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Secretome improves anti-Müllerian hormone level and ovarian function in a premature ovarian insufficiency mice model

Sekretom, prematüre yumurtalık yetmezliği fare modelinde anti-Müllerian hormon düzeyini ve yumurtalık fonksiyonunu iyileştiriyor

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Abstract

Objective: To evaluate the efficacy of secretome in improving the anti-Müllerian hormone (AMH) level and ovarian weight and restoring ovarian function in the premature ovarian insufficiency (POI) model mice.

Materials and Methods: A randomized, post-test-only control-group design was conducted on 18 mice, which were divided into three groups: A control group and two case groups injected with a secretome. Blood samples were analyzed for the AMH level with an enzyme-linked immunosorbent assay kit; ovarian weight was measured; and hematoxylin-eosin staining was used to measure and categorize follicles at each stage.

Results: The cyclophosphamide (CTX) group showed significant differences in ovarian weight, AMH, and the numbers of primary, secondary, antral, and atretic follicles compared with the control group, indicating induction of premature ovarian failure. Follicular development was improved in the CTX-secretome group compared to the CTX group, with significantly increased ovarian weight and AMH, increased numbers of primary, secondary, and antral follicles, and decreased numbers of atretic follicles. However, the results showed a significant difference between the CTX-secretome and the control group.

Conclusion: Our findings show that secretome therapy improved POI management, but the results have not yet restored normal ovarian function. It still does not achieve the same functional state as normal ovarian function. Further research, particularly involving different doses of secretome, is necessary to validate these findings.

Keywords: Primary ovarian insufficiency, anti-Müllerian hormone, secretome, ovarian function tests

Öz

Amaç: Prematüre over yetmezliği (POY) model farelerde anti-Müllerian hormon (AMH) düzeyini ve over ağırlığını iyileştirmede ve over fonksiyonunu geri kazandırmada sekretomenin etkinliğini değerlendirmektir.

Gereç ve Yöntemler: On sekiz fare üzerinde, yalnızca test sonrası kontrol grubu tasarımı uygulanarak, fareler üç gruba ayrıldı: Bir kontrol grubu ve sekretom enjekte edilmiş iki olgu grubu. Kan örnekleri, enzim bağlantılı immünosorbent test kiti ile AMH düzeyi açısından analiz edildi; yumurtalık ağırlığı ölçüldü; ve her aşamada folikülleri ölçmek ve kategorize etmek için hematoksilen-eozin boyama yöntemi kullanıldı.

PRECIS: We have evaluated that secretome therapy improved premature ovarian insufficiency management in mice model experimental design.

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Bulgular: Siklofosfamid (CTX) grubu, kontrol grubuyla karşılaştırıldığında over ağırlığı, AMH ve primer, sekonder, antral ve atretik folikül sayıları açısından anlamlı farklılıklar gösterdi ve bu da erken over yetmezliğinin indüklendiğini gösterdi. CTX-sekretom grubunda foliküler gelişim, CTX grubuna kıyasla iyileşti; over ağırlığı ve AMH anlamlı şekilde arttı, primer, sekonder ve antral folikül sayıları arttı ve atretik folikül sayıları azaldı. Ancak sonuçlar, CTX-sekretom grubu ile kontrol grubu arasında anlamlı bir fark olduğunu gösterdi.

Sonuç: Bulgularımız, sekretom tedavisinin POY yönetimini iyileştirdiğini, ancak sonuçların henüz normal yumurtalık fonksiyonunu geri kazandırmadığını göstermektedir. Yine de normal yumurtalık fonksiyonuyla aynı işlevsel duruma ulaşmamaktadır. Bu bulguları doğrulamak için, özellikle farklı sekretom dozlarını içeren daha fazla araştırmaya ihtiyaç vardır.

Anahtar Kelimeler: Primer over yetmezliği, anti-Müllerian hormon, sekretoma, over fonksiyon testleri

Introduction

Premature ovarian insufficiency (POI) is defined as the cessation of ovarian function before the age of 40[1]. The incidence is approximately 1% in women under 40 years of age and 0.1% in women under 30 years of age[2]. Due to the side effects of menopause at an earlier age, women are advised to take hormonal replacement therapy (HRT) until the natural age of menopause, which is around 51 years[3]. However, some contraindications and side effects related to HRT may occur. One of the most challenging issues is whether this occurs in women who want to preserve their fertility or are trying to conceive.

