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Case Control Study

Significance of oxidative stress and antioxidant capacity (diacron-reactive oxygen metabolites and biological antioxidant potential) tests as biomarkers of premature ovarian insufficiency: A case-control study

Oxidative stress in premature ovarian insufficiency

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Abstract

BACKGROUND

Premature ovarian insufficiency (POI) is a condition that causes secondary amenorrhea due to ovarian hypofunction at an early stage. Early follicular depletion results in intractable infertility, thereby significantly reducing women's quality of life. Given the continuum in weakened ovarian function, progressing from incipient ovarian failure (IOF) to transitional ovarian failure (TOF) and further to POI, it is necessary to develop biomarkers for predicting POI. A comprehensive evaluation of the oxidative stress state in IOF and POI from both oxidative stress (diacron-reactive oxygen metabolites [d-ROMs]) test and antioxidant capacity (biological antioxidant potential [BAP]) was conducted.

AIM

To explore the possibilities of oxidative stress and antioxidant capacity as biomarkers for early detection of POI.

METHODS

Women presenting with secondary amenorrhea over 4 mo and an follicle stimulating hormone (FSH) level of above 40 mIU/mL were categorized as the POI group; women presenting with a normal menstrual cycle and an FSH level above 10.2 mIU/mL were the IOF group; and normal women without ovarian hypofunction were the control group. Among women under 40 who visited our hospital from January 2021 to June 2022, we recruited 11 women in the POI group and 11 women in the IOF group. For potential antioxidant capacity, the relative oxidase stress index (BAP/d-ROMs x 100) was calculated, and the oxidative stress defense system was comprehensively evaluated.

RESULTS

d-ROMs were significantly higher in the POI group and the IOF group than in the control group, with 341.1 ± 35.1 U.CARR in the control group, 434.5 ± 60.6 U.CARR in the IOF group, and 478.2 ± 58.7 U.CARR in the POI group, but no significant difference was found between the POI group and the IOF group. Regarding BAP, no significant difference was found between the three groups, with 2,078.5 ± 157.4 μmol/L in the control group, 2,116.2 ± 240.2 μmol/L in the IOF group, and 2029.0 ± 186.4 μmol/L in the POI group. The oxidase stress index was significantly higher in the POI group and the IOF group than in the control group, with 16.5 ± 2.1 in the control group, 23.7 ± 3.3 in the POI group, and 20.7 ± 3.6 in the IOF group, but no significant difference was found between the POI group and the IOF group.

CONCLUSION

The high levels of oxidative stress suggest that the evaluation of the oxidative stress state is useful as an indicator for the early detection of POI.

Key Words: Premature ovarian insufficiency; Oxidative stress; Diacron-reactive Oxygen metabolites test; Biological antioxidant potential; Infertility; Biomarker

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Core Tip: The majority of POI cases are idiopathic, but regardless of the cause, the disease results in a rapid decrease in the number of remaining follicles in the ovary and extremely intractable infertility. The process leading to POI is marked by a decline in ovarian function starting with IOF, then TOF, and finally POI. d-ROMs and OSI were significantly higher in the IOF and POI groups than in the control group, indicating that evaluating oxidative stress status is useful for early diagnosis of POI.

INTRODUCTION

Among the 35 participants, two smokers were excluded. There were 11 participants in the POI group with secondary amenorrhea over 4 mo and an FSH level above 40 mIU/mL, 11 participants in the IOF group with a normal menstrual cycle and an FSH level above 10.2 mIU/mL, and 11 participants in the control group without ovarian hypofunction. Table 1 shows the backgrounds of the participants. The mean age and Body Mass Index (BMI) were 35.8 ± 3.0 years and 20.1 ± 1.9, respectively, in the control group; 37.5 ± 1.7 years and 20.1 ± 2.1, respectively, in the IOF group; and 35.8 ± 2.7 years and 19.4 ± 2.5, respectively, in the POI group. In addition, gravidity and parity were investigated, and they were 0.6 ± 0.7 and 0.5 ± 0.5, respectively, in the control group; 0.4 ± 0.5 and 0.2 ± 0.4, respectively, in the IOF group; and 0.6 ± 0.9 and 0.3 ± 0.5, respectively, in the POI group. No significant difference was found among the groups in terms of mean age, BMI, gravidity, and parity (Table 1)

