

Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate

Abstract: We investigated the relationship between oxidative stress and poor oocyte quality and whether the antioxidant melatonin improves oocyte quality. Follicular fluid was sampled at oocyte retrieval during in vitro fertilization and embryo transfer (IVF-ET). Intrafollicular concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in women with high rates of degenerate oocytes were significantly higher than those with low rates of degenerate oocytes. As there was a negative correlation between intrafollicular concentrations of 8-OHdG and melatonin, 18 patients undergoing IVF-ET were given melatonin (3 mg/day), vitamin E (600 mg/day) or both melatonin and vitamin E. Intrafollicular concentrations of 8-OHdG and hexanoyl-lysine adduct were significantly reduced by these antioxidant treatments. One hundred and fifteen patients who failed to become pregnant with a low fertilization rate ($\leq 50\%$) in the previous IVF-ET cycle were divided into two groups during the next IVF-ET procedure; 56 patients with melatonin treatment (3 mg/day) and 59 patients without melatonin treatment. The fertilization rate was improved by melatonin treatment compared to the previous IVF-ET cycle. However, the fertilization rate was not significantly changed without melatonin treatment. Oocytes recovered from preovulatory follicles in mice were incubated with H_2O_2 for 12 hr. The percentage of mature oocytes with a first polar body was significantly reduced by addition of H_2O_2 (300 μM). The inhibitory effect of H_2O_2 was significantly blocked by simultaneous addition of melatonin. In conclusion, oxidative stress causes toxic effects on oocyte maturation and melatonin protects oocytes from oxidative stress. Melatonin is likely to improve oocyte quality and fertilization rates.

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Introduction

Despite great advances in assisted reproductive technology, poor oocyte quality remains a profound problem for female infertility. Reactive oxygen species (ROS) are produced within the follicle, especially during the ovulatory process [1]. It is believed that oxidative stress may be a cause of poor oocyte quality. The role of ROS and antioxidants in relation to female reproductive function has been a subject of recent research interest [1, 2]. Reduced levels of glutathione peroxidase (GSH-Px) are reported in the follicular fluid of women with unexplained infertility [3]. Yang et al. [4] found higher levels of the oxidant, H_2O_2 in fragmented embryos compared with nonfragmented embryos, while Paszkowski et al. [5] reported the elevated consumption of antioxidants, which suggests increased ROS levels, during incubation of poor quality embryos.

There is evidence that melatonin plays an important role in the regulation of reproductive activity in seasonal breeders [6] with some potential effects in humans as well

[7]. The effects of melatonin on seasonal reproductive functions are thought to be mediated by G-protein coupled melatonin receptors in the pars tuberalis [8] while the role of melatonin in influencing the hypothalamic-pituitary-gonadal axis in humans is less clear [7]. In nonhuman mammals, it is generally believed that the reproductive actions of melatonin are mediated via regulating gonadotropin release subsequent to changes in hypothalamic monoamine metabolism and gonadotropin releasing hormone (GnRH) regulation [9–12] and possibly on 3',5'-cyclic adenosine monophosphate cAMP and Ca^{2+} -dependent intracellular mechanisms in the hypophysis [13, 14].

However, there is also evidence to suggest that melatonin acts on the ovary to modify its function [15, 16]. High levels of melatonin which may undergo seasonal variations [17] are found in human preovulatory follicular fluid in concentrations which are almost three-fold higher than serum levels [18, 19]. At this time, the physiological role of intrafollicular melatonin is not fully understood.

Several antioxidant enzymes protect oocytes and embryos against oxidative stress; these enzymes include superoxide dismutase (SOD), catalase and GSH-Px [20]. Cu,Zn-SOD and Mn-SOD dismutate the superoxide anion radical to the nonradical species, H₂O₂. Melatonin as well as its metabolites are potent direct free radical scavengers [21–24] and indirect antioxidants by virtue of their ability to modulate gene transcription for antioxidant enzymes [25]. The antioxidant properties of melatonin have been extensively studied and the use of this molecule as a cell protector and as a potential disease preventing agent has been summarized [26–30].

