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Vitamin D's Impact on Serum Exosome IL-8 Gene Expression, Biochemical and Microscopic Study in PCOS Rats: An Experimental Study

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ABSTRACT

Introduction: Polycystic ovary syndrome (PCOS) is a prevalent endocrine disorder affecting 5-15% of women of reproductive age, presenting symptoms such as elevated androgen levels and irregular ovulation. Recent research highlights the significance of inflammatory markers, particularly interleukin-8 (IL-8). Additionally, vitamin D deficiency may worsen inflammation, suggesting therapeutic benefits of supplementation.

This research aimed to examine the impact of vitamin D therapy on the ovaries of female rats with induced PCOS using biochemical and microscopic methodologies.

Methods: In experimental study, twenty-four pre-pubertal female Wistar rats (4-5 wk, 90-100 gr) were divided into four groups: control, laboratory control, PCOS, and treatment. The PCOS group received subcutaneous injections of 6 mg/kg/day dehydroepiandrosterone (DHEA) in sesame oil and ethanol. The treatment group received the same DHEA dosage with 120 ng/100 g/week of 1,25 (OH)2D3. The laboratory control group was injected with sesame oil and ethanol, while the control group received only the vehicle. After 28 days, blood samples and ovarian tissues were collected for analysis.

Results: Elevated levels of FSH, LH, and testosterone were noted in the PCOS group compared to controls. The treatment group exhibited lower hormone levels than the PCOS group. Light microscopy revealed increased atretic and cystic follicles in the PCOS group, while vitamin D treatment significantly reduced these follicle types. IL-8 levels were higher in the PCOS group, but vitamin D treatment significantly decreased them.

Conclusion: The findings suggest that vitamin D treatment plays a therapeutic role in addressing androgen excess in a rat model of PCOS, benefiting hormonal and structural alterations associated with the condition.

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Introduction

Polycystic ovary syndrome (PCOS) is a multifaceted and common endocrine condition that affects an estimated 5% to 15% of women of reproductive age globally. This syndrome is marked by a range of symptoms, including elevated androgen levels, irregular ovulation, and the presence of multiple cysts on the ovaries. The diverse clinical manifestations of PCOS can have profound effects on various aspects of a woman's health, including reproductive, metabolic, and psychological well-being (1). The exact causes of PCOS are not fully understood, but it is thought to arise from a complex interplay of genetic, environmental, and hormonal influences. Insulin resistance is particularly significant in the development of this disorder, contributing to its overall pathophysiology. As a result, women with PCOS may experience a variety of health challenges that require comprehensive management strategies (2).

Research into the fundamental mechanisms of PCOS has uncovered a complex relationship involving hormonal imbalances, persistent inflammation, and metabolic irregularities. Present treatment approaches emphasize personalized strategies that include lifestyle

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changes, medication, and reproductive therapies designed to meet the specific requirements of each patient. As knowledge of PCOS advances, new research directions are being investigated, such as the influence of genetic factors, the gut microbiome, and dietary interventions in the management of this intricate condition (1).

Recent studies have placed significant emphasis on the involvement of inflammatory markers and cytokines in the pathophysiology of PCOS. Among these, interleukin-8 (IL-8) has been identified as a key factor in driving inflammatory responses within the ovarian microenvironment. This pro-inflammatory cytokine plays a vital role in various physiological functions, such as immune regulation, angiogenesis, and the development of ovarian follicles. The presence of elevated IL-8 levels has been associated with the intensity of symptoms experienced by individuals with PCOS (3).

Vitamin D, a fat-soluble vitamin that is mainly produced in the skin when exposed to sunlight, has gained significant interest due to its diverse biological functions, especially in regulating immune responses and inflammation. Emerging research suggests that many women with PCOS experience vitamin D deficiency, which may worsen the inflammatory conditions linked to this disorder (4). The connection between vitamin D levels and PCOS has prompted discussions about the potential benefits of vitamin D supplementation. By addressing this deficiency, there is hope that it could serve as a therapeutic strategy to alleviate the symptoms associated with PCOS and help lower inflammation markers in affected individuals (5).

This study aims to comprehensively evaluate the impact of vitamin D on the expression of the IL-8 gene in serum exosomes within a rat model of PCOS. By exploring the potential regulatory effects of vitamin D on IL-8 expression, the research seeks to elucidate the connection between vitamin D levels and the inflammatory responses linked to PCOS. The outcomes of this study may provide insights into the mechanisms that relate vitamin D concentrations to inflammation in the context of this syndrome. Additionally, the research will assess levels of LH, FSH, and testosterone, along with conducting microscopic examinations. By gaining a better understanding of these interactions, the study could enhance our knowledge of how vitamin D influences inflammatory pathways that are often altered in individuals with PCOS.

Materials and Methods

This experimental study took place during the initial half of 2024 at the Faculty of Sciences, Islamic Azad University of Mashhad. In line with findings from comparable studies, the sample size was established at 24 rats. A total of 24 immature female Wistar rats, aged 4 to 5 weeks and weighing between 90 and 100 grams, participated in this experimental study. These rats were sourced from the Animal Breeding and Experimental Research Center at Ferdowsi University in Mashhad, Iran. They were provided with adequate food and water while being maintained under regulated environmental conditions, which included a temperature of 23 ± 2°C, a 12-hour light/dark cycle, and a humidity level of 55 to 60%. All experimental procedures involving these animals adhered to the ethical guidelines established by the Ethical Committee of the Islamic Azad University, Mashhad Branch, Faculty of Medicine (IR.IAU.MSHD.REC.1401.042). The study was conducted with a commitment to ensuring the welfare and humane treatment of the animals throughout the research process. The careful management of the rats' living conditions and adherence to ethical standards underscores the importance of responsible research practices in the field of medical sciences. This approach not only ensures the validity of the experimental results but also reflects a dedication to the ethical treatment of research subjects. Experimental design.