AMH was significantly lower in the IOF and POI groups than in the control group, with 2.8 ± 1.4 ng/mL in the control group, 1.3 ± 1.3 ng/mL in the IOF group, and 0.4 ± 0.3 ng/mL in the POI group, but no significant difference was found between the POI

and IOF groups (Figure 1). AFC was significantly lower in the POI and IOF groups than in the control group and significantly less in the POI group than in the IOF group, with 9.5 ± 2.4 in the control group, 5.6 ± 1.8 in the IOF group, and 0.7 ± 0.9 in the POI group (Figure 2). d-ROMs were significantly higher in the POI and IOF groups than in the control group, with 341.1 ± 35.1 U.CARR in the control group, 434.5 ± 60.6 U.CARR in the IOF group, and 478.2 ± 58.7 U.CARR in the POI group, but no significant difference was found between the POI and IOF groups (Figure 3). Regarding BAP, no significant difference was found among the three groups, with 2,078.5 ± 157.4 µmol/L in the control group, 2,116.2 ± 240.2 µmol/L in the IOF group, and 2029.0 ± 186.4 µmol/L in the POI group (Figure 4). OSI was significantly higher in the POI and IOF groups than in the control group, with 16.5 ± 2.1 in the control group, 23.7 ± 3.3 in the POI group, and 20.7 ± 3.6 in the IOF group, but no significant difference was found between the POI and IOF groups (Figure 5).

MATERIALS AND METHODS

POI is a disorder diagnosed as ovarian amenorrhea under the age of 40. When normal ovarian function is lost, decreased ovarian function results in low estradiol levels, infertility, climacteric symptoms, low bone density and the accompanying fracture, circulatory diseases, and mental disorders such as depression, anxiety, and cognitive disorders that are likely to appear; thus, appropriate management is essential. Causes of POI include heredity, iatrogenicity, autoimmunity, metabolism, infection, and environmental factors, but the majority of POI cases are idiopathic without a clear cause[2,3].

However, regardless of the cause, the remaining follicle count in the ovary decreases rapidly, exceeding the physiological level and resulting in extremely intractable infertility. In POI, the lifetime pregnancy rate with own eggs is extremely low, and at present, infertility treatment using eggs donated by a third party instead of own eggs is the most effective and the only evidence-based infertility treatment in terms of time, efficiency, and live birth rate[2,3]. Follicle reduction in POI is progressive, and there is no

means to increase the remaining follicle count. For patients who wish to have children, in addition to guidance to begin infertility treatment as soon as possible, an early diagnosis before the transition to POI is desirable.

The ovary is considered to be one of the organs with the earliest decrease in function compared to other organs, and decreased ovarian function causes a decreased remaining follicle count and decreased egg quality, leading to infertility[35,38]. Apoptotic pathways have been suggested to be involved in these [39,40]. It has been suggested that mitochondrial function in eggs and cytotoxicity caused by accompanying reactive oxygen are involved as factors inducing apoptosis[28,29].

Oxidative stress is defined as the difference between the oxidative damage ability of ROS generated *in vivo* and the antioxidant potential of the *in vivo* antioxidant system[16,17]. It is thought that most reactive oxygen is generated in mitochondria, impairing lipids, proteins, and DNA as well as mitochondria themselves and, as a result, causing various diseases such as senility at the cellular level, arteriosclerosis, diabetes mellitus, and malignant diseases[20].

In a study on the mitochondrial membrane potential of human eggs, the mitochondrial membrane potential of eggs harvested from elderly women with decreased remaining follicle count and egg quality due to reduced ovarian function was significantly lower than the mitochondrial membrane potential of young eggs[36]. Mitochondria have mtDNA, which is a double-stranded circular DNA with its own genome, and it has been reported that the percentage of deletions and point mutations in mtDNA is higher in eggs harvested from elderly women than in eggs from young women[41,42]. In a study on differential gene expression by microarray analysis using ovulation eggs of elderly mice and young mice, the expression of genes involved in mitochondrial function, oxidative stress regulation, and stabilization of DNA and chromosomes was found to be decreased in the eggs of old mice[43], and in a similar study using human eggs, the expression of genes belonging to the same category was decreased[44]. These reports suggest that mitochondrial dysfunction occurs. Mitochondria are intracellular organelles that play a central role in energy metabolism.

