Research Article

Peripheral Serum AMH and FSH Levels for Determining Ovarian Reserve in Experimental Mouse Model

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ABSTRACT

Objective: To determine peripheral serum levels of AMH and FSH in experimental animals for predicting ovarian reserve.

Methods: This is an experimental study was conducted from March 2022 to May 2023 at the Indonesia Medical Education and Research Institute (IMERI) and Integrated Laboratory and Histology Laboratory of the Faculty of Medicine, Universitas Indonesia (FKUI) and involving 20 female Sprague-Dawley rats aged 8–10 weeks with normal estrus cycles and 5 female rats aged 28–30 weeks. The experiment is divided into 5 groups, the young age control group, the old age control group, the 1x cisplatin group, the 2x cisplatin group, and the 3x cisplatin group. Cisplatin is one of the chemotherapeutic agents that has moderate toxicity employed to reduce ovarian reserve in a rat model. Intraperitoneal injection of cisplatin at 2 mg/kg/day twice a week for five weeks significantly decreases ovarian reserve, as evidenced by a notable reduction in Anti-Mullerian Hormone (AMH) levels. After the treatment, tissue collection, histological staining, peripheral and central blood collection through the retro-orbital bleeding (ROB) and heart were performed.

Conclusion: In the follicle count, the group treated with cisplatin 2x exhibited a significantly lower number of live follicles compared to the cisplatin 1x and 3x groups. The average concentrations of serum AMH and FSH in the ROB and heart were higher in the 2x cisplatin group compared to the other groups. The more cisplatin added the more ovarian reserve reduced and it corresponds to the old age control group. The increase in the follicles count and decrease in serum level in the cisplatin 3x group was due to the burn out phenomenon. Blood collection through the ROB is considered a less invasive alternative technique in the treatment group, requiring serial observation.

Keywords: AMH, animal model, follicle pool, ovarian aging, ROB.

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INTRODUCTION

Menopause is the end of a woman's reproductive phase. However, a decline in fertility can be seen in the last 20 years before menopause, and 10 years before menopause, the ability to become pregnant becomes significantly low. Ovarian aging stands out as a primary factor in the decline of female fertility associated with age. It involves a progressive decrease in the quantity and quality of oocytes, while the physiology of ovarian aging means a specific decline in the functional ovarian reserve over a defined period. The female ovarian reserve diminishes progressively with advancing chronological age.[1] The passage of time since birth is what determines chronological age. In contrast, physiological rather than chronological factors determine biological age. Chronological and biological aging are different from one another, with genetic and environmental factors influencing biological age. Reproductive function is more significantly influenced by biological age than chronological age. Knowing the biological age of a woman's ovaries and the subsequent pattern of decline is very important for assisted reproductive technology (ART), as it can determine the subsequent course of management. Ovarian reserve serves as a reliable marker for determining biological age.[2] The true ovarian reserve can be determined through the histological counting of ovarian follicles. However, this method is invasive, necessitating less invasive measures, one of which is through serum levels of AMH (Anti-Müllerian Hormone) and FSH (Follicle-Stimulating Hormone).[3]

Anti-Mullerian Hormone (AMH) and antral follicle count (AFC) are widely used as ovarian reserve markers based on Bologna and Poseidon criteria.[3] Granulosa cells start producing AMH from the primary follicle stage, reaching peak expression during the secondary follicle stage. The synthesis decreases as the follicle matures, continuing through to the preovulatory stage. In women, serum AMH levels remain relatively steady during infancy, childhood, puberty, and the initial phases of adulthood. However, there is a gradual decrease starting from the fourth decade of life, eventually becoming undetectable a few years prior to menopause. [4,5]

FSH has a crucial role in the development and control of the male and female reproductive systems, exerting its effects through FSHR, primarily found in granulosa and Sertoli cells. In females, FSH stimulates the growth and maturation of follicles and plays a role in LH-induced ovulation and luteinization.[4]

This research aims to assess ovarian reserve in poorly responsive mouse based on AMH and FSH serum levels collected from peripheral (ROB), and central (heart), also from ovarian tissue.