The current study was undertaken to examine the relationship between oxidative stress and poor oocyte quality and to test whether melatonin improves oocyte quality because of its antioxidant activity.

Materials and methods

The protocol for this study was reviewed and approved by Institutional Review Board of Yamaguchi University Graduate School of Medicine. All patients gave their informed consent to participate. One hundred eighty nine patients were recruited in this study. The mean age \pm S.D. of the patients was 34.8 ± 3.8 yr, with a range of 24–45 yr. Patients were nonsmokers and free from major medical illness including hypertension; all were interested in becoming pregnant. Patients were excluded if they had myoma, adenomyosis, a congenital uterine anomaly, ovarian tumors or if they used estrogens, progesterone, androgens, or chronic use of any medication, including nonsteroidal anti-inflammatory agents or anticonvulsants.

Patients underwent in vitro fertilization and embryo transfer (IVF-ET) with a standardized ovarian-stimulation protocol as reported previously [31] consisting of GnRH agonist (900 mg/day buserelin acetate, Suprecur; Mochida Pharmaceutical Co. Ltd., Tokyo, Japan) beginning in the mid-luteal phase, followed by 300 IU follicle stimulating hormone (FSH, Fertinorm P; Serono-Japan Co. Ltd., Chiba, Japan) on the second and third day, 225 IU FSH on day 4 and 5, and thereafter by 150 IU human menopausal gonadotropins (hMG, HMG-teizo; Asuka Co. Ltd., Tokyo, Japan). When at least three follicles reached 18 mm or more in diameter by ultrasonography 10,000 IU human chorionic gonadotropin (Gonotropin; Asuka Pharmaceutical Co., Ltd., Tokyo, Japan) was administered and an ultrasound-guided trans-vaginal oocyte retrieval was performed 34 hr later. Care was taken to completely aspirate each follicle to one tube. Each follicle was aspirated separately and follicular fluid containing the oocyte was collected. The oocytes were inseminated with sperm, and fertilization results were assessed 16–18 hr after insemination. Embryo transfer was carried out approximately 48 hr after insemination.

Immediately after removal of the oocyte, each follicular fluid was centrifuged at 300 g for 15 min to remove cellular components and the supernatant was kept at -80°C until assayed. Concentrations of melatonin or biomarkers for oxidative stress in mature follicles (more than 18 mm in diameter) were determined for each patient. Clinical pregnancies were identified by the presence of a gestational sac on ultrasonography 5 wks after oocyte retrieval.

Clinical studies

To investigate the relationship between oocyte quality and intrafollicular oxidative stress, the numbers of retrieved oocytes and degenerate oocytes were counted in 56 patients undergoing IVF-ET. Degenerated oocytes were characterized by multiple abnormal morphological aspects, such as darkened, vacuolated and irregular ooplasm. As a biomarker of oxidative stress, intrafollicular concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) were measured by an enzyme-linked immunosorbent assay (ELISA) kit as described below. Intrafollicular concentrations of melatonin were also determined by radioimmunoassay as described below.

To examine the effects of melatonin and vitamin E as antioxidants on intrafollicular oxidative stress, some patients were treated with melatonin, some with vitamin E, and some received both melatonin and vitamin E. Eighteen patients undergoing IVF-ET who failed to become pregnant in the previous IVF-ET cycle were enrolled. Four patients were given a 3 mg tablet of melatonin (KAL, Park City, UT, USA) orally at 22:00 hr, 10 patients were given vitamin E (α -tocopherol, 600 mg/day, 200 mg capsule three times a day) orally and 15 patients were similarly given both melatonin and vitamin E. These treatments were carried out from the fifth day of the previous menstrual cycle until the day of oocyte retrieval. Intrafollicular concentrations of melatonin, 8-OHdG and hexanoyl-lysine adduct (HEL, a biomarker of lipid peroxidation) were determined and were compared to the data in the previous IVF-ET cycle (control cycle).

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 and 59 patients without melatonin treatment. 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).

