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Review

Melatonin as a free radical scavenger in the ovarian follicle

Hiroshi Tamura[1)], Akihisa Takasaki[2)], Toshiaki Taketani[1)], Manabu Tanabe[1)], Fumie Kizuka[1)], Lifa Lee[1)], Isao Tamura[1)], Ryo Maekawa[1)], Hiromi Asada[1)], Yoshiaki Yamagata[1)] and Norihiro Sugino[1)]

[1)] *Department of Obstetrics and Gynecology, Yamaguchi University Graduate School of Medicine, Ube 755-8505, Japan*
[2)] *Department of Obstetrics and Gynecology, Saiseikai Shimonoseki General Hospital, Shimonoseki 759-6603, Japan*

Abstract. This review summarizes new findings related to beneficial effects of melatonin (N-acetyl-5-methoxytryptamine) on reproductive physiology. Recently many researchers have begun to study the local role of melatonin as an antioxidant. We focused on intra-follicular role of melatonin in the ovary. Melatonin, secreted by the pineal gland, is taken up into the follicular fluid from the blood. Reactive oxygen species (ROS) are produced within the follicles, during the ovulatory process. Melatonin reduces oxidative stress as an antioxidant, and contribute to oocyte maturation, embryo development and luteinization of granulosa cells. Our 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 also improves progesterone production by corpus luteum in infertile women with luteal phase defect. Melatonin treatment could become a new cure for improving oocyte quality and luteal function in infertile women.

Key words: Melatonin, Ovarian follicle, Reactive oxygen species, Oocyte, Granulosa cell

MELATONIN (N-acetyl-5-methoxytryptamine) is secreted during the dark hours at night by pineal gland, and it regulates a variety of important central and peripheral actions related to circadian rhythms and reproduction. It has been believed that melatonin regulates ovarian function by the regulation of gonadotropin release in the hypothalamus-pituitary gland axis *via* its specific receptors. However, the discovery of melatonin as a direct free radical scavenger has greatly broadened the understanding of melatonin's mechanisms which benefit reproductive physiology. Reactive oxygen species (ROS), which are locally produced during the ovulatory process, seem to have an essential role on follicle rupture. However, excess ROS can also be responsible for oxidative stress; they can damage oocytes and granulosa cells within the follicle. We focused on the direct role of melatonin on oocyte maturation and luteinization of granulosa cells as an anti-oxidant to reduce oxidative stress induced by ROS. This review also discusses the first application of melatonin to the clinical treatment of infertile women with poor oocyte quality or luteal phase defect.

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Correspondence to: Hiroshi Tamura, M.D., Ph.D., Department of Obstetrics and Gynecology, Yamaguchi University Graduate School of Medicine, Minamikogushi 1-1-1, Ube 755-8505, Japan.
E-mail: hitamura@yamaguchi-u.ac.jp

Synthesis of melatonin

Melatonin (N-acetyl-5-methoxytryptamine), the hormone of the pineal gland, has a circadian rhythm which is generated by the circadian pacemaker situated in the suprachiasmatic nucleus (SCN) of the hypothalamus, and synchronized to 24 hours primarily by the light-dark cycle acting *via* the SCN [1, 2]. During the day, serum melatonin concentrations are low, and significantly increase at night. The synthesis of melatonin is strictly controlled by lighting conditions. Photosensory information arrives at the pineal gland *via* the polyneuronal pathway that begins in the retina and involves the retinohypothalamic tract, SCN, paraventricular nuclei, intermediolateral cell column of the spinal cord and the superior cervical ganglia [3, 4]. Noradrenalin plays the crucial role in the control of melatonin synthesis [5]. It is released from postganglionic sympathetic nerve fibers that end in the pineal gland, and binds to pinealocyte α-adrenergic receptors, activates adenylate cyclase, and increases cAMP levels leading to the stimulation of activity of arylalkulamine N-acetyltransferase (AA-NAT), the key enzyme in

melatonin synthesis [6, 7]. The biosynthesis of melatonin is initiated by the uptake of the amino acid L- tryptophan from blood circulation into the gland. Within the pinealocyte, it is catalyzed to 5-hydroxytryptophan which is then decarboxylated to serotonin. The next step, N-acetylation of serotonin to N-acetylserotonin is completed by AA-NAT. The final step in the pathway is the O-methylation of N-acetylserotonin to melatonin by hydroxyindole O-methyltransferase (HIOMT) [8, 9].