METHODS

The research was conducted from March 2022 to May 2023 at the Indonesia Medical Education and Research Institute (IMERI) within the Animal Research Facility (ARF) Cluster and the Human Reproductive, Fertility, and Family Planning Research (HRIFP) Cluster, as well as at the Integrated Laboratory and Histology Laboratory of the Faculty of Medicine, Universitas Indonesia (FKUI). This research has been approved by the Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia, with the ethical clearance letter number: KET-177/UN.2F1/ETIK/PPM.00.02/2022.

The research design used in this study is an experimental study utilizing a rat model, specifically Sprague-Dawley rats. The short and precisely timed estrous cycle, typically lasting 4–5 days, makes the mouse the preferred mammalian model for human-related research. Additionally, mice have a very brief reproductive age, typically reaching sexual maturity at 4–7 weeks following birth. The reproductive cycle in mice is referred to as the estrous cycle. During estrus, ovarian target tissues are prepared for gestation.[6]

In the experimental setting with mouse, cisplatin will also be administered. Cisplatin is a chemotherapeutic agent used to diminish ovarian reserve in a mouse model. Various doses of cisplatin will be administered to determine the most effective dosage for establishing the model of poorly responsive mouse.[7]

Female Sprague-Dawley rats, aged 8-10 weeks and 28-30 weeks, were utilized in this research. The selected rats exhibited normal estrous cycles lasting 4-7 days, with an average of 5 days. The total number of rats involved in the study was 25. The research was divided into five groups, as follows: Group A: Old-age control group (28–30 weeks); Group B: Cisplatin 1x (administration of cisplatin at 2.5 mg/kgBW/day for 5 days); Group C: Cisplatin 2x (administration of cisplatin at 2.5 mg/kgBW/day for 2 weeks, followed by 1.25 mg/kgBW/day for 5 days); and Group D: Cisplatin 3x (administration of cisplatin at 2.5 mg/kgBW/

day for 2 weeks, followed by 1.25 mg/kgBW/day for 1 week and 1.25 mg/kgBW/day for 5 days); Group E: Young-age control group (8-10 weeks). Ovaries were collected on the 5th day after cisplatin administration.

Peripheral blood was collected through retro-orbital bleeding (ROB) and central blood from the heart. The volume of blood collected amounted to 8% of the body weight. Safe percentages were estimated at 7.5% every 7 days and 1% every 24 hours. Anesthetized rats had 2 cc of blood drawn from the retro-orbital vein using a heparin-containing capillary tube and 2 cc from the heart using a 2.5 cc syringe needle. The collected blood was placed in serum separator tubes (SST) for centrifugation, and the serum was then collected. AMH and FSH concentrations were quantified using immunoassay methods, such as Enzyme-Linked Immunosorbent Assay (ELISA).[8]

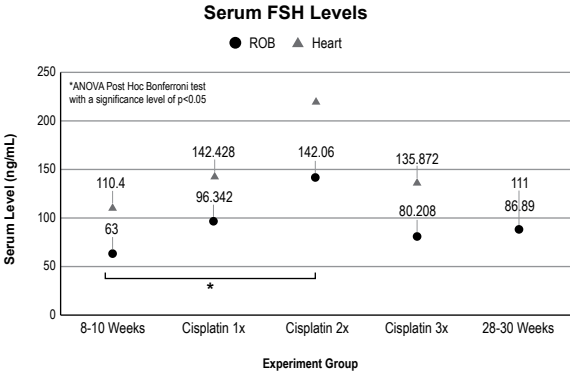
The ovaries of the mice were collected through laparotomy, followed by weighing and histological examination to determine the follicle count using hematoxylin-eosin staining. Follicle counting was performed using one ovary per animal. Paraffin blocks are cut with a microtome to a thickness of 5–6 mm. Follicles were classified based on their histological pattern. They are primordial follicles with one-layer squamous granulosa cells; primer follicles with one-layer cuboid cells; secondary follicles with layers of cuboid cells; antral follicles with layers of cuboid cells; and space with fluid and cumulus granulosa cells. Primordial follicle belongs to the non-growing follicle category, while the primary, secondary, and antral follicle belong to the growing follicle category.[9]

Statistical analysis was conducted using the SPSS vs 20. A significance level of $P<0.05$ was employed to determine statistical significance. ANOVA with subsequent multiple comparisons was applied.