Collection and culture of oocytes

The experimental protocol was reviewed by the Committee for Ethics on Animal Experimentation and was performed under the Guidelines for Animal Experiments at Yamaguchi University Graduate School of Medicine under Law (No.105) and Notification (No. 6) of the Japanese Government. Immature (3 wks) ICR mice (Japan SLC Inc., Hamamatsu, Japan) were housed in a controlled room with a 14:10 hr light:dark photoperiod and free access to standard mouse chow and water. All mice received a subcutaneous injection of 20 units of pregnant mare serum gonadotropin (PMSG, Sigma Chemical Co., St. Louis, MO, USA) to stimulate the development of multiple follicles. All mice received laparotomy under deep ether anesthesia 48 hr after PMSG injection; the ovaries were quickly removed for the following experiments, and mice

were killed by exsanguinations. The ovaries were transferred to the minimum essential medium (MEM) without phenol red (Sigma) supplemented with 3 mg/mL bovine serum albumin (BSA) (Sigma) and 21 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma). Oocytes were collected by puncturing ovarian follicles with a 26-gauge needle under a dissecting microscope and then the surrounding cumulus cells were removed by pipetting gently with a fine-bore pipette. Germinal vesicle (GV)-stage oocytes (cumulus cell-free) were incubated with the incubation medium [MEM medium supplemented with 3 mg/mL BSA, 0.1 mIU/mL FSH (Sigma) and 5 ng/mL human epidermal growth factor (Sigma)] under mineral oil for 1 hr at 37°C in a humidified 5% O₂, 5% CO₂, 90% N₂.

To investigate the effect of H₂O₂ on oocyte maturation, denuded oocytes were cultured in the incubation medium with various concentrations of H₂O₂ (0, 100, 200, 300, 400, and 500 μM). After 12 hr incubation, oocytes with first polar body (MII stage oocytes) were counted. To investigate the protective effect of melatonin on oocyte maturation, denuded oocytes were incubated with various concentrations of melatonin (0, 0.1, 1, and 10 ng/mL) in the presence of 300 μM H₂O₂. After 12 hr incubation, oocytes with first polar body were counted.

Melatonin assay

Intrafollicular concentrations of melatonin were measured by radioimmunoassay as reported previously [32]. Briefly, serum melatonin (500 mL) was extracted with 2 mL of chloroform. The 1.5 mL organic phase was evaporated and dissolved in 250 mL of assay buffer (0.01 M sodium phosphate, 0.15 M NaCl, 0.1% BSA, 0.1% NaN₃). Melatonin antiserum (100 mL, 1:30,000; HAC-AA92-03RBP86, Endocrinology Research Center, Gunma University, Japan) and ³H-melatonin (5,000 cpm/100 mL; Du Pont Co., Wilmington, DE, USA) were added into the tubes containing standards or samples, and these were incubated for 24 hr at 4°C. The bound/free separation was performed by the dextran-charcoal method, and the radioactivity of supernatant was measured by a liquid scintillation counter (LSC5100, Aloka Co. Ltd., Tokyo, Japan). The sensitivity of the assay was 2.1 pg/tube, and the intra- and inter-assay coefficients of variation were less than 10%.

8-OHdG and HEL assays

Intrafollicular concentrations of 8-OHdG were measured using an ELISA kit (Japan Institute for the Control of Aging, Nikken SEIL Co. Ltd., Shizuoka, Japan). Minimal detectable concentration of 8-OHdG (sensitivity of the assay) was estimated to be 0.5 ng/mL. Intrafollicular concentrations of HEL were measured using an ELISA kit (Japan Institute for the Control of Aging). Minimal detectable concentration of HEL was estimated to be 2 nM.

Statistical analysis

Statistical analysis was carried out using the computer program SPSS for windows 13.0. The Mann-Whitney *U*-test, Kruskal-Wallis *H*-test, Mann-Whitney *U*-test with

Bonferroni correction, chi-squared test and single regression analysis were used as appropriate. A value of *P* < 0.05 was considered significant.

