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Review article

Antioxidative action of melatonin and reproduction

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Abstract

Melatonin secreted via the pineal gland has been proven to have strong antioxidant activity. In addition to its activity as a neuroendocrine hormone through receptors, its relationship with the aging phenomena and various diseases has received particular attention. Reactive oxygen species (ROS) play crucial roles in reproductive functions, such as the ovulatory process; however, in excess, they may adversely affect oocytes as oxidative stress and cause of infertility. Melatonin existing in follicles may possibly protect oocytes from ROS with its antioxidant activity. Recent reports have clarified the role of melatonin in the maturation of oocytes; fertilization and embryonic development. The research using the oocytes of mice and cows shows the possibility that melatonin addition in culture medium improves oocyte growth and embryonic development. As a clinical application in humans, the trials to improve the outcome of assisted reproductive medicine, represented by *in vitro* fertilization and embryo transfer, have been conducted by administering infertile patients with melatonin.

KEY WORDS: melatonin, antioxidant, reactive oxygen species (ROS), oxidative stress, ovum

Introduction

Melatonin is an indoleamine derivative (molecular weight: 232.2) produced by the pineal gland on the back wall of the third ventricle. It is mainly secreted at night and almost none in the daytime; the rhythm of melatonin secretion is controlled by light and dark cycles and photic stimulation. The secretion rhythm of melatonin plays important roles in the formation and adjustment of the biological clock, generating circadian rhythm, controlling body temperature, secretions of various hormones, and sleep-wake rhythm. Since melatonin has the characteristic property of lipid-soluble and water-soluble, it can easily pass through cell membranes. It exists not only in blood, but also in body fluids such as cerebral spinal fluid, follicular fluid, and semen. Melatonin receptors exist in various organs of the entire body, and the involvement of melatonin and its receptors in various function including, biological rhythm, secretion of various hormones, immune functions, lipid and glucose metabolisms, and bone metabolism, and their relationships with aging, carcinogenesis, and various diseases have been clarified. Recently, it was clarified that melatonin has a strong antioxidant ability to scavenge free radicals, such as reactive oxygen species (ROS). In addition to the neuroendocrine effect of melatonin through its receptors, its direct antioxidant activity as a free radical scavenger without through its receptors produces various functions.

Reactive oxygen species (ROS) production mechanism and oxidative stress

ROS is a general term for highly reactive compounds generated when oxygen molecules are activated, and it is considered that they cause the aging phenomena, carcinogenesis and various diseases due to its cytotoxicity. A free-radical is a kind of ROS, and it is defined as a molecule having an unpaired electron in an orbit. Compared with the molecule having two electrons in an orbit, a free radical is energetically unstable; therefore, it steals an electron from a surrounding molecule (reduction) and becomes stable. As a result, the molecule robbed of an electron becomes unstable (oxidation).

The causes generating ROS are cellular respiration generating energy from oxygen within mitochondria,

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environmental factors such as ultraviolet rays and chemical materials, and living factors such as stress, smoking, and dietary habits. Even though ROS, such as superoxide (O_2-) and hydrogen peroxide (H_2O_2), are generated by cellular respiration, they can be detoxified with antioxidant enzymes such as superoxide dismutase (SOD) and catalase. However, when H_2O_2 reacts with iron molecules in a body, hydroxyl radical (·OH), ROS with the strongest cytotoxicity, is generated. Hydroxyl radical (·OH) accounts for 70% of ROS generated in a body and it causes damage to protein molecules, damage to DNA, and peroxidation of cell membranes. Oxidative stress is a state of imbalance between free radical production and their degradation by antioxidant systems with increased accumulation of ROS in the tissues and organs.

