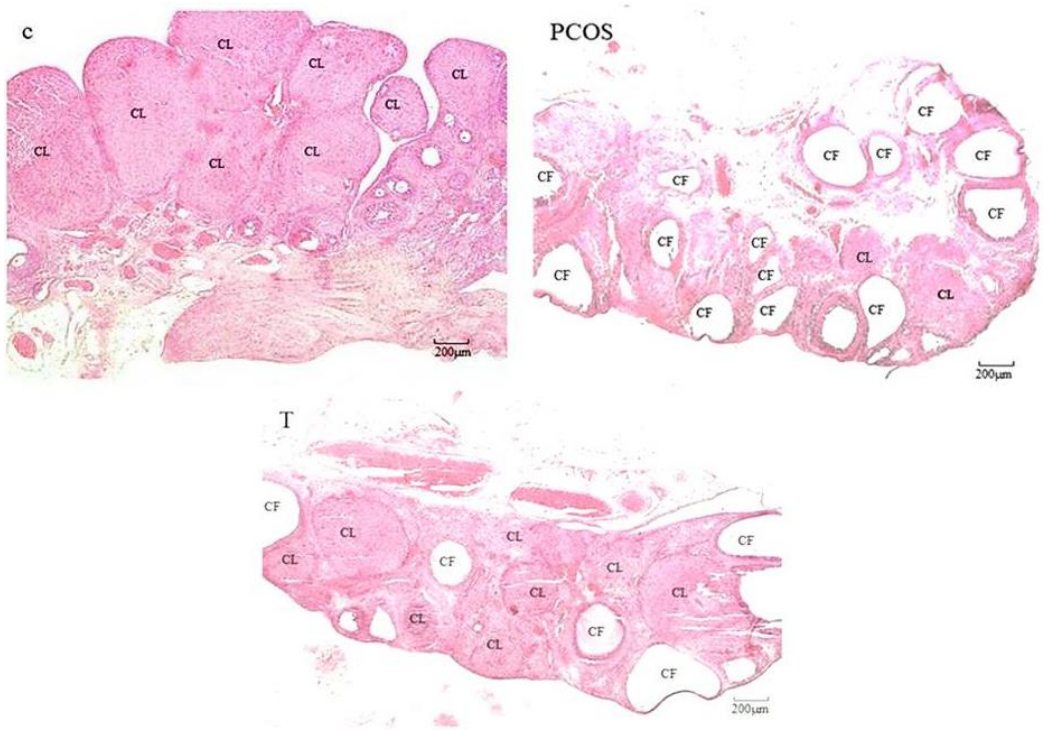

Figure 3. Images of microscopic sections of ovaries in studied groups (control (C), polycystic ovary syndrome (PCOS), test (T) (CF (Cystic Follicle), CL (Corpus luteum), and H&E Staining) (8X magnification)

Discussion

In the present study, the effects of EXOs extracted from hESCs derived MSCs on PCOS in an animal model were examined. The results indicated that treatment with EXOs can significantly reduce the size and weight of ovaries, the testosterone hormone and Cystic Follicles count, It can also increase the corpus luteum count.

PCOS is a multifaceted genetic, endocrine and metabolic heterogeneous disorder and the most prevalent endocrine illness and metabolic disorder among reproductive age children and the most important cause of infertility due to ovulation in women (8).

The use of animal models to evaluate various aspects of PCOS is one of the methods to research this disorder. In the present study, aromatase inhibitors namely LTZ used to induce PCOS due to their greater ability to inhibit the aromatase enzyme than other inhibitors. LTZ is a non-steroid aromatase that blocks conversion of androgen to strogen. In adult rat, it causes cyclical irregularity, anovulation and the development of ovaries that have a large number of large follicular cysts and no corpus luteum or few corpus luteum for at least 21 consecutive days. LTZ can also increase gonadotropin and testosterone levels and decrease estrogen levels (2).

Numerous studies have been conducted to minimize and prevent the complications of this disease but nowadays researchers use cell therapy as the best treatment method, looking for a better way to treat it. Cell therapy is a treatment based on stem cells, and MSCs are known as promising candidates for it. The secretome from these cells includes growth factors, cytokines and extracellular vesicles (exosomes, microvesicles and apoptotic bodies), which are effective in the treatment of PCOS (3).

