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Impact of alcoholic extract from sea cucumber (*Holothuria parva*) on letrozole-induced polycystic ovary syndrome in adult female rats

Samaneh Askari¹, Mohammad Reza Jafarzadeh Shirazi^{1✉}, Masoumeh Ahmadi¹, Arezoo Khoradmehr², Nadiar M. Mussin³, Asset A. Kaliyev³, Farzad Mohammad Rezazadeh¹, Mohammad Dadpasand Taromsari¹, Ali Bustani⁴, Nader Tanideh^{5,6,7}, Kulyash R. Zhilibayeva⁸, Amin Tamadon^{6,7,9✉}

¹Department of Animal Science, College of Agriculture, Shiraz University, Shiraz, Iran²The Persian Gulf Marine Biotechnology Research Center, The Persian Gulf Biomedical Sciences Research Institute, Bushehr University of Medical Sciences, Bushehr, Iran³Department of Surgery No. 2, West Kazakhstan Marat Ospanov Medical University, Aktobe, 030019, Kazakhstan⁴Department of Animal Science, Science and Research Branch, Islamic Azad University, Tehran, Iran⁵Department of Pharmacology, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran⁶Stem Cells Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran⁷PerciaVista R&D Co., Shiraz, Iran⁸Department of Scientific Work, West Kazakhstan Marat Ospanov Medical University, Aktobe, Kazakhstan⁹Department of Natural Resources, West Kazakhstan Marat Ospanov Medical University, Aktobe, Kazakhstan

ABSTRACT

Objective: To explore the potential therapeutic impact of an alcoholic extract derived from the sea cucumber (*Holothuria parva*) on letrozole-induced polycystic ovary syndrome (PCOS) in adult female Sprague-Dawley rats.

Methods: Sixteen female rats, aged 8 to 10 weeks, with a mean weight of (200±20) g, were randomly assigned to four groups: the control, PCOS, and two treatment groups receiving sea cucumber extract and metformin, respectively. PCOS was induced by administering letrozole at a dose of 1 mg/kg to initiate the treatment period at 60 days of age. The study spanned four weeks, during which ovarian and uterine tissues were collected for histological examination, and blood samples were collected for hormonal levels.

Results: Significant decreases in luteinizing hormone/follicle stimulating hormone (LH/FSH) and testosterone and increases progesterone levels among groups treated with sea cucumber extract were observed. While no significant differences were observed in follicle-stimulating hormone (FSH) and testosterone levels, the distinctive variations in key hormones suggested a potential role for sea cucumber alcoholic extract in ameliorating PCOS symptoms.

Conclusions: This research highlights the moderate efficacy of sea cucumber extract as a therapeutic intervention for PCOS, providing a novel perspective in the search for effective remedies. The observed hormonal alterations, particularly in LH, estradiol, and progesterone, underscore the need for further exploration into the underlying mechanisms and for optimizing the application of sea cucumber extract in PCOS management.

KEYWORDS: Polycystic ovary syndrome; Sea cucumber; Letrozole; Metformin; Rats

Significance

Polycystic ovary syndrome (PCOS) is a common reproductive disorder affecting a significant percentage of women in their reproductive age. In the study, the observed hormonal changes, particularly in luteinizing hormone, estradiol, and progesterone levels, suggest a promising role for sea cucumber extract in PCOS management. These findings underscore the need for further research to elucidate the mechanisms and optimize treatment strategies for PCOS. This study adds to existing knowledge by investigating the therapeutic potential of sea cucumber extract in alleviating PCOS symptoms.

✉To whom correspondence may be addressed. E-mail: jafarzd@shirazu.ac.ir; amintamaddon@yahoo.com

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1. Introduction

Polycystic ovary syndrome (PCOS) is a multifactorial disorder affecting 10%-15% of women during their reproductive years, characterized by disturbances in the reproductive, endocrine, and metabolic systems, notably recognized as a leading cause of anovulatory infertility[1]. PCOS manifests through symptoms such as menstrual irregularities, hirsutism, obesity, and the presence of ovarian cysts[2]. The underlying pathophysiology involves hyperinsulinemia and hyperandrogenism, contributing to oligomenorrhea or amenorrhea, hirsutism, and polycystic ovaries, resulting in anovulation[2]. Despite ongoing research, the precise etiology of PCOS remains elusive, implicating genetic predisposition, insulin dysregulation, neuroendocrine abnormalities, hyperandrogenism, cortisol metabolism disturbances, contraceptive use, and stress as potential contributing factors[3,4]. Notably, PCOS is associated with aberrant endocrine profiles, featuring altered gonadotropin secretions, heightened ovarian steroidogenesis, and insulin resistance[5].

Current therapeutic interventions for PCOS encompass contraceptive pills, clomiphene citrate, metformin, tamoxifen, aromatase inhibitors (anastrozole, letrozole), glucocorticoids (dexamethasone), and gonadotropins[6,7]. In the contemporary era, natural compounds, particularly those derived from marine sources, have gained prominence in medical and pharmaceutical research[8,9]. With a history spanning over 3.5 billion years, marine life offers a vast source of natural compounds with remarkable biological activities. Despite less than four decades of exploration into the biological properties of marine chemical compounds, their abundance surpasses those from other sources significantly. Marine organisms present an extensive repertoire of bioactive compounds, with marine-derived compounds often surpassing terrestrial counterparts in abundance and biological efficacy[10]. The sea cucumber, a fascinating marine invertebrate belonging to the class Holothuroidea, has long captivated scientific interest for its diverse biological compounds and potential therapeutic properties[11,12]. With a spindle-shaped body and a unique anatomy featuring a ventral mouth surrounded by numerous tentacles, sea cucumbers inhabit marine ecosystems worldwide[13]. Beyond their ecological role, sea cucumbers are recognized for their rich biochemical composition, including peptides, collagen, and various bioactive molecules[14]. Traditional medicine has often embraced sea cucumbers for their purported health benefits, and contemporary research continues to unveil their pharmacological potential[14].

