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ORIGINAL ARTICLE

Retrospective Cohort Study

# Analysis of oxidative stress and antioxidative potential in premature ovarian insufficiency

Kaoru Kakinuma, Toshiyuki Kakinuma

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## Abstract

### *BACKGROUND*

Premature ovarian insufficiency (POI) is characterized by an early decline in ovarian function, inducing secondary amenorrhea. While the cause of POI has not yet been identified, the function of mitochondria in the ovaries and the cytotoxicity associated with reactive oxygen species (ROS) have been implicated in follicle pool depletion and a decline in follicle quality. Recently developed tests have enabled easy measurement of diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP). The combination of these two tests is used to comprehensively assess oxidative stress in the blood.

### *AIM*

To comprehensively assess the oxidative stress of d-ROMs and BAP in POI.

### *METHODS*

Participants were classified into two groups: A POI group of 11 women aged < 40 years examined between January 2021 and June 2022 with a history of secondary amenorrhea for at least 4 mo in our hospital and an FSH value of ≥ 40 mIU/mL; and a control group of healthy women of the same age with normal ovarian function in our hospital. Plasma d-ROMs and BAP were measured in both these groups underwent. Differences between groups were assessed using the *t*-test.

### *RESULTS*

The mean age and mean body mass index (BMI) were 35.8 ± 3.0 years and 20.1 ± 1.9 kg/m$^2$ in the control group and 35.8 ± 2.7 years and 19.4 ± 2.5 kg/m$^2$ in the POI group, respectively. The mean gravidity and parity in control and POI groups were 0.6 ± 0.7 and 0.4 ± 0.5 and 0.6 ± 0.9 and 0.3 ± 0.5, respectively. The two groups did not differ significantly in terms of mean age, BMI, gravidity, or parity. The d-ROMs level was significantly higher in the POI group than in the control group (478.2 ± 58.7 *vs* 341.1 ± 35.1 U.CARR; $P < 0.001$); however, the BAP level did not significantly differ between the two groups (2078.5 ± 157.4 *vs* 2029.0 ± 186.4

μmol/L). The oxidase stress index (d-ROMs/BAP × 100) was significantly higher in the POI group than in the control group (23.7 ± 3.3 *vs* 16.5 ± 2.1; $P < 0.001$).

***CONCLUSION***

Oxidative stress was significantly greater in the POI group than in the control group, suggesting oxidative stress as a factor that can serve as a POI biomarker.

**Key Words:** Premature ovarian insufficiency; Reactive oxygen species; Oxidative stress; Ovary; Antioxidant



**Core Tip:** We hypothesized that oxidative stress, suggested to be a factor in premature ovarian insufficiency (POI) and a potential POI biomarker, is likely a useful target for the treatment and early intervention for various conditions, including early POI diagnosis and infertility treatment. In this retrospective study, we discovered that oxidative stress was significantly higher in patients with POI than in healthy controls, suggesting the use of this measurement as a biomarker of POI.

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## INTRODUCTION

Premature ovarian insufficiency (POI) is a syndrome in which women aged < 40 years experience amenorrhea involving persistent hypergonadotropic hypoestrogenemia for at least 4 mo[1,2]. Primary ovarian insufficiency naturally develops in 1 in 100 women[3], with an incidence of approximately 1% at ages 30-39 years, 0.1% at ages 20-29 years, and 0.01% at < 20 years[3,4]. However, with many women now marrying and conceiving later in life, those who had previously achieved pregnancy prior to POI onset may now require fertility treatment, although other recent studies have indicated that the incidence of POI itself may be on the rise[5-8].

Early ovarian depletion is thought to be caused by various factors, such as chromosomal abnormalities, genetic mutations, polyendocrine syndrome (adrenal insufficiency, hypothyroidism), autoimmune abnormalities (chiefly rheumatoid arthritis and systemic lupus erythematosus), metabolic disease, and iatrogenic factors associated with pelvic surgery, anticancer agents, and pelvic radiotherapy; nonetheless, many factors influencing POI have not yet been sufficiently determined[9-17].