RESULTS

Serum AMH Level in ROB and Heart

The serum AMH levels from ROB in the young age control group, cisplatin 1x group, cisplatin 2x group, cisplatin 3x group, and old age control group were 1151, 1818, 2782.960, 1381.352, and 1544 ng/mL, respectively. The average serum AMH level from ROB was significantly higher in the cisplatin 2x group compared to the other groups. The serum AMH levels from the heart in the young age control group, cisplatin 1x group, cisplatin 2x group, cisplatin 3x group, and old age control group were 2138, 2595.760, 4057.040, 2633.520, and 2062 ng/mL, respectively (figure 1). Similarly, the average concentration of serum AMH in the heart was significantly higher in the cisplatin 2x group compared to the young age control group.



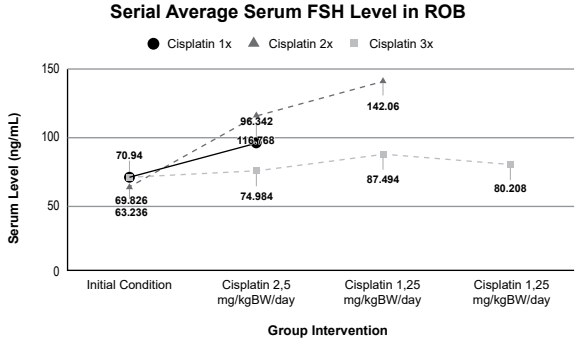


Figure 1. (A) Average Serum FSH Levels for Each Experiment Group.
B Serial Average Serum FSH Levels from Intervention Group: Cisplatin 1x, 2x, and 3x.

Follicle Count

In the follicle count analysis, the group treated with cisplatin 2x exhibited a significantly lower number of live follicles compared to the cisplatin 1x and 3x groups. In the old-age control group, the number of follicles was significantly lower than in the cisplatin 2x group. Conversely, the young-age control group had the highest number of follicles compared to the other groups. Meanwhile, the highest number of atretic follicles was observed in the old age control group, followed sequentially by the cisplatin 3x, cisplatin 2x, cisplatin 1x, and young age control groups

(Figure. 2). The number of non-growing follicles for the young age control group, cisplatin 1x group, cisplatin 2x group, cisplatin 3x group, and the old age control group were 7, 6, 3, 6, and 0, respectively. The number of growing follicles for the young age control group, cisplatin 1x group, cisplatin 2x group, cisplatin 3x group, and the old age control group were 50, 37, 24, 32, and 8, respectively. The number of atresia follicles for the young age control group, cisplatin 1x group, cisplatin 2x group, cisplatin 3x group, and the old age control group were 4, 19, 21, 28, and 30, respectively.

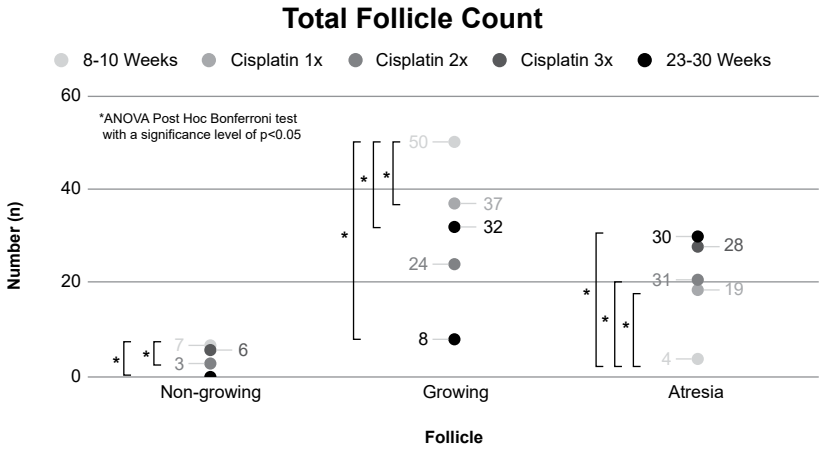


Figure 2. Total Follicle Count For Primordial, Primary, Secondary, Antral, and Atresia Follicle In Each Experimental Group In Mouse Model Study.

