Melatonin levels in follicular fluid as markers for IVF outcomes and predicting ovarian reserve

Jing Tong^{1,2}, Shile Sheng³, Yun Sun^{1,2}, Huihui Li^{1,2,4}, Wei-Ping Li^{1,2}, Cong Zhang^{1,2,5} and Zi-Jiang Chen^{1,2,6,7,8,9}

¹Center for Reproductive Medicine, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China, ²Shanghai Key Laboratory for Assisted Reproduction and Reproductive Genetics, Shanghai, China, ³Department of Nuclear Medicine, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China, ⁴Department of Obstetrics and Gynecology, Center for Reproductive Medicine, Qilu Hospital of Shandong University, Jinan, China, ⁵Key Laboratory of Animal Resistance Research, College of Life Science, Shandong Normal University, Ji'nan, Shandong, China, ⁶Center for Reproductive Medicine, Shandong Provincial Hospital, Shandong University, Jinan, China, ⁷National Research Center for Assisted Reproductive Technology and Reproductive Genetics, Jinan, China, ⁸The Key Laboratory for Reproductive Endocrinology of Ministry of Education, Jinan, China and ⁹Shandong Provincial Key Laboratory of Reproductive Medicine, Jinan, China

Correspondence should be addressed to C Zhang or W-P Li or Z-J Chen; Email: zhangxinyunlife@163.com or liweiping@renji.com or chenzijiang@hotmail.com

Abstract

Good-quality oocytes are critical for the success of *in vitro* fertilization (IVF), but, to date, there is no marker of ovarian reserve available that can accurately predict oocyte quality. Melatonin exerts its antioxidant actions as a strong radical scavenger that might affect oocyte quality directly as it is the most potent antioxidant in follicular fluid. To investigate the precise role of endogenous melatonin in IVF outcomes, we recruited 61 women undergoing treatment cycles of IVF or intracytoplasmic sperm injection (ICSI) procedures and classified them into three groups according to their response to ovarian stimulation. Follicular fluid was collected to assess melatonin levels using a direct RIA method. We found good correlations between melatonin levels in follicular fluid with age, anti-Müllerian hormone (AMH) and baseline follicle-stimulating hormone (bFSH), all of which have been used to predict ovarian reserve. Furthermore, as melatonin levels correlated to IVF outcomes, higher numbers of oocytes were collected from patients with higher melatonin levels and consequently the number of oocytes fertilized, zygotes cleaved, top quality embryos on D3, blastocysts obtained and embryos suitable for transplantation was higher. The blastocyst rate increased in concert with the melatonin levels across the gradient between the poor response group and the high response group. These results demonstrated that the melatonin levels in follicular fluid is associated with both the quantity and quality of oocytes and can predict IVF outcomes as well making them highly relevant biochemical markers of ovarian reserve.

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Introduction

Infertility is defined as the inability to achieve pregnancy within a year of unprotected intercourse (Rad *et al.* 2015). It is not only a medical concern, but also a social issue with increasing prevalence in both developed and developing countries. Over the past 35 years, IVF as an effective treatment for infertility has become widely accepted. However, poor oocyte quality remains one of the most intractable causes of the failure of IVF procedures (Tamura *et al.* 2009). The ovarian reserve, which indicates female's reproductive potential, is associated with both oocyte quantity and quality. Age-related quality decrease in the ovary reserve occurs as

a result of two processes: cellular malfunction caused by the accumulation of reactive oxygen species (ROS) in cells (Stadtman 2002) and compromised antioxidant defense mechanisms. Furthermore, not only aging itself but also standard IVF procedures such as superovulation, oocyte cryopreservation and freezing of surplus embryos could lead to the accumulation of ROS (Ali *et al.* 2003, Chao *et al.* 2005, Tamura *et al.* 2012).

It is acknowledged that excess ROS in follicular fluid will deteriorate oocyte quality (Goud *et al.* 2008) by inducing apoptosis of oocyte and granulosa cells (Zhang *et al.* 2006). These harmful effects of ROS can be inhibited by intracellular antioxidant systems such as

glutathione (GSH) and ascorbic acid (Wang *et al.* 2002), and the balance between ROS production and their detoxification therefore becomes essential for the quality of oocytes. Melatonin (N-acetyl-5-methoxytryptamine) is famous as a potent free radical scavenger and a broad-spectrum antioxidant (Manchester *et al.* 2015). Melatonin, which is an ubiquitously distributed and functionally diverse molecule, can modulate the physiology and molecular biology of cells via various mechanisms (Reiter *et al.* 2010). In humans, melatonin is the principle secretory product of the pineal gland and is involved in many of the physiology manifestations (Chen *et al.* 2011). Although investigators observed the phenomenon of melatonin-exerted effects on mammalian reproductive activities decades ago (Reiter *et al.* 2009), contradictory reports on the effect of melatonin on the reproductive axis exist. Early studies have shown that melatonin can influence the reproductive function at the level of the brain and pituitary by activating the receptor sites within the hypothalamic–pituitary–gonadal axis (Malpau *et al.* 2001). However, other studies have demonstrated that melatonin did not have a direct influence on GnRH neurons (Kauffman *et al.* 2007).

As for oocytes, they are protected and nourished by the surrounding follicular fluid that is regarded as a biological ‘window’ that reflects the metabolic and hormonal processes of oocytes and granulosa cells (Wiener-Megnazi *et al.* 2004), and the actual environment in which these cells exist. Interestingly, a high concentration of melatonin, almost threefold higher than that in serum (Brzezinski *et al.* 1987), has been detected in human follicular fluid (Reiter *et al.* 2014). These concentrations are maintained presumably not only by active ovarian uptake (Tamura *et al.* 2008) from the general circulation but also by ovarian synthesis (Itoh *et al.* 1999). Melatonin synthesized by ovarian cells, such as rat oocytes (Sakaguchi *et al.* 2012), bovine cumulus cells (Reiter *et al.* 2013) and human granulosa cells (Reiter *et al.* 2009), would then diffuse into follicular fluid. As melatonin exerts its antioxidant actions as a direct free radical scavenger and by regulation of gene transcription for antioxidative enzymes (Rodriguez *et al.* 2004), melatonin in follicular fluid can protect the oocyte from oxidative stress (Reiter *et al.* 2014) and may act as a direct modulator of oocyte quality.

Currently, much attention is paid on the beneficial impacts of supplement of melatonin on culture medium in animal experiments such as in vitro oocyte protection (Kang *et al.* 2009, Nakano *et al.* 2012) and embryo development (Dehghani-Mohammadabadi *et al.* 2014, Niknafs *et al.* 2014). However, the precise role of endogenous melatonin in human follicular fluid, and, in particular, a focus on its association with the IVF outcomes for infertile females remains to be determined.

The goal of the present study was to investigate the roles of intrafollicular melatonin. We designed a retrospective study to verify the roles of endogenous

melatonin with a focus on the relationship between melatonin levels and other clinical parameters linked to ovarian reserve and the IVF outcomes.

Materials and methods

Study population

Participants were recruited from the Center for Reproductive Medicine, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University between June 2015 and August 2015. This research was conducted with patients after informed consent according to the guidelines of the Ministry of Public Health of China (MPH) and was approved by the Reproductive Ethics Committee of the Ren Ji Hospital. Sixty-one women undergoing a treatment cycle of IVF or intracytoplasmic sperm injection (ICSI) were retrospectively studied. Patients were nonsmokers and were free from major medical illness. Patients with a congenital uterine anomaly, ovarian tumors or chronic use of any medication were excluded. All patients presented with tubal, male or mixed factor infertility.

The corresponding demