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Melatonin Protects Oocyte from Reactive Oxygen Species

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Abstract Melatonin (N-acetyl-5-methoxytryptamine) is secreted during the dark hours at night by the pineal gland, and it regulates a variety of important central and peripheral actions related to circadian rhythms and reproduction. Melatonin is believed to regulate ovarian function by the regulation of gonadotropin release in the hypothalamus-pituitary gland axis via its specific receptors. However, it has been discovered that melatonin is a powerful direct free radical scavenger and a broad-spectrum antioxidant. High concentrations of melatonin have been found in human preovulatory follicular fluids. This study focused on the intra-follicular role of melatonin as an antioxidant in the ovary. Reactive oxygen species (ROS), which are locally produced during the ovulatory process, seem to play an essential role on follicle rupture. However, excess ROS can also be responsible for oxidative stress; they can damage oocytes within the follicle. This review shows how melatonin protects oocyte from ROS in the follicle, and contributes to oocyte maturation and embryo development. This review also demonstrated the benefit of melatonin treatment for infertile women to improve oocyte quality.

Key words: melatonin, oocyte, ovulation, reactive oxygen species, antioxidant

Introduction

Melatonin is a neuroendocrine hormone secreted by the pineal gland. The secretion is regulated by light-and-dark stimuli, and the hormone influences circadian rhythm, such as the sleep-cycle and body temperature.[1] Melatonin also plays a key role in a variety of important physiological functions, including reproduction,[2] neuroendocrine,[3] cardiovascular,[4] neuroimmunological,[5] and oncostatic actions.[6] We already reported that the role of melatonin on lipid metabolism,[7] pregnancy and parturition time,[8-10] and corpus luteum (CL) function.[11] Although some of these functions of melatonin are mediated through specific receptors (MT1/MT2), its specific high affinity membrane receptors, a considerable amount of melatonin's actions are dependent on its ability as an antioxidant. A growing number of studies have demonstrated that melatonin is a powerful direct free radical scavenger. In contrast to the majority of other known radical scavengers, melatonin is multifunctional and a universal antioxidant. The high lipophilicity and hydrophilicity of melatonin permits its rapid transfer into other organs and fluids, and melatonin can easily pass through cell membranes. Interestingly, high levels of melatonin have been found in human follicular fluid.[12,13] Our previous study demonstrated that human preovulatory follicular fluids contain higher concentrations of melatonin

than of plasma and the melatonin concentrations in follicular fluids increased depending on follicular growth.[14]

Although the physiological roles of melatonin in follicular fluid have not been understood, it is possible that melatonin plays as an antioxidant in the follicle. This review focused on the direct role of melatonin on oocyte maturation as an antioxidant to reduce oxidative stress induced by ROS. This review also discusses the first application of melatonin for the clinical treatment of infertile women with poor oocyte quality.

Ovulation and reactive oxygen species (ROS)

ROS is locally produced during follicular rupture and may be involved in the ovulation process. Luteinizing hormone (LH) surge induces a dissolution of the basement membrane between the granulosa and theca interna layers and an expansion of the theca capillaries into the avascular granulosa cell layer to form a dense network of capillaries. Macrophages and neutrophiles are well-documented to reside in follicles; it is also well-documented that they are taken into the follicles.[15,16] Tremendous amounts of free radicals are produced within the follicle not only by macrophages and neutrophiles but also by the endothelial cells of the capillaries. Locally produced ROS seems to have an essential role on follicle rupture, and ROS also have an important role as second messengers modulating the expression of genes that govern physiological processes of oocyte maturation.[17,18] However, excess ROS can also be responsible for oxidative stress; they can damage molecules and structures of oocyte and granulosa cells within the follicle. Accumulating data have shown that ROS accelarate oocyte aging and deteriorate oocyte quality.[19,20] ROS such as superoxide radical ($O2^{-}$), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2) are known to be detrimental to the oocyte. They cause deterioration of cell membrane lipids, destroy DNA and induce two-cell block, apoptosis, and inhibition of fertilization in mouse and hamster.[21-23] Also, higher levels of the oxidant H_2O_2 have been reported in fragmented human embryos compared with non-fragmented embryos and unfertilized oocytes.[20] These results suggested that excessive oxidative stress may be a cause of poor oocyte quality.