A recent study explored that EXOS derived from MSCs hold great potential for treating PCOS, similar to that of the complete MSCs from which they are sourced. This research suggests that EXOs derived from MSCs are the primary factors controlling androgen production in an in vitro setup and rejuvenating fertility in a PCOS mouse model. Both intravenous and intraovarian injections show new therapeutic promise for PCOS conditions. Intravenous injection demonstrates a more efficient effect in systemic regulation, including control of blood glucose levels. Conversely, intraovarian injection demonstrates greater effectiveness in reinstating ovarian function (9).

Numerous studies have explored the role of EXOs in relation to metabolic disorders in PCOS. For example, a study conducted in 2020 analyzed human follicular fluid (HFF) from people with and without PCOS focusing particularly on EXOs. The research identified ten miRNAs that showed a significant rise in expression among PCOS patients (miR-6087, miR-193b-3p, miR-4745-3p, miR-199a-5p, miR-199a-3p, miR-4532, miR-629-5p, miR-199b-3p, miR-25-3p, and miR-143-3p). In contrast, these miRNAs (miR-200c-3p, miR-483-5p, miR-382-5p, miR-98-5p, miR-23b-3p, miR-200a-3p, miR-10a-5p, miR-141-3p, miR-3911, and miR-483-3p) exhibited a significant decrease in expression levels in patients with PCOS. These findings offer important understanding regarding the possible roles of EXOs and miRNAs in the metabolic changes linked to PCOS (10).

While additional clinical trials are necessary in our upcoming research, EXOs derived

from MSCs may serve as a hopeful treatment alternative for patients with PCOS.

Conclusion

Our study indicated that LTZ can act successfully in the creation of an experimental model of PCOS and EXOs can be used to reduce the complications of prescribed LTZ since there are well-founded and solid studies that prove the effects of EXOs as proven in our study.

Conflict of interest

The authors have no conflict of interest to declare.

References

1. Bai H, Ding H, Wang M. Polycystic Ovary Syndrome (PCOS): Symptoms, Causes, and Treatment. Clinical and Experimental Obstetrics & Gynecology. 2024;51(5):126.

2. Kamada S, Yamamoto Y, Aoki H, Tamura K, Takeda A, Minato S, et al. A novel PCOS rat model and an evaluation of its reproductive, metabolic, and behavioral phenotypes. Reproductive medicine and biology. 2022;21(1):e12416.

3. Margolis L, Sadovsky Y. The biology of extracellular vesicles: The known unknowns. PLoS biology. 2019;17(7):e3000363.

4. Zhou M, Weber SR, Zhao Y, Chen H, Sundstrom JM. Methods for exosome isolation and characterization. Exosomes. 2020:23-38.

5. Izadi M, Rezvani ME, Aliabadi A, Karimi M, Aflatoonian B. Mesenchymal stem cells-derived exosomes as a promising new approach for the treatment of infertility caused by polycystic ovary syndrome. Frontiers in pharmacology. 2022;13:1021581.

6. Javidpou M, Seifati S-M, Farashahi-Yazd E, Hajizadeh-Tafti F, Golzadeh J, Akyash F, et al. Mesenchymal stem/stromal-like cells from diploid and triploid human embryonic stem cells display different gene expression profiles. Iranian Biomedical Journal. 2020;25(2):99.

7. Barazesh F, Mirzaei A, Abbasian Z, Ghavami Zadeh M. The effect of hydroalcoholic extract of glycyrrhizaglabra l.(licorice) root on serum level of glucose, triglyceride and cholesterol in polycystic ovary syndrome induced by letrozole in Rats. Armaghane Danesh. 2016;21(2):148-59.

8.Mukerjee N. Polycystic Ovary Syndrome (PCOS) symptoms, causes & treatments-a review. International Journal of Science and Research. 2020;9(7):1949-57.

9. Park HS, Cetin E, Siblini H, Seok J, Alkelani H, Alkhrait S, Liakath Ali F, Mousaei Ghasroldasht M, Beckman A, Al-Hendy A. Therapeutic potential of mesenchymal stem cell-derived extracellular vesicles to treat PCOS. International Journal of Molecular Sciences. 2023 Jul 6;24(13):11151.

10. Hu J, Tang T, Zeng Z, Wu J, Tan X, Yan J. The expression of small RNAs in exosomes of follicular fluid altered in human polycystic ovarian syndrome. PeerJ. 2020 Feb 19;8:e8640.