Antioxidative Capacity of Melatonin in Follicular Fluid of Aged IVF Patients: Beneficial Effects on Oocytes and Embryo

Alessandro Pacchiarotti1, Claudia Valeri1, Gabriele Antonini2, Giulio Biagiotti1, Nikos Prapas4, Arianna Pacchiarotti1*3

1Praxi Provita IVF Center, Praxi DS, Rome, Italy
2Department of urology U. Bracci, Sapienza University of Rome, Italy
3Department of Obstetrics and Gynecology, Infertility and Assisted Reproduction Unit, Sapienza University of Rome, Italy
4IVF Unit, Aristotle University of Thessaloniki, Greece

Abstract
The aim of the present study was to evaluate the role of melatonin supplementation on the main IVF outcomes in aged patients underwent IVF. 358 infertile women aged over 40 underwent a short-down-regulation protocol and were randomized into two groups: 178 patients who received 5mg melatonin (group A) and 180 patients who did not received melatonin (group B). Oxidative stress values, mature oocytes, embryo quality, pregnancy rates and implantation rates, intrafollicular concentration of melatonin and progesterone were measured. There were significant statistical differences comparing group A with group B in terms of mature oocytes (48.2% vs 35.0%), oxidative stress (CARR U) (190 ± 41 vs 388 ± 64), antioxidative capacity (AOCs) (1.76±0.4 vs 0.89 ± 0.2), progesterone concentration in follicular fluid (10.4±1.1 ml vs 4.3±0.8 ml) and grade I embryos (45.7% vs 30.4%, p=0.0045). Melatonin intrafollicular concentrations were significantly increased after melatonin treatment (213±35 pg/ml versus 69 ± 23 pg/ml).

In conclusion melatonin supplementations during ovarian stimulation in aged patients improve oocyte and embryo quality, increasing progesterone production and scavenging free radicals. Furthermore we demonstrated that exogenous administrated melatonin is able to accumulate efficiently in the follicular fluid.

Keywords: Embryos quality; Oocytes quality; Intrafollicular melatonin; Oxidative stress; Antioxidative capacity

Introduction
Reduction of oocyte and embryo quality is the main burden that IVF protocols have to face. Indeed, several studies have been carried out in order to identify predictive factors for IVF outcomes41. The most common cause of IVF-ET failure is reduced oocyte and embryo quality and several factors, such as social-environmental, aging and/or pathological factors, can negatively affect it1-2. The free radical theory of aging hypothesizes that oxygen-derived free radicals are responsible for the age-related damage at the cellular and tissue levels. Free radical species are unstable and highly reactive. They become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates or any nearby molecule causing a cascade of chain reactions resulting in cellular damage and disease3-4. There are two major types of free radical species: reactive oxygen species (ROS) and reactive nitrogen species (NOS). Oxidative stress(OS) influences the entire reproductive span of women’s life and even thereafter (i.e. menopause). It has been suggested that the age-related decline in fertility is modulated by OS15. It plays a role during pregnancy6-7 and normal parturition7,8 and in initiation of preterm labor9,10. The pathological effects are exerted by various mechanisms including lipid damage, inhibition of protein synthesis, and depletion of ATP11. There is some understanding of how ROS affect a variety of physiologic functions (i.e. oocyte maturation, ovarian steroidogenesis, ovulation, implantation, formation of blastocyst, luteolysis and luteal maintenance in pregnancy)12-13. Exogenous gonadotropin has a stimulatory effect on the follicular content of iron, which is a potent oxidant, catalyses generation of free radicals in Haber-Weiss reaction. At ovarian level melatonin have been shown to induce LH receptor expression that reach in an increase concentration of progesterone (P) and therefore, it is involved in follicle maturation and it has been speculated to be involved in dominant follicle selection10. Furthermore, melatonin is a documented powerful free radical scavenger and a broad spectrum antiox-
 Beneficial effects of melatonin on Oocyte and embryo quality

Therefore, it is proposed that, in addition to the previously reported free radical scavenging cascade, melatonin is involved in a concurrent “chelating cascade” thereby contributing to a reduction in oxidative stress[18].