Experimental Groups

Vaginal smears were employed to assess the synchrony of the estrous cycle. Rats exhibiting two to three consecutive and regular estrous cycles were chosen for the subsequent phases of the research.

The adult female rats were assigned to four distinct groups, each comprising six individuals, categorized as control, laboratory control, PCOS, and treatment groups. The control group received no interventions, while the laboratory control group was administered a subcutaneous injection of 0.2 ml sesame oil combined with 0.01 ml of 95% ethanol for 35-day period. In the PCOS

group, a daily dose of 6 mg/kg DHEA (from Sigma-Aldrich Co.) was administered for a duration of 35 consecutive days, using the same vehicle of 0.2 ml sesame oil and 0.01 ml 95% ethanol. The treatment group was administered a dosage of DHEA along with an extra 120 ng/kg of vitamin D3 weekly, starting one week after the initial injection, also delivered via the same vehicle, over the same 35-day period (6). Throughout the experiment, all animal groups were maintained under standardized conditions in separate cages to ensure consistency and reliability of the results. This controlled environment was crucial for accurately assessing the effects of the various treatments on the animals.

Collection of Samples

At the conclusion of the experiment, all rats were subjected to anesthesia using Xylazine (Alfazyne 2%, Atafen) and Ketamine (Alfamine 10%, Ege-Vet) administered via intraperitoneal injection. Blood samples were subsequently collected from the heart into centrifuge tubes that did not contain anticoagulants, allowing the blood to clot naturally. Following the clotting process, the samples were centrifuged at a speed of 3000 rpm for a duration of 20 minutes. This procedure facilitated the separation of serum, which was then promptly stored at a temperature of −80°C to preserve its integrity for future biochemical analyses. The measurement of serum levels of FSH and LH testosterone was conducted by Epoch Biotek in the USA. Serum FSH concentrations were assessed using the Double Antibody Sandwich method, while serum levels of LH and Testosterone were evaluated through Competitive Inhibition Enzyme Immunoassay techniques.

Technique for Smear Extraction

The estrous cycle was monitored through daily microscopic analysis of vaginal smears, which were conducted at specific times each morning and consistently maintained throughout the experiment. To facilitate this process, the rat was securely held with one hand around its waist, ensuring the ventral surface faced downward for stability, while the other hand operated the pipette. Initially, approximately 0.2 ml of normal saline was drawn into the pipette, which was then carefully inserted into the vaginal canal at varying depths to obtain the smear. After the saline was introduced into the vaginal canal, it was subsequently retracted back into the pipette, capturing the smear in the process. This sample was then placed onto a clean glass slide and covered with a cover slip. The cover slip was essential for maintaining a uniform orientation of the smear, which facilitated easier focusing under the microscope and prevented the smears from merging during handling. The collected smear was subjected to Giemsa staining to enhance visibility and contrast. Following the staining process, the smear was examined under a microscope, allowing for detailed observation of the cellular components and providing insights into the estrous cycle of the rat. This methodical approach ensured accurate and reliable results throughout the duration of the study.

Morphometric Assessment

For the morphometric evaluation, five micrometer step sections were prepared and mounted onto microscope slides at 50 µm intervals to ensure that the same structure was not counted multiple times. The slides underwent staining with hematoxylin-eosin (H&E) to facilitate the enumeration of ovarian follicles and corpora lutea (CL) within each ovary section. Follicles were categorized based on specific criteria: primary follicles were identified by the presence of one or more layers of granulosa cells surrounding a primary oocyte, secondary follicles were recognized by the formation of an antrum, and tertiary follicles were distinguished by the complete development of the antrum along with the association of the primary oocyte with the cumulus oophorus. The morphological features of atretic follicles were characterized by the degeneration and separation of the granulosa cell layer from the basement membrane, the occurrence of pyknotic nuclei, and signs of oocyte degeneration. These features indicate a decline in follicular health and viability. In contrast, follicles that exhibited four or five layers of granulosa cells encasing a significantly large antrum, or those that presented as large fluid-filled structures with a diminished granulosa cell layer and a thickened theca layer, were classified as cystic follicles. This systematic approach to follicle classification and assessment provides valuable insights into ovarian morphology and health. By employing precise staining techniques and careful sectioning, researchers can obtain a clearer understanding of the dynamics of ovarian

follicle development and atresia. Such detailed morphometric analysis is essential for advancing knowledge in reproductive biology and related fields (7).

Techniques for the Characterization and Isolation of Exosomes

The supernatant was carefully collected, and exosome extraction was performed using the SBI ExoQuick method, adhering to the manufacturer's instructions with some modifications. The serum was first centrifuged at 3000×g for 30 minutes. Following this, it was diluted with PBS in a 1:1 ratio and subjected to a second centrifugation at 10,000×g for 40 minutes at 4 °C. The resulting supernatant was then transferred to an ultrafiltration tube (Millipore Amicon Ultra 10 kDa) and centrifuged again at 3000×g for an additional 40 minutes at 4 °C. The filtrate obtained from the ultrafiltration process was combined with the ExoQuick exosome precipitation solution in a ratio of 250:63, and the mixture was incubated for 30 minutes to promote exosome precipitation. After this incubation period, the mixture underwent centrifugation at 1500×g for 30 minutes. The supernatant was carefully discarded, and the remaining sample was centrifuged once more at 1500×g for 5 minutes to ensure the complete removal of any residual supernatant. Ultimately, the pellet containing the exosomes was resuspended in PBS and stored at either 4 °C or −20 °C for future analysis. This detailed procedure guarantees the efficient extraction and preservation of exosomes for subsequent research purposes (8).