ROS must be continuously deactivated to keep only the small amount necessary to maintain normal cell function. It is well recognized that endogenous antioxidant, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, and non-enzymatic antioxidants, such as vitamin E, vitamin C, glutathione, uric acid and albumin, are present in the follicles.[19,24,25] Reduced antioxidant enzyme levels, such as GPx, are reported in the follicular fluids of women with unexplained infertility.[26] A higher level of SOD activity in follicular fluid efficiently reduced DNA damage caused by oxidative stress in porcine oocytes and cumulus cells, resulting in successful fertilization and development to the blastocyst stage after in vitro insemination; however, these abilities were interrupted by the SOD inhibitor.[27] When mice were given antioxidant supplements (vitamins C and E), an increased number of normal MII oocytes and decreased percentage of apoptotic oocytes were observed in comparison with the control group.[28] The balance between ROS and antioxidants within the follicle therefore seems to be critical for oocyte maturation. It is thus likely that this critical balance is involved in poor oocyte quality, and it may also be an important factor associated with female infertility

Melatonin as a free radical scavenger

Although melatonin exerts effects through its receptors, melatonin also can act as a powerful direct free radical scavenger. In 1993, melatonin was discovered to function as a direct free radical scavenger when it was shown to detoxify the highly reactive hydroxyl radical (·OH).[29,30] Since then, many reports have confirmed the ability of melatonin to reduce oxidative stress.[31,32] In these investigations, melatonin was found to scavenge both oxygen- and nitrogen-based reactants[33,34] in several subcellular organelles.[35] Melatonin works in a variety of ways to reduce the levels of oxidative stress. It has been shown that melatonin has the capability of quenching reactive oxygen as well as reactive nitrogen species including superoxide radical ($O2^{-}$), hydroxyl radical (·OH), singlet oxygen ($^1O2$), hydrogen peroxide (H_2O_2), hypochlorous acid

(HOCl), nitric oxide (NO·) and the peroxynitrite anion ($ONOO^-$).[35-38] Three key players are involved in ROS damage to cells: hydrogen peroxide (H_2O_2), superoxide radical ($O2^-$), and hydroxyl radical (·OH). H_2O_2 and superoxide radicals ($O2^-$) are thought to create less damage than hydroxyl radical (·OH), however, in the presence of free iron, specifically ferrous iron, H_2O_2 is converted to hydroxyl radical (Fenton reaction). Hydroxyl radical (·OH) is the most potent free radicals and is known to produce damage to all biological membranes and DNA. Melatonin can easily pass through cell membranes because of its properties of lipophilicity and hydrophilicity, and it has been demonstrated that a high levels of melatonin exist not only in cytoplasm but also beside the nucleus. The antioxidant properties of melatonin as a cell protector have been extensively studied and a previous report demonstrated that the melatonin's ability to detoxify the hydroxyl radical (·OH) was higher than well-known scavengers including vitamin C and vitamin E.[31]

Not only is melatonin itself a direct free radical scavenger, but also metabolites that are formed during these interactions, i.e., cyclic 3-hydroxmelatonin, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), are likewise excellent scavengers of reactive species.[34,35,39-41] In addition, melatonin has a high capability to detoxify ROS and suppresses the oxidative effect indirectly by enhancing the production of endogenous antioxidants. Melatonin has been stimulates activities and mRNA levels of antioxidative enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase.[42,43] Thereby, these multiple actions of melatonin protect cells from ROS-mediated lipid peroxidation, protein destruction and nuclear DNA damage.[44-48]