Although the scavenging action of melatonin is not ovarian specific it plays a crucial role during ovulation indeed, the ovulation process has different traits in common with an inflammation process and several reactive species are generated and released in the follicular fluid. Melatonin concentration in the growing follicle may be an important factor in avoiding atresia, because melatonin in the follicular fluid reduces apoptosis of critical cells[19].

The aim of the study was to test the synergistic effect melatonin when integrated in the common IVF protocols of aged patients on the main IVF outcomes.

Materials and Methods

Patients enrollment and randomization

From July 2009 to December 2013, in Praxi Pro Vita IVF Center in Rome, 358 patients were assessed for eligibility in this prospective double blind randomized controlled trial. Women, aged over 40, have had the following inclusion criteria: 1) Absence of uterine and genetics causes of infertility 2) Serum levels of FSH on day 3 of the ovarian cycle <12IU/L 3) Normal uterine cavity 4) Body mass index (BMI) of 20 to 26 kg/m² and 5) First IVF treatment, in order to limit the heterogeneity of the patients and to minimize any confounding variables that may affect the results.

Randomization was performed using a computer-based random assignment schedule for each patient. Sealed and numbered envelopes were used to conceal the treatment allocation until randomization. The physicians were blinded to the randomization. All patients were counseled about the nature of the study and gave their written informed consent for their participation in the randomization procedure. All patients were blinded after assignment to interventions. The patients were randomized and blindly divided into two groups: group A (study group) 178 patients who received melatonin (5mg) from the first day of the cycle until 14 days after embryo transfer; group B (control group) 180 women who did not receive melatonin.

Treatment and protocols of stimulation

All the participating patients underwent a short down-regulation protocol with a gonadotropin-releasing hormone (GnRH) analogue hormone (Triptorelin, Decapeptyl; Ipsen, Milan, Italy) at 0.1 mg/day from the first day of their cycle. Moreover they received combined protocol[20-22] starting with 225 IU of acidic HMG (Meropur; Ferring Italy) for the first 6 days starting from day 2 of the cycle and followed by 225 IU of less-acidic recombinant FSH (Puregon, MSD, Rome, Italy) until hCG administration.

Treatment monitoring was conducted throughout gonadotropin administration. Every other day (until hCG day) a blood sample was drawn between 8 and 9AM in a regular manner to measure serum estradiol (E2). Transvaginal pelvic ultrasound (Sonoace 8000 SE) was performed during gonadotropin treatment. The participants were reviewed at the same time intervals and received the same amount of attention from researchers and staff. Human chorionic gonadotropin (hCG) 10,000 IU (Gonasi HP, IBSA CH ) was given intramuscularly (IM) when 50% of the follicles had reached 20 mm of diameter and E2 level 250 pg/ml.

Transvaginal ultrasound-guided oocyte retrieval (Sonoace 8000 SE) was done 36 hours after hCG injection. Each follicle was aspirated separately and follicular fluid containing the oocyte was collected. Cumulus oocyte complex was assessed according to the oocyte maturation score established criteria. The oocytes were then inseminated in vitro by conventional intracytoplasmic sperm injection, and the resultant embryos were scored according to established criteria. Ultrasound-guided ET (Sonoace 8000 SE) was performed at day 2. The luteal phase was supplemented with progesterone (P) 50 mg IM daily.

Calculation of outcome measures

Immediately after retrieval of the oocyte, half follicular fluid of each patient was analyzed to determine melatonin and progesterone concentrations in mature follicles (more than 18 mm in diameter) for each patient. Intrafollicular concentrations of melatonin were measured by immunoassay (Melatonin Sulfate Elisa Test, DRG international inc. EIA 1431). The sensitivity of the assay was 2.1 pg/tube, and the intra- and inter-assay coefficients of variation were less than 10%. Progesterone concentration was measured by immunoassay (Immulite, Siemes Healthcare Global). The sensitivity of the assay was 2.1 ng/tube, and the intra- and inter-assay coefficients of variation were less than 10%.