Once synthesized, melatonin is not stored in pineal cells but is quickly released into the bloodstream and then into other body fluids, such as bile [10], cerebrospinal fluid [11], saliva [12], semen [13], amniotic fluid [14] and ovarian follicular fluid [15]. It has been reported that melatonin has many functions in organisms, i.e., helping to synchronize circadian rhythms [16, 17], sleep promotion [18, 19], immune stimulation [20, 21], blood pressure regulation [22, 23], seasonal reproductive regulation [24-26], oncostatic function [27-29], and antidepressive function [30, 31].

Some of the various physiological actions of melatonin are mediated by two G-protein-coupled MT1 and MT2 receptors widely distributed throughout the body. The activation of these receptors reduces forskolin-stimulated cAMP formation, protein kinase A activity and phosphorylation of cAMP-responsive element binding, even if the receptors can also interact with other intracellular signaling pathways, including the phosphorylation of mitogen-activated protein kinase kinases 1 and 2 (MEK1 and MEK2), extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) and protein kinase C [32]. Depending on the tissue, organ and species, melatonin activates different second messenger cascades by interacting with the same receptor subtype. Although some of melatonin's actions are mediated through its specific receptors, MT1/MT2, a considerable amount of melatonin's actions are dependent on its ability as an antioxidant.

Melatonin as an antioxidant

ROS are involved in a variety of cellular processes ranging from physiological to pathological responses. It is well-known that ROS can promote not only cell survival, proliferation and differentiation at the physiological level, but also cell death by apoptosis or necrosis at the higher level [33, 34]. Oxidative stress can be defined as the imbalance between cellular oxidant species like ROS and antioxidants, and has a direct toxic effect on cells, which leads to lipid peroxidation, protein oxidation or DNA damage. Oxidative stress plays a causative or adjuvant role in almost all human pathologies, including cancer [35, 36] and neurodegeneration [37, 38], and is involved in aging [39, 40] and chronic inflammatory pathologies [41, 42].

A small percentage (an estimated 1-4%) of the oxygen that enters cells is metabolized to derivatives which are often referred to as free radicals or ROS [43]. Mitochondria seem to be the most important subcellular site of ROS production. The mitochondrial electron transport chain with molecular oxygen directly generates the superoxide anion radical ($O_2^{\bullet-}$) [44, 45]. $O_2^{\bullet-}$ is not considered to be highly reactive and, therefore, does minimal direct structural damage to molecules. $O_2^{\bullet-}$ is scavenged by a specific enzyme, manganese -superoxide dismutase (Mn-SOD), to form hydrogen peroxide (H_2O_2) [46, 47]. The direct toxicity of H_2O_2 to cells is limited, however, have a long half-life (> 4 s) and it can readily pass the membranes. Although H_2O_2 is also changed into H_2O by glutathione peroxide (GPx) in mitochondria [48], some part and/or excess of H_2O_2 can diffuse to cytosol, membranes and nucleus, and can be a source to increase in concentrations of ROS. H_2O_2 is readily converted to the hydroxyl radical ($^{\bullet}OH$) when it is in the presence of a transition metal, e.g. Fe (Fenton reaction) [49]. $^{\bullet}OH$ has a half - life shorter than 1 ns in an aqueous environment [50], but it is highly reactive. Thus when it is produced *in vivo*, it harms or destroys any molecule in the vicinity of where the radical is generated [51]. The formation of $^{\bullet}OH$ in the vicinity of DNA might lead to this radical reacting with DNA bases or with the deoxyribose backbone of DNA to produce damaged bases or strand breaks. Since the most sensitive DNA base is G (guanine), hydroxyl radical reacts with guanine to form 8-hydroxy-2'-deoxyguanosine (8-OHdG). 8-OHdG is one of the major products of DNA oxidation and a sensitive genotoxic marker of oxidatively damaged DNA [52].