Results

To examine relationship between oocyte quality and intrafollicular oxidative stress, the percentage of degenerate oocytes and concentrations of 8-OHdG as a biomarker of oxidative stress were determined in the follicular fluid sampled at oocyte retrieval in 54 patients undergoing IVF-ET. As shown in Fig. 1A, intrafollicular 8-OHdG concentrations in women with high rates (≥30%) of degenerate oocytes were significantly higher than those in women with low rates (< 30%) of degenerate oocytes. Intrafollicular concentrations of 8-OHdG were significantly and negatively correlated with intrafollicular concentrations of melatonin ($y = 141.95 - 2.4954x$, $r = -0.257$, $P < 0.05$) (Fig. 1B).

To examine the effect of melatonin on intrafollicular oxidative stress, some patients were treated with melatonin. As shown in Fig. 2A, administration of melatonin significantly ($P < 0.05$) decreased intrafollicular 8-OHdG concentrations compared with the previous IVF-ET cycle (control). Intrafollicular concentrations of melatonin were significantly increased by melatonin treatment (control cycle: 112 ± 51 pg/mL versus melatonin cycle: 432 ± 260 pg/mL, mean \pm SEM, $n = 15$, $P < 0.01$, Mann-Whitney *U*-test). In addition, we examined the effect of another antioxidant, vitamin E on intrafollicular oxidative stress. Intrafollicular concentrations of 8-OHdG were significantly decreased by administration of vitamin E (Fig. 2A).

We further examined the effect of melatonin and vitamin E on intrafollicular oxidative stress using another biomarker of oxidative stress HEL, which is a biomarker of lipid peroxidation. HEL was significantly reduced by treatment with vitamin E or combination of vitamin E and melatonin compared with the previous IVF-ET cycle (control), whereas melatonin alone tended to decrease intrafollicular HEL concentrations but had no statistically significant effect (Fig. 2B).

To investigate the clinical usefulness of melatonin administration, we examined the effect of melatonin treatment on clinical outcome of IVF-ET for 115 patients who failed to become pregnant in the previous IVF-ET cycle with a low fertilization rate ($\leq 50\%$). In 56 patients with melatonin treatment, the fertilization rate was markedly ($P < 0.01$) improved compared with the previous IVF-ET cycle and 11 of 56 patients (19.6%) achieved pregnancy (Table 1). On the other hand, in 59 patients who were not given melatonin, the fertilization rate was not significantly changed and 6 of 59 patients (10.2%) achieved pregnancy (Table 1). There was no statistically significant difference in pregnancy rates between the two groups.

We further studied the effect of oxidative stress on oocyte quality in vitro. The percentage of the mature oocytes (MII stage oocytes with a first polar body) was significantly decreased by addition of H₂O₂ in a dose-dependent manner (Fig. 3A). When oocytes were incubated with melatonin (0, 0.1, 1, and 10 ng/mL) in the presence of H₂O₂ (300 μM), melatonin dose-dependently blocked the inhibitory effect of