Considering the adverse effects associated with chemical drugs for PCOS treatment[15,16], identifying marine-derived alternatives becomes imperative. Sea cucumbers, recognized in traditional medicine for their therapeutic properties, represent a valuable marine organism with diverse bioactive compounds[11,12]. Studies

on sea cucumber extracts have demonstrated cytotoxic, antioxidant, antibacterial, anti-inflammatory, antiviral, and anticancer properties[17–20]. Considering the documented impact of sea cucumber extract on reproductive functions[21], this study posits the potential efficacy of the alcoholic extract of *Holothuria (H.) parva* in treating PCOS. The aims of the present study were: 1) to investigate the potential therapeutic effects of sea cucumber extract in ameliorating PCOS; 2) examine morphometric and morphological changes in ovarian follicles induced by sea cucumber *H. parva* extract; and 3) assess hormonal variations [estrogen, estradiol, progesterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH)] and quantify insulin resistance.

2. Materials and methods

2.1. Identification of sea cucumber as *H. parva*

H. parva is characterized by its small size (<10 cm), spindle-shaped body with relatively thick extremities, and integument. It features a ventral mouth surrounded by 20 small tentacles and a terminal anus. The identification of sea cucumbers was facilitated by scrutinizing the skeletal elements (ossicles) present in different body parts. The confirmation of *H. parva* species was established through the examination of separated ossicles, aligning with identification keys[19,22].

2.2. Preparation of sea cucumber alcoholic extract

Upon removal from the freezer and thawing, the sea cucumber was meticulously cleaned. Subsequently, longitudinal incisions were made from the mouth towards the anus, facilitating the evacuation of the visceral cavity. The wall segments were further reduced into smaller fragments. Following this, the sample underwent freeze-drying at 45 °C for a duration of 2 days to achieve complete desiccation. The dried specimen was then finely crushed using a grinder and subsequently pulverized into a homogeneous powder using a mortar. All stages of the extraction process were conducted under sterile conditions and within a fume hood. The prepared powder was combined with 70% ethanol, subjected to filtration for 6 h using a Soxhlet apparatus, and then transferred to a rotary evaporator for hydroalcoholic extraction under vacuum conditions.

2.3. Animal model and experimental design

Sixteen 56-day-old female Sprague-Dawley rats with an average weight of (200±20) g, obtained from the Center of Comparative and Experimental Medicine at Shiraz University of Medical Sciences, were used in this study. The rats were acclimatized in the

controlled animal housing environment for at least 7 days before any therapeutic interventions. PCOS was induced in 12 rats by administering 1 mg/kg body weight of letrozole *via* gavage for 21 days. After PCOS induction, the rats were randomly assigned to four groups: the control, PCOS, sea cucumber treated (500 µg/kg body weight for 28 days after establishing the reproductive cycle), and metformin treated (150 mg/kg body weight for 28 days after establishing the reproductive cycle), with 4 rats in each group.

The animals were kept in standard laboratory cages, with each cage housing no more than four rats to ensure adequate space and comfort. The housing environment was controlled to maintain consistent conditions. The temperature was maintained at $(22\pm 2)^{\circ}\text{C}$ with a relative humidity of $(50\pm 10)\%$. The light-dark cycle was set at 12 h of light and 12 h darkness, with lights on at 7:00 a.m. and off at 7:00 p.m. Rats were provided with *ad libitum* access to standard rodent chow and fresh water. The diet and water were checked and replenished daily to ensure continuous availability.

The reproductive cycle of the rats was determined. In proestrus, numerous small, round, nucleated epithelial cells with similar morphology, basophilic nuclei, and often seen in clusters are present, along with some cornified epithelial cells. During estrus, an abundance of cornified epithelial cells without nuclei is observed, along with a few well-developed nucleated epithelial cells. Metestrus is identified by a mixture of predominantly cornified epithelial cells, both with and without nuclei, along with some neutrophils. The smear during this phase shows mostly cornified epithelial cells, neutrophils, and a few nucleated epithelial cells. In diestrus, there is a higher number of neutrophils and a lower number of cornified epithelial cells.

To determine the sample size for this study, an analysis of variance (ANOVA) power analysis was conducted. The calculation involved estimating the effect size, power, and significance level. Effect size was assumed to be moderate, with a Cohen's *d* of 0.5, based on previous studies or pilot data. Power was set at 0.8 (80%), indicating the probability of detecting a true effect if it exists. The significance level (α) was set at 0.05, representing the probability of rejecting the null hypothesis when it is true.