It has been discovered that melatonin is a powerful free radical scavenger and a broad-spectrum antioxidant [53, 54]. Because of its small size and highly lipophilic properties [53, 55], melatonin crosses all cell membranes and easily reaches subcellular compartments, including mitochondria and nuclei, where it seems to accumulate in high concentrations [56, 57]. Melatonin prevents lipid peroxidation [58, 59], protein [60], and DNA damage [61, 62]. In particular, melatonin has been found to preserve optimal mitochondrial function

[63-66] and homeostasis by reducing and preventing mitochondrial oxidative stress, thereby curtailing subsequent apoptotic events and cell death [53, 67, 68]. The ability of melatonin to scavenge the $^{\bullet}OH$ is much higher than other antioxidants including mannitol, glutathione and vitamin E [69]. Melatonin is a powerful and broad antioxidant, since it has been shown to scavenge different types of free radicals including $O_2^{\bullet-}$, $^{\bullet}OH$, singlet oxygen ($^1O_2$), H_2O_2, hypochlorous acid (HOCl), nitric oxide ($NO^{\bullet}$), and the peroxynitrite anion ($ONOO^-$) [63-66]. Not only is melatonin itself a direct free radical scavenger, but metabolites that are formed during these interactions (i.e., cyclic 3-hydroxmelatonin, N1-acetyl-N2-formyl-5-methoxykynuramine, and N1-acetyl-5-methoxykynuramine), are likewise excellent scavengers of toxic reactants [64, 70-72]. Furthermore, melatonin plays an important role in activating antioxidant defenses such as SOD, catalase (CAT), GPx, glutathione reductase (GSH-Rd) and glucose-6-phosphate dehydrogenase (G6PD) [73, 74].

Ovulation and oxidative stress

Ovulation is initiated by an LH surge and is accompanied with a variety of changes in the ovulatory follicle. The mechanism of ovulation has been compared to an inflammatory reaction [75]. Components of inflammation that are found in ovulation include increases in prostaglandin synthesis and cytokine production, the action of proteolytic enzymes, and increased vascular permeability [76-78]. ROS are important mediators of these inflammatory reactions, and they have been reported to be involved in ovulation [79]. Macrophages, neutrophils, and vascular endothelial cells reside in follicles [80], and ROS are produced by these cells during ovulation [81].

Although ROS play a role in follicle rupture during ovulation, they potentially damage oocyte and granulosa cells undergoing luteinization. ROS have been reported to inhibit progesterone production by luteal cells through the inhibition of steroidogenic enzymes [81] and intracellular carrier proteins involving transport of cholesterol to mitochondria [82]. ROS also disrupt the plasma membrane of luteal cells because of lipid peroxidation, and, as a consequence, membrane damage is often seen in the regressing corpus luteum [83, 84]. ROS are also essential for oocyte maturation; the oxidative phosphorylation within mitochondria provides a major source of energy needed for oocyte maturation from the germinal vesicle (GV) stage to mature metaphase II (MII), which is identified by the appearance of the first polar body. MII oocytes consume high levels of adenosine triphosphates (ATP) [85]. However, over production of ROS inhibit ATP synthase [86] and damage mitochondria so that ATP content of oocytes decreases [86, 87]. The relationship between low ATP content in oocyte and poor oocyte quality has been recognized [88-90]. Excess amounts of ROS may be involved in oxidative stress and poor oocyte quality. ROS such as $O_2^{\bullet-}$, $^{\bullet}OH$, and 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 mice and hamsters [91-93]. Also, higher levels of the oxidant H_2O_2 have been reported in fragmented human embryos compared with non-fragmented embryos and unfertilized oocytes [94].