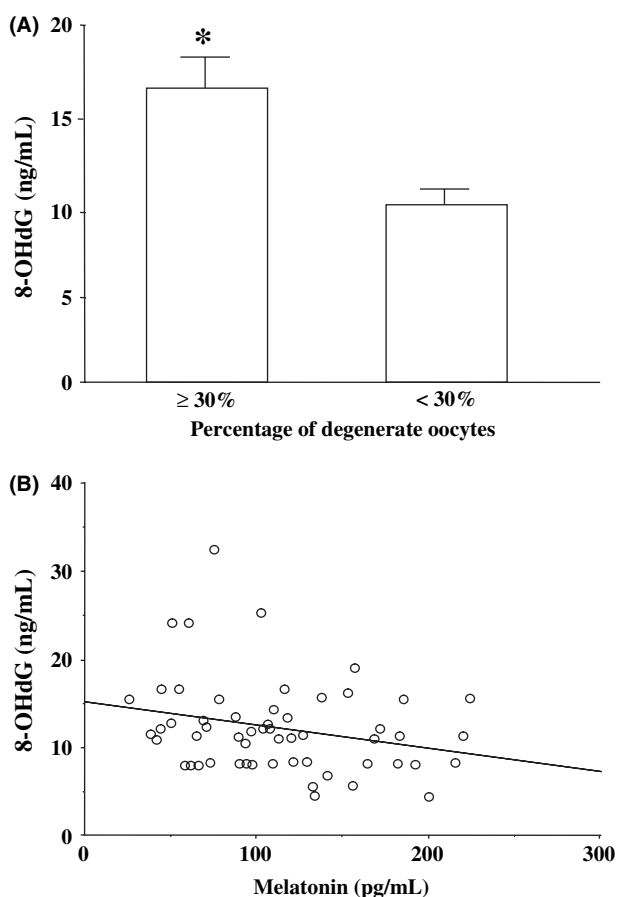


Fig. 1. Relation between the rate of degenerate oocytes and intrafollicular concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) (A) and the correlation between intrafollicular concentrations of melatonin and 8-OHdG (B). (A) To examine relationship between oocyte quality and intrafollicular oxidative stress, the percentage of degenerate oocytes in the total number of retrieved oocytes and concentrations of 8-OHdG as a biomarker of oxidative stress were determined in the follicular fluid sampled at the oocyte retrieval in 54 patients undergoing in vitro fertilization and embryo transfer (IVF-ET). Degenerated oocytes were characterized by multiple abnormal morphological aspects, such as darkened, vacuolated and irregular ooplasm. Intrafollicular 8-OHdG concentrations were compared between women with high rates ($\geq 30\%$, $n = 10$) and low rates ($< 30\%$, $n = 44$) of degenerate oocytes. Data are shown as the mean \pm S.E.M. $*P < 0.01$ (Mann-Whitney *U*-test). (B) Intrafollicular concentrations of melatonin were also measured in the patients. There was a significant negative correlation between intrafollicular concentrations of 8-OHdG and melatonin ($y = 141.95 - 2.4954x$, $r = 0.257$, $P < 0.05$, single regression analysis).

H_2O_2 on oocyte maturation and there was a significant effect at the concentration of 10 ng/mL of melatonin (Fig. 3B).

Discussion

This report is the first large-scale clinical trial of melatonin use in infertile patients. Melatonin treatment with a 3 mg tablet which increases intrafollicular melatonin concentrations four times above patients who did not receive

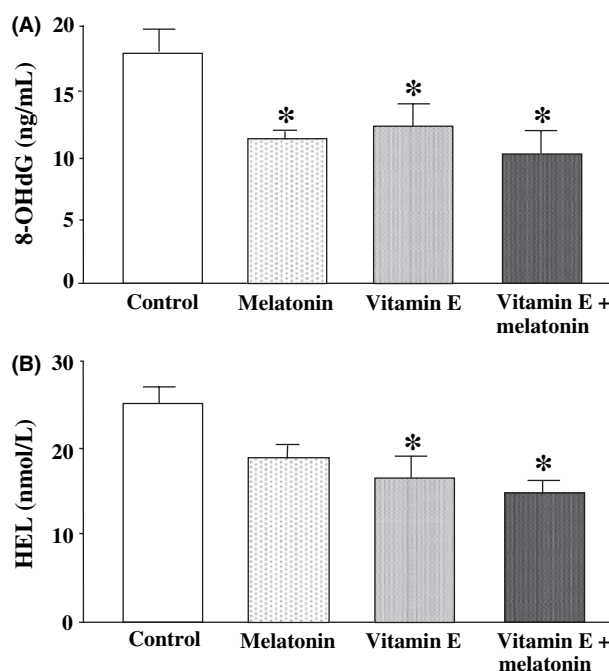


Fig. 2. Effects of melatonin and vitamin E on intrafollicular oxidative stress. To examine the effects of melatonin and vitamin E on intrafollicular oxidative stress, some patients were treated with melatonin, some with vitamin E and some received both melatonin and vitamin E. Eighteen patients undergoing in vitro fertilization and embryo transfer (IVF-ET) who failed to become pregnant in the previous IVF-ET cycle were enrolled. Four patients were given melatonin (3 mg/day) orally at 22:00 hr, 10 patients were given vitamin E (600 mg/day) orally, and 15 patients were given both melatonin and vitamin E. These treatments were carried out from the fifth day of the previous menstrual cycle until the day of oocyte retrieval. Intrafollicular concentrations of (A) 8-hydroxy-2'-deoxyguanosine (8-OHdG) and (B) hexanoyl-lysine adduct (HEL) were determined as biomarkers of oxidative stress and were compared to the data in the previous IVF-ET cycle (control). Data are shown as the mean \pm S.E.M. $*P < 0.05$ compared with control (Kruskal-Wallis *H*-test and Mann-Whitney *U*-test with Bonferroni correction).