Regardless of the cause of POI, early follicular depletion and impaired follicular development cause a decrease in the number or complete disappearance of residual oocytes and follicles[11,12]. Generally, when the number of residual follicles in the ovaries falls below 1000, the primordial follicles cease to activate, thereby stopping the recruitment of developing follicles and preventing the remaining follicles from achieving ovulation. Consequently, granulosa cells, the primary source of estrogen production, disappear almost completely owing to the absence of developing follicles, thereby reducing estrogen levels in the blood. Consequently, the endometrium does not thicken, the absence of the corpus luteum following ovulation halts the secretion of progesterone, and withdrawal bleeding associated with regression of the corpus luteum does not occur, resulting in amenorrhea.

In summary, in POI, the loss of ovarian function leads to low estradiol levels, which can trigger the development of some mental disorders, such as climacteric symptoms, depression, anxiety, and cognitive disorders. Therefore, appropriate POI management is essential to improve the quality of life and prevent fractures caused by bone and circulatory diseases and decreased bone density[1,18-22].

A major problem associated with POI is extremely severe infertility. In POI, amenorrhea and anovulation associated with follicular pool depletion may result in major infertility[23]. Although only approximately 25% of patients with POI demonstrate decreased ovulation, the lifetime pregnancy rate among these patients with their own ova is only 5%-10%[11,12,23,24], causing intractable infertility in patients with POI. Currently, no reliable treatment is available for infertility caused by POI. The European Society of Human Reproduction and Embryology (ESHRE) Guidelines on POI state that other than oocyte donation, there are currently no medical interventions worth recommending[25,26]. This situation demands the determination of the cause of POI and early diagnosis and intervention.

Oxidative stress, which is defined as a breakdown in the balance between the generation of reactive oxygen species (ROS) in the body and the antioxidant mechanisms that counteract these ROS[27-29], has

been implicated in the pathogenesis of many diseases. ROS and free radicals are byproducts of oxygen-consuming energy metabolism reactions. These metabolites are unstable and highly reactive, and their excessive production results in the oxidation of proteins, lipids, nucleic acids, and other biopolymers, thereby increasing the risk of dysfunction. The body is equipped with a defense system (antioxidant potential) that uses antioxidant enzymes and antioxidants to remove and neutralize free radicals and ROS to avoid this type of damage. Oxidative stress occurs when the production of ROS and free radicals (degree of oxidation) surpasses the antioxidant potential and reportedly triggers senescence, cardiovascular disease, neurodegenerative disease, cancer, and other refractory diseases[30-33]. The function of mitochondria in the ovaries and cytotoxicity associated with ROS production by this mitochondrial function have been indicated as factors in follicular pool depletion and the decline in oocyte quality observed with age[34-36].

Active oxygen is a state in which oxygen has become chemically active and is generally very unstable, complicating its measurement method. Recently developed tests have enabled the easy measurement of diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP), and the combination of these two tests is now used to comprehensively assess oxidative stress in the blood[37-41]. The d-ROM test uses a color reaction to measure the levels of blood compounds, such as hydroperoxide, which is generated by ROS and free radicals[42]. In contrast, the BAP test assesses antioxidant potential by measuring the reducing ability of antioxidants, which involves donating electrons to ROS and free radicals, to terminate oxidation reactions[42].

In this study, we aimed to comprehensively assess oxidative stress in POI in terms of oxidative stress (d-ROMs) and antioxidant potential (BAP) in the blood to determine the cause of POI and investigate the potential of d-ROMs and BAP as biomarkers of POI.

## MATERIALS AND METHODS

This study was approved by the Institutional Review Board of the International Health and Welfare Hospital (approval no. 21-Im-075, approved on 3/22/2022). The participants were patients aged < 40 years who were examined at the International Health and Welfare Hospital between January 2021 and June 2022. All participants received oral and written explanations of the study and provided verbal and written informed consent to participate. The following patients were excluded due to the consideration of the potential effects of different conditions on oxidative stress and antioxidant capacity: patients with obstetric and gynecological diseases, patients with severe menstruation-associated symptoms requiring analgesics, patients using other pharmaceuticals or supplements, and smokers.

Participants were divided into two groups: The POI group, comprising women aged < 40 years who had experienced secondary amenorrhea for at least 4 mo and demonstrated a follicle-stimulating hormone (FSH) level of ≥ 40 mIU/mL; and a control group, comprising healthy women aged < 40 years with normal ovarian function.