They supply intracellular Adenosine triphosphate (ATP) *via* oxidative phosphorylation, and mitochondrial dysfunction is known to be the cause of various diseases[45,46]. In mitochondria, reactive oxygen is generated in the process of oxidative phosphorylation, but the reactive oxygen is promptly scavenged, and is regulated to avoid excessive oxidative stress. However, it has been reported that molecules and enzymes associated with the reactive oxygen scavenging system decrease or become dysfunctional due to the aging of eggs[43,44,47].

In addition, it has been reported that increased oxidative stress exacerbates intracellular Ca^{2+} regulatory mechanisms[48]. Eggs show a cyclical increase/decrease in intracellular Ca^{2+} during fertilization; the phenomenon is called Ca^{2+} oscillation. Ca^{2+} oscillation during fertilization in eggs is associated with the release of surface granules, the resumption of meiosis, and the activation of eggs. It has been reported that increased oxidative stress reduces mitochondrial function and ATP production and that changes in the intracellular Ca^{2+} regulatory mechanism centering on the endoplasmic reticulum are associated with abnormal embryo growth[49,50].

In recent years, it has become possible to comprehensively evaluate the blood oxidative stress state conveniently by combining d-ROMs and BAP.

In the process to POI, there is a continuum of weakened ovarian function, starting from IOF, then TOF, and finally POI[16]. Thus, in the present study, by comprehensively evaluating the blood oxidative stress of patients with IOF and POI, we examined whether they could be biomarkers for the early diagnosis of POI. d-ROMs, an indicator of oxidative stress, compared to that in the control group at the stage of IOF. On the contrary, in the examination of antioxidant capacity, BAP was constant regardless of disease progression; no significant difference was found among the three groups (the control, IOF, and POI groups); and no decrease in antioxidant capacity was observed. Meanwhile, relative OSI increased from the stage of IOF, as with d-ROMs, suggesting that by combining blood d-ROMs and BAP, it can be an indicator for early diagnosis of POI. As reactive oxygen and free radicals are unstable, the measurement is complex and difficult. In the present study, rather than directly measuring reactive oxygen and free

radicals themselves in the examined d-ROMs, we measured the ROOH produced by them. This more clearly reflects the state of blood oxidative stress, and it is thought that the increase in oxidative stress could be evaluated from the state of IOF exhibiting mild ovarian function.

In the future, it is necessary to examine in detail what kind of influence the blood oxidative stress state demonstrated in this study has on the pathological conditions of IOF and POI and, as a local factor, how it reflects cytotoxicity caused by reactive oxygen, which is considered to be the trigger of decreased remaining follicle count and decreased egg quality associated with ovarian hypofunction.

RESULTS

Compared to those in the control group, d-ROMs and OSI are significantly higher in the IOF group, the prestage of POI transition, and in the POI group, suggesting that evaluating oxidative stress state is useful as a biomarker for the early diagnosis of POI, and it is expected to be useful for early intervention of treatment, including infertility treatment.

DISCUSSION

This study was conducted with the approval of the ethics committee of our hospital (Ethics Review Committee, International University of Health and Welfare, approval number: 21-Im-075, approval date: 2022.3.22). The participants were patients under the age of 40 who visited the International University of Health and Welfare Hospital from January 2021 to June 2022. They were given written and oral explanations about the contents of this study, and the participants provided their consent. Considering the effects on oxidative stress and antioxidant capacity, patients with gynecological diseases and severe paramenstrual symptoms requiring analgesics, patients taking other medications and supplements, and smokers were excluded.

Women presenting with secondary amenorrhea over a period of 4 mo and an FSH level of above 40 mIU/mL were categorized as the POI group; women presenting with

a normal menstrual cycle and an FSH level above 10.2 mIU/mL were categorized as the IOF group; and normal women without ovarian hypofunction were categorized as the control group.