Inclusion and exclusion criteria for animals and data points were established *a priori*. Female Sprague-Dawley rats were selected from the Center of Comparative and Experimental Medicine at Shiraz University of Medical Sciences. Animals exhibiting signs of disease or abnormalities that could interfere with study outcomes were excluded. Data points included in the analysis were those obtained from eligible animals that met the inclusion criteria. Data from animals that did not complete the full study period or did not meet the inclusion criteria at the time of data collection were excluded from the analysis.

Randomization was used to allocate experimental units to control and treatment groups, with the randomization sequence generated

using a computer-based random number generator to ensure unbiased group assignments. To minimize potential confounders, the order of treatments and measurements was randomized, and the location of animal cages was rotated regularly to avoid any location bias. If any potential confounders were identified during the study, they were controlled as much as possible to maintain the integrity and reliability of the study results.

To ensure the integrity of the study, blinding was implemented at various stages. During the allocation phase, only the researcher responsible for generating the randomization sequence and assigning the animals to the control and treatment groups was aware of the group allocations. This individual was not involved in the conduct of the experiment, outcome assessment, or data analysis. During the conduct of the experiment, the researchers administering the treatments and handling the animals were blinded to the group allocations. This was achieved by labeling the cages with codes that did not reveal the treatment groups. Outcome assessments were performed by researchers who were also blinded to the group allocations. This ensured that the measurements and evaluations were conducted without bias. Finally, the data analysis was carried out by a separate team of researchers who were blinded to the group assignments. The data were coded and anonymized before analysis to maintain the blinding throughout the entire process. By implementing these blinding procedures, we aimed to minimize bias and ensure the reliability and validity of the study outcomes.

2.4. Blood sampling, hormonal analysis, and tissue histopathology

Blood samples were collected from rats *via* cardiac puncture under ether anesthesia. Serum concentrations of testosterone, estradiol, progesterone, FSH, and LH were measured using radioimmunoassay. Ovarian and uterine tissues from rats were fixed, sectioned, and stained with hematoxylin and eosin. Histomorphometric evaluations of ovarian structures and uterine layers were performed using ImageJ software.

2.5. Statistical analysis

GraphPad Prism software (GraphPad Prism 9.5.1.733, Boston, US) and IBM SPSS Statistics (IBM Corp. Released 2019, Version 26.0, Armonk, US) were utilized for data analysis and graphical representation. Data were assessed for normal distribution using the Kolmogorov-Smirnov test. *Chi-square* test was used to compare the portions of estrus cycle. Other logarithmically normalized data were subjected to one-way ANOVA analysis and LSD *post hoc* test. Data are presented as mean and standard deviation or percent in graphs. Statistical significance was set at $P < 0.05$.

2.6. Ethics statement

This research adhered to the pertinent guidelines and regulations set forth by the Animal Care Committee of Shiraz University Studies. All experimental procedures received ethical approval from the Research Committee of Shiraz University, with the assigned approval number 98gcbm148075 on June 18, 2020.

3. Results

3.1. Sea cucumber and metformin treatment influence estrous cycles

The examination of vaginal smears during PCOS induction and subsequent treatment revealed irregular estrous cycles in the PCOS group, primarily dominated by the diestrus phase (Figure 1A). However, both metformin and sea cucumber treatment groups exhibited a gradual normalization of estrous cycles, with the control group surpassing completed cycles (Figure 1B). Significant differences were observed in estrous cycles between the control and PCOS groups ($P<0.05$). The irregular cycles and preponderance of diestrus stages in the PCOS group were markedly improved by both metformin and sea cucumber treatments ($P<0.05$).

3.2. Impact of PCOS induction on body weights

Weight measurements at various time points revealed a significant increase in PCOS-induced rats' weight compared to the control group ($P<0.05$) (Figure 1C). Conversely, metformin and sea cucumber treatments resulted in a significant reduction in weight, emphasizing the potential therapeutic effect of both treatments compared to the PCOS group ($P<0.05$).

3.3. Sea cucumber modulates hormonal imbalances induced by PCOS

Significant variations in mean serum LH concentrations were observed, with metformin-treated rats differing significantly from the control, PCOS, and sea cucumber-treated groups ($P<0.05$) (Figure 2A). Sea cucumber treatment did not significantly differ serum LH concentrations compared to the control and PCOS groups. Sea cucumber treatment significantly increased FSH compared to the control and PCOS groups ($P<0.01$) (Figure 2B). A notable difference in LH to FSH ratio was observed in the metformin and sea cucumber-treated group ($P<0.05$) (Figure 2C). Sea cucumber-treated rats in the control, and metformin-treated groups exhibited significantly lower testosterone levels compared to the PCOS group ($P<0.001$) (Figure 2D). Additionally, estrogen levels were reduced in

the sea cucumber-treated group compared to the PCOS group but the difference was not significant ($P>0.05$) (Figure 2E). Progesterone levels, while significantly different between the control and PCOS groups, showed no significant change in the metformin and sea cucumber-treated groups (Figure 2F).

3.4. Ovarian follicle counts and morphology

Histological examination revealed significant differences in atretic follicles among groups ($P<0.05$) (Figure 3). While the PCOS group exhibited limited corpus luteum, metformin and sea cucumber-treated groups displayed increased counts of primary follicles and corpus luteum ($P<0.05$). Sea cucumber treatment also significantly reduced cystic follicles compared to the PCOS group ($P<0.05$).