Table 1. Effects of melatonin treatment on clinical outcome of IVF-ET

	Melatonin treatment 56 cycles	No melatonin treatment 59 cycles
Fertilization rate in previous IVF-ET cycle	20.2 \pm 19.0%	20.9 \pm 16.5%
Fertilization rate	50.0 \pm 38.0% *	22.8 \pm 19.0%
Pregnancy rate	11/56 (19.6%)	6/59 (10.2%)

IVF-ET, in vitro fertilization and embryo transfer.

Values are mean \pm S.E.M.

* $P < 0.01$ compared with the previous IVF-ET cycle (Mann-Whitney *U*-test).

exogenous melatonin was effective for improvement of oocyte quality. Melatonin enters all tissues; however, it reportedly specifically concentrates in the ovary, eye and pineal gland when injected systemically [33]. Intrafollicular

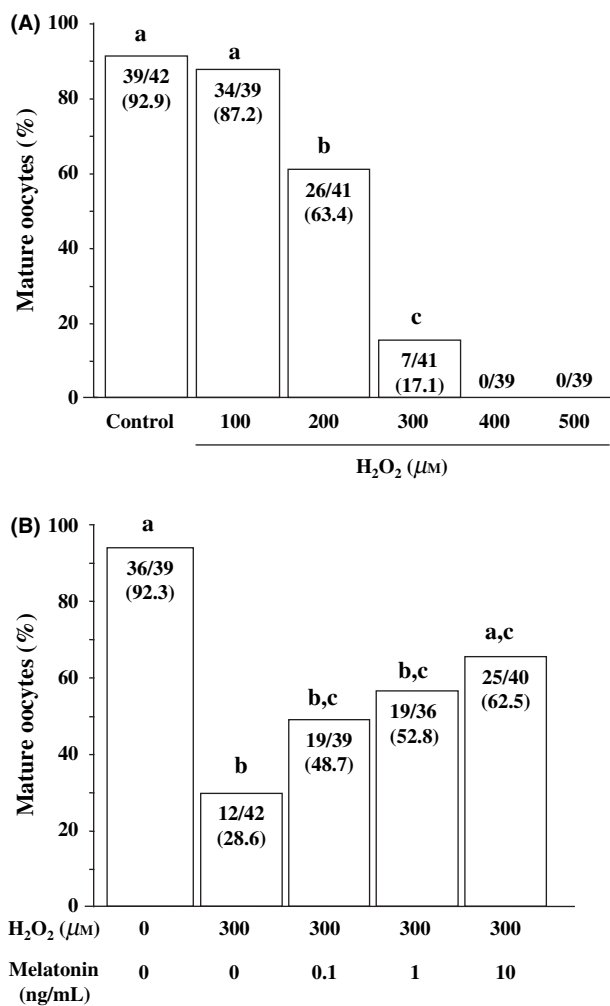


Fig. 3. Toxic effect of H₂O₂ on oocyte maturation (A) and protective effect of melatonin against the inhibitory effect of H₂O₂ on oocyte maturation (B). (A) Oocytes were incubated with H₂O₂ (100, 200, 300, 400, and 500 μM). (B) Oocytes were incubated with melatonin (0, 0.1, 1, and 10 ng/mL) in the presence of H₂O₂ (300 μM). After 12 hr incubation, oocytes with a first polar body (MII stage oocytes) were defined as mature oocytes. Data are expressed as a percentage of the mature oocytes in the total number of oocytes. Different letters indicate statistically significant differences between groups (*P* < 0.01, chi-squared test).

melatonin concentrations are higher in mature follicles than in small atretic follicles [15]. An active uptake of melatonin by the ovary may explain why its levels are higher in preovulatory follicles. Melatonin seems to have an essential role in protecting oocytes from ROS.