Melatonin and reproduction

The roles of melatonin in reproduction are focused on its direct actions in the ovary. Melatonin can pass through all cell membranes and enter all tissues because of its lipophilic property, however, it specifically concentrates in the ovary when injected systemically.[49] High levels of melatonin are found in human preovulatory follicular fluids, and the concentrations are higher than in serum.[12,13] A previously report documented the melatonin concentrations in the ovaries at mid-light and mid-dark during the estrous cycle in the cyclic hamster.[50] Melatonin concentrations in the ovary show a phasic variation as in the pineal gland and serum; they were high at mid-dark and low at mid-light. The melatonin concentrations in the ovaries at mid-dark were significantly higher on proestrus than the other estrous cycle. In addition, the melatonin concentrations in human follicular fluids have been measured in patients undergoing in vitro fertilization and embryo transfer (IVF-ET) program.[14] Melatonin concentrations are higher in the fluid of large follicles (> 18mm) than in the small follicles (10-12mm), thus suggesting that increased melatonin in preovulatory follicles may have an important role in the ovulation processes. Melatonin production in the ovarian follicle seems to be negative, because mRNA of arylalkulamine N-acetyltransferase (AA-NAT), the rate-limiting enzyme of melatonin, have not detected in granulosa cells of rats and humans. In addition, the concentrations of melatonin in human follicular fluids were measured in patients who were given melatonin treatment. The melatonin concentrations increased depending on the dose of melatonin (1 mg, 3 mg and 6 mg tablet). These findings suggest that the melatonin in the follicular fluid is derived from the circulation and the uptake of melatonin by the ovarian follicles increases depending on follicular growth. Increased melatonin in follicular fluid seems to have an important role in ovulation.

Melatonin, oocyte quality, and embryo development

High quality oocytes produce well-developed embryos. After fertilization, ooplasm becomes the embryo cytoplasm, but the spermatozoon's participation in this process is minimal. It has been thought that the first steps of embryogenesis are controlled exclusively by maternal information present in the oocyte. For this reason, the quality of oocytes is a key factor in determining the quality of the early steps of embryo development. Oocyte maturation begins with the resump-

tion of meiosis, and oocytes are arrested at prophase of the first meiotic division. Only fully grown oocytes can resume meiosis in response to LH surge. Oocytes pass through the first meiotic division and then become arrested at metaphase of the second meiotic division until fertilization. During this long period of meiotic maturation, oocyte accumulate molecules of mRNA, proteins, lipid and sugars as well as oxidative stress.

Oxidative stress in the oocyte caused by ROS must be limited in order for a good embryo to be produced. ROS induce lipid peroxidation of membranes and DNA damage in the oocyte and are expected to cause harmful effects in cell division, metabolite transport, and mitochondrial function.[51] We recently reported the direct effect of ROS and melatonin on oocyte maturation.[52] To investigate the effects of H_2O_2 on oocyte maturation, the denuded oocytes from immature mice treated with PMSG were cultured in the incubation medium with various concentrations of H_2O_2. After 12 hr incubation, oocytes with the first polar body (MII stage oocytes) were counted. The percentage of the mature oocytes (MII stage oocytes with a first polar body) was significantly decreased by the addition of H_2O_2 in a dose-dependent manner (>200 μM). When oocytes were incubated with melatonin in the presence of H_2O_2 (300 μM), melatonin dose-dependently blocked the inhibitory effect of H_2O_2 on oocyte maturation, and there was a significant effect at the concentration of 10 ng/mL of melatonin. To further investigate the intra-cellular role of melatonin, 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. These results suggest that H_2O_2 inhibits oocytes maturation by producing ROS, but melatonin demonstrated protective activity against oxidative stress caused by H_2O_2. Recently, Kang et al.,[53] investigated the effects of melatonin on the maturation of porcine oocytes. Oocytes from antral follicles were incubated in the medium with or without melatonin supplementation. Melatonin supplementation (10 ng/ml) during in-vitro maturation resulted in a greater proportion of oocytes extruding the polar body, and melatonin-treated oocytes had significantly lower levels of ROS than control (without melatonin treatment) oocytes.