Reactive oxygen species (ROS) and reproductive functions

ROS play important roles in reproductive functions such as follicular growth, oocyte maturation, ovulation, fertilization, implantation and embryonic development [1]. During the ovulation process after luteinizing hormone (LH) surge, a great amount of ROS is generated from vascular endothelial cells and macrophage in association with promotion of vascularization within follicles. Even though the ROS generated from follicles become the stimulus necessary for oocyte maturation and the rupture of follicles, the excessive ROS also have cytotoxicity. Meanwhile, antioxidant enzymes and antioxidant substances exist in follicles as a defense mechanism against ROS, and they are protecting oocytes and granulosa cells from oxidative stress. If ROS and antioxidant defence become imbalanced, oocytes and granulosa cells may be easily damaged by oxidative stress and could possibly lead to a decline in the quality of oocytes. In the animal model experiment with mouse, when the super-ovulation stimulation to induce follicle growth was conducted by pregnant mare serum gonadotropin (PMSG) and ovulation was induced by human chorionic gonadotropin (hCG), the increase of concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is a DNA damage marker, and hexanoyl-lysine (HEL), which is a lipid peroxide marker, in follicles before ovulation was observed [2]. In the human clinical analysis, infertile women with a high degenerated oocyte (oocytes in bad condition) rate in the *in vitro* fertilization (IVF) program showed the high concentration of 8-OHdG in their follicular fluid, and the oocytes retrieved from high concentration of 8-OHdG in follicular fluid also showed a low fertilization rate [3]. Therefore, the ROS generated during the ovulation process in follicles become a cause of oxidative stress of the oocyte and granulosa cells, which degrades the quality of oocytes and can also become a cause of infertility.

Antioxidant effect of melatonin

A new development in melatonin was discovered in a finding by Reiter Laboratory in 1993. They proved that melatonin has a powerful antioxidant effect [4]. Melatonin is a direct free radical scavenger, and it is reported that its potency is stronger than those of antioxidant materials such as vitamin C and E, mannitol and glutathione. Because melatonin is lipid soluble and water soluble and can easily pass through cell membrane, it exists not only in cytoplasm but also in mitochondria and cell nuclei in high concentrations. It works as a scavenger against either of the ROS and reactive nitrogen species generated locally in cells, and possibly protects DNA and cell components. The antioxidant effects of melatonin are very broad, such as superoxide anion ($O_2^{\cdot -}$), hydroxyl radical (·OH), singlet oxygen ($^1O_2$), H_2O_2, hypochlorous acid (HOCl), nitric monoxide (NO), and peroxynitrite ($ONOO^-$) [5]. Melatonin is a valuable substance with a strong scavenging effect against hydroxyl radical (·OH), in particular. If melatonin scavenges free radicals, it becomes a metabolic product, such as cyclic 3-hydroxmelatonin (C3OHM), N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), and N1-acetyl-5-methoxykynuramine (AMK). It has been proven that these metabolic products also have a powerful antioxidant effect, which is similar to melatonin [6]. Furthermore, melatonin has the effect of indirectly increasing the activities of antioxidant enzymes such as SOD and glutathione peroxidase (GPx), and it is considered that it increases the activities and mRNA expressions of antioxidant enzymes through the membrane receptor of melatonin (MT1 and MT2) [7].

Melatonin in ovaries

It has been clarified that melatonin may directly act on the ovaries. Melatonin exists in human follicular fluid in high concentrations, and it increases proportionally with the growth of follicles [8]. According to a report on administering melatonin to cats and discussing tissue transitivity, it was found that melatonin is accumulated in greater volume in the ovaries than in other organs; thus, it is considered that there is a mechanism which melatonin takes up into follicle and melatonin levels in ovarian follicles to increase depending on follicular growth [9]. The physiological significance of why melatonin is taken up into follicles and exists there in high concentration has not been clarified for a long time. The ovulation is understood as an inflammation-like phenomenon. Antioxidant enzymes and antioxidant substances exist as a defense mechanism against ROS generated in follicles, and they protect oocyte and granulosa cells from ROS. Melatonin possibly plays an important role as an antioxidant substance in follicles. According to research conducted on the follicular fluid collected at the time of oocyte retrieval from women who underwent *in vitro* fertilization and embryo transfer (IVF-ET) program, although no significant correlation was recognized between the concentration of 8-OHdG and those of antioxidant enzymes of Cu, Zn-SOD, and glutathione, a significant negative correlation was observed between the concentration of melatonin and that of 8-OHdG. Furthermore, in the infertile women who orally took a melatonin agent (3 mg/day, taken orally at 22:00), the concentration of melatonin in the follicular fluid significantly increased after melatonin treatment compared to the previous cycle of without melatonin administration, and the concentration of 8-OHdG in melatonin-treatment cycle significantly decreased after melatonin treatment compared to the previous cycle [3]. These results suggest the possibility that melatonin decreases

oxidative stress in follicular fluid with its antioxidant effect and protects oocytes and granulosa cells.