3.5. Follicular layers' thickness and antral areas

The PCOS group demonstrated a non-significant larger antral area compared to the other groups (Figures 4 and 5). Metformin and sea cucumber-treated groups exhibited a non-significant decrease in granulosa area, with no significant change in theca area. The thickness of the theca layer increased significantly in the PCOS group, while metformin treatment led to a reduction in thickness ($P<0.05$).

3.6. Uterine tissue modifications

Sea cucumber treatment resulted in a significant reduction in luminal area and endometrial glands, and increased total uterine area compared to the PCOS group ($P<0.05$) (Figure 6). The mean gland count per area was significantly lower in the sea cucumber-treated group compared to the PCOS group ($P<0.05$). Comparisons of different uterine layers relative to the whole uterus indicated significant reductions in the perimetrial layer and increase in endometrial layer in sea cucumber group ($P<0.05$) (Figure 7).

4. Discussion

In this study, we investigated the efficacy of sea cucumber hydro-alcoholic extract, recognized for its flavonoids with antioxidant properties and anti-inflammatory attributes, in mitigating the symptoms of letrozole-induced PCOS. Individuals with PCOS typically exhibit elevated LH to FSH ratios and increased insulin resistance[23]. Prior research, such as that involving sea cucumber, has demonstrated therapeutic effects of sea cucumber on female infertility[7,24]. Our findings suggest that the observed alleviation of PCOS symptoms in rats treated with sea cucumber may be linked to a reduction in LH and potentially other hormone levels.

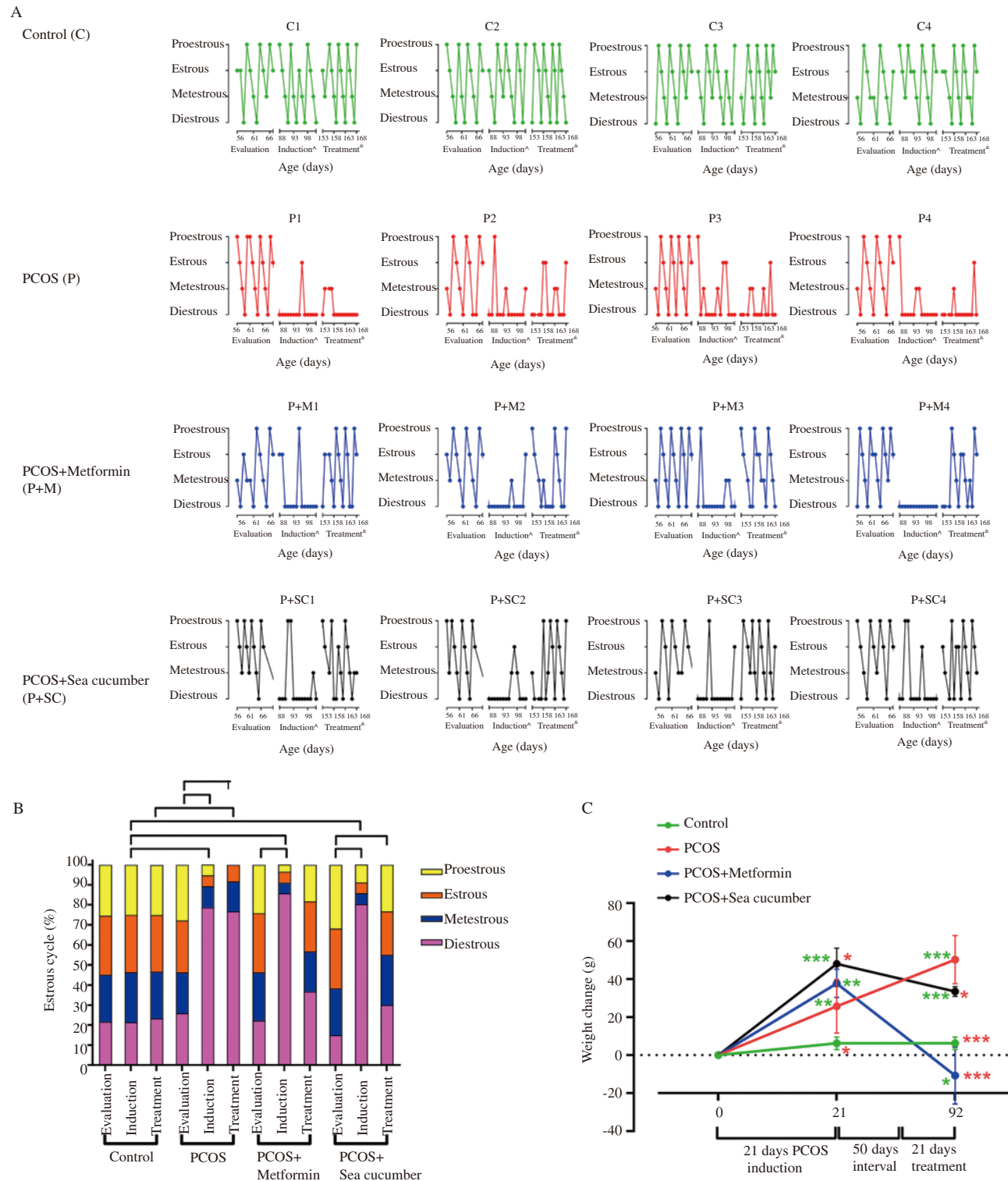


Figure 1. The therapeutic impact of sea cucumber (*Holothuria parva*) on the stages of the estrus cycle and body weights following the induction of polycystic ovary syndrome (PCOS) in adult rats through letrozole administration. A) Diagrams depict the various stages of the estrus cycle in the control group, PCOS group, metformin-treated PCOS rats, and sea cucumber extract-treated PCOS rats. Induction^Δ: The last 14 days of induction; Treatment^Δ: The last 14 days of treatment. B) The proportion of estrus cycle stages is presented for three study periods: before induction (evaluation), during induction (induction), and after induction (treatment) in the four study groups. Lines positioned above the columns signify significant differences between the groups ($P<0.05$). C) Mean and standard deviation of body weights in rats are showcased across different study periods for the four groups. Stars matching the lines' colors indicate statistical differences on each day. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