The ability of melatonin to promote embryo development in different species has been reported. When inseminated mouse embryos were cultured in the medium with melatonin (10^{-8}-10^{-4} M), increased fertilization and blastocyst rates were observed.[54] Rodriguez-Osorio et al.[55] demonstrated the effects of melatonin on in-vitro porcine embryo development. Melatonin supplementation (10^{-9} M) had a positive effect on the fertilization rates of inseminated porcine embryos that were cultured. Although blastocyst rates were not increased by melatonin, the number of blastocyst cells in the melatonin-supplemented group was significantly higher than in the control group. When the oocytes recovered from porcine follicles were incubated in the medium with melatonin (10^{-7} M), fertilization rate, blastocyst rate and the number of blastocyst cells were significantly higher than that of the control (without melatonin).[56] The effect of melatonin on embryo development seems to be, at least in part, caused by its action as an antioxidant, as Papis et al.[57] demonstrated that the beneficial effects of melatonin on bovine embryo development was observed not in a low oxygen environment but in a high oxygen environment where free radicals are easily produced. We recently confirmed the benefit of melatonin treatment to infertilite women who underwent an IVF-ET program. When women were treated with 3 mg of melatonin daily from day 5 of the previous menstrual cycle until the day of oocyte retrieval, the percentage of good embryos (day 2 after insemination) was significantly higher compared to the control (without melatonin treatment) cycle. These data suggest that melatonin may be involved in oocyte maturation and embryo development.

Clinical trial of melatonin for infertility patients

As summarized above, a growing amount of literature has demonstrated that melatonin and/or melatonin treatment may have a beneficial effect on oocyte maturation and embryo development. Poor oocyte quality is one of the most intractable causes of infertility in women, and no effective treatment to improve oocyte quality has established.

To document the association between melatonin and ovarian oxidative stress, human follicular fluids were sampled at oocyte retrieval during IVF-ET program, and concentrations of melatonin and 8-hydroxy-2'-deoxyguanosine (8-OHdG), an oxidative stress marker, were analyzed. The study revealed an inverse correlation between intra-follicular concentrations of melatonin and 8-OHdG, suggesting that melatonin reduces oxidative stress in the follicles and may protects oocyte from free radical damage. When patients 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, intra-follicular concentrations of melatonin rose from 112 pg/ml in the control cycle (without melatonin treatment) to 432 pg/ml after daily melatonin treatment. Intra-follicular concentrations of 8-OHdG and hexanoyl-lysine adduct (HEL), a damaged lipid product, were decreased after melatonin treatment compared to those in the prior cycle. The result demonstrates that melatonin treatment reduces intra-follicular oxidative damage. To investigate the clinical usefulness of melatonin administration, the effect of melatonin treatment on clinical outcome of IVF-ET was examined for 115 patients who failed to become pregnant in the previous IVF-ET cycle with a low fertilization rate (< 50%). In 56 patients with melatonin treatment, the fertilization rate (50.0 ± 38.0%) was markedly improved compared with the previous IVF-ET cycle (20.2 ± 19.0%), and 11 of 56 patients (19.6%) achieved pregnancy. On the other hand, in 59 patients who were not given melatonin, the fertilization rate (22.8 ± 19.0% vs 20.9 ± 16.5%) was not significantly changed, and only 6 of 59 patients (10.2%) achieved pregnancy. These results show that melatonin administration increases intra-follicular melatonin concentrations, reduces intra-follicular oxidative damage and elevates fertilization and pregnancy rates.

To our knowledge, our study represents the first clinical usefulness of melatonin treatment for infertilite patients. Melatonin is likely to become a treatment for improving oocyte quality for women who cannot become pregnant because of poor oocyte quality.