Assessment of Exosome Morphology through TEM

Transmission electron microscopy (TEM) has been employed to investigate the structural properties of exosomes. The process began with the fixation of the exosomal pellet using 1% glutaraldehyde (Sigma). After fixation, 20 µL of the exosomes was applied to a carbon-coated grid and allowed to air dry at room temperature for 30 minutes. The examination was performed using a LEO 906 TEM (Zeiss: Germany), which was set to operate at 80 kV. Prior to imaging, the grids underwent two washes with PBS for 5 minutes each and were then stained with 1% uranyl acetate for 10 minutes to enhance contrast.

The imaging process involved the use of Digital Micrograph Software (Gatan Inc) to capture the TEM images, employing an Orius 200 camera (Gatan Inc., Washington: DC). This meticulous procedure ensured that the exosomal morphology was accurately represented and analyzed. The combination of fixation, drying, washing, and staining was critical in preparing the samples for high-resolution imaging. Furthermore, the examination extended to the surface morphology of the particles, providing insights into their structural features. This comprehensive approach to TEM analysis allows for a detailed understanding of exosomal characteristics, which is essential for further research and applications in the field (9).

The Analysis of Exosome Size and Distribution Using DLS

The dynamic light scattering (DLS) method has been utilized to investigate solvent nanoparticles, providing a quick and uncomplicated means of assessing particles in solution without requiring any sample preparation. In this research, the isolated exosomes were reconstituted in 330 µl of phosphate-buffered saline (PBS). After thoroughly mixing the solution, the Malvern Zetasizer Nano ZS was employed to measure the size of the exosomes. This approach is particularly beneficial due to its effectiveness and user-friendliness, enabling real-time evaluation of particle size distribution. The DLS technique functions by examining the variations in light scattering that occur as particles move within the solution, yielding important information about the properties of the exosomes. This capability makes DLS a valuable tool for researchers seeking to understand the characteristics of nanoparticles in a variety of applications, enhancing the overall analysis process.

The use of PBS as a resuspension medium ensures that the exosomes remain stable and well-dispersed during measurement. By employing the Malvern Zetasizer Nano ZS, researchers can obtain precise measurements of exosome size, which is crucial for understanding their biological functions and potential applications in various fields, including drug delivery and diagnostics. The ability to conduct these measurements without extensive sample preparation further enhances the practicality of the DLS method in nanoparticle analysis (10).

RNA Extraction and cDNA Synthesis

Total RNA exosomes were extracted using the Trizol reagent (Invitrogen, CA, USA), following the instructions provided by the manufacturer. To

eliminate any residual genomic DNA, an RNase-Free DNase Set (Qiagen, Hilden, Germany) was utilized. The concentration and quality of the isolated RNA were evaluated with the NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., USA) and the Experion system (Bio-Rad, CA, USA). Each RNA sample was then stored at -80°C until it was needed for cDNA synthesis. After adjusting the RNA concentrations, cDNA synthesis was performed using the cDNA synthesis kit from TaKaRa (Otsu, Shiga, Japan). This process is vital for preparing RNA for various applications in molecular biology. The synthesized cDNA was then stored at -20°C until required for quantitative real-time PCR, ensuring that the material remained stable and ready for analysis. The meticulous handling and processing of both RNA and cDNA are critical for achieving reliable and reproducible outcomes in subsequent applications. By adhering to these protocols, researchers can enhance the accuracy of their molecular biology experiments, ultimately leading to more dependable results (11).

Quantitative Real-Time PCR

The quantitative real-time polymerase chain reaction (qPCR) was conducted utilizing the standard SYBER Green premix EX Tag2 (TAKARA) within a total reaction volume of 20 µl to assess the mRNA expression levels of the IL-8 gene. Each qPCR cycle comprised 40 repetitions, with the thermal cycling conditions set at 95 °C for 30 seconds, followed by 64 °C for 30 seconds, and concluding with 72 °C for an additional 30 seconds. This process was executed on the ABI 7300 Real-Time PCR system (Applied Biosystems, USA). Most experiments were performed in triplicate, and the specificity of the PCR products was verified through melt curve analysis. To normalize the expression levels of the target gene, GAPDH was selected as the endogenous control. This choice is vital for ensuring precise comparisons of gene expression across various samples. The expression levels of the IL-8 gene were subsequently determined using the $2^{-\Delta\Delta}$Ct method, a widely recognized technique for quantifying relative gene expression. Comprehensive details regarding the primer characteristics utilized in this study are presented in Table 1. This table contains critical information that underpins the reproducibility and reliability of the qPCR results, thereby enhancing the overall validity of the findings related to IL-8 gene expression (12).

Table 1. Quantitative Real-time PCR primers.