a. Granulosa cells and melatonin

Tanabe *et al.* reported that they investigated how melatonin alleviates the oxidative stress of granulosa cells and protects them [10]. They collected granulosa cells from mature follicles of mice and added H_2O_2 (0.1-10 m/M) as ROS in the culture media and cultured them in the existence (100 μg/mL) and absence of melatonin. DNA damage was assessed by fluorescence-based immunocytochemistry using specific antibodies for 8-OHdG, an indicator of oxidative guanine base damage of DNA, and for histone H2AX phosphorylation (γH2AX), a marker of double-strand breaks of DNA. As a result, the fluorescence intensity of 8-OHdG and γH2AX were dose-dependently increased by H_2O_2 treatment, and the increases in 8-OHdG and γH2AX intensities induced by H_2O_2 were blocked by melatonin treatment. Mitochondrial function of granulosa cells was assessed by the fluorescence intensities of MitoTracker Red probes (active mitochondria maker). The intensity of MitoTracker Red was significantly decreased by H_2O_2, whereas the decrease in MitoTracker Red intensities caused by H_2O_2 was significantly reversed by melatonin treatment. Lipid peroxidation of plasma membranes in granulosa cells was evaluated by measuring HEL, a stable oxidative stress marker for lipid peroxidation. The HEL concentrations were significantly increased by H_2O_2, and the increase was significantly blocked by melatonin treatment. Furthermore, apoptosis of granulosa cells was assessed by nuclear fragmentation using DAPI staining and by the caspase-3/7 activities. The percentage of apoptotic cells and caspase 3/7 were dose-dependently increased by H_2O_2, and the increase in numbers of apoptotic cells and caspase 3/7 induced by H_2O_2 were blocked by melatonin treatment. From these results, melatonin reduces the oxidative stress-induced DNA damage, mitochondrial dysfunction, lipid peroxidation, and apoptosis of granulosa cells, suggesting that melatonin protects these cells by reducing free radical damage of cellular components including nuclei, mitochondria, and plasma membranes.

Furthermore, Taketani *et al.* have reported the effects of ROS and melatonin on the progesterone production of granulosa cells during luteinization by using human granulosa cells [11]. The luteinized granulosa cells collected at the time of oocyte retrieval from women who underwent IVF-ET program were cultured with H_2O_2 in the presence or absence of melatonin. As a result, although no significant difference in the production of progesterone was observed by adding melatonin only, the progesterone production was dose-dependently decreased by H_2O_2. The decrease in the production of progesterone by granulosa cells induced by H_2O_2 was blocked by melatonin treatment. These results suggest that melatonin protects granulosa cells undergoing luteinization from ROS in the follicle and contributes to luteinization for progesterone production during ovulation.

b. Oocytes and melatonin

Tamura *et al.* is studying the effects of ROS and melatonin in the maturing process of oocytes using mice oocytes [3]. The oocytes in a germinal vesicle (GV) stage were collected from mice ovulation-stimulated by PMSG and cultured in a culture medium with H_2O_2 as ROS. In the maturing process of the oocytes, the first meiotic division re-starts and the release of the first polar body can be observed, so the rate of the first polar body release after twelve hours of culture showed the dose-dependently decrease by H_2O_2. Meanwhile, the decreased release of the first polar body caused by H_2O_2 was significantly improved by melatonin treatment. In other words, although oocyte maturation was impaired by oxidative stress, melatonin protected oocytes from ROS. Furthermore, in order to investigate whether melatonin exerts an antioxidant effect within oocyte cells, oocytes were incubated with dichlorofluorescein (DCF-DA). The nonfluorescent DCF-DA was oxidized by intracellular ROS to form the highly fluorescent DCF, intracellular ROS formation was visualized by fluorescence image, and fluorescence intensity was analyzed. When oocytes were incubated without H_2O_2, there was no observable fluorescent intensity. However, high fluorescence intensities were observed in the presence of H_2O_2 (300 μm). The increased fluorescence intensity of oocytes incubated with H_2O_2 was significantly decreased by melatonin treatment. Therefore, it was clarified that melatonin has the antioxidant effect to decrease ROS within oocyte cells.

From the above results, it is considered that melatonin that was produced in the pineal gland and secreted into blood is taken in within follicles, locally scavenges ROS in follicles and reduces oxidative stress, and as a result, it protects oocytes and granulosa cells and contributes to the oocytes maturation and luteinization of granulosa cells (***Fig. 1***).