The other half of follicular fluid from each patient was analyzed with two test: The d ROM Test (Diacon, Grosseto, Italy) colorimetric assay, and the AntiOxidant Capacity (AOC) as well as the FORD test (Callegari, Parma, Italy). The first is based on the ability of transition metals, such as iron, to catalyze the breakdown of hydroperoxides (ROOH) into derivative radicals, according to the Fenton reaction. When 20μl of follicular fluid is dissolved in an acidic buffer provided by the manufacturer (R2), the hydroperoxides react with the transition metal ions liberated from the proteins in the acidic medium and are converted to alkoxyl (RO•) and peroxy (ROO•) radicals (reactions A and B). The radical species produced by the reaction interact with an additive (phenylenediamine derivative, 2CrNH2) that forms a colored solution (reaction C). The red blood cells (RBCs) are then spun down (∼960 ×g, 60 s) and the cuvette is placed into the spectrophotometer. Following six min at 37°C (standardized temperature for accurate and reproducible measurements), the color is estimated at 505 nm (linear kinetic-based reaction). The intensity of the color correlates directly with the quantity of radical compounds and with the hydroperoxide concentration accordingly to the Lambert-Beer law:

(A) R-OOH+Fe2+→RO+OH+Fe3+
(B) R-OOH+Fe3+→ROO+H+Fe2+
(C) RO+ROO+2CrNH2→ROO+RO+ [2CrNH2+]

Results are expressed as CARR U (units), whereby 1CARR U is equivalent to 26 mg/dl H2O2. Oxidative stress classifications are as follows: no oxidative stress <300 CARR U, intermediate 300-320 borderline range; 321-340 CARR U low level OS, 341-400 CARR U middle level oxidative stress; 401-500 CARR U high level OS; >500 very high level OS.

The second test is based on the decrease in absorbance
Beneficial effects of melatonin on Oocyte and embryo quality

that is proportional to the total AOCs of follicular fluid in accordance to the Lambert-Beer law. The linearity range is from 0.25 to 3.0 mmol/L Trolox. The assay is usually completed within six minutes. Classification of the AOCs is as follows: good AOCs (> 1.53 mmol/L Trolox and normal is between 1.07 and 1.53 mmol/L Trolox, where as values below 1.07 are considered as reduced AOCs.

Primary end points were: intra follicular melatonin concentration, values from tests of oxidative stress, progesterone concentration, oocyte (number of mature oocytes) and embryo (grade I quality, clinical pregnancy and implantation rates. Clinical pregnancies were identified by the presence of a gestational sac on ultrasonography 5 weeks after oocyte retrieval. Secondary outcomes were: FSHIU administered, days of stimulation, serum estradiol levels, endometrial thickness on the day of hCG administration, and incidence of moderate or severe Ovarian Hyper Stimulation Syndrome (OHSS).

Statistical analysis

Statistical analysis was performed using the JMP software (version 4.0.4; SAS, Cary, NC). The parameters were compared using the two-tailed Student’s t test for independent data and the chi-square test, setting the significance level at P<0.05. The analysis of variance two-way test was also used to analyze continuous variables, including primary and secondary outcome parameters.

Statistical power calculation was based on a level of 0.05 with 80% power to detect a 20% difference with 50 evaluable patients per group. Sample size needed was 214 (Confidence Interval 4; Confidence level 95%). The difference between two groups was not statistically significant. 313 patients underwent oocyte retrieval: 165 in group A, 166 in group B.

As expected, melatonin intrafollicular concentrations were significantly higher in the group A (213±51 pg/ml versus non melatonin cycle: 69±23 pg/ml, P=0.0013). Furthermore an increased number of mature oocytes were obtained in Group A vs Group B (48.2% in group A and 35.0% in group B). Statistically difference were found in: oxidative stress value CARR U (190±41 vs 388±64 in group A and B respectively), in antioxidant capacity (AOCs) (1.76±0.4 vs 0.89±0.2 in group A and B respectively), in follicular fluid progesterone concentration (10.4±1.1 vs 4.3±0.8 in group A vs B) and in the number of grade I embryos (45.7 vs 30.4 respectively in group A and B, P=0.0045). Melatonin intrafollicular concentrations were significantly increased after melatonin treatment (213±51 pg/ml vs 69±23 pg/ml, P=0.0013) (Table 2).