The incidence of cancer has been increasing recently, especially in younger age groups, with approximately 10% of the 6.6 million young adults aged 15-39 years diagnosed with cancer annually[4]. One current approach to cancer management is the use of chemoradiotherapy to improve survival. However, chemoradiotherapy can alter DNA synthesis and the RNA transcriptome, and indirectly accelerate cell death, particularly by reducing the number of dormant follicles in the ovary[5,6]. This process is recognized as an emerging cause of POI in women younger than the typical age at menopause.

Some studies on stem cells have emerged that aim to improve ovarian function. Because of limitations of stem cells, such as the risk of rejection and high cost, the application of the secretome for certain diseases is emerging. The secretome, which consists of proteins, including extracellular matrix proteins, vesicle proteins, and proteins shed from the cell membrane, has the potential to promote angiogenesis, reduce inflammation, and evade immune responses, which together may facilitate restoration of ovarian function and fertility[7].

Anti-Müllerian hormone (AMH) is recognized as a biomarker for assessing functional ovarian reserve[8]. In the ovary, AMH is expressed by granulosa cells of preantral and early antral follicles, indicating the presence of an active follicular pool. A decline in AMH levels reflects a reduction in the number of primordial and developing follicles. Unlike other hormonal markers, AMH is minimally affected by menstrual cycle fluctuations, making it a reliable parameter for evaluating ovarian reserve[8-10].

This research aims to evaluate ovarian function by assessing ovarian reserve through AMH serum levels measured in peripheral blood and by counting follicles on hematoxylin-eosin (H&E)-stained histological sections after administration of the secretome in a cyclophosphamide (CTX)-induced menopausal mouse model.

Materials and Methods

Study Design

The institution at Universitas Udayana approved this experimental study. The Research Ethics Committee, Faculty of Medicine, Universitas Udayana (approval number: 2713/UN14.2.2.VII.14/LT/2024, date: 11.11.2024). This research was conducted from December 2024 to May 2025 at the Faculty of Veterinary, Universitas Udayana, and at the Histology Laboratory of the Faculty of Medicine, Universitas Udayana Denpasar, Bali.

This study used a posttest-only experimental group design and employed wild-type (n=18) female mice aged 6 weeks (20-22 g), which were housed on a 12-hour light/12-hour dark cycle with free access to mouse food and water. Animal procedures and treatments were conducted at the Veterinary Lab of Universitas Udayana in strict accordance with provisions for the protection of experimental animals.

The sample size was determined based on previous studies using a similar CTX-induced POI model[11], in which six animals per group were sufficient to detect significant hormonal and histological changes with statistical power >0.8 and α=0.05. No formal a priori power analysis was conducted. However, the chosen group size aligns with standard practice for reproducible outcomes in rodent POI models.

The secretome used in this study was generated by Regenic KALBE Laboratory Indonesia [certified under current Good Manufacturing Practices (cGMP)] and manufactured by PT Bifarma Adiluhung, Jakarta, Indonesia (batch number RUCM-SFP-080125-1). The product, known as Regenic Hypoxia UCMSC-Secretome, was derived from human umbilical cord mesenchymal stem cells cultured under hypoxic and serum-free conditions to stimulate secretion of paracrine factors. The conditioned medium was collected, centrifuged to remove cell debris, filtered through a 0.22 µm membrane, and concentrated under cGMP standards. Each vial contained 1.5 mL of sterile secretome, confirmed to be free of cells, mycoplasma, and endotoxin (<0.25 EU/mL).

The protein profile consisted primarily of pro-collagen I (889,550 pg/mL), keratinocyte growth factor (111.93 pg/mL), vascular endothelial growth factor (25.5 pg/mL), basic fibroblast

growth factor (20.69 pg/mL), and stromal cell-derived factor-1 (823.5 pg/mL).

After a two-week acclimatization period, the rats were administered CTX for two weeks. One week after the final CTX dose (day 21), the animals received secretome by intramuscular injection (0.1 mL per injection) every two days (days 22, 24, 26, and 28). Samples for data analysis were collected on day 29.