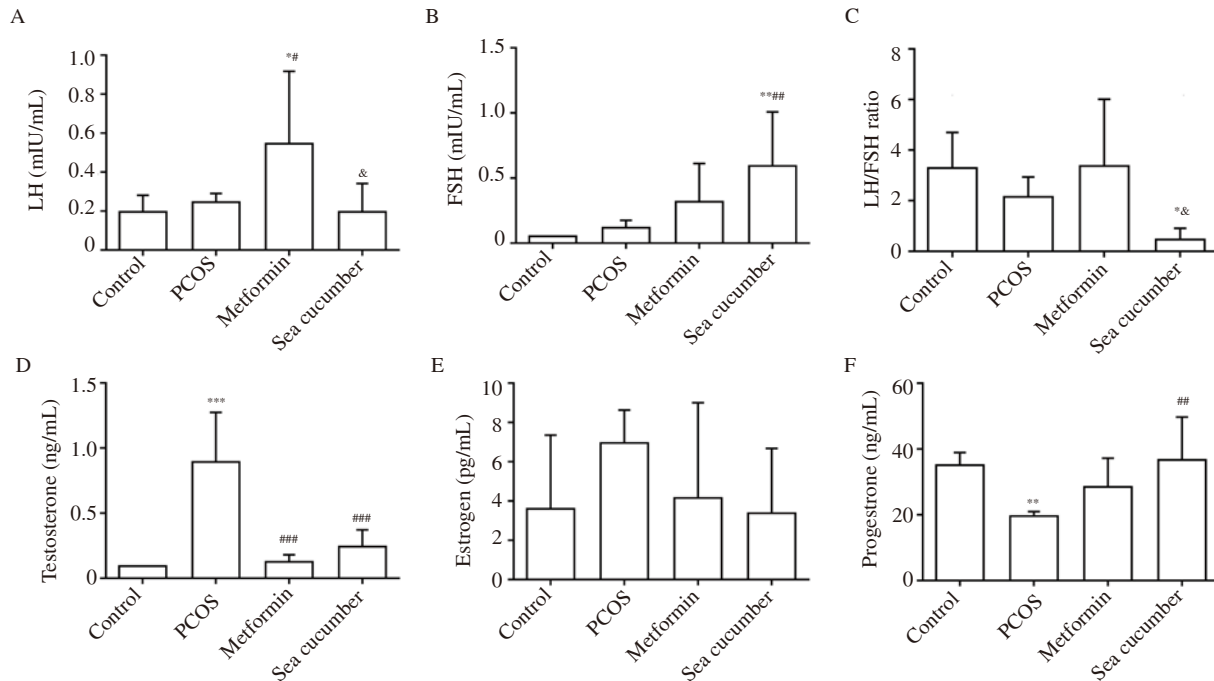


Figure 2. Serum hormone concentrations observed in various groups. A) Luteinizing hormone (LH), B) Follicle-stimulating hormone (FSH), C) LH/FSH, D) Testosterone, E) Estrogen, and F) Progesterone. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: Compared to the control group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$: Compared to the PCOS group; & $P < 0.05$: Compared to metformine group.

