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Research Article

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

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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 shortdown-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 stimulationin aged patients improve oocyte and embryo quality, increasing progesterone production and scavenging free radicals. Furthermorewe 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

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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 outcomes[1]. 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 it[1-2]. 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 disease[3,4]. 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 OS[5]. It plays a role during pregnancy[6] and normal parturition[7,8] and in initiation of preterm labor[9,10]. The pathological effects are exerted by various mechanisms including lipid damage, inhibition of protein synthesis, and depletion of ATP[11]. 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)[12-15]. 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 selection[16].

Furthermore, melatonin is a documented powerful free radical scavenger and a broad spectrum antiox-

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idant[17]. 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/m^2 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 accordingto 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 sensitivityof 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 (Diacron, Grosseto, Italy) colorimetric assay, and the AntiOxidant Capacity (AOC) as with 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 alkoxy (RO•) and peroxy (ROO•) radicals (reactions A and B). The radical species produced by the reaction interact with an additive (phenylenediamine derivative, $2CrNH_2$) 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) $R\text{-}OOH+Fe^{2+} \rightarrow RO\cdot+OH^{-}+Fe^{3+}$
(B) $R\text{-}OOH+Fe^{3+} \rightarrow ROO\cdot+H^{+}+Fe^{2+}$
(C) $RO\cdot+ROO\cdot+2CrNH_2 \rightarrow ROO^{-}+RO^{-}+ [2CrNH2^{+}]$

Results are expressed as CARR U (units), whereby 1CARR U is equivalent to 26 mg/dl H_2O_2. Oxidative stress classifications are asfollows: 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

that is proportional to the total AOCs of follicular fluid in accordance to the Lambert-Beer law. The linearity range is from 0.25 to3.0 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 treatments was evaluated using a two-sided, 95% confidence interval.

The study was registered on clinical trials having the registration number NCT01540747.

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, primaryinfertility), as well as sterility factors (tubal, male, and idiophatic) and main cycle parameters(Table 1).

Table 1: Demographic and clinical characteristics of study groups.

	Group A	Group B	P value
	N=165	N=166	
Age [years]	39 ± 3,6[a]	38.5±2.8[a]	NS
BMI [kg/m^2]	22.8 ± 1.3[a]	23.1±1.7[a]	NS
Cycle length [days]	28 ± 1,1[a]	27.6±2.5[a]	NS
Duration of sterility [years]	2.1 ± 1.6[a]	2.5±2.3[a]	NS
Primarysterility [N(%)]	133 (80.6)[b]	130 (78.3)[b]	NS
Secondarysterility [N(%)]	32 (19.4)[b]	36 (21.7)[b]	NS

[a]The data are expressed like mean ± standard deviation.

[b] The data represent the number of patients with the parenthetical percentage.

Twentyseven patients dropped out: 13 in group A of which 7 low responders (estradiol level <1000 pg/ml and less than two follicles developed), and 6 because of excessive ovarian response; 14 in group B of which 8 low responders and 6 for excessive ovarian response. The differences in two groups were 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 antioxidative 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[a]	10.5 ± 3.2[a]	NS
Total FSH per cycle (IU)	2987 ± 233[a]	2876 ± 345[a]	NS
Estradiol level at hCG day (pg/ml)	2389 ± 221[a]	2198 ± 331[a]	NS
Endometrial thickness at hCG day (mm)	10.8 ± 2.3[a]	11.2 ± 3.1[a]	NS
Intrafollicularmelatoninconcentration (pg/ml)	213 ± 35[a]	69 ± 23[a]	0.0013
Oxidative stress test (CARR U)	190 ± 41[a]	388 ± 64[a]	0,014
Antioxidativecapacity (AOC)	1,76 ± 0,4[a]	0,89 ± 0,2[a]	0,018
Progesterone (ng/ml)	10,4 ± 1.1[a]	4,3 ± 0,8[a]	0,001
N oocytesretrieval	5.1 ± 1.8[a]	5.2 ± 2.3[a]	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[a]	2.7 ± 0.8[a]	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	NS

[a]The data are expressed like mean ± standard deviation.

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[23]. In particular, high nocturnal melatonin levels were found in children with delayed puberty, while low nocturnal melatonin levels were found in children with precocious pubertywhen 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[25].

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 trough two membrane receptors MT_1-R and MT_2-R, one nuclear receptor or directly by entering the cell[28]. Melatonin receptors have been identified in hypothalamic neurons that govern the release of pituitary gonadotropins, in the anterior pituitary, and in both female and male gonads, as well as in other reproductive organs[29]. A previous work has clearly shown that both the melatonin receptor isoforms, mt_1-R and MT_2-R, are expressed in human granulosa-luteal cells (hGLCs)[30].

Melatonin interaction with its receptors lead to the expression of several enzymes such as: superossido dismutasi (SOD), glutathione peroxides (GPx); furthermore, as already mention, it leads to the increase of LHr and P production[25].
To support this theory in our study we demonstrated a significantly higher concentration of intrafollicular progesterone in the group of patient treated with melatonin.

Ovulation involves processes similar to a local inflammatory response[31], in which both reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced, impairing oocyte quality. Therefore, since both melatonin and its metabolites are able to quench ROS and RNS, they might be involved in the protection of granulosa cells and oocyte during ovulation[25].

The age related decline in oocyte quality results in reduced number and quality of follicles and in increased incidence of congenital anomalies in children. The ageing of the oocytes affects many biochemical pathways which have a deleterious effect on pre and post-implantation development of the embryo. Oxidative stress occurs at menopause because of loss of estrogens, which have antioxidant effect on low-density lipoproteins. Estrogens have oxidation and antioxidant properties[32]. This study showed a significantly reduction of free radicals in melatonin group and therefore a higher concentration of intrafollicular melatonin in the same group. These data were supported by recent study that demonstrates like supplement of metformin or D-chio-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.

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