Blood was collected during the follicular phase (within 5 d of the initiation of the menstrual cycle). Ovarian function was measured in terms of FSH, anti-Müllerian hormone (AMH), and antral follicle count (AFC), measured using a diagnostic ultrasound device (Voluson S10 Expert; GE Healthcare Japan, Tokyo, Japan). Oxidative stress was assessed using a test for d-ROMs, a marker of oxidative stress, and a test for BAP, a marker of antioxidant potential. To comprehensively assess the oxidative stress defense system, the latent antioxidant potential was calculated using the oxidase stress index (OSI) (BAP/d-ROMs × 100).

### *Assessment of ovarian function*

FSH levels were measured using a chemiluminescent immunoassay (CLIA) (CL AIA-PACK®FSH TEST; Tosoh Corporation, Tokyo, Japan), and AMH levels were measured using a chemiluminescent enzyme immunoassay (CLEIA) (Access AMH®; Beckman Coulter, Tokyo, Japan).

### *Measurement of oxidative stress and antioxidant potential*

Oxidative stress in the blood during the follicular phase (d-ROM) and antioxidant potential (BAP) was measured using a free radical analysis device (FREE Carrio Duo; Diacron International, Grosetto, Italy). Measurements using this device have been reported to be valid and reproducible in previous studies[43-45].

d-ROMs were measured as follows: After blood samples were centrifuged, 20 μL of serum was collected and mixed into a pH 4.8 acidic buffer solution. A colorless aromatic amine solution (color reaction chromogen) was added, and the mixture was placed in a photometer in the free radical analysis device. After 5 min, the reduction in absorbance at 505 nm was measured, and the rate of change was used to calculate the concentration of hydroperoxide in the serum. D-ROMs are measured using color reactions to measure the blood levels of hydroperoxide (functional group: ROOH), which is generated from ROS and free radicals in the body, serving as a comprehensive assessment of the degree of oxidative stress in the body[37,46-48]. The unit of d-ROM is U.CARR, with 1 U.CARR equivalent to 0.08 mg/dL hydrogen peroxide. The reference values were as follows: normal, 200-300 U.CARR; borderline,

301-320 U.CARR; mild oxidative stress, 321-340 U.CARR; moderate oxidative stress, 341-400 U.CARR; severe oxidative stress, 401-500 U.CARR; and highly severe oxidative stress, ≥ 501 U.CARR[37,46-48].

BAP was measured as follows: A reagent containing a tricyanic acid derivative was mixed with a reagent containing iron ions, which was placed in the photometer in the free radical analysis device, and the absorbance at 505 nm was measured. Next, 10 μL of serum was added to this mixture, which was incubated at 37 °C for 5 min, after which absorbance was measured again. The concentration of oxidized iron ions was calculated based on the change in absorption over 5 min. The BAP represents the capacity of the serum solution to reduce iron from ferric ($Fe^{3+}$) to ferrous ($Fe^{2+}$) form (reduction reaction). The unit of BAP is μmol/L, with reference values as follows: Optimal value, > 2200 μmol/L; borderline, 2000-2200 μmol/L; slight reduction, 1800-2200 μmol/L; moderate reduction, 1600-1800 μmol/L; strong reduction, 1400-1600 μmol/L; very strong reduction, < 1400 μmol/L[49-51].

### *Statistical analysis*

Differences between groups were assessed using the *t*-test. All results are shown as the mean ± SD, with $P < 0.05$ considered to indicate statistical significance. Statistical analyses were performed using JMP® version 14.2 (SAS Institute Japan Co. Ltd, Tokyo, Japan), and IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, United States) was used for statistical processing.

## RESULTS

Of the 23 participants, 1 was excluded for being a smoker. Finally, the POI group included 11 participants with secondary amenorrhea persisting for ≥ 4 mo and an FSH level ≥ 40 mIU/mL, whereas the control group comprised 11 participants with normal ovarian function (Figure 1).

Table 1 presents the participants' basic characteristics. The control group had a mean age of 35.8 ± 3.0 years and a mean body mass index (BMI) of 20.1 ± 1.9 kg/m$^2$, whereas the POI group had a mean age of 35.8 ± 2.7 years and a mean BMI of 19.4 ± 2.5 kg/m$^2$. The mean gravidity and parity in the control and POI groups were 0.6 ± 0.7 and 0.4 ± 0.5, respectively, *vs* 0.6 ± 0.9 and 0.3 ± 0.5, respectively. The two groups did not differ significantly in terms of mean age, BMI, gravidity, or parity (Table 1).

The mean AMH levels in the control and POI groups were 2.8 ± 1.4 ng/mL and 0.4 ± 0.3 ng/mL, respectively; the mean AMH level was significantly lower in the POI group than in the control group ($P < 0.001$) (Figure 2A). The mean AFC in the control and POI groups were 9.5 ± 2.4 and 0.7 ± 0.9, respectively, indicating that the mean AFC was significantly lower in the POI group ($P < 0.001$) than in the control group (Figure 2B).

The mean d-ROM values in the control and POI groups were 341.1 ± 35.1 U.CARR and 478.2 ± 58.7 U.CARR, respectively. The mean d-ROM value was significantly higher in the POI group than in the control group ($P < 0.001$) (Figure 2C). Conversely, the mean BAP values were 2078.5 ± 157.4 μmol/L and 2029.0 ± 186.4 μmol/L, respectively, in the control and POI groups, and there were no significant differences between the two groups ($P = 0.28$) (Figure 2D). The mean OSI (d-ROMs/BAP× 100) in the control and POI groups was 16.5 ± 2.1 and 23.7 ± 3.3, respectively, indicating a significantly higher OSI in the POI group than in the control group ($P < 0.001$) (Figure 2E).

## DISCUSSION

POI is diagnosed as ovarian amenorrhea in women aged < 40 years. In patients with POI, normal ovarian function is lost, and a decline in ovarian function leads to a hyperestradiolic state, which is likely to result in infertility, menopausal symptoms, reduced bone density and associated fractures, cardiovascular disease, and mental disorders (depression, anxiety, and cognitive impairment); thus, proper management of this condition is essential to maintain quality of life. Although genetic factors, iatrogenesis, autoimmunity, metabolism, infection, and environmental factors have been proposed as causes of POI, most cases are idiopathic, and a clear cause has not yet been identified[11,12].

The ovaries are believed to decline in function earlier than other organs. As women age, the follicular pool and quality of oocytes decline, resulting in infertility[52,53]. These processes are thought to involve the activation of apoptotic pathways[54,55], which is believed to be triggered by the dysfunction of mitochondria in oocytes and the resulting ROS cytotoxicity[34-36].

Oxidative stress is defined as the difference between the oxidative damage potential of ROS generated in the body and the antioxidant potential of the antioxidant systems in the body[27-29]. Most ROS are produced in the mitochondria; these reactive molecules damage the mitochondria themselves, along with lipids, proteins, and DNA, which is considered to result in senescence at the cellular level, further triggering arteriosclerosis, diabetes, malignant disease, and various other diseases[30-33].

The membrane potential of mitochondria in oocytes harvested from older women, which demonstrates follicular pool depletion and diminished oocyte quality, has been found to be significantly lower than the mitochondrial membrane potential of oocytes in younger women[56]. Mitochondria

**Table 1 Characteristics of the study participants**

| | Control group (*n* = 11) | POI group (*n* = 11) | *P* value |
|---|---|---|---|
| Age (yr) | 35.8 ± 3.0 | 35.8 ± 2.7 | 0.50 |
| BMI (kg/m$^2$) | 20.1 ± 1.9 | 19.4 ± 2.5 | 0.24 |
| Gravidity (times) | 0.6 ± 0.7 | 0.6 ± 0.9 | 0.50 |
| Parity (times) | 0.4 ± 0.5 | 0.3 ± 0.5 | 0.36 |

Values are expressed as means, with the error bar representing the standard deviation. There were no significant differences between the two groups. POI: Premature ovarian insufficiency; BMI: Body mass index.

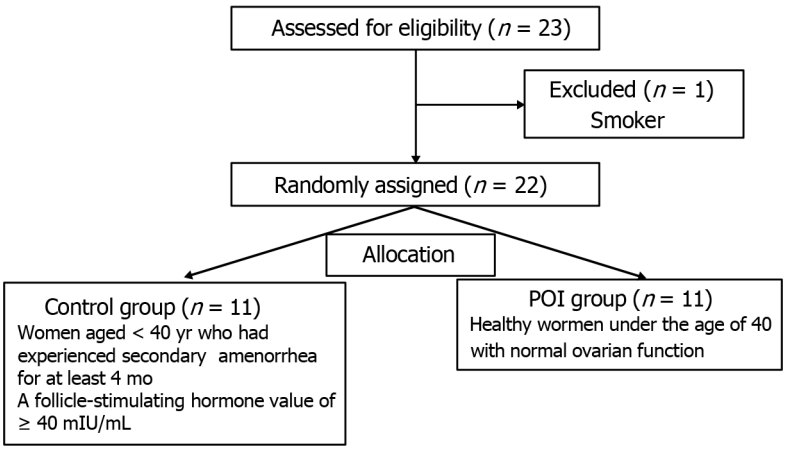


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**Figure 1 Participant distribution in this study.** POI: Premature ovarian insufficiency.

contain mtDNA, double-stranded circular DNA comprising a unique genome; oocytes retrieved from older women reportedly have greater percentages of mtDNA deletion and point mutations than oocytes from younger women[57,58]. In a study in which microarrays were used to examine differences in gene expression in oocytes collected from aged and younger mice, the oocytes of old mice demonstrated reduced expression of genes involved in mitochondrial function, inhibition of oxidative stress, and stabilization of DNA and chromosomes[59]. A similar study conducted on human oocytes also demonstrated reduced expression of genes in the same categories[60]. These studies have all suggested the importance of mitochondrial dysfunction in ovary degradation. Mitochondria are cellular organelles that metabolize energy and supply intracellular ATP *via* oxidative phosphorylation. Mitochondrial dysfunction is known to cause various diseases[61,62]; although ROS are generated during oxidative phosphorylation, they are quickly eliminated and regulated to prevent exposure to excessive oxidative stress. However, the molecules and enzymes associated with the ROS elimination system decline in number and function as oocytes age[58,59,63].

In this study, we comprehensively assessed the levels of oxidative stress in the blood of patients with POI to determine the cause of POI and examine whether oxidative stress in the blood can serve as a biomarker of POI. The d-ROM levels, which indicate oxidative stress, were significantly higher in the POI group than in the control group. In contrast, BAP, which reflects antioxidant potential, did not differ significantly between the two groups, indicating that antioxidant capacity did not decline. However, OSI, similar to d-ROMs, was significantly higher in the POI group than in the control group, indicating that the POI group presented a state of oxidative stress. Factors suggested to be involved in age-related decline in the quantity and quality of oocytes include the function of mitochondria and attendant ROS cytotoxicity[34-36]. Although patients with POI do not demonstrate a diminished antioxidant potential, excessive oxidative stress is suggested to be involved in early depletion of follicular and inhibition of follicle development.

Additionally, continuity is observed in the attenuation of ovarian function in the pathogenesis of POI. Knauff *et al*[64] previously described the process of POI as the following steps: (1) Menstruation is normal at first but subsequently shifts to incipient ovarian failure (IOF), in which FSH is slightly elevated (> 10.2 IU/L); (2) transitional ovarian failure, which presents with elevated FSH and irregular menstruation; (3) amenorrhea, in which FSH is ≥ 40 IU/L for at least 4 mo; and (4) follicle depletion and/or a halt in follicular development[64]. Extremely severe infertility is a major problem associated with POI. Although only approximately 25% of patients with POI demonstrate decreased ovulation, the

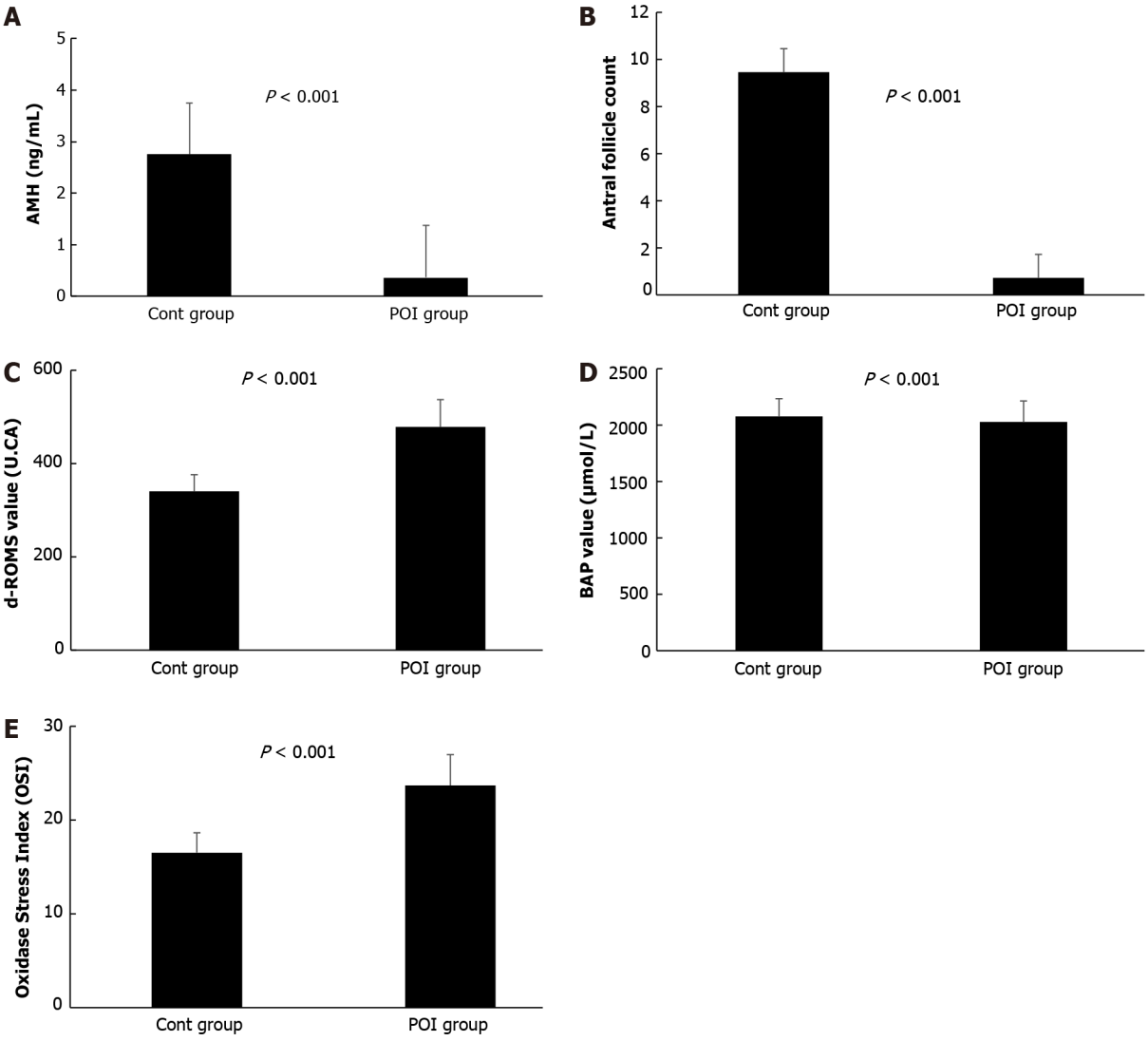


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**Figure 2 Comparison of anti-Müllerian hormone, antral follicle count, mean plasma levels of diacron-reactive oxygen metabolites, mean plasma levels of biological antioxidant potential, and the mean oxidase stress index between the control and premature ovarian insufficiency groups.** A: Anti-Müllerian hormone; B: Antral follicle count; C: Mean plasma levels of diacron-reactive oxygen metabolites; D: Mean plasma levels of biological antioxidant potential; E: Mean oxidase stress index. Values are expressed as means, with the error bar representing the standard deviation. Significant difference between the control and premature ovarian insufficiency groups, $P < 0.001$. POI: Premature ovarian insufficiency; Cont: Control; AMH: Anti-Müllerian hormone; d-ROMs: Diacron-reactive oxygen metabolites; BAP: Biological antioxidant potential; OSI: Oxidase stress index.

lifetime pregnancy rate with their own ova among these patients is only 5%-10%[11,12,23,24], suggesting intractable infertility in patients with POI. To date, no reliable treatment option is available to maintain reproductive capacity in POI, and the ESHRE guidelines hold that there is no medical intervention worth recommending other than oocyte donation[25,26]. Progressive ovarian insufficiency is a persistent condition, and women with POI who wish to conceive require treatment before their remaining follicles are depleted. This situation requires early diagnosis before the patient's condition progresses to POI. In this study, we comprehensively assessed POI in terms of oxidative stress in the blood (d-ROMs) and antioxidant potential (BAP), which have been suggested as potential biomarkers of POI; however, ROS and free radicals are unstable, complicated, and hinder their measurements. The d-ROM test does not directly measure ROS or free radicals but instead measures the level of blood hydroperoxide generated by ROS and free radicals, thereby conceivably reflecting oxidative stress in the blood more sensitively. In the future, we intend to examine whether the comprehensive assessment of d-ROMs and BAP in the blood in IOF and transitional ovarian failure (the stages before POI) can be used as a biomarker to facilitate the early diagnosis of POI and investigate the potential for early diagnosis and therapeutic intervention for POI.

This study was limited by the small sample size. However, as the number of target cases at this time was small, we will continue to accumulate data and examine a larger sample size in the future and investigate how the state of oxidative stress in the blood and local factors, such as a decrease in the number of remaining follicles associated with ovarian dysfunction and an increase in egg quality, affects

POI pathogenesis. Moreover, in future studies, we intend to examine in detail how the cytotoxicity of ROS, which is thought to be the cause of this decrease, is reflected.

## CONCLUSION

Oxidative stress (d-ROM, OSI) was significantly greater in the POI group than in the control group, suggesting that oxidative stress status is a factor in POI and could serve as a biomarker of POI. This result suggests that the oxidative stress status is likely useful for fertility treatment and other forms of early therapeutic intervention. In the future, we plan to investigate the detailed mechanism underlying how the state of oxidative stress in the blood affects the pathology of POI.

## ARTICLE HIGHLIGHTS

### *Research background*

Premature ovarian insufficiency (POI) is characterized by the premature decline of ovarian function, inducing secondary amenorrhea and leading to severe infertility. Excessive production of reactive oxygen species (ROS) induces DNA damage, lipid peroxidation, and protein denaturation, while oxidative stress causes or exacerbates various diseases. The function of mitochondria in the ovaries and the cytotoxicity associated with ROS have been implicated in follicle pool depletion and follicle quality decline. Diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) can be easily measured, and tests have been developed for the comprehensive evaluation of blood oxidative stress by combining the d-ROMs and BAP tests.

### *Research motivation*

Most cases of POI are idiopathic, and no definitive cause has yet been identified. Investigation of the cause of POI, early diagnosis, and early intervention are warranted.

### *Research objectives*

This study sought to comprehensively assess the oxidative stress status with d-ROMs and BAP tests in POI and to investigate whether these can be biomarkers for POI.

### *Research methods*

To comprehensively assess oxidative stress status, we measured plasma d-ROM and BAP in POI and control groups.

### *Research results*

The d-ROMs level and the oxidase stress index were significantly higher in the POI than in the control group. However, the BAP level did not significantly differ between the two groups.

### *Research conclusions*

Oxidative stress (d-ROMs, OSI) in the POI group was significantly higher than in the control group, suggesting that the oxidative stress state may be a factor in POI and a potential biomarker. Therefore, it may be useful for early intervention for treatment, including infertility treatment.

### *Research perspectives*

Oxidative stress was significantly higher in patients with POI than in healthy controls, suggesting the use of this measurement as a biomarker of POI. In the future, we plan to investigate whether these markers are useful for the early diagnosis of POI and how the state of oxidative stress affects the pathology of POI.

## FOOTNOTES

**Author contributions:** Kakinuma K and Kakinuma T contributed to the methodology, software design, validation, and formal analysis, writing-original draft preparation, writing-review and editing, visualization, supervision, and project administration.

**Institutional review board statement:** The present study was approved by the Institutional Review Board of the International Health and Welfare Hospital (approval No. 21-Im-075, approved on 3/22/2022).

**Informed consent statement:** All study participants or their legal guardian provided informed written consent about

personal and medical data collection prior to study enrolment.

**Conflict-of-interest statement:** All the Authors have no conflict of interest related to the manuscript.

**Data sharing statement:** The original anonymous dataset is available on request from the corresponding author at tokakinuma@gmail.com.

**STROBE statement:** The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.



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