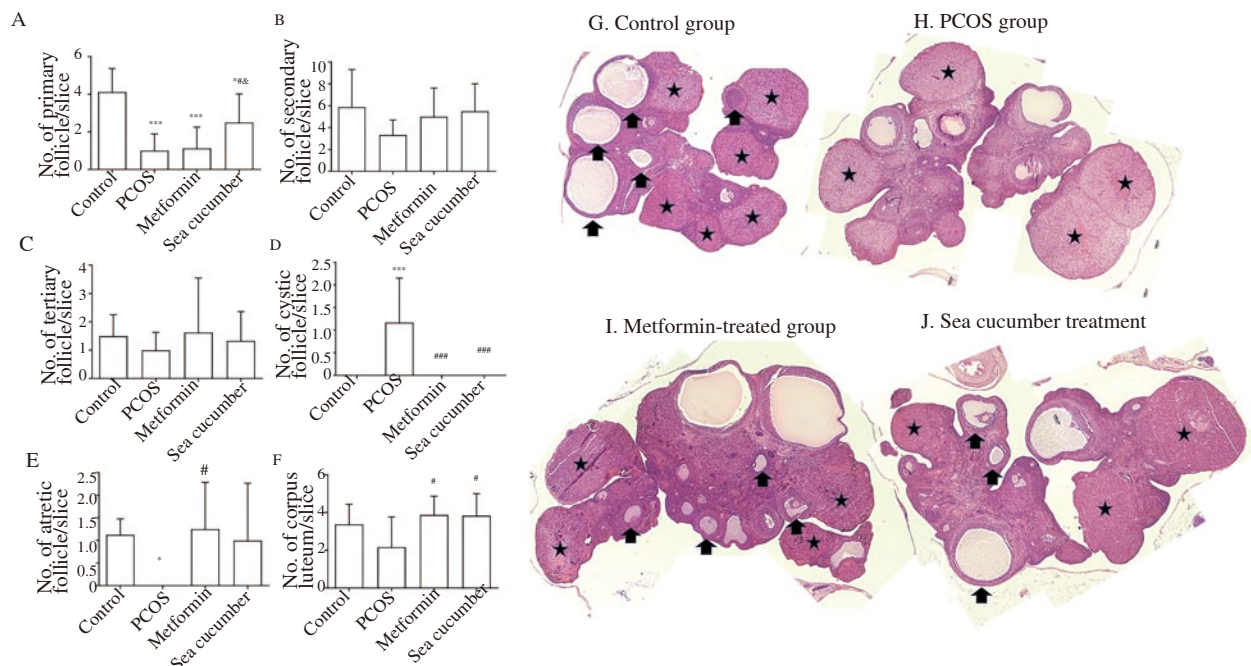


Figure 3. Ovarian follicle counts (A-F) observed in different groups. Hematoxylin and eosin (H&E) stained images of histological changes in the ovaries of rats with letrozole-induced polycystic ovary syndrome (PCOS) are shown in (G) to (J), highlighting the identification of corpus luteum (black stars) and normal tertiary follicles (arrows). The groups depicted are as follows: G. Control group, H. PCOS group, I. Metformin-treated group, and J. Sea cucumber treatment group. * $P < 0.05$, *** $P < 0.001$: Compared to the control group; # $P < 0.05$, ### $P < 0.001$: Compared to the PCOS group; & $P < 0.05$: Compared to metformine group.

The consistent elevation in LH poses a significant challenge in PCOS, contributing to continuous LH elevation, secondary follicle development, and the formation of ovarian cysts[25]. Our results align with this pattern, indicating a decrease in LH levels in the sea cucumber-treated group compared to the PCOS group, potentially resulting in a reduction in the number of follicles, a crucial factor in

diminishing cysts and improving ovarian symptoms in PCOS.

Women with PCOS often experience abnormal production of progesterone and estradiol in granulosa cells, leading to altered responses to gonadotropins[26]. Our study demonstrated a significant reduction in testosterone levels in the sea cucumber-treated group, attributed to the plant's impact on decreasing the proliferation and

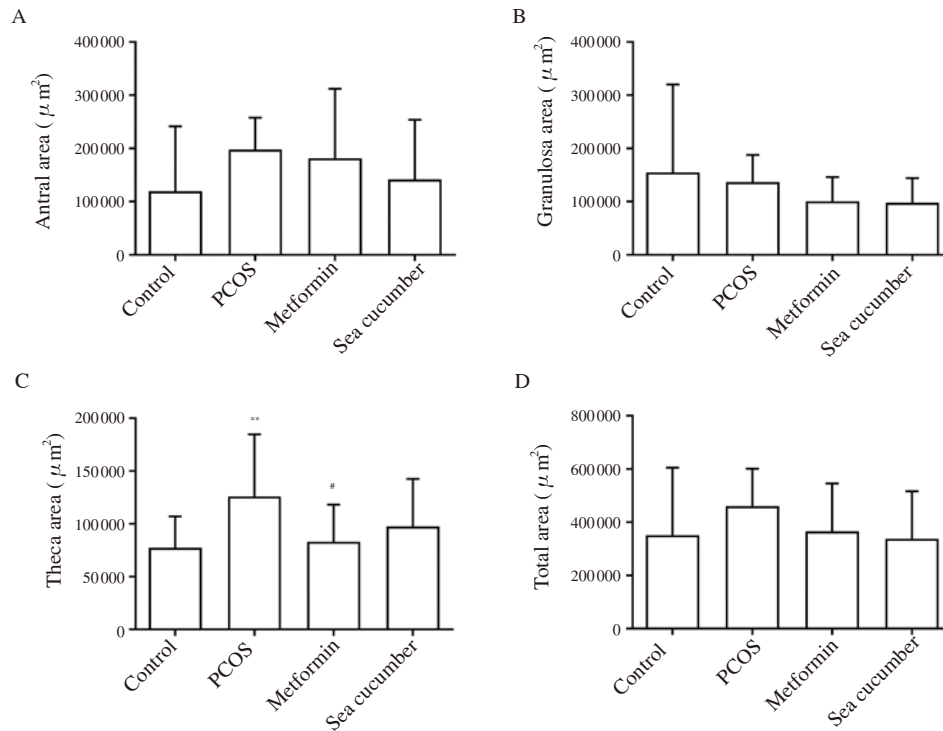


Figure 4. The area of various layers of tertiary follicles observed across different groups. ** $P<0.01$: Compared to the control group; * $P<0.05$: Compared to the PCOS group.