Gene name	Sequence	Length	Annealing temperature
GAPDH	Forward Primer: 5′ - CATCACTGCCACCCAGAAGACTG-3′	23	71 °C
	Reverse Primer: -5′ ATGCCAGTGAGCTTCCCGTTCAG-3′	23	71 °C
IL-8	Forward Primer: 5′- GGTGATATTCGAGACCATTTACTG-3′	24	64 °C
	Reverse Primer: 5′ - GCCAACAGTAGCCTTCACCCAT-3′	22	64 °C

Ethical Approval

The study was conducted in accordance with ethical standards for the treatment of laboratory animals, ensuring compliance with applicable regulations designed to protect animal welfare. Ethical clearance for this experimental study involving animals was obtained from the Research Deputy Ethics Committee at the Islamic Azad University of Mashhad Medical Sciences Branch in Mashhad, Iran. This approval is recorded under the code IR.IAU.MSHD.REC.1401.042 and the approval number 992589. By strictly following these ethical guidelines, the rights and welfare of the animals participating in this research are upheld and prioritized.

Statistical Analyses

The Biostatistics Department of Azad University Medical Faculty conducted the statistical analyses. To assess the normality of each continuous variable, the Shapiro-Wilk test was utilized. For non-parametric data, continuous variables were compared using the Kruskal-Wallis test, followed by post hoc analysis with the Mann-Whitney U test. A significance level of $p < 0.05$ was established for other analyses. To evaluate differences between groups, either the Chi-square test or Fisher's exact test was employed. This approach ensured a comprehensive examination of the data across various categories. The results were interpreted with careful consideration of the statistical methods applied. The data were summarized and presented as mean ± standard deviation, along with median and range values. This format provided a clear understanding of the distribution and variability of the continuous variables analyzed in the study.

Result

Evaluation of Body Weight and Hormonal Profiles Related to Sex

The body weight of the rats across all groups exhibited a consistent upward trend over the four-week period. Specifically, the average (±SD) body weight in the control group rose from 18.6 ± 1.12 g in the first week to 52.34 ± 6.95 g by the fourth week. Similarly, the laboratory control group showed an increase from 18.75 ± 1.23 g to 53.56 ± 8.21 g, while the PCOS group experienced a rise from 19.21 ± 1.12 g to 57.45 ± 3.86 g. The treatment group also demonstrated growth, with weights increasing from 20.76 ± 1.32 g to 55.61 ± 4.96 g. Despite the observed increases in body weight across all groups, statistical analysis revealed no significant differences among them. This suggests that while the rats gained weight consistently, the variations in weight between the different groups were not substantial enough to indicate a meaningful distinction. The data indicates a general trend of weight gain that is consistent across the experimental conditions.

The serum levels of FSH showed no notable differences when the control group was compared to the laboratory control. However, the FSH levels in the PCOS group were markedly elevated in comparison to the other groups. Treatment with vitamin D resulted in a significant reduction in FSH levels among the rats, indicating a potential therapeutic effect on hormone regulation in this context. The analysis revealed no notable differences in serum LH levels when the control group was compared to the laboratory control. However, the serum LH levels in the PCOS group were markedly elevated in comparison to the other groups. In the treatment group, a significant reduction in LH levels was observed. Similarly, there were no significant differences in serum testosterone levels between the control group and the laboratory control. In contrast, the PCOS group exhibited significantly higher serum testosterone levels compared to the other groups. Following vitamin D treatment, a substantial decrease in testosterone levels was noted. The serum LH/FSH ratio was significantly elevated in the PCOS group relative to the other groups. Conversely, the treatment group showed a decrease in the LH/FSH ratio, indicating a positive response to the intervention. These findings are summarized in Table 2.

Table 2. The concentrations of serum hormones observed in the examined groups

Groups	FSH (IU/L)	LH (mIU/mL)	LH/FSH	Testosterone (ng/mL)
Control group	6.97 ± 1.15 a	6.84 ± 2.45 a	0.98 ± 0.45 a	0.65 ± 0.25 a
laboratory control group	6.82 ± 1.25 a	6.65 ± 3.51 a	0.97 ±0.52 a	0.70 ± 0.35 a
PCOS group	9.15 ± 0.83 b	64.41 ± 27.84 b	7.03 ±2.75 b	15.79 ± 8.64 b
treatment group with vit D	7.27 ± 0.86 a	38.37 ± 13.45 c	5.27 ±1.92 c	5.15 ± 2.68 c

There are treatments identified by common letters, which do not exhibit statistically significant differences. In contrast, distinct letters signify significant differences at the 5% error level. Data were analyzed using one-way ANOVA with Tukey's post hoc test.

Morphometric Observations

The vaginal smear cytology analysis conducted on the rats prior to the commencement of the experiment revealed no significant alterations in cell size or type across the various groups (Figure 1). The examination consistently identified leukocytes in all tested groups, suggesting that the animals were not experiencing an estrous cycle but were instead in the metaestrous phase. This finding indicates a lack of reproductive readiness among the subjects at the outset of the study. Following a series of consecutive smears, the emergence of nucleated epithelial cells was noted, signifying the onset of the proestrus phase. This progression continued until keratinized epithelial cells were detected, which marked the transition into the estrus phase. The presence of these cells indicated that the animals were now prepared for the induction of the disease, reflecting a shift in their reproductive status. Upon verification of the estrous phase, the disease was induced in a methodologically sound manner. On the final day of the smearing process, a notable disarray in the cellular composition was observed, which suggested a disruption in the expected pattern of the estrous cycle. This confusion in the smear cell arrangement was indicative of the induction of cystic ovarian syndrome, highlighting the impact of the experimental conditions on the reproductive health of the subjects.