Oxidative stress, a state characterized by an imbalance between pro-oxidant molecules, including reactive oxygen and nitrogen species, and antioxidant defenses, has been identified to play a key role in the pathogenesis of subfertility in both males and females. However, in females, the impact of oxidative stress on oocytes and the reproductive functions remains unclear. It has been shown that extremes of body weight and lifestyle factors such as cigarette smoking, alcohol use and recreational drug use can promote excess free radical production, which can affect fertility [95].

On the other hand, antioxidants are scavengers that detoxify excess ROS, thereby helping to maintain the body's delicate oxidant/antioxidant balance. There are two types of antioxidants: enzymatic and nonenzymatic. It is well-documented that antioxidant enzymes, such as SOD, GPx and CAT, and non-enzymatic antioxidants, such as vitamin E, vitamin C, glutathione, uric acid and albumin, are present in the follicles [79, 96, 97]. Reduced antioxidant enzyme levels, such as reduced levels of GPx, are reported in the follicular fluids of women with unexplained infertility [98]. Another report demonstrated that higher levels of SOD activities 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 [99]. When mice were given antioxidant supplements (vitamins C and E), an increased number of normal MII oocytes and decreased percent-

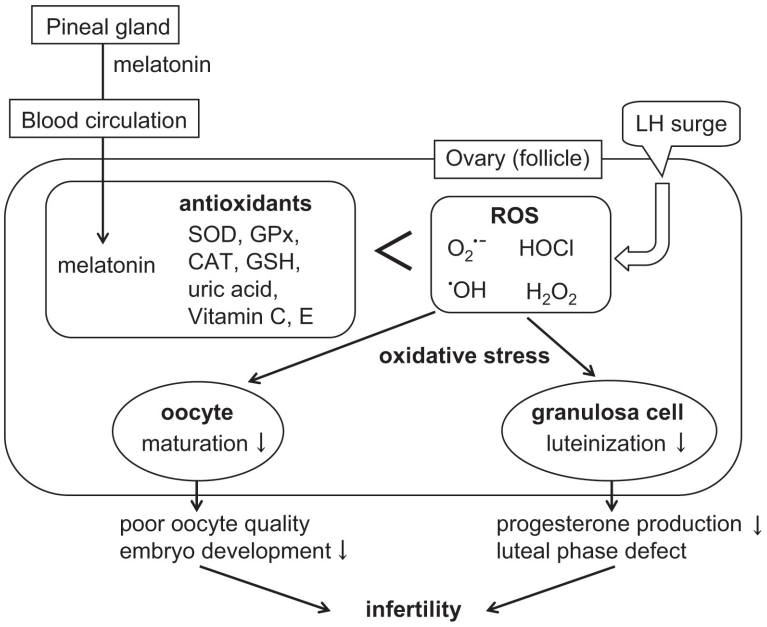

Fig. 1 The balance between ROS and antioxidants in the follicle.
ROS are produced within the follicles, especially ovulation process induced by LH surge. Antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, and non-enzymatic antioxidants, such as vitamin E, vitamin C, glutathione, uric acid and albumin, are present in the follicles. Melatonin, secreted by pineal gland and is taken up into the follicular fluid from the blood, is one of the antioxidant in the follicle. Excess amounts of ROS may be involved in oxidative stress of oocyte and granulosa cells. The balance between ROS and antioxidants within the follicle may be critical for oocyte maturation and luteinization of granulosa cells.

age of apoptotic oocytes were observed in comparison to the control group [100].

Infertile females seem to have an imbalance status involving increased oxidative stress and decreased antioxidants, although the underlying mechanism is unclear. However, the balance between ROS and antioxidants within the follicle seems to be critical for oocyte maturation and granulosa cells functions undergoing luteinization during ovulation. Therefore, it is likely that the balance is involved in poor oocyte quality and luteal phase defect, and it may be an important factor of female infertility (Fig. 1).