Table 2: Outcome measure.

|                      | Group A | Group B | P value |
|----------------------|---------|---------|---------|
| N patients           | 178     | 180     | NS      |
| N dropout patients   | 13      | 14      | NS      |
| N patients undergoing egg retrieval | 165 | 166 | NS |
| N patients undergoing embryo transfer | 157 | 158 | NS |
| Days of stimulation  | 11.3±2.1 | 10.5±3.2 | NS |
| Total FSH per cycle (IU) | 2987±233 | 2876±345 | NS |
| Estradiol level at hCG day (pg/ml) | 2389±221 | 2198±331 | NS |
| Endometrial thickness at hCG day (mm) | 10.8±2.3 | 11.2±3.1 | NS |
| Intrafollicular melatonin concentrations (pg/ml) | 213±35 | 69±23 | 0.0013 |
| Oxidative stress test (CARR U) | 190±41 | 388±64 | 0.014 |
| Antioxidative capacity (AOCs) | 1.76±0.4 | 0.89±0.2 | 0.018 |
| Progesterone (ng/ml) | 10.4±1.1 | 4.3±0.8 | 0.001 |
| N oocytes retrieval  | 5.1±1.8 | 5.2±2.3 | NS |
| MII oocytes (%)      | 48.2    | 35.0    | 0.008   |
| MI oocytes (%)       | 23.6    | 30.5    | NS      |
| Immature oocytes (%) | 28.2    | 34.5    | NS      |
| N embryos transferred/patient | 2.5±0.6 | 2.7±0.8 | NS |
| Grade I embryos (%)  | 45.7    | 30.4    | 0.0045  |
| Grade II embryos (%) | 33.3    | 39.5    | NS      |
| Grade III embryos (%)| 17.5    | 24.6    | NS      |
| Grade IV embryos (%) | 3.5     | 5.5     | NS      |
| Clinical Pregnancy (%)| 41.4    | 36.7    | NS      |
| Implantation rate (%)| 13.8    | 12.2    | NS      |
| Abortion rate (%)    | 10.8    | 8.6     | NS      |
| Twin pregnancy rate (%) | 20.8   | 20.0    | NS      |

*The data are expressed like mean ± standard deviation.

Table 1: Demographic and clinical characteristics of study groups.

|                      | Group A | Group B | P value |
|----------------------|---------|---------|---------|
| N=165                | N=166   |         |         |
| Age [years]          | 39±3.6* | 38.5±2.8 | NS      |
| BMI [kg/m²]          | 22.8±1.3 | 23.1±1.7 | NS      |
| Cycle length [days]  | 28±1.1* | 27.6±2.5 | NS      |
| Duration of sterility [years] | 2.1±1.6 | 2.5±2.3 | NS      |
| Primary sterility [N(%)] | 133 (80.6) | 130 (78.3) | NS |
| Secondary sterility [N(%)] | 32 (19.4) | 36 (21.7) | NS |

*The data are expressed like mean ± standard deviation.

Results

Recruitment of patients lasted from July 2010 to December 2013 and follow-up was conducted until the 5th week of gestational age. Both groups were comparable for the main demographic characteristics (mean age, body mass index, duration of sterility, primary infertility), as well as sterility factors (tubal, male, and idiopathic) and main cycle parameters (Table 1).
Conclusions

In the present manuscript we showed that by oral melatonin supplementation it is possible to increase its follicular fluid concentration.

Human pathological studies have shown that high nocturnal melatonin levels have a suppressive effect on GnRH pulsatile secretion, ovarian function and pubertal development[21]. In particular, high nocturnal melatonin levels were found in children with delayed puberty, while low nocturnal melatonin levels were found in children with precocious puberty when compared to age and weight matched controls. Moreover, hypothalamic amenorrhea was associated with high melatonin concentrations[24].

At ovarian level, it was shown that melatonin increases LH receptor (LHr) expression (but not FSH receptor)[25], likely being involved in dominant follicle selection process and ovulation[26].

Ovulation is a complex process by which a preovulatory follicle ruptures and releases a fertilizable oocyte into the oviductal lumen. This process occurs as a result of a dynamic interaction between the LH surge and local factors including steroids, nitric oxide (NO), prostaglandins, and peptides in a time-dependent manner. The LH surge triggers structural and biochemical changes that lead to rupture of the Graafian follicles, resulting in expulsion of the oocyte and subsequent development of the corpus luteum. After hCG injection, follicular steroidogenesis quickly shifts from E2 dominance to P dominance by the inhibition of 17a-hydroxylase-C17-20 lyase activity[27]. This acute increase of P production is essential for luteinization and ovulation. In Human P and E2 concentrations are significantly higher in the larger follicles than in the smaller one. Similarly melatonin concentrations are higher in follicles containing mature oocyte[28].