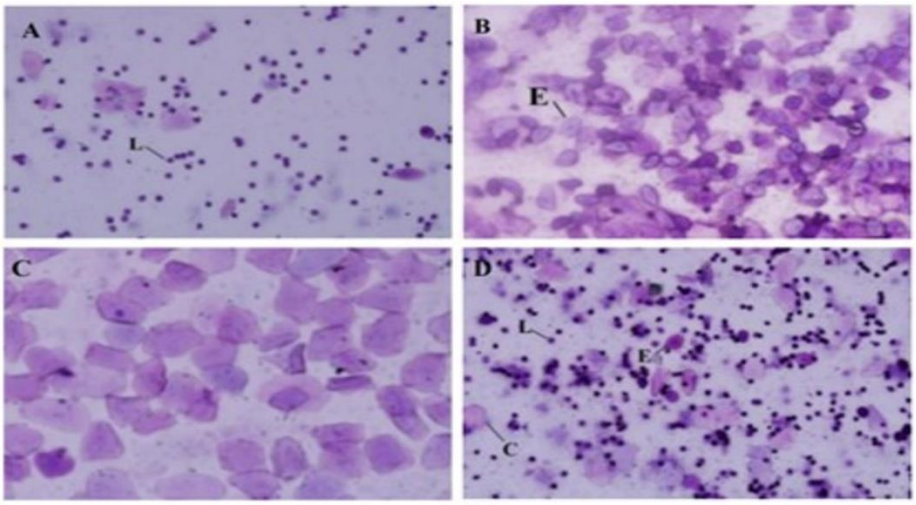

Figure 1. Vaginal smear cytology can be categorized into several distinct phases: A) metaestrous phase, B) proestrus phase, C) estrous phase, and D) induction of PCOS phase. Nucleated epithelial cells (E), leukocytes (L), and keratinized epithelial cells (C).

Morphometric Measurement

The assessment of follicle counts in each ovary was conducted to evaluate the influence of vitamin D on follicular development changes. The analysis revealed no significant differences in the counts of primary follicles, secondary follicles, and corpus luteum among the various groups. However, within the PCOS group, a notable reduction in the number of tertiary follicles was observed compared to the other groups. In contrast, the ovaries of rats administered vitamin D exhibited a significant increase in tertiary follicle counts when compared to the PCOS group. Additionally, the incidence of atretic follicles was markedly higher in the PCOS group relative to the other experimental groups. Upon treatment with vitamin D, there was a significant decrease in the number of atretic follicles, bringing their levels in line with those observed in the control group. This finding aligns with expectations, as the control group did not present any follicular cysts, indicating a healthy follicular development profile. In terms of cystic follicles, the PCOS group displayed a significant increase compared to the control groups. Following vitamin D treatment, while the number of cystic follicles decreased, it remained elevated relative to the control groups. These results underscore the potential role of vitamin D in modulating follicular dynamics, particularly in the context of PCOS, as illustrated in the data presented in Table 3.

Table 3. Morphometric analysis of ovary

Groups	Primary Follicle	Secondary Follicle	Tertiary Follicle	Atretic Follicle	Cystic Follicle	Corpus Luteum
Control group	10.4 ± 4.25 a	3.9 ± 0.94 a	0.7 ± 0.87 a	4.9 ± 1.23 a	0 a	0.5 ± 0.68 a
laboratory control group	11.3 ± 3.65 a	4.1 ± 1.15 a	0.6 ± 0.65 a	5.2 ± 0.95 a	0 a	0.4 ± 0.55 a
PCOS group	11.5 ± 3.74 a	4.4 ± 1.45 a	0.2± 0.15 b	11.5 ± 2.52 b	1.5 ± 0.87 b	0.3 ± 0.23 a
treatment group with vit D	10.1 ± 2.24 a	3.5± 1.43 a	0.6 ± 0.73 a	5.1 ± 0.86 a	0.6 ± 0.55 c	0.4 ± 0.95 a

There are treatments identified by common letters, which do not exhibit statistically significant differences. In contrast, distinct letters signify significant differences at the 5% error level. Data were analyzed using one-way ANOVA with Tukey's post hoc test.

Assessment of Exosome Quality and Structure Utilizing TEM

The research methodology involved the isolation of exosomes from serum through differential centrifugation, which was subsequently characterized using transmission electron microscopy (TEM), as illustrated in Figure 2. The ultra-structural examination of the exosome pellets via TEM revealed a significant enhancement of the characteristic spherical morphology of the exosomes, which measured between 50 and 200 nm in diameter, as depicted

in Figure 2. Additionally, the TEM analysis confirmed that the average diameter of the exosomes remained within the specified limit of 200 nm, while preserving their distinct round particle morphology. This observation underscores the integrity of the exosomal structure throughout the isolation process, ensuring that the characteristics of the exosomes were accurately represented. The findings further indicated that the exosomes possess a membrane, as shown in Figure 2. This membrane presence is crucial for understanding the functional properties of exosomes and their potential applications in various biomedical fields.

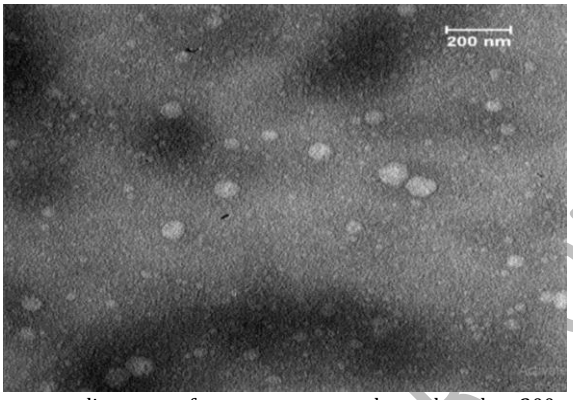

Figure 2. TEM validated that the average diameters of exosomes are equal to or less than 200 nm, while maintaining their intact spherical shape.