Blood collection was done in the follicular phase (within 5 days from the start of menstruation). For ovarian function, FSH and Anti-Mullerian hormone (AMH) were measured, and Antral follicle count (AFC) was measured with a transvaginal ultrasound imaging diagnostic machine (Voluson S10 Expert, GE Healthcare Japan, Tokyo, Japan). For the evaluation of the oxidative stress state, d-ROMs, the marker for oxidative stress, and BAP, the marker for antioxidant capacity, were measured. For potential antioxidant capacity, the Oxidase stress index (OSI) (BAP/d-ROMs x 100) was calculated, and the oxidative stress defense system was comprehensively evaluated.

Methods for evaluating ovarian function

FSH was measured by chemiluminescent immunoassay (CL AIA-PACK®FSH TEST, Tosoh Corporation, Tokyo, Japan). In addition, AMH was measured using the chemiluminescent enzyme immunoassay (Access AMH®, Beckman Coulter, Tokyo, Japan).

Methods for measuring oxidative stress and antioxidant capacity

Blood oxidative stress (d-ROMs) and antioxidant capacity (BAP) in the follicular phase were measured using a free radical analyzer (FREE Carrio Duo: Diacron International, Grosseto, Italy). The validity and reproducibility of the measurement method using this instrument have previously been reported in other papers[34]. To measure d-ROMs, 20 µL of plasma was collected from postcentrifugation blood and mixed into an acidic buffer of pH 4.8. Thereafter, colorless aromatic amine aqueous solution (color liquid chromogen) was further added and mixed; the mixture was transferred into the photometer in the instrument; the decrease in absorbance at 505 nm was measured after 5 min; and plasma hydroperoxide concentration was calculated and measured from the rate of change. d-ROMs assess the degree of OSI by measuring

blood hydroperoxide (ROOH) concentrations produced by *in vivo* reactive oxygen and free radicals *via* color reaction[34]. The unit used is U.CARR, and 1 U.CARR is equivalent to 0.08 mg/dL of hydrogen peroxide. Reference values applied are: normal values: 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 markedly severe oxidative stress: over 501[35].

To measure BAP, a reagent containing thiocyanic acid derivatives and a reagent containing iron ions were mixed and placed in the photometer of the instrument, and absorbance at 505 nm was measured. Furthermore, 10 µL of plasma was added to this mixture, which was then incubated for 5 min at 37°C, and the absorbance was measured again. The oxidized iron ion concentration was calculated from the change in absorbance in 5 min. BAP demonstrates the ability of plasma solution to reduce trivalent iron (Fe3＋) to bivalent iron (Fe2＋) (redox). The unit is µmol/L, and the reference values applied are as follows: optimal values: over 2,200, borderline: 2000–2,200, mildly deficient antioxidant capacity: 1800–2000, deficient antioxidant capacity: 1600–1,800, markedly deficient antioxidant capacity: 1400–1,600, and severely deficient antioxidant capacity: under 1,400)[36,37].

Data analysis

All measurement values were expressed as mean ± standard deviation. One-way analysis of variance and the Tukey–Kramer multiple comparison test were used for statistical analysis. JMP® version 14.2 (SAS Institute Japan Co., Ltd., Tokyo Japan) statistical processing software (IBM SPSS Statistics 21) was used for statistical processing. A P-value of less than 0.05 was determined to be statistically significant.

CONCLUSION

Premature ovarian insufficiency (POI) is a disorder diagnosed as ovarian amenorrhea in patients under the age of 40. POI is characterized by the loss of normal ovarian function and is defined as hypergonadotropic amenorrhea[1]. Causes of POI include chromosome

abnormalities, iatrogenicity, autoimmune disorders, metabolic disorders, infections, and environmental factors, but the majority of POI cases are idiopathic without a clear cause[2,3]. POI develops naturally in one out of 100 women[4], and its onset frequency by age is reported to be approximately 1% in patients under the age of 40, 0.1% in patients under the age of 30, and 0.01% in patients under the age of 20[4]. However, in today's modern society where the trends of late marriage and late childbirth are prevalent, patients with POI who achieve pregnancy before onset have been targets of infertility treatment, and recent reports have indicated that the incidence itself may be increasing[5,6].