Conclusions

Melatonin is applicable to the regulation of reproductive events. The regulation of reproductive events seems to be mediated through its receptors (MT1/MT2) located in hypothalamus and pituitary gland. However, many researchers have recently begun to study the local role of melatonin as an antioxidant. This study focused on intra-follicular role of melatonin in the ovary. Melatonin, secreted by pineal gland, is taken up into the follicular fluid from the blood. ROS produced within the follicles, especially during the ovulation process, are scavenged by melatonin, and reduced oxidative stress may thus be involved in oocyte maturation and embryo development (Fig. 1). This clinical study demonstrated that melatonin treatment for infertile women increases intra-follicular melatonin concentrations, reduces intra-follicular oxidative damage and elevates fertilization and pregnancy rates. Melatonin treatment could become a new strategy for improving oocyte quality in infertile women.

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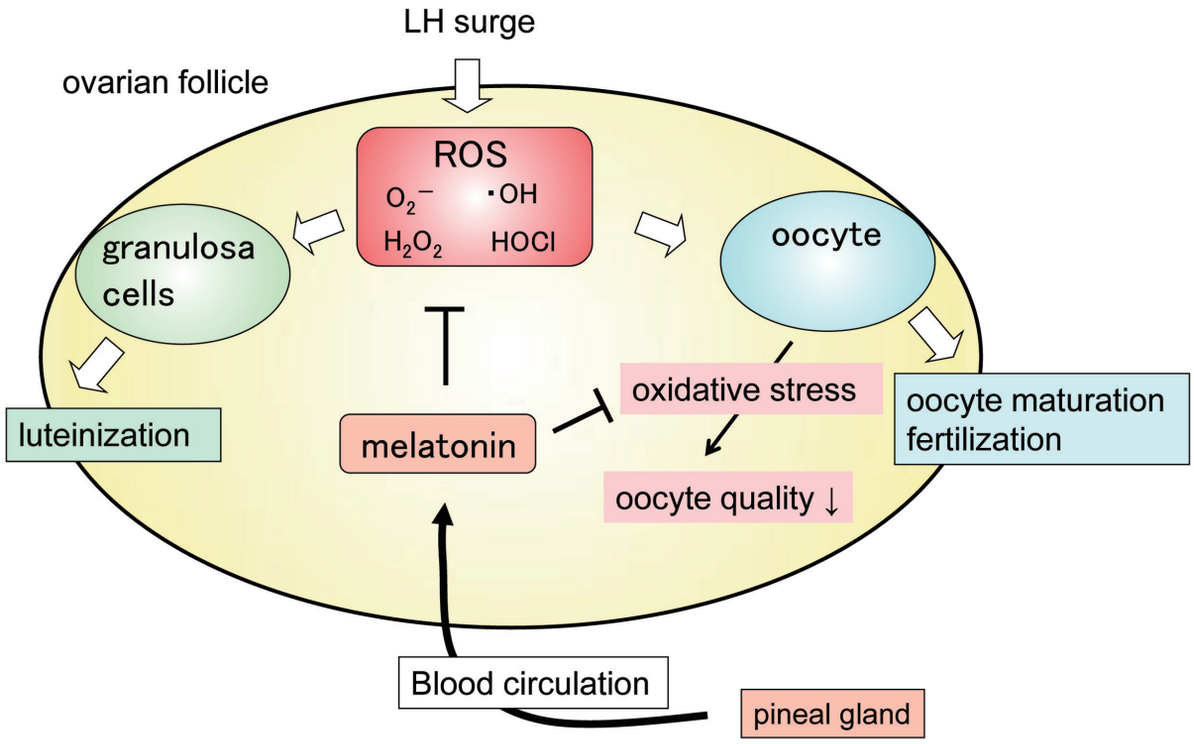

Fig. 1 Schematic representation of the presumed roles of melatonin in ovarian antral follicle.

Melatonin, secreted by pineal gland, is taken up into the follicular fluid from the blood. ROS produced within the follicles, especially ovulation process induced by LH surge, were scavenged by melatonin, and reduced oxidative stress may be involved in oocyte maturation and fertilization. Oxidative stress in oocyte may be a caused of poor oocyte quality.

Conflict of Interest

The authors state no conflict of interest.

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