Premature Ovarian Insufficiency Mice Model Preparation

The 6 weeks old Wistar mice (n=18) were observed for the first 1 week to ensure that all mouse was in their estrous cycle, before they were divided into three groups of 6 animals as follow: Group A: Control group; Group B: CTX group (CTX injection) as the POI group, and Group C: POI with secretome group (CTX followed by secretome injection). All injections were administered intraperitoneally, and the chemotherapy dose was given continuously for 14 days. The control group received 0.2 mL of physiological saline daily, compared with the other two groups. Mice in the POI group were given 50 mg/kg CTX on the first day, then received 4 mg/kg per day from day 2 through day 14 to reliably induce a model of POI while minimizing sample loss. This regimen was selected based on a study by Elahi et al.[11], which showed that a high loading dose followed by lower daily maintenance doses of CTX effectively induces follicle loss, endocrine disruption, and impaired ovarian reserve in rodents, without unacceptable mortality.

Morphological Analysis of Ovarian Follicles

At the end of the experiment, the mice were euthanized. The ovaries were preserved in 4% paraformaldehyde and embedded in paraffin, serially sectioned at 5-6 µm, mounted on glass slides, and stained with H&E for morphological analysis by light microscopy. Follicles with a visible nucleus were recorded to avoid counting the same follicle more than once, and follicle classification was based on published criteria.

Follicles were classified by histological pattern into primary follicles characterized by a single layer of cuboidal cells. secondary follicles with layers of cuboid cells with no visible antrum; antral follicles had layers of cuboidal cells, a fluid-filled space, and a cumulus of granulosa cells. Atretic follicles were defined as follicles with zona pellucida remnants[12,13].

Enzyme-linked Immunosorbent Assay (ELISA) of AMH

The AMH was quantified using the Rat AMH ELISA kit (cat. no. EA0083Ra) from Bioassay Technology Laboratory. Following the experiment, peripheral blood samples were obtained, allowed to clot at room temperature for 10-20 minutes, centrifuged at 2,000-3,000 rpm for 20 minutes, and the supernatant was collected free of sediment. After dilution, add the sample to the sample well and 50 µL of biotinylated antigen to each well. Incubate for 60 minutes at 37 °C. Add 50 µL of avidin-HRP to the standard and sample wells; then add 50 µL of substrate solution A and 50 µL of substrate solution B to each well. Lastly, 50 µL of stop solution was added to each well, and the optical density values were measured at 450 nm using a microplate reader within 10 minutes of adding the stop solution[14].

Statistical Analysis

Each experiment in this research was conducted separately. The findings are presented as mean ± standard deviation and were analyzed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The normality of the data was assessed using the Shapiro-Wilk test, and data with a normal distribution were analyzed using one-way ANOVA followed by a post-hoc test. In contrast, the Kruskal-Wallis test followed by the Mann-Whitney test were employed to analyze data that were not normally distributed. Statistical significance was defined as $p<0.05$.

Results

Ovarian Weight

The mean ovarian weight was 0.56±0.01 mg in the control group, 0.31±0.04 mg in the CTX model group, and 0.41±0.01 mg in the CTX with secretome group (Figure 1). The post hoc comparisons among all groups showed that mean ovarian weight in the CTX group was significantly decreased compared with the control group ($p<0.05$), whereas mean ovarian weight in the CTX-secretome group was significantly increased compared with the CTX group ($p<0.05$). Despite this, a significant difference was observed between the CTX-secretome and control groups ($p<0.05$).

Ovarian Follicles

The follicles analyzed in this study were primary, secondary, antral, and atretic (Figure 2).