Clinical application of melatonin in reproductive medicine

Recently, great progress has been made in assisted reproductive technology (ART) technologies such as IVF-ET in the field of reproductive medicine. However, a satisfying pregnancy rate has not been obtained. It is considered that poor oocyte quality is the major cause of low pregnancy rate [12, 13]. We often experience that infertile women with unsuccessful results of IVF-ET program from poor fertilization and embryo development due to poor oocyte quality in spite of a large enough number of retrieved oocytes. Even though the mechanism of an oocyte being degraded has not been clearly identified, it is considered that the oxidative stress caused by ROS in the ovulatory process is an important factor. Meanwhile, for IVF-ET, an oocyte is cultured *in vitro* and an embryo is transferred to the uterus after the processes of oocyte maturation, insemination (incubation of oocyte and sperm), fertilization, and embryonic development. Various conditions for *in vitro* incubation over a long term are factors greatly affecting the qualities of the oocyte and embryo, so that great attention should be paid to oxidative stress caused by ROS during incubation.

As the application of melatonin having an antioxidant effect to reproductive medicine, there are two possibilities. One is *in vivo* melatonin administration to patients before ovulation (ovum pick up in IVF-ET program) to improve the oocyte quality. Another possibility is melatonin supplementation added to *in vitro* culture media to enhance the oocyte maturation, fertilization, and embryonic development (***Fig. 2***).