Assessment of Exosome Distribution and Size through the Application of Dynamic Light Scattering (DLS) Techniques

The dynamic light scattering (DLS) technique has been employed to examine the solvent nanoparticles, offering a rapid and straightforward approach to measuring particles in solution without the need for sample preparation. In this study, the extracted exosomes were resuspended in 330 µl of phosphate-buffered saline (PBS). Following the resuspension, the solution was agitated to ensure homogeneity, after which the Malvern Zetasizer Nano ZS was utilized to determine the size of the exosomes (Figure 3). This method allows for efficient characterization of the nanoparticles, providing valuable insights into their properties. The use of DLS in this context highlights its effectiveness in analyzing exosomes, facilitating a better understanding of their behavior in solution. The ability to conduct measurements without extensive sample preparation further underscores the practicality of this technique in nanoparticle research.

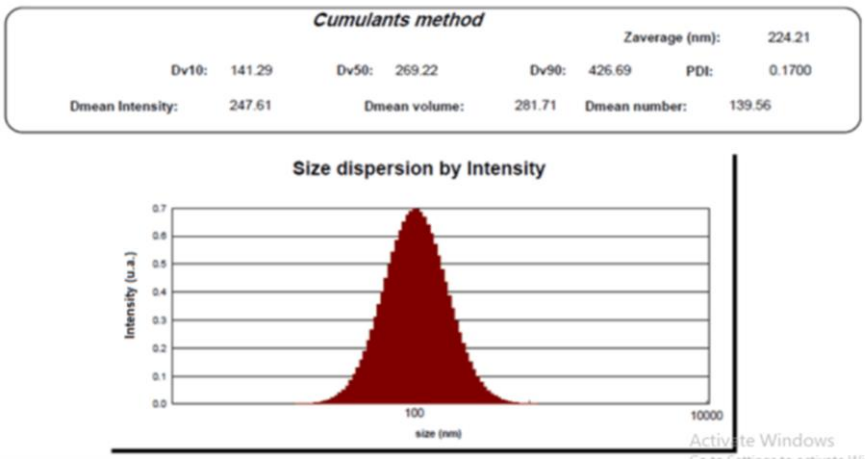

Figure 3. The findings from the DLS analysis indicated that nearly half of the components in the solution exhibited an average particle size of 139.56 nm.

Expression Levels of IL-8

The RNA purity test results indicated that the sample met the required purity standards, as illustrated in Figure 4. The levels of IL-8 in exosome samples were assessed and compared across different groups through quantitative real-time PCR analysis. The evaluation of IL-8 expression indicated that there was no significant difference between the control group and the experimental control group. As illustrated in Figure 5, a marked increase in IL-8 expression levels was noted in the PCOS group when compared to the control groups. In the treatment group, a significant reduction in IL-8 expression levels was observed. This finding suggests that the treatment may have a substantial impact on the modulation of IL-8 levels in the context of the studied conditions. The results highlight the potential differences in inflammatory markers between the various groups analyzed. Overall, the data underscores the importance of examining IL-8 expression in exosome samples, particularly in relation to PCOS and treatment effects. The contrasting expression levels between the PCOS group and the control groups, along with the decrease in the treatment group, provide valuable insights into the inflammatory processes involved and the potential therapeutic implications.

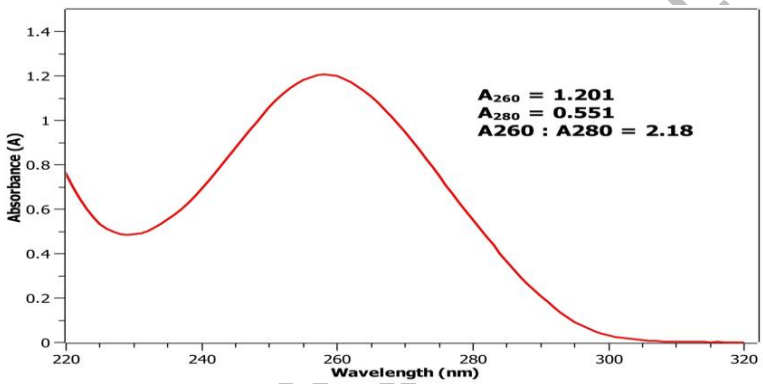

Figure 4. The UV spectrum with the A260 and A280 values and A260 : A280 ratio

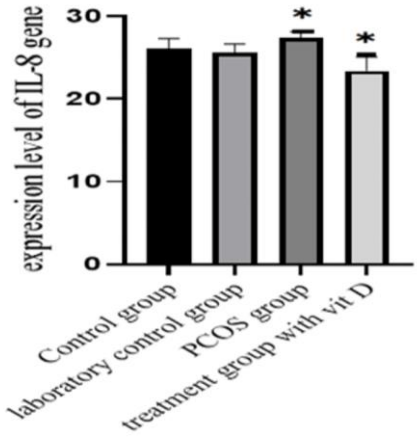

Figure 5. Analysis of IL- 8 gene expression levels in serum-derived exosomes. *Iindicated a significant difference of 0.05%.

Discussion

The research examined body weight and hormonal profiles associated with sex in different groups of rats over a four-week duration, demonstrating a steady increase in body weight across all groups with no significant differences observed. Hormonal assessments revealed that FSH and LH levels were higher in

the PCOS group compared to the control group, while vitamin D treatment resulted in a decrease in these hormones, indicating possible therapeutic benefits. Vaginal smear cytology initially showed no signs of reproductive readiness, but later evaluations indicated a transition towards estrous phases, reflecting changes in reproductive status. Morphometric analyses revealed a significant reduction in tertiary follicles and an increase in atretic follicles within the PCOS group, whereas vitamin D treatment appeared to enhance follicular dynamics. The evaluation of exosomes confirmed their size and structure, ensuring their integrity after isolation, and highlighted significant alterations in inflammatory markers, particularly IL-8, emphasizing the potential influence of vitamin D on reproductive health and inflammation in the context of PCOS.