Compared with the CTX group, the CTX-secretome group showed significant increases in the numbers of primary follicles (147.50±12.94 vs. 56.83±2.92, $p<0.05$; b), secondary follicles (57.00±2.28 vs. 17.33±2.58, $p<0.05$; b), and antral follicles

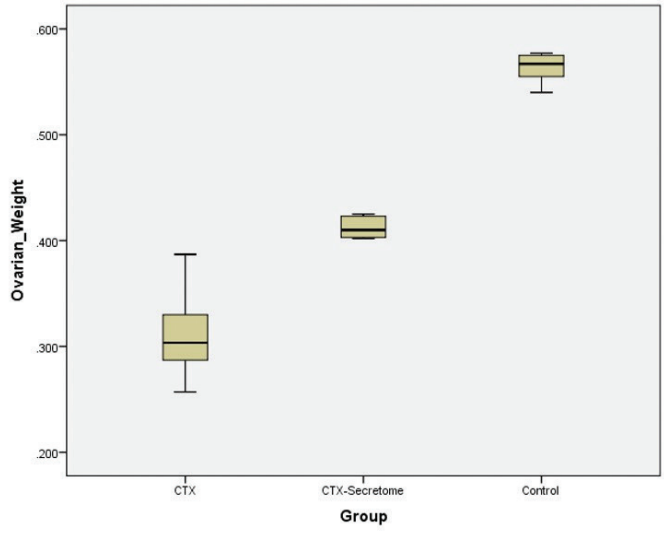

Figure 1. Boxplot graph of ovarian weight

CTX: Cyclophosphamide

(25.33±3.44 vs. 7.50±2.25, p<0.05; b). Compared with the control group, the CTX group demonstrated a significant reduction in all follicle stages: primary follicles (175.33±14.20 vs. 56.83±2.92), secondary follicles (81.50±6.12 vs. 17.33±2.58), and antral follicles (34.50±3.39 vs. 7.50±2.25) (p<0.05; a) (Figure 3).

Meanwhile, the CTX-secretome group showed partial restoration, but remained significantly lower than those of the control group for primary (147.50±12.94 vs. 175.33±14.20), secondary (57.00±2.28 vs. 81.50±6.12), and antral follicles (25.33±3.44 vs. 34.50±3.39) (p<0.05; c).

For atretic follicles, the CTX group showed the highest count (559.17±14.63), which was significantly higher than the counts in the control (354.17±31.53) and CTX-secretome (374.17±12.41) groups (p<0.05; a, b). Secretome treatment reduced the number of atretic follicles to levels not significantly different from the control group (p=0.19; Figure 3c).

Anti-Müllerian Hormone

The AMH, which roughly reflects the number of active follicles in the ovary, also shows improvement following secretome treatment. The AMH was significantly higher in the control group than in the CTX group (11.78±3.51 vs. 5.92±0.74; p<0.05), and was also significantly higher in the CTX-secretome group

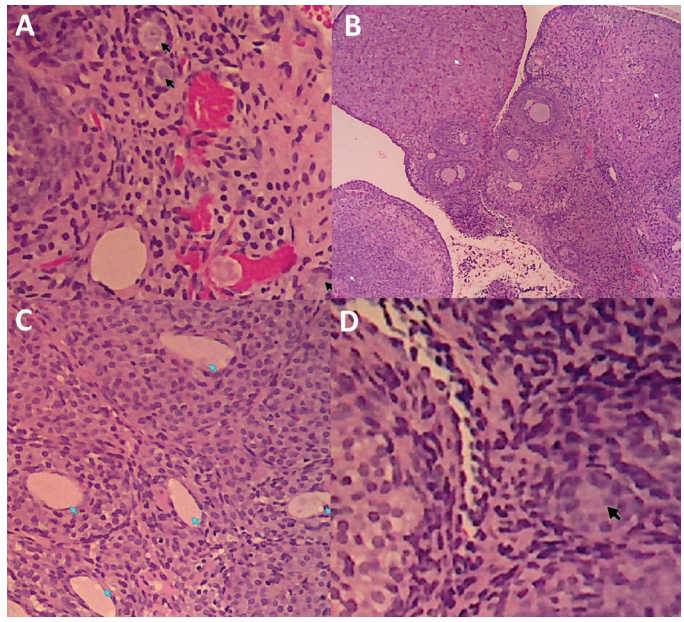

Figure 2. The histology of mouse ovary in the CTX-secretome group with magnification 100x, (A) the distribution of primary follicles (black arrows); (B) the distribution of secondary follicles (purple arrows); (C) the predominance of atretic follicles (blue arrows); (D) the minimal amount of primary follicle (black arrows)