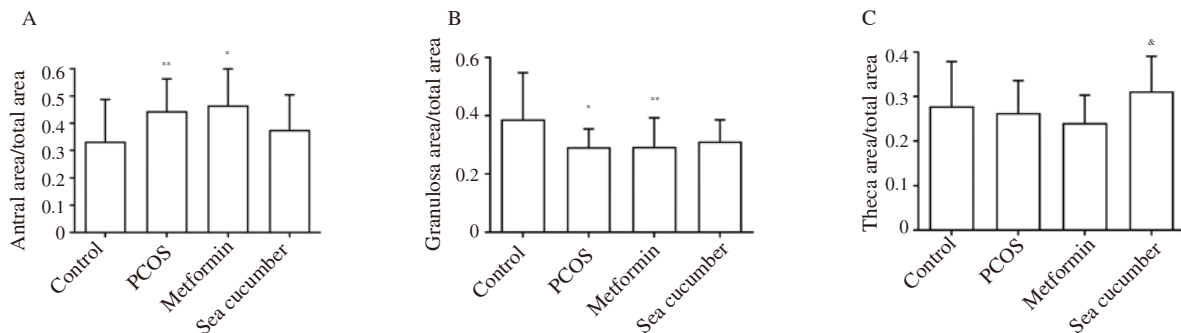


Figure 5. The area of different layers of tertiary follicles relative to total follicles in various groups. * $P<0.05$, ** $P<0.01$: Compared to the control group; & $P<0.05$: Compared to metformine group.

thickness of the follicular layers. Additionally, sea cucumber extract reduced estradiol levels and increased progesterone, possibly due to an elevation in corpus luteum in treated rats. The observed increase in the growth and development of primary follicles in response to the sea cucumber extract further indicates its potential in ovarian activation.

The role of sea cucumber extract as a hormone secretion regulator is a significant aspect of its therapeutic potential in managing PCOS[27]. Our findings indicate that the extract can modulate the levels of key hormones involved in the pathophysiology of PCOS, such as estrogen, progesterone, and testosterone. By normalizing these hormone levels, sea cucumber extract helps restore ovarian function and alleviate symptoms associated with hormonal imbalances[28]. The regulatory effect on hormone secretion is likely mediated through its bioactive compounds, which interact with endocrine pathways to promote hormonal homeostasis. This

hormonal regulation is crucial for managing PCOS, as it directly addresses the underlying hormonal disturbances that contribute to the development and progression of the condition. Therefore, while the antioxidant and anti-inflammatory properties of sea cucumber extract are vital, its ability to act as a hormone secretion regulator adds a critical dimension to its therapeutic efficacy, making it a promising candidate for holistic PCOS treatment.

Furthermore, sea cucumber extract exhibited hypoglycemic effects, reducing glucose, triglyceride, and lipid levels in rats[29]. Given the association between hyperglycemia and markers of lipid peroxidation in diabetes, sea cucumber's impact on inhibiting glucose-6-phosphatase and gluconeogenesis[30] suggests a potential mechanism for symptom reduction in PCOS. The role of sea cucumber as an anti-inflammatory agent, inhibiting COX-2 and lipoxygenase enzymes[31], may contribute to the observed reduction in inflammation associated with PCOS.

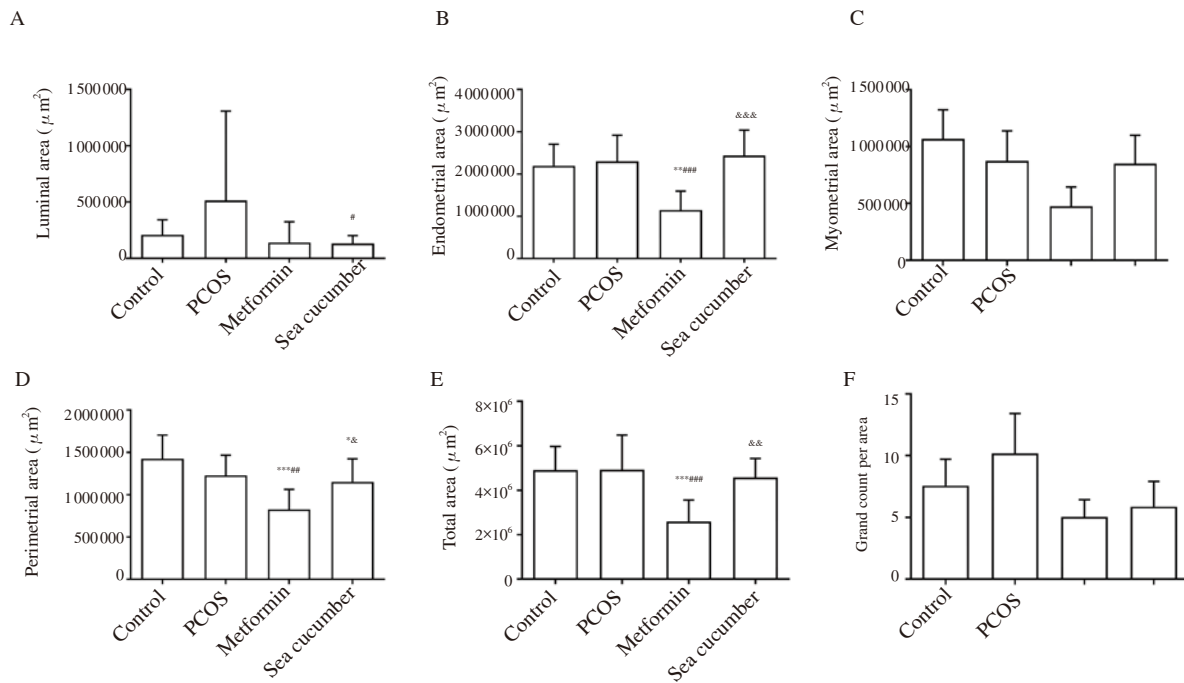


Figure 6. The area of different regions of uterine tissue in various groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: Compared to the control group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$: Compared to the PCOS group; & $P < 0.05$, && $P < 0.01$, &&& $P < 0.001$: Compared to metformine group.