Melatonin and Reproduction (ovary)

Circadian rhythms are driven by biological oscillators (circadian clocks) that entrain to the light:dark cycle. Melatonin is a well-known transducer of light information, defining the length of the light. Melatonin plays an essential role in reproduction rhythms by acting on the hypothalamus and pars tuberalis of the pituitary, where melatonin receptors are highly expressed. Melatonin regulates gonadotropin releasing hormone (GnRH) secretion in the hypothalamus and gonadotropin release in the pituitary gland. In addition, melatonin has recently been discovered to stimulate gonadotropin-inhibitory hormone (GnIH), a neuropeptide that inhibits gonadotropin synthesis, release and production in the hypothalamus in birds [101]. Rats ovulate during the night after the proestrus day, and rupture of the ovarian follicles is triggered by the release of a surge of LH. However, the role of melatonin in influencing the hypothalamic-pituitary-gonadal axis in humans is less clear.

It is worth noting that melatonin concentration in human fluids can be higher than that measured in blood [102-104]. Although high concentration of melatonin in ovarian follicular fluids has been reported [15, 105], the role of melatonin in follicle has not been analyzed. We focused on the effect of melatonin as an antioxidant within the ovarian follicle. Previous report demonstrated the tissue distribution of H^3-melatonin when given by intravenous injection to cat [106]. It was found that the concentration of H^3-melatonin in the ovary was ten times higher than plasma, and high uptake of H^3-melatonin by the ovary was demonstrated compared

to other peripheral tissues. Since circulating melatonin is highly concentrated by the ovary, it is of interest in view of the relationship between melatonin and ovarian function. We previously reported the melatonin concentrations in the ovary at mid-light and mid-dark during the estrous cycle in the cyclic hamster [107]. Melatonin concentrations in the ovary showed a phasic variation as in the pineal gland and serum; they were high at mid-dark and low at mid-light. The ovarian melatonin concentration at mid-dark was significantly higher on proestrus than the other days of the estrous cycle. The ovary on proestrus has preovulatory follicles, therefore, it is likely that melatonin is taken up from the circulation by the ovary during follicular growth. We also demonstrated the melatonin concentrations in human follicular fluids in patients undergoing *in-vitro* fertilization and embryo transfer (IVF-ET) program [108]. Melatonin concentrations are higher in the fluid of large follicles than in the small follicles suggesting that increased melatonin in preovulatory follicles may have an important role in ovulation processes.

Some peripheral tissues like retina [109], gastrointestinal tract [110], skin [111], leukocytes [112] and bone marrow [113, 114] synthesize melatonin. When we checked the NAT expression of granulosa cells by PCR in rats and humans, we did not detect active NAT expression. We also measured the melatonin concentrations in human follicular fluids from the patients who were given melatonin administrations. The melatonin concentrations were increased depending on the dose of melatonin (1mg, 3mg and 6mg) (unpublished data). These findings suggest that the melatonin in follicular fluid is derived from circulation and the uptake of melatonin by the ovarian follicles increased depending on follicular growth. However, the mechanism is unclear, and further studies are needed to better understand the role of melatonin in follicles.

To investigate the relationship between oxidative stress and sex steroid production, we analyzed the concentrations of an oxidative stress marker (8-OHdG), antioxidants (Cu,Zn-SOD, glutathione, melatonin), and sex steroids (progesterone, testosterone, estradiol) in mature follicles from the patients undergoing an IVF-ET program [115]. Melatonin concentrations in the follicular fluid showed a negative correlation with 8-OHdG, whereas Cu,Zn-SOD and glutathione did not show any significant correlation with 8-OHdG. Progesterone concentrations in the follicular fluid showed a positive correlation with melatonin, whereas estradiol and testosterone did not show any significant correlation with melatonin. The progesterone concentrations in the follicular fluid were negatively correlated with 8-OHdG concentrations. Our data suggest that melatonin is an important antioxidant within the follicle and contributes to progesterone production by luteinized granulosa cells.