Ovulation is one of the most dramatic and dynamic events in the reproductive process. The rupture of the follicular wall can be modeled as a short inflammatory process. Near the time of ovulation, an increase in various substances in the follicle which can induce oxidative stress has been measured; these free radical generating agents include histamine, bradykinin, angiotensin, prostaglandins, eicosanoids, proteolytic enzymes, nitric oxide, superoxide. Cu,Zn-SOD transcripts are present in human and mouse oocytes at germinal vesicle (GV) and metaphase II (MII)

stages and GSH-Px and Mn-SOD transcripts are detected in mouse and human MII oocytes [34]. The presence of antioxidant enzyme transcripts at the GV/ MII stage in mouse oocytes and MII stage in human oocytes [34] suggests that these defense mechanisms are important for further oocyte maturation. ROS are produced within the follicle during the ovulatory process [35–39] and oxidative stimulation has important physiological roles which promote oocyte maturation and follicular wall rupture within the follicle. However, the excessive production of ROS may lead to an increased risk of poor oocyte quality. Our results using mouse oocyte cultures confirmed the harmful effects of H₂O₂ on oocyte maturation. These data are consistent with a previous report, showing that oxidative stress induces apoptosis of human oocytes [40]. In the present study, intrafollicular 8-OHdG concentrations in women with high rates (≥ 30%) of degenerate oocytes were significantly higher than those in women with low rates (< 30%) of degenerate oocytes. Also, a negative correlation was observed between intrafollicular melatonin concentrations and 8-OHdG levels, suggesting a close relationship between oxidative stress and poor oocyte quality.

Melatonin is a direct free radical scavenger [23], and also its regulation of gene transcription for antioxidant enzymes is of special interest [41]. The antioxidant properties of melatonin have been extensively studied and the use of this molecule as a cell protector and as a potential disease preventing agent have been summarized [26–30]. Mitochondrial DNA is a major target for oxygen radicals because of its location near the inner mitochondrial membrane where oxidants are formed and DNA repair activity is lacking. Mitochondria have been identified as a target for melatonin actions, and melatonin lowers mitochondrial protein damage, improving electron transport chain activity and reducing mitochondrial DNA damage [42]. Melatonin may directly prevent mitochondrial damage in oocytes, resulting in improvement of oocyte quality. In the present study, although intrafollicular 8-OHdG concentrations were significantly reduced by melatonin treatment, the reduction of intrafollicular HEL was not statistically significant. 8-OHdG is a sensitive indicator of DNA damage as a result of oxidative stress, and HEL may be a useful biomarker for the initial stage of lipid peroxidation. The main role of melatonin within the follicle may be a free radical scavenger which reduces oocyte DNA damage.

Melatonin also influences both antioxidant enzyme activity and cellular mRNA levels for these enzymes. Ozturk et al. [43] found increased SOD activities in the rat liver after administration of 10 mg/kg of melatonin for 7 days, while Liu and Ng [44] reported enhancement of SOD activity in the rat kidney, liver and brain after a single melatonin injection (5 mg/kg). Baydas et al. [45] observed that melatonin deficiency caused by pinealectomy reduced GSX-Px activity levels in several tissues of rats. Melatonin has also been shown to influence antioxidant enzyme gene expression. Antolin et al. [46] first reported that melatonin causes incremental changes in mRNA levels for both Cu,Zn-SOD and Mn-SOD after its exogenous administration (500 μg/kg). Mayo et al. [47] provided an insight into the mechanism by which melatonin regulates the

antioxidant enzyme gene expression using cultured dopaminergic cells; they found that melatonin-induced synthesis of new proteins as a condition for regulation of gene expression of all the three antioxidant enzymes, i.e., Cu,Zn-SOD, Mn-SOD and GSH-Px.