CTX: Cyclophosphamide

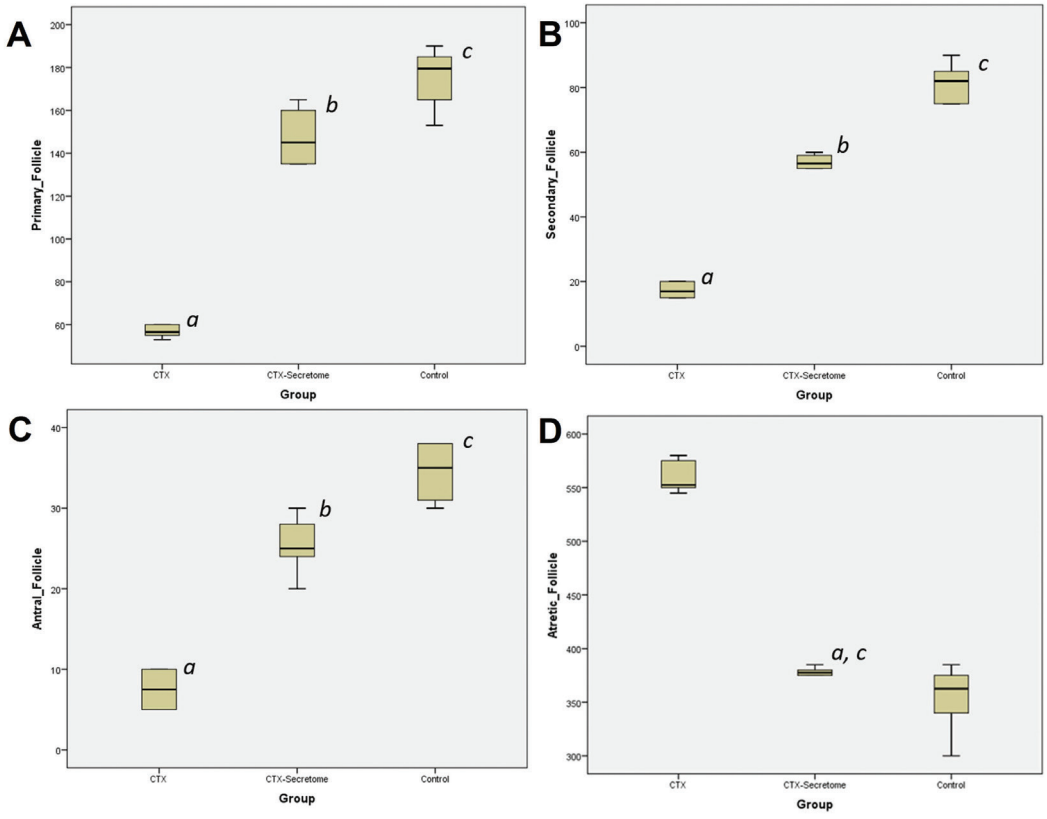

Figure 3. Boxplot graph of (A) primary follicle, (B) secondary follicle, (C) antral follicle, (D) atretic follicle. Data are presents as mean ± SD (n=6 per group). a = p<0.06 vs control, b = p<0.05 vs CTX, c = p<0.05 vs. CTX+secretome

CTX: Cyclophosphamide, SD: Standard deviation

than in the CTX group (9.98±2.8 vs. 5.92±0.74; p<0.05). The control group differed significantly from the CTX-secretome result, with values of 11.78±3.51 ng/mL and 9.98±2.8 ng/mL, respectively.

Discussion

This study established the POI mouse model based on the preceding experiment that used several doses of CTX. The optimal dose that did not cause more than a dropout rate in the mouse population was 50 mg/kg on the first day, followed by 4 mg of CTX for the next 13 days. The preceding experiment was performed to ensure that the animals survived to undergo the subsequent experiment, and it showed an aging effect compared with the control group.