Interestingly, melatonin is able to induce P production[25]. Therefore, during oogenesis, elevated concentrations of melatonin are involved in the induction of LH sensibility of the developing follicle, inducing LHr expression and to P production resulting in luteinization and ovulation.

The mechanisms trough which melatonin exerts its action are different, indeed, melatonin can work through two membrane receptors MT1-R and MT2-R, one nuclear receptor or D-chiro-inositol (DCI) decrease the oxidative damage on follicular fluid proteins in women with polycystic ovary syndrome (PCOS)[33]. In conclusion it suggest an important scavenger role of melatonin which results in better oocytes and embryo quality in aged women, supported by previous study on murine[34].

Our suggestion is to give a melatonin supplementation to aged patients under went IVF.

References

1. van Loendersloot, L.L., van Wely, M., Limpens, J., et al. Predictive factors in invitro fertilization (IVF): a systematic review and meta-analysis. (2010) Hum Reprod Update 16(6): 577-589.
2. Chattopadhayay, R., Ganesh, A., Samanta, J., et al. Effect of follicular fluid oxidative stress on meiotic spindle formation in infertile women with polycystic ovarian syndrome. (2010) Gynecol Obstet Invest 69(3): 197-202.
3. Szczepanska, M., Kozlik, J., Skrzypczak, J., et al. Oxidative stress may be a piece in the endometriosis puzzle. (2003) Fertil Steril 79(6): 1288–1293.
4. Attaran, M., Pasqualotto, E., Falcone, T., et al. The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. (2000) Int J Fertil Womens Med 45(5): 314–320.
5. Debruin, J.P., Dorland, M., Spek, E.R., et al. Ultrastructure of the resting ovarian follicle pool in healthy young women. (2002) Biol Reprod 66(4): 1115–1160.
6. Myatt, L., Cui, X. Oxidative stress in the placenta. (2004) Histochem Cell Biol 122(4): 369–382.
7. Fainaru, O., Almog, B., Pichuch, I., et al. Active labour is associated with increased oxidisability of serum lipids ex vivo. (2002) BJOG 109(8): 938–941.
8. Mocatta, T.J., Winterbourn, C.C., Inder, T.E., et al. The effect of gestational age and labour on markers of lipid and protein oxidation in cord plasma. (2004) Free Radic Res 38(2): 185–191.
9. Wall, P.D., Pressman, E.K., Woods, J.R. Preterm premature rupture of the membranes and antioxidants: the free radical connection. (2002) J Perinat Med 30(6): 447–457.
10. Pressman, E.K., Cavanaugh, J.L., Mingione, M., et al. Effects of maternal antioxidant supplementation on maternal and fetal antioxidant levels: a randomized, double-blind study. (2003) Am J Obstet Gynecol 189(6): 1720–1725.
11. Ray, S.D., Lam, T.S., Rotollo, J.A., et al. Oxidative stress is the master operator of drug and chemically-induced programmed and unprogrammed cell death: Implications of natural antioxidants in vivo. (2004) BioFactors 21(1-4): 223–232.
12. Sugino, N., Takiguchi, S., Kashida, S., et al. Superoxide dismutase expression in the human corpus luteum during the menstrual cycle and in early pregnancy. (2000) Mol Hum Reprod 6(1): 19–25.
13. Suzuki, T., Sugino, N., Fukaya, T., et al. Superoxide dismutase in...
Beneficial effects of melatonin on oocyte and embryo quality

14. Jozwik, M., Wolczynski, S., Szamatowicz, M. Oxidative stress markers in preovulatory follicular fluid in humans. (1999) Mol Hum Reprod 5(5): 409–413.

15. Ashok, A., Sajal, G., Rakesh, K.S. Role of oxidative stress in female reproduction. (2005) Reprod Biol Endocrinol 3: 28.