PCOS is a prevalent and multifaceted condition that affects the reproductive, endocrine, and metabolic systems in women of adolescent and reproductive age. The underlying causes and mechanisms of PCOS are highly intricate, and the management of this condition continues to be a topic of debate among healthcare professionals. Consequently, treatment strategies are tailored to address the specific symptoms experienced by each individual patient (13). In recent years, the role of vitamin D supplementation has gained attention within the treatment protocols for PCOS, with varying reports on its therapeutic effectiveness among patients. Due to ethical and practical challenges associated with conducting human studies, researchers often turn to animal models of PCOS for their investigations. While no animal model has been identified that perfectly replicates the characteristics of PCOS in women, these models have proven valuable for preclinical research aimed at understanding the disorder's etiology and symptomatology (14). Animal studies have facilitated the exploration of hormonal imbalances and ovarian function related to PCOS, providing insights that may not be easily obtainable through human trials. As research continues, the findings from these models may contribute to the development of more effective treatment options and a deeper understanding of the complexities surrounding PCOS. This ongoing investigation is crucial for improving the quality of care for women affected by this condition (15).

Numerous studies indicate that animal models of PCOS induced by DHEA exhibit a range of symptoms akin to those observed in human cases of the condition. Furthermore, DHEA-induced PCOS has been associated with an early onset, typically around 15 years of age, mirroring the timeline of human PCOS development (16). Research to date has primarily concentrated on the metabolic disturbances resulting from vitamin D supplementation in both human and animal subjects. However, there is a notable scarcity of studies examining the impact of vitamin D treatment on ovarian morphology in PCOS, with no ultrastructural investigations conducted thus far. Consequently, our study aimed to explore the effects of vitamin D treatment on the ovaries of a pre-pubertal rat model of PCOS induced by DHEA, employing both light and electron microscopy techniques. In our current study, we found no significant differences in body weights among the various groups of rats. Previous research has indicated that postnatal DHEA treatment in rodents can induce characteristics of human PCOS, such as anovulation, polycystic ovaries, and hyperandrogenism, without affecting the animals' weight. At the conclusion of our experiment, biochemical analysis of blood samples revealed significantly elevated levels of LH, LH/FSH ratio, and testosterone in the DHEA-treated rats, consistent with findings from other studies utilizing DHEA-induced PCOS models (17).

One of the key features of PCOS is hyperandrogenism, with numerous studies suggesting that elevated levels of LH may significantly contribute to the abnormal production of androgens in the ovaries. It has been observed that a marked increase in LH levels leads to a higher LH/FSH ratio, while a relative deficiency in FSH can hinder the maturation of ovarian follicles (18). In our investigation of a PCOS model, we found that vitamin D treatment resulted in a reduction of elevated serum hormone levels. In the treatment group, serum FSH levels were comparable to those observed in the control group, whereas serum levels of LH, the LH/FSH ratio, and testosterone were reduced, although they remained elevated compared to the control group. Several studies have indicated that vitamin D treatment can lead to improvements in hormonal and metabolic parameters in women

with PCOS, although the underlying mechanisms remain to be fully elucidated. Further research is necessary to clarify how vitamin D influences these hormonal changes and to explore its potential therapeutic benefits for managing PCOS (19).

The morphometric analysis conducted in our study revealed no statistically significant differences in the counts of primary follicles, secondary follicles, and corpus luteum across the various groups. However, a notable reduction in the number of tertiary follicles was observed in the PCOS group, while a significant increase was recorded in the group receiving vitamin D treatment when compared to the PCOS group. Additionally, the counts of atretic and cystic follicles were elevated in the PCOS group, but these numbers decreased following vitamin D administration. These findings suggest that atresia is particularly pronounced in follicles that are at more advanced stages of development, leading to the formation of cystic structures in antral follicles and a reduction in tertiary follicle counts. In fact, the treatment group exhibited a decrease in both atretic and cystic follicles, alongside an increase in the number of tertiary follicles. This indicates a potential therapeutic effect of vitamin D on follicular health in the context of PCOS. The findings from our research suggest a link between the elevated testosterone levels observed in the PCOS group and the increased presence of atretic follicles when compared to the control group. This correlation underscores the impact of hormonal imbalances on follicular development and atresia, further contributing to the understanding of the pathophysiology of PCOS. The structural alterations noted in the follicles provide valuable insights into the mechanisms underlying this condition and highlight the need for further investigation into therapeutic interventions (20).

The findings from our research revealed a significant rise in atretic follicles associated with higher testosterone levels in the PCOS group compared to the control group. A variety of studies have demonstrated the essential role of androgens in the regulation of follicular atresia. It has been suggested that testosterone promotes somatic cell atresia in the ovaries of rats and mitigates the antiapoptotic influence of estrogens on the granulosa cells of maturing follicles (21). Additionally, a marked reduction was observed in the junctional complexes between granulosa cells, indicating a disconnection among these cells. This disconnection may further contribute to the impaired communication and support necessary for healthy follicular development. Overall, these results underscore the complex interplay between androgens and ovarian follicle dynamics in the context of PCOS (22).