Melatonin receptors (MT1, MT2) are expressed in ovaries in various mammalian species, including humans. Melatonin may regulate the ovarian function *via* activation of multiple receptors and signaling pathways in different target cell types, especially theca and granulosa cells [116]. MT1 and MT2 receptors expressed in antral follicles and the corpus luteum may affect steroidgenesis *via* cAMP-mediated signaling in rats [116]. An analysis of chicken ovaries revealed that only MT2, and not MT1, is expressed in small follicles. In contrast, both the MT1 and MT2 expressions are detected in preovulatory follicles [117]. In addition, the MT1 expression is detected in the thecal layer, but not in the granulosa layer. In contrast, MT2 is expressed in both granulosa and theca layers. In humans, the MT1 and MT2 gene expressions in granulosa cells have been documented with a prevalence observed in the MT1 expression [118]. Further studies are needed to better understand the mechanisms of MTs in the ovarian function.

The role of melatonin in folliculogenesis has also been reported. Melatonin combined with FSH plays an important role in early folliculogenesis to promote follicular growth in *in vitro* cultures of goat preantral follicles [119]. The number of large follicles is increased in melatonin-treated ewes, suggesting that melatonin is a potent regulator of follicular development [120]. Another report demonstrated that ovaries exhibit increased numbers of atretic follicles after pinealectomy [116]. The evidence suggests that melatonin is involved in folliculogenesis and follicle selection; however, little is still known about the influence of melatonin on follicle development in humans.

Melatonin reduces oxidative stress in the follicle

It would be interesting to know the physiological role of melatonin in follicular fluid, especially the high concentrations in preovulatory follicles, during ovulation processes. Oxidative stress caused by ROS during the ovulatory process is detrimental to oocytes and

granulosa cells. The protective role of melatonin as an antioxidant within the follicle is discussed below.

a) *Oocyte*

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 [121]. We recently reported the direct effect of ROS and melatonin on oocyte maturation [122]. 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 [123]. 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*., [124] 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 [125]. Rodriguez-Osorio *et al*. [126] 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) [127]. 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*. [128] 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 infertility 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 [123]. These data suggest that melatonin may be involved in oocyte maturation and embryo development.

b) Granulosa cells

In ovaries, the corpus luteum is formed after ovulation and produces progesterone, which is necessary for establishment and maintenance of pregnancy. Although ROS play a role in follicle rupture during ovulation, they potentially damage granulosa cells undergoing luteinization. ROS have been reported to inhibit progesterone production by luteal cells [81], mediated by inhibition of steroidogenic enzymes and intracellular carrier proteins involving transport of cholesterol to mitochondria [82]. ROS also disrupt the plasma membrane of luteal cells because of lipid peroxidation, and, as a consequence, damaged membrane changes are often seen in the regressing corpus luteum [83, 84].

To examine the effect of melatonin on progesterone production, luteinized granulosa cells were obtained at the time of oocyte retrieval in women undergoing IVF-ET. Luteinized granulosa cells were incubated with or without H_2O_2 (30, 50, or 100 μM) in serum-free incubation medium for 12 h in the presence or absence of melatonin (1, 10, 100 μg/mL). After incubation, the progesterone concentration in culture medium was measured. Progesterone production was significantly inhibited by H_2O_2 (30 μM: 54.9 ± 18.8%; 50 μM: 30.1 ± 18.8%; 100 μM: 17.4 ± 6.0%, values are mean ± SEM), and the inhibitory effect of H_2O_2 on progesterone production was reversed by addition of melatonin (0 μg/mL: 21.4 ± 2.8%; 1 μg/mL: 38.0 ± 7.8%; 10 μg/mL: 65.5 ± 22.1%; 100 μg/mL: 99.7 ± 31.4%, values are mean ± SEM) [115]. Our results also showed that ROS reduced progesterone production by luteinized granulosa cells, however, melatonin abolished the inhibitory effect of H_2O_2 on progesterone production. Therefore, the study suggests that melatonin protects granulosa cells from ROS in the follicle during ovulation and contributes to luteinization of granulosa cells.

Melatonin treatment for infertility women

a) ***Oocyte quality***

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. Melatonin treatment can be a useful infertility treatment and, therefore recently has been applied to infertility patients for the first time.