Not only is melatonin itself a direct free radical scavenger, but metabolites that are formed during these interactions, i.e., cyclic 3-hydroxymelatonin, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK) are likewise excellent scavengers of reactive species [21–24]. These metabolites are generated from melatonin via several pathways including enzymatic, pseudo-enzymatic and because of interaction with a variety of ROS [23]. This cascade reaction is a novel property of melatonin and explains how it differs from other conventional antioxidants. It appears that melatonin's interaction with ROS is a prolonged process that involves many of its metabolites; this makes melatonin highly effective in protecting cells from oxidative stress.

Vitamin E is a fat-soluble vitamin that exists in eight different forms, and acts to protect cell membranes from free radicals, which are generated by heavy metal poisoning, radiation, toxic chemicals, drugs and alcohol [48]. Alpha-tocopherol is traditionally recognized as the most active form of vitamin E in humans, and it is a powerful biological antioxidant [49]. ROS incite the generation of lipid hydroperoxides from cell membrane phospholipids. These reactive lipids can function as signaling molecules and also can induce apoptosis. Alpha-tocopherol helps to maintain homeostatic levels of ROS by terminating the chain reaction of oxidizing events [50]. Numerous clinical studies have demonstrated that vitamin E has some value in a number of chronic, oxidative stress-induced pathologies, e.g., heart disease, atherosclerosis, Alzheimer's disease and cancers [51]. Our data documenting that intrafollicular HEL concentrations were significantly decreased by vitamin E treatment indicating that vitamin E works as a scavenger of lipid peroxyl radicals and suppresses the generation of lipid hydroperoxides from cell membrane phospholipids. Vitamin E works synergistically with other antioxidant nutrients including selenium, vitamin C, carotene and melatonin to quench free radicals. We chose melatonin and vitamin E for infertile patients in this study and this combination showed the largest decrease in the intrafollicular 8-OHdG and HEL concentrations. Therefore, we believe that this combination of the antioxidants is useful for improving oocyte quality in infertile patients.

In the present study, oxidative stress inhibited oocyte maturation while the antioxidant melatonin showed a protective role against the inhibitory effect of oxidative stress on oocyte maturation. Additionally, melatonin administration increased intrafollicular melatonin concentrations and reduced intrafollicular 8-OHdG and HEL concentrations. After noting these changes, melatonin was used for infertile patients who failed to become pregnant in a previous IVF-ET cycle and whose fertilization rate was less than 50%. The fertilization and pregnancy rates were improved by melatonin administration compared to the patients without melatonin treatment. Subsequently, we have continued the use of 3 mg melatonin tablets in several hundred patients. No side effects have been noted and no

infant showed any abnormality; indeed, melatonin is reported to have minimal negative consequences [52].

It is well recognized that oocyte and embryo aneuploidy is significantly increased with advancing age in humans [53]. Most aneuploidies associated with maternal ageing are believed to derive from nondisjunctions and meiotic errors initiated at meiosis I (MI) [54]. It has been demonstrated that 95% of Down's syndrome children receive their extra chromosome from their mother, and in 80% of these, the nondisjunction occurred in the first meiotic division which is completed in the ovary before ovulation [55]. In addition, oxidative stress induces disturbances in spindle organization and aneuploidy in mouse oocytes [56]. We believe that the reduction of oxidative damage of oocytes is important not only for the outcome of IVF-ET but also for the prevention of chromosomal abnormalities of the fetus.

It should be pointed out that the amounts of melatonin (3 mg/day) and vitamin E (600 mg/day) used to reduce oxidative damage differed markedly suggesting that the indole is a much more powerful antioxidant than vitamin E, at least under the conditions of this study. When melatonin has been compared to vitamin E in other experiments, it has been commonly observed that melatonin is superior in terms of reducing molecular damage resulting from free radicals, especially at the DNA level [57].

In conclusion, the current results document that oxidative stress causes toxic effects on oocyte maturation and melatonin protects oocytes from oxidative stress. Melatonin administration increases intrafollicular melatonin concentrations, reduces intrafollicular oxidative damage and elevates fertilization and pregnancy rates. Melatonin is likely to become the medicine of choice for improving oocyte quality for women who cannot become pregnant because of poor oocyte quality. Melatonin and vitamin E combination would be a great benefit to women who suffer from infertility.

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