The connection between vitamin D and IL-8 highlights the regulatory influence of vitamin D on immune responses. Research has shown that vitamin D may play a role in downregulating the production of IL-8, a pro-inflammatory cytokine. Vitamin D receptors (VDR) are found in various immune cells, and when vitamin D binds to these receptors, it can alter the expression of several cytokines, including IL-8. Studies suggest that maintaining sufficient vitamin D levels may lead to a reduction in IL-8 production when exposed to inflammatory triggers (23). The anti-inflammatory effects of vitamin D are closely associated with its impact on IL-8 levels. Elevated IL-8 is known to contribute to the development of numerous inflammatory diseases, making its regulation crucial. By lowering IL-8 concentrations, vitamin D may help alleviate chronic inflammation and oxidative stress, both of which are significant factors in the progression of various health conditions (24). Understanding the interplay between vitamin D and IL-8 carries important clinical implications for managing chronic inflammatory diseases. Individuals with low vitamin D levels often show increased IL-8 production, which can worsen conditions like asthma, chronic obstructive pulmonary disease (COPD), and inflammatory bowel disease. Therefore, vitamin D supplementation in these individuals may not only reduce IL-8 levels but also help in managing inflammation more effectively (25). Our findings indicate a notable increase in IL-8 levels among the pcos group. Furthermore, the administration of vitamin D resulted in a significant reduction of IL-8 levels, suggesting a potential therapeutic effect. Numerous studies have established a link between vitamin D levels and IL-8. For instance, certain research has demonstrated that vitamin D supplementation can lead to lower IL-8 concentrations in both serum and bronchoalveolar lavage fluid. Additionally, other investigations have shown that elevated serum vitamin D levels are associated with decreased IL-8 levels in individuals suffering from

inflammatory disorders (26). The relationship between vitamin D and IL-8 exists within a broader immune framework, involving a variety of cytokines and immune signals. Vitamin D not only affects IL-8 but also regulates other cytokines, including IL-6, IL-10, and tumor necrosis factor-alpha (TNF-α), thereby influencing the overall inflammatory environment. Understanding this relationship is crucial for advancing our knowledge of immune responses and inflammation. While current evidence supports the role of vitamin D in reducing IL-8 production, additional research is necessary to clarify the underlying mechanisms and assess the potential of vitamin D as a treatment for inflammatory diseases marked by high IL-8 levels. It is advisable for individuals to seek guidance from healthcare professionals before altering their vitamin D intake or treatment strategies, particularly in relation to specific health issues (27).

Research indicates that vitamin D deficiency is a prevalent risk factor for individuals with PCOS. Furthermore, vitamin D levels have been linked to the process of follicular development. Insufficient vitamin D can result in calcium dysregulation, which may lead to follicular arrest and contribute to menstrual irregularities and fertility issues in women diagnosed with PCOS (28). The correlation between vitamin D deficiency and PCOS is supported by various studies, highlighting the importance of adequate vitamin D levels for reproductive health. Low vitamin D levels can disrupt calcium balance in the body, which is crucial for normal follicular function. This disruption can ultimately result in complications such as irregular menstrual cycles and challenges with fertility (29). Addressing vitamin D deficiency may be essential for improving reproductive outcomes in women with PCOS. By ensuring sufficient vitamin D levels, it may be possible to enhance follicular development and mitigate the risks of menstrual and fertility dysfunction. Therefore, monitoring and managing vitamin D levels could play a significant role in the overall treatment and management of PCOS (30).

The research faced multiple limitations. Although the sample size was established based on similar studies, it only included six rats per group, which may have restricted the statistical power and the generalizability of the results. The 35-day duration of the experiment, while consistent with previous DHEA-induced PCOS models, did not consider the long-term impacts of vitamin D3 treatment. Additionally, the use of subcutaneous DHEA injections in a sesame oil/ethanol vehicle raised potential confounding issues, as interactions with the vehicle were not entirely ruled out, even with a laboratory control group present. Furthermore, the vitamin D3 treatment commenced one week after DHEA administration, preventing the possibility of concurrent treatment, which could have affected the results. Financial limitations also hindered further molecular analysis.

Conclusion

This study highlights the therapeutic potential of vitamin D in mitigating ovarian dysfunction in polycystic ovary syndrome (PCOS), demonstrating its favorable effects on endocrine parameters, inflammatory immune responses, and ovarian follicular architecture; however, the 35-day intervention period may have limited the assessment of long-term benefits, necessitating longitudinal studies to elucidate dose-duration dynamics and sustained efficacy. Future research should prioritize mechanistic investigations into vitamin D's role in ovarian steroidogenesis and immune modulation, alongside clinical trials to validate preclinical findings and establish evidence-based guidelines. The findings underscore vitamin D's promise as an adjunct therapy for PCOS, advocating for its integration into multimodal strategies to address reproductive and metabolic sequelae, though further exploration of temporal dynamics and long-term impacts is critical to optimize therapeutic applications and improve patient outcomes.

Artificial Intelligence Utilization for Article Writing

Artificial intelligence (AI) has not been used for writing this article.

Declarations

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Conflict of Interest

The authors declare that they have no competing interests.

Data Availability

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

Author Contributions

MP was instrumental in the collection of samples and the execution of tests, in addition to aiding in the development of the methodology and the preliminary draft of the manuscript. **AS** contributed by assisting in sample collection and manuscript writing. **JKH** and **SZ** played significant roles in the study's conception and experimental design. **MP** took charge of data curation, validation, statistical analysis, and the preparation of the manuscript, whereas JKH concentrated on reviewing and refining the written material.

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