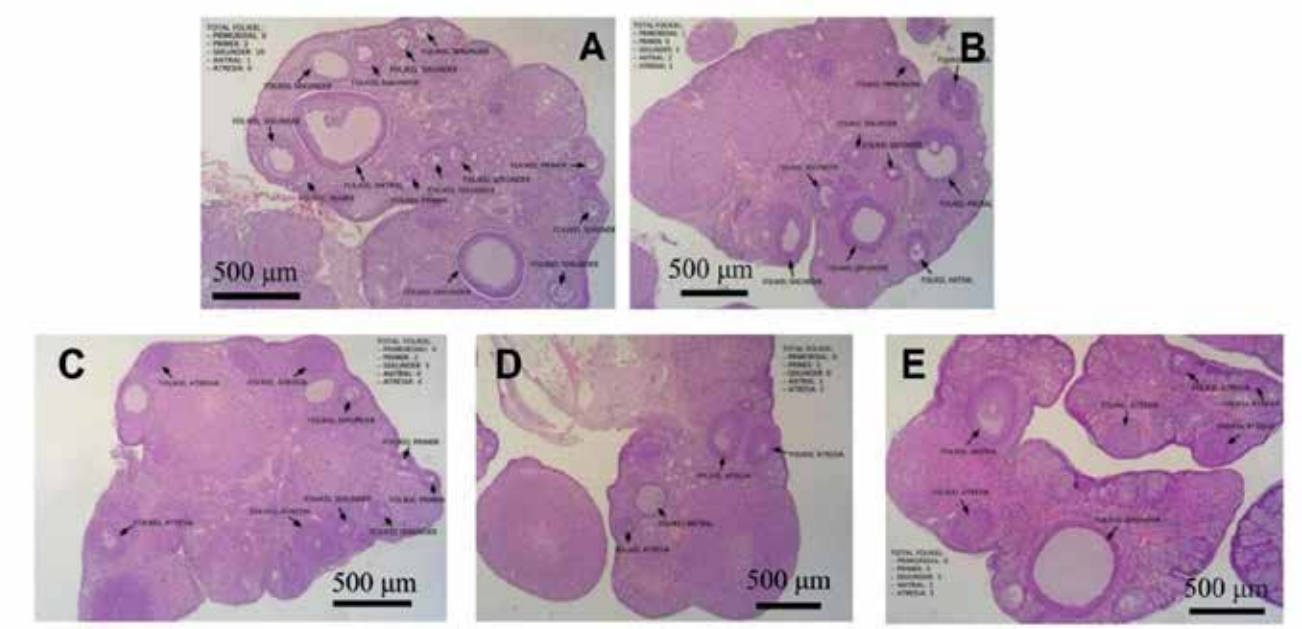


Figure 3. Histological View of Follicle Count for Each Experimental Group With Magnifications x400. Follicle Count Includes Primordial, Primary, Secondary, Antral, and Atresia Follicle. (A) Young age control group. (B) Cisplatin 1x group. (C) Cisplatin 2x group. (D) Cisplatin 3x group. (E) Old age control group.

Ovarian Weight

There were no significant differences in the ovarian weights of the young age control group, the cisplatin 1x group, the cisplatin 2x group, the cisplatin 3x group, and the old age control group. The ovarian weights were 0.555 grams, 0.509 grams, 0.487 grams, 0.387 grams, and 0.191 grams, respectively (Figure. 5).

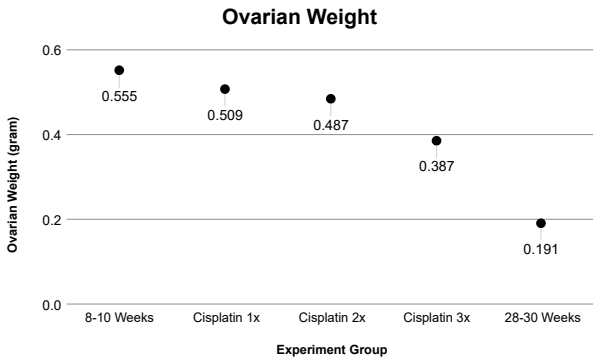


Figure 4. Ovarian Weight In Mouse Model Study.