16. Tamura, H., Takasaki, A., Miwa, I., et al. Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. (2008) J Pineal Res 44(3): 280-287.

17. Rizzo, P., Raffone, E., Benedetto, V. Effect of the treatment with myo-inositol plus folic acid plus melatonin in comparison with a treatment with myo-inositol plus folic acid on oocyte quality and pregnancy outcome in IVF cycles. A prospective, clinical trial. (2010) Eur Rev Med Pharmacol Sci 14(6): 555-561.

18. Galano, A., Medina, M.E., Tan, D.X., et al. Melatonin and its metabolites as copper chelating agents and their role in inhibiting oxidative stress: a physicochemical analysis. (2015) J Pineal Res 58(1): 107-116.

19. Cruz, M.H., Leal, C.L., Cruz, J.F., et al. Essential actions of melatonin in protecting the ovary from oxidative damage. (2014) Theriogenology 82(7): 925-932.

20. Selman, H., Pacchiarotti, A., El-Danasouri, I. Ovarian stimulation protocols based on follicle-stimulating hormone glycosylation pattern: impact on oocyte quality and clinical outcome. (2010) Fertil Steril 94(5): 1782-1786.

21. Pacchiarotti, A., Aragona, C., Gaglione, R., et al. Efficacy of a combined protocol of urinary and recombinant follicle-stimulating hormone used for ovarian stimulation of patients undergoing ICSI cycle. (2007) J Assist Reprod Genet 24(9): 400-405.

22. Selman, H., Pacchiarotti, A., Rinaldi, L., et al. Simultaneous administration of human acidic and recombinant less acidic follicle-stimulating hormone for ovarian stimulation improves oocyte and embryo quality, and clinical outcome in patients with repeated IVF failures. (2013) Eur Rev Med Pharmacol Sci 17(13): 1814-1819.

23. Srinivasan, V., Spence, W.D., Pandi-Perumal, S.R. Melatonin and human reproduction: shedding light on the darkness hormone. (2009) Gynecol Endocrinol 25(12): 779-785.

24. Arendt, J. Melatonin and the pineal gland: influence on mammalian seasonal and circadian physiology. (1998) Rev Reprod 3(1): 13-22.

25. Tamura, H., Nakamura, Y., Korkmaz, A., et al. Melatonin and the ovary: physiological and pathophysiological implications. (2009) Fertil Steril 92(1): 328-343.

26. Reiter, R.J., Tamura, H., Tan, D.X., et al. Melatonin and the circadian system: contributions to successful female reproduction. (2014) Fertil Steril 102(2): 321-328.

27. Roy, S.K., Greenwald, G.S. In vitro steroidogenesis by primary to antral follicles in the hamster during the periovulatory period: effects of follicle-stimulating hormone, luteinizing hormone, and prolactin. Biol Reprod 37(1): 39-46.

28. Mori, T., Nonoguchi, K., Watanabe, H., et al. Morphogenesis of polycystic ovaries as assessed by pituitary-ovarian androgenic function. (2009) Reprod Biomed Online 18(5): 635-643.

29. Reiter, R.J., Tan, D.X., Fuentes-Broto, L. Melatonin: a multitasking molecule. (2010) Prog Brain Res 181: 127-151.

30. Niles, L.P., Wang, J., Shen, L., et al. Melatonin receptor mRNA expression in human granulosa cells. (1999) Mol Cell Endocrinol 156(1-2): 107-110.

31. Espey, L.L. Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. (1994) Biol Reprod 50(2): 233-238.

32. Arteaga, E., Villaseca, P., Rojas, A., et al. Comparison of the antioxidant effect of estril and estradiol on low density lipoproteins in post menopausal women. (1998) Rev Med Chil 126(5): 481–487.

33. Piomboni, P., Focarelli, R., Capaldo, A., et al. Protein modification as oxidative stress marker in follicular fluid from women with polycystic ovary syndrome: the effect of inositol and metformin. (2014) J Assist Reprod Genet 31(10): 1269-1276.

34. Wang, F., Tian, X., Zhang, L., et al. Melatonin promotes the in vitro development of pronuclear embryos and increases the efficiency of blastocyst implantation in murine. (2013) J Pineal Res 55(3): 267-274.