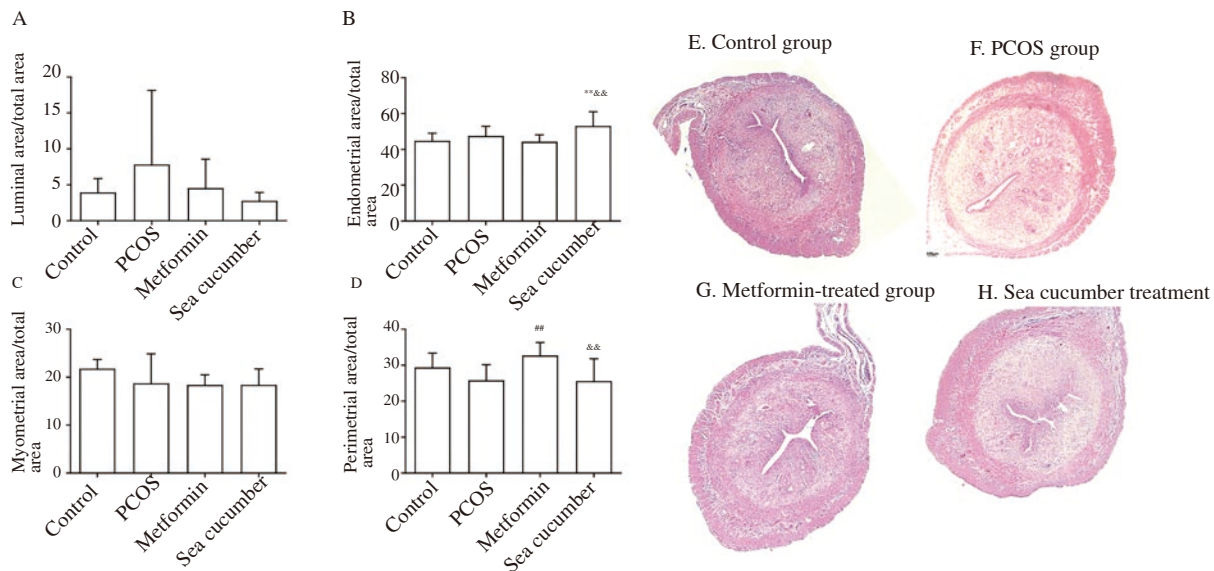


Figure 7. Uterine tissue relative to the entire uterus in various groups. ** $P < 0.01$: Compared to the control group; ## $P < 0.01$: Compared to the PCOS group; && $P < 0.01$: Compared to metformine group. H&E-stained images are shown in E to H. The groups depicted are as follows: E. Control group, F. PCOS group, G. Metformin-treated group, and H. Sea cucumber treatment group.

The present study primarily focused on the effects of sea cucumber hydro-alcoholic extract on specific aspects of letrozole-induced PCOS. The complex nature of PCOS, involving multifaceted hormonal, metabolic, and reproductive changes, was not comprehensively explored, leaving aspects such as long-term impacts, genetic factors, and broader metabolic implications unaddressed. In summary, while this study provides valuable insights into the potential benefits of sea cucumber in alleviating PCOS symptoms, these findings are constrained by the outlined limitations.

Addressing these limitations in future research endeavors will enhance the robustness and applicability of the outcomes to clinical scenarios.

In conclusion, our results suggest that sea cucumber, with its antioxidant and anti-inflammatory effects, holds promise in improving ovarian follicle conditions and alleviating tissue symptoms associated with PCOS. The observed reduction in PCOS symptoms may be attributed to the antioxidant, anti-inflammatory, and hypoglycemic properties of sea cucumber, highlighting its

potential therapeutic benefits in PCOS treatment. While the findings of this study contribute valuable information to the understanding of PCOS and may have implications for human biology, further research is needed to confirm and extend these findings to other species and experimental conditions, including human populations. However, the limitations of our study should be considered, and further research is warranted to explore the full spectrum of sea cucumber's effects on PCOS.

Conflict of interest statement

Author Amin Tamadon was employed by PerciaVista R&D Co. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Samaneh Askari, Mohammad Reza Jafarzadeh Shirazi, and Amin Tamadon conceptualized and designed the study. Samaneh Askari, Masoumeh Ahmadi, Arezoo Khoradmehr, and Farzad Mohammad Rezazadeh were involved in data collection and laboratory work. Mohammad Dadpasand Taromsari and Ali Bustani contributed to the animal management and data acquisition. Nadiar M. Mussin, Asset A. Kaliyev, and Kulyash R. Zhilibayeva assisted with the experimental design and provided critical insights during the data analysis. Nader Tanideh supervised the histological and pharmacological aspects of the research. Mohammad Reza Jafarzadeh Shirazi and Amin Tamadon oversaw the project and provided guidance throughout. All authors contributed to the drafting and revision of the manuscript and approved the final version for publication.

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