DISCUSSION

In the selection of control groups, it is evident that the young age control group has a significantly higher number of non-growing and growing follicles compared to the old age control group, indicating the appropriateness of the control group that was used in the study. Cisplatin, as a primary platinum-based chemotherapy agent for ovarian cancer, possesses moderate toxicity that can lead to damage to the ovaries and gonads. Administration of cisplatin induces Premature Ovarian Failure (POF) due to death or accelerated primordial follicle depletion, atresia in growing follicles, damage to stromal tissue, blood vessels, inflammation, and ovarian fibrosis. DNA damage also occurs due to the activation of the P53 apoptosis signaling pathway, oxidative stress, and free radical production.[10]

The administration of cisplatin in the treatment groups reduces the number of follicles in both cisplatin 1x and 2x groups and increases the number of atretic follicles significantly different from the young age control. This finding aligns research that cisplatin and similar compounds induce aneuploidy in mature oocytes correlating with embryonic mortality rates.[11] However, in cisplatin 3x administration, there is an increase in the number of follicles caused by the "burnout" phenomenon. The PI3K/PTEN/Akt pathway brings an end to the dormancy of follicles, directly impacting the oocytes and pre-granulosa cells (pre-GCs) within primordial follicles (PFs). Additionally, it indirectly leads to the breakdown of large follicles. The result of follicle destruction causes a disruption in AMH and diminishes the inhibition of the PF pool. Subsequently, this triggers the activation of PFs in an effort to compensate for the decline in the number of developing follicles. Other studies support these findings, indicating that exposure to cisplatin increases the phosphorylation of proteins in the PTEN/Akt/FOXO3a pathway, including PTEB, Akt, GSK 3, FOXO 3a, and ERK. These changes result in nuclear translocation of FOXO3a, suppressing transcriptional activity, activating PFs, and reducing ovarian reserve. [12-14]

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The evaluation of ovarian reserve was also conducted by examining serum AMH and FSH levels. The research results indicate an increase in AMH and FSH with cisplatin 1x administration, peaking with cisplatin 2x before declining with cisplatin 3x. This condition contradicts the follicle count, where cisplatin 2x was found to be the least compared to cisplatin 1x and 3x. The higher number of follicle count and lower concentration in AMH and FSH in cisplatin 3x was likely due to the burnout phenomenon.

The research also supports that the chemotherapy agent cyclophosphamide (Cy) reduces ovarian follicle reserve by triggering an increase in the PI3K pathway, initiating waves of follicle recruitment and growth, and causing apoptosis of growing follicles within 24 hours of exposure. The study also indicates that AMH expression drops below control concentrations 12 hours after Cy treatment, indicating the loss of growing follicles at that time. However, between 3 and 7 days after treatment, AMH secretion doubles its relative expression and persists for at least 14 days post-treatment. This illustrates an increase in early-growing follicles as a result of follicle activation induced by Cy. The decrease in AMH expression, along with the activation of the PI3K pathway occurring within 24 hours after Cy treatment, combines to create an "irregular growth window" for the dormant PF population. [12,14,15]

The levels of AMH and FSH show significant results with cisplatin 2 times compared to young control and the same pattern, but AMH serves as a more reliable predictor of ovarian reserve compared to FSH because AMH is not influenced by the menstrual cycle, whereas FSH is cycle-dependent. AMH is expressed by growing follicles and can be detected in serum, making it a reliable marker.[16]

Serial blood sampling through the ROB technique and heart blood collection at necropsy yielded similar results. Thus, blood collection via ROB is considered an alternative, less invasive technique for treatment groups requiring serial observations.

CONCLUSION

In the follicle count, the group treated with cisplatin 2x exhibited a significantly lower number of live follicles compared to the cisplatin 1x and 3x groups. The average concentrations of serum AMH and FSH in the ROB and heart were higher in the 2x cisplatin group compared to the other groups. The more cisplatin added the more ovarian reserve reduced and it corresponds to the old age control group. The increase in the follicles count and decrease in serum level in the cisplatin 3x group was due to the burn out phenomenon. Blood collection through the ROB is considered a less invasive alternative technique in the treatment group, requiring serial observation.

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