Genetic factors account for approximately 10%–17% of POI cases. Many of them are abnormalities associated with the X chromosome, and numerical abnormalities, structural abnormalities, and genetic abnormalities on the X chromosome have been reported[7-10]. Numerical abnormalities include 45, X (Turner syndrome), 47, XXX, and their mosaic. In addition, structural abnormalities include partial deletion, translocation, ring chromosome, isochromosome, and additional chromosome. It has been reported that DIAPH2, DACH2, XIST, *etc.*, which are genes on the X chromosome, are involved in the onset of POI. Besides X chromosome abnormalities, blepharophimosis, ptosis, and epicanthus inversus syndrome, which develops due to mutations in FOXL2 found on an autosome, have also been reported[7-9]. In a study using genetically modified mice, many genetic abnormalities exhibiting the phenotype of POI have been reported; in fact, NOBOX, BMP15, FSHR, *etc.* have been confirmed in humans[10].

In POI, regardless of the cause, the activation of primordial follicles stops, and as a result, the recruitment of growing follicles vanishes. Generally, when the remaining follicle count in the ovary is under 1,000, the activation of primordial follicles stops, and consequently, the recruitment of growing follicles vanishes and follicles leading to ovulation are lost. Because follicles leading to ovulation are lost due to early follicular depletion, inhibition of follicle development, and destruction of the follicle pool, the remaining eggs and follicles either become extremely few or completely disappear[2,3].

Because there are no growing follicles, there are almost no granulosa cells, the main source of estrogen, thereby causing blood estrogen levels to fall. As a result, the endometrium does not thicken. Furthermore, as there is no post-ovulatory corpus luteum, progesterone secretion and withdrawal bleeding caused by the regression of the corpus luteum do not occur, resulting in amenorrhea. Therefore, in POI, the loss of ovarian function causes low estradiol levels, and mental disorders such as climacteric symptoms, depression, anxiety, and cognitive disorders are likely to appear; thus, it is essential to appropriately manage it by improving the quality of life as well as preventing fractures caused by bone and circulatory diseases and decreased density[1,11].

Furthermore, major issues related to POI include extremely severe infertility. In this disorder, amenorrhea or anovulation associated with decreased follicle count is the main cause of infertility[12]. Approximately 25% of patients with this disorder have decreased ovulation, but the lifetime pregnancy rate with own eggs is reported to be 5%–10%[2,3,12,13], leading to intractable infertility. At present, there is no reliable reproductive healthcare for this disorder, and in the European Society of Human Reproduction and Embryology Guidelines, there is no recommended medical intervention other than egg donation[14,15]. This disorder is a progressive disorder that requires treatment before remaining follicles are depleted if pregnancy is desired, and early diagnosis before reaching POI is required.

However, oxidative stress has been reported to cause various diseases. Oxidative stress is defined as a state where *in vivo* oxidative capacity exceeds antioxidant capacity[16,17]. Reactive oxygen species (ROS) is the general term for oxygen derivatives with high oxidative capacities, such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. It is known that there are many ROS-generating systems *in vivo* such as the mitochondrial electron transport system, xanthine oxidase, and NADPH oxidase[18]. Today, it has been clarified that redox signaling by ROS plays an important role in regulating various life phenomena, such as immune response and wound healing[19]. On the contrary, as ROS are highly toxic substances that cause nonspecific injury to cells due to their high reactivity, *in vivo* redox balance breakdown induced by

ROS dysregulation causes oxidative stress, and DNA damage, lipid peroxidation, and protein denaturation result in intractable diseases such as senility, cardiovascular diseases, neurodegenerative diseases, and cancer[20]. Furthermore, in germ cells, they have been reported to increase the risk of the incidence of hereditary diseases, infertility, and miscarriage[21,22]. It has also been suggested that mitochondrial function in eggs and cytotoxicity due to accompanying reactive oxygen are involved as factors leading to decreased egg count or decreased egg quality observed in aging[23,24].