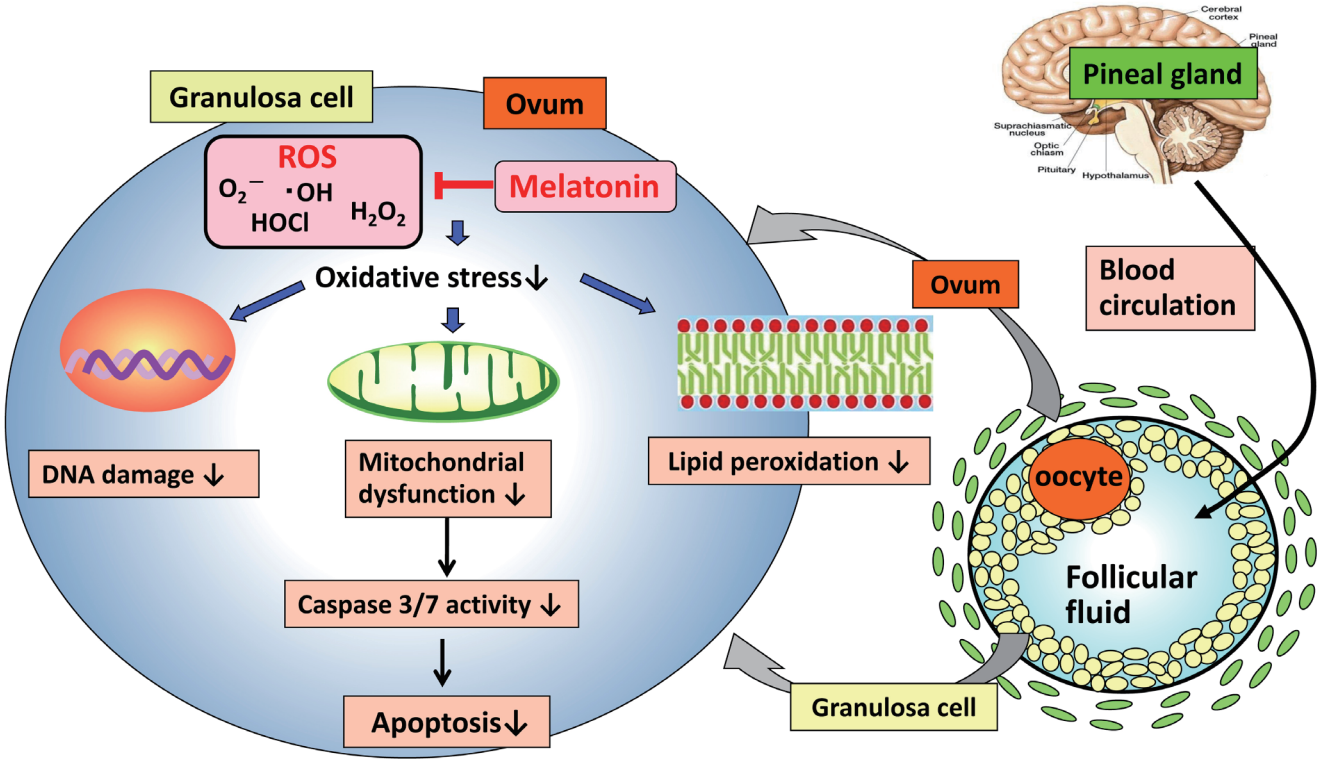

Fig. 1. **Presumed activities of melatonin in follicles.**

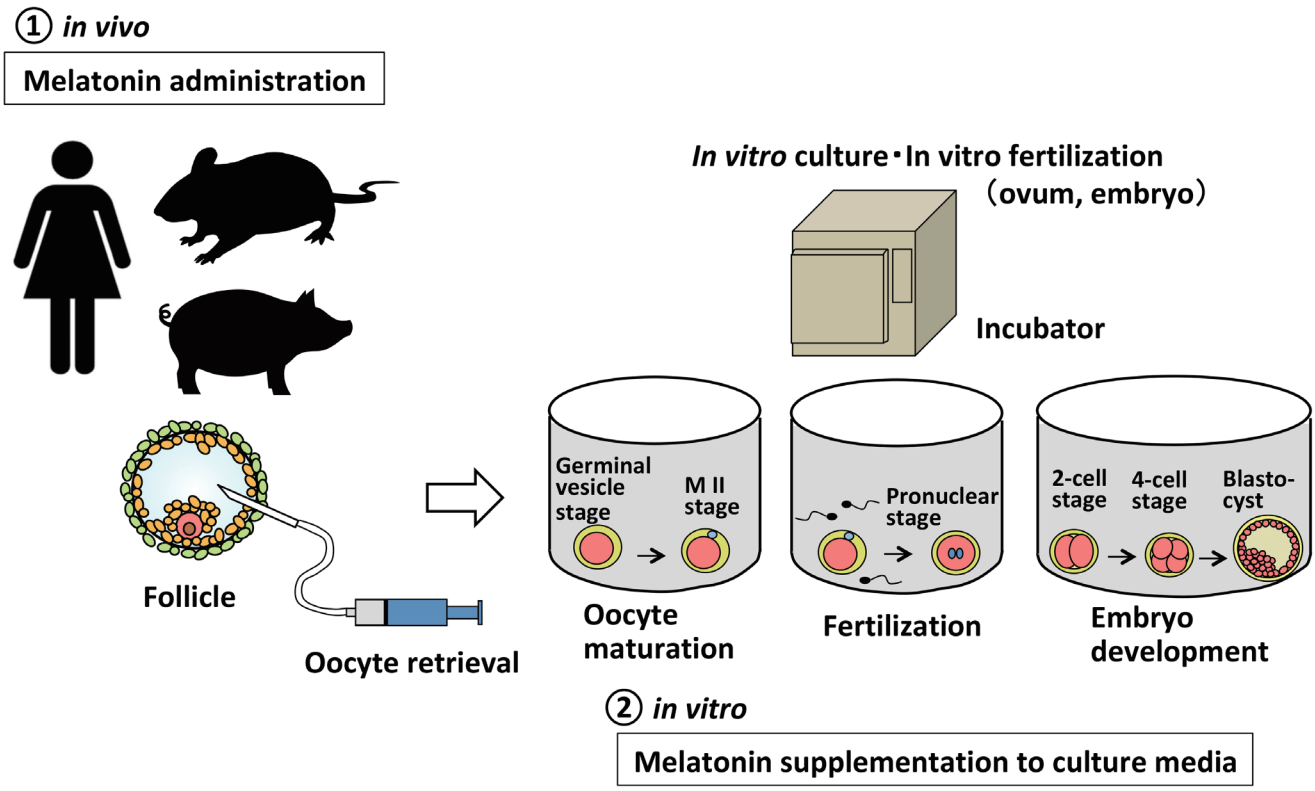

Fig. 2. **Possibility of use of melatonin in reproductive medicine.**

a. Melatonin for assisted reproductive treatment (ART)

As shown so far, melatonin exists in follicular fluid in high concentration, it increases in proportion to the development of follicles, oxidative stress is involved in the degradation of the quality of oocytes in follicles in the process of ovulation, and melatonin inhibits oxidative stress and enhances the oocytes maturation. Therefore, it is possible that melatonin administration to infertility patients whose quality of oocytes is poor may improve oocyte quality and outcome of IVF-ET program by reducing oxidative stress.

To investigate the clinical usefulness of melatonin administration, 115 patients who failed to become pregnant in the previous IVF-ET cycle with a low fertilization rate (50%) were divided into two groups during the next IVF-ET procedure; 56 patients underwent IVF-ET with melatonin treatment (daily 3 mg tablet of melatonin orally at 22:00 hr about one month until oocyte retrieval) and 59 patients without melatonin treatment (control cycle). The patients in the melatonin treatment group were given a 3 mg tablet of melatonin orally at 22:00 hr from the fifth day of the previous menstrual cycle until the day of oocyte retrieval. Degenerate oocyte rates, fertilization rates and pregnancy rates were compared to the data in the previous IVF-ET cycle (control cycle). The fertility rate of the melatonin group was approximately 50% (20% for control group) and the pregnancy rate was approximately 20% (10% for control group), suggesting an improvement of the outcome of IVF-ET was recognized [3].