To document an association between melatonin and ovarian oxidative stress, human follicular fluids were sampled during oocyte retrieval for the purpose of IVF-ET and concentrations of melatonin and 8-OHdG were measured. The study revealed an inverse correlation between intra-follicular concentrations of melatonin and 8-OHdG, suggesting that melatonin in the follicle diffuses into the cumulus and oocytes to protect them 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 [122, 129]. 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 [122, 129]. 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 infertility patients. Melatonin is likely to become a treatment for improving oocyte quality for women who cannot become pregnant because of poor oocyte quality.

b) Luteal function

Luteal phase defect has been implicated as a cause of infertility and spontaneous miscarriage. However, the etiology of luteal phase defect is complicated by the fact that the causes of luteal phase defect are highly varied. Not only Low blood flow of the corpus luteum [130, 131] but also oxidative stress [132, 133] are associated with luteal phase defect. Serum lipid peroxide levels were significantly elevated in patients with luteal phase defect compared to women with normal luteal function [134]. In addition, ascorbic acid (antioxidant) supplementation improved serum progesterone levels in patients with luteal phase defect [135]. These findings suggest that oxidative stress caused by ROS is one of the important causes of luteal phase defect.

To analyze the clinical effectiveness of melatonin administration in patients with luteal phase defect, twenty-five infertility patients (aged 26-42 yr) with luteal phase defect who did not have decreased luteal blood flow were enrolled [115]. These patients were

diagnosed as having luteal phase defect (serum progesterone concentrations during the mid-luteal phase were < 10 ng/mL), and were not diagnosed as having decreased luteal blood flow. Patients were divided into two groups during the subsequent treatment cycle; 14 women were given 3 mg/day of melatonin orally at 22:00h after hCG injection (5,000 IU, im) throughout the luteal phase, and 11 women were given no medication after hCG injection as a control. Venous blood samples were obtained for progesterone assay during the mid-luteal phase (6-8 days after ovulation). When 14 women who had luteal phase defect were given melatonin throughout the luteal phase of the subsequent menstrual cycle, nine patients (64.3%) showed improved serum progesterone concentrations of more than 10 ng/mL, and the mean progesterone concentration was 11.0 ± 2.6 ng/mL. In the control group without melatonin treatment, only two patients out of 11 (18.2%) showed normal serum progesterone concentrations, and the mean progesterone concentration was 8.9 ± 2.2 ng/mL. The improvement rates of the two groups were significantly different ($p < 0.05$). The result strongly suggests that oxidative stress is one of the causes of luteal phase defect, and melatonin protects luteinized granulosa cells and increases progesterone production of corpus luteum by reducing oxidative stress. Melatonin supplementation can be a useful treatment for luteal phase defect that is related to oxidative stress.

Conclusions

The discovery of melatonin as a direct free radical scavenger has greatly broadened the understanding of its multiple physiological roles. The new findings regarding the potential role of melatonin in reproductive physiology have also been increasing. Melatonin is applicable to the regulation of seasonal reproductive events in photoperiod dependent breeding mammals. The regulation of seasonal reproductive events seems to be mediated by receptors (MT1/MT2) in the hypothalamus and pituitary gland. However, recently many researchers have begun to study the local role of melatonin as an antioxidant. We focused on intrafollicular role of melatonin in the ovary. Melatonin, secreted by the pineal gland, is taken up into the follicular fluid from the blood. ROS produced within the follicles, especially during the ovulation process, were scavenged by melatonin, and reduced oxidative stress may be involved in oocyte maturation, embryo development and luteinaization of granulosa cells (Fig. 1). Our 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 also improves progesterone production by corpus luteum in infertile women with luteal phase defect. The safety of exogenous melatonin for humans has been shown in many studies [136, 137]. It has also been reported that melatonin has no detrimental effects on mouse and rat embryo development during toxicity tests that were performed both *in-vitro* and *in-vivo* [124, 138, 139]. It should be noted that melatonin treatment could become a new cure for improving oocyte quality in infertile women.

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