In this study, we found that ovarian weight in the CTX group was significantly lower than the control and CTX-secretome groups. Histological examination also revealed significant changes in the CTX group: Decreased numbers of primary, secondary, and antral follicles and increased numbers of atretic follicles. These findings are consistent with the studies by Song et al.[6] and Pouladvand et al.[16], who reported that CTX promotes follicular atresia by inducing granulosa cell apoptosis, overstimulating dormant primordial follicles (PMFs) via the PTEN/Akt/FOXO3 and mTOR pathways, and generating DNA double-strand breaks that overactivate the ATM–CHK2-Tap63 pathway, leading to apoptosis of PMF oocytes[15]. Another mechanism underlying CTX-induced POI is excessive oxidative stress, which causes mitochondrial dysfunction and upregulates pro-apoptotic and pro-inflammatory mediators such as nuclear factor kappa, tumor necrosis factor alpha, cyclooxygenase-2, and inducible nitric oxide synthase, thereby amplifying cellular injury and death[16]. In addition, CTX disrupts the endocrine balance by reducing circulating levels of estrogen and progesterone, while increasing circulating levels of follicle-stimulating hormone and luteinizing hormone. This hormonal imbalance accelerates follicular depletion and atresia, ultimately reducing the number of mature follicles in the ovary[9,16,17]. At the tissue level, CTX also impaired angiogenesis (resulting in reduced ovarian blood supply) and induced fibrosis within the ovarian cortex, further impairing follicular survival and ovarian function[6].

Secretome treatment resulted in partial restoration of ovarian morphology and function. Ovarian weight increased after secretome injection compared with CTX, reflecting the increased number of primary, secondary, and antral follicles in the ovary. This recovery correlates with a significant increase in AMH levels in the CTX-secretome group compared with the CTX group, indicating better preservation of the follicular pool. Mechanistically, FGF signalling—especially that mediated by FGF2, FGF8, FGF18, and FGF21—could restore PI3K/AKT activity and counteract FOXO3-driven dormancy, thereby rescuing granulosa cell proliferation and angiogenesis[15]. Simultaneously, transforming growth factor-beta/SMAD signalling may attenuate the overactivation of the ATM-CHK2-TAp63 pathway, essential for balancing oocyte elimination and survival. Moreover, FGF21 induces GC proliferation and estradiol production and activates crosstalk between the PI3K/AKT and mTOR pathways. Secretome-derived factors may restore homeostasis within the ovarian microenvironment, mitigating CTX-induced injury at multiple checkpoints[15].

However, the result for the CTX-secretome group still shows substantial differences from the control group. It can serve as the basis for a subsequent study evaluating different doses of secretome to achieve an optimal value approximating the normal value.

Study Limitations

This research has several limitations. We did not incorporate biochemical or molecular markers that would have further strengthened the secretome in POI. The present study focuses on an experimental approach to demonstrate morphological and endocrine recovery following secretome therapy. Furthermore, a single dose of secretome administered exclusively via the intraperitoneal route limits conclusions regarding dose optimization and may overlook differences associated with alternative routes of administration. The lack of long-term fertility assessments, such as mating success and offspring viability, also limits the study. Nevertheless, this study offers novel insights and underscores secretome-based therapy as a promising strategy to preserve ovarian reserve against chemotherapy-induced damage.

Conclusion

The POI condition was induced in normal mice by CTX injection, and secretome injection significantly improved follicle number, ovarian weight, and AMH level. However, the result has not yet reached the normal value observed in the control group. It can be concluded that the secretome improved ovarian quality compared with the POI group. However, it had not yet returned to the quality of the normal ovary. Notably, the following study requires testing different doses of secretomes to determine the optimal dose.

Ethics

Ethics Committee Approval: The Research Ethics Committee, Faculty of Medicine, Universitas Udayana (approval number: 2713/UN14.2.2.VII.14/LT/2024, date: 11.11.2024).

Informed Consent: Retrospective study.

Footnotes

Authorship Contributions

Surgical and Medical Practices: S.K., I.G.E.W., I.N.G.B., Concept: S.K., I.G.E.W., I.W.P.S.Y., Design: I.G.E.W., I.W.P.S.Y., Data Collection or Processing: S.K., I.W.P.S.Y., I.N.G.B., Analysis or Interpretation: S.K., I.W.P.S.Y., I.N.G.B., Literature Search: S.K., I.W.P.S.Y., Writing: S.K., I.G.E.W., I.N.G.B.

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