After this report, many clinical trials of melatonin administration to improve the outcome of IVF-ET were carried out. These reports showed that melatonin administration to infertile women who underwent an IVF-ET program increased the number of mature oocytes, fertility rate, the number of good quality embryos, and the outcome of IVF-ET. The clinical application of melatonin to reproductive medicine may contribute via its great benefits to women who suffer from infertility.

b. Oocyte maturation, embryonic development and melatonin

It has been reported that the *in vitro* maturation of oocytes and embryonic development (blastocyst) after fertilization can be promoted by adding melatonin in culture media via research using the oocytes of various animals such as mice, cows and swine. Due to the concentration of oxygen being high in the condition of *in vitro* culture compared with *in vivo* physiological condition, the oxidative stress caused by the ROS generated during culture adversely affects oocyte maturation and embryonic development. However, it is possible to protect oocytes and granulosa cells by scavenging ROS and alleviating oxidative stress by adding melatonin in the culture media. *In vitro* experiments using mice and swine oocytes demonstrated that the oxidative stress and apoptosis are alleviated by melatonin supplementation, and mitochondria function is also improved by melatonin supplementation. As a result, oocyte maturation, fertility rate and the blastocyst rate (number of cells of blastocyst) are improved by melatonin supplementation [18-20]. The adverse effect to oocytes by oxidative stress induced by materials generating ROS such as bisphenol A (BPA) and aflatoxin B1 (AFB1) can be alleviated by melatonin supplementation [21, 22].

These actions of melatonin are to be expected as a direct antioxidant effect to alleviate active oxygen species and oxidative stress. Meanwhile, it is reported that antioxidant enzyme activity of oocytes, the expression of apoptosis-related factors, expression of genes involved in oocyte maturation and embryonic development, and epigenome changes such as DNA methylation and histon acetylation can be recognized by melatonin supplementation in the culture media [23-25]. These indirect activities of melatonin via cell membrane receptors (MT1, MT2) and nuclear receptor (RORα) of melanin also are considered to be very important for oocyte maturation and embryonic development. Melatonin membrane receptors (MT1, MT2) exist also in oocytes and granulosa cells, and there are some reports to investigate the detailed intra-cellular signalling of melatonin action [24, 26]. In addition to a direct antioxidant effect, it is considered to be important to clarify the mechanisms of melatonin via its receptors in oocytes and granulosa cells as the essential target in the future.

Ovarian aging and melatonin

Similarly with general aging, the main cause of ovarian aging is considered to be oxidative stress caused by ROS. The number and quality of oocytes rapidly decrease after latter part of the 30s. The frequencies of chromosome abnormality during meiosis and the miscarriage rate increases, and fertilization rate, blastocyst rate, and implantation rate decrease according to the age of women. Melatonin is focused on as an anti-aging hormone due to its antioxidant effect, and that it may be useful for anti-aging of ovaries.

A recent report showed the effect of long-term melatonin treatment on ovarian aging in mice [27]. The number of ovulated oocytes decreased with age; however, long-term melatonin administration (10-43 weeks) to mice increased the number of ovulated oocytes more than that from control mice. Old control mice (43 weeks) exhibited a markedly reduced number of follicles at different developmental stages including primordial, primary, secondary, and antral follicles, whereas the age-matched old mice treated with melatonin (43 weeks) had significantly more follicles than the old controls. The decreased fertilization rate and blastocyst rate during aging also were higher in the melatonin- treated mice than in the controls. Furthermore, transcriptome analysis demonstrated that melatonin alleviates ovarian aging by multiple mechanisms including ribosome function, activation of antioxidant mechanism, DNA repair, and regulation of aging-related proteins (telomere length, sirtuins).

Many women are suffering age-related infertility because the age for marriage has increased. Melatonin treatment may become a new management to prevent the decrease in number and quality of oocytes with age.

Conclusion

Melatonin, a pineal hormone, possibly scavenges ROS locally generated in follicles and protects oocytes. Many trials have been conducted to enhance oocyte maturation and embryonic development by expecting results from the antioxidant effect of melatonin. A detailed mechanism via melatonin receptors, may possibly contribute to the drastic development of reproductive medicine.

Conflict of interest statement

The authors claim no conflict of interest in this study.

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