However, in the body, there is an antioxidant mechanism that scavenges constantly generated ROS, thereby protecting the body from oxidative capacity induced by ROS. The ROS scavenging mechanism includes endogenous antioxidant enzymes and exogenous antioxidants. Endogenous antioxidant enzymes include superoxide dismutase and catalase that scavenge superoxide and hydrogen peroxide, as well as glutathione peroxidase that reduces lipid hydroperoxide[25]. There are many types of exogenous antioxidants, including carotenoids (lycopene and astaxanthin), polyphenols (carotenoids, catechin, and curcumin), and antioxidant vitamins (vitamin C, vitamin E, and β-carotene)[26-29]. As a result, oxidative capacity and antioxidant capacity are balanced, maintaining homeostasis without developing oxidative stress and preventing injuries[16,17].

In recent years, tests for diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP), which can be easily measured, have been developed. The combination of the d-ROMs and BAP tests is used for the comprehensive evaluation of blood oxidative stress[30,31]. The d-ROMs test comprehensively evaluates the state of oxidative stress *in vivo* by measuring primarily the hydroperoxide concentration in the blood caused by reactive oxygen and free radicals by color reaction vivo[32]. The BAP test evaluates antioxidant capacity by measuring the reducing ability of antioxidants to donate electrons to reactive oxygen and free radicals to stop oxidation[32].

There is a continuum in the weakened ovarian function of POI. According to Knauff *et al*, it begins with incipient ovarian failure (IOF) with normal menstruation but a

slightly high follicle stimulating hormone (FSH) level (>10.2 IU/L), progresses to transitional ovarian failure (TOF) with a high FSH level and irregular menstruation, and when it progresses further, the patient presents with amenorrhea over 4 mo and an FSH level of above 40 IU/L and finally transitions to a condition where follicles are depleted, or growth stops[33].

This study aims to comprehensively evaluate oxidative stress state in IOF and POI using both the oxidative stress and antioxidant capacity and investigate whether they can be used as biomarkers to aid in the early detection of POI.

ARTICLE HIGHLIGHTS

Research background

+ADw-html+AD4APA-p+AD4-Premature ovarian insufficiency (POI) is a condition that causes secondary amenorrhea due to decreased ovarian function before the age of 40. Early follicle depletion causes intractable infertility and significantly reduces a woman's quality of life. Mitochondrial function within the egg and cytotoxicity caused by the accompanying active oxygen have been suggested to be involved as one of the factors contributing to the decrease in the number of remaining follicles and the decline in oocyte quality due to decline in ovarian function. There is a continuum in the decline of ovarian function, including incipient ovarian failure (IOF), transitional ovarian failure (TOF), and, as it progresses, transition to POI.+ADw-/p+AD4APA-/html+AD4-

Research motivation

There is a need to discover biomarkers for early detection of POI and to investigate the etiology.

Research objectives

The purpose of this study was to comprehensively evaluate the oxidative stress state in IOF and POI from both oxidative stress (Diacron-Reactive Oxygen Metabolites test: d-

ROMs) and antioxidant potential (Biological Antioxidant Potential: BAP). , to explore its potential as a biomarker for early detection of POI.

Research methods

11 women with secondary amenorrhea for 4 mo or more and an FSH value of 40 mIU/mL or more were included in the POI group, and 11 women with normal menstrual cycles and an FSH value of 10.2 mIU/mL or more were included in the IOF group. d-ROMs and BAP in the plasma of each group were measured using normal women of the same age without ovarian function decline as a control group.

Research results

In the POI and IOF groups, d-ROMs and Oxidase Stress Index (OSI) were significantly higher than in the control group. Regarding BAP, no significant difference was observed between the three groups.

Research conclusions

Oxidative stress (d-ROMs, OSI) in the IOF and POI groups was significantly higher than in the control group, suggesting that evaluation of oxidative stress status is useful as an indicator for early detection of POI.

Research perspectives

d-ROMs and OSI were significantly higher in the IOF group, which is the pre-POI transition stage, as well as in the POI group compared to the control group, and evaluation of oxidative stress status is useful as a biomarker for early diagnosis of POI. It is expected that this finding will be useful for early intervention in treatments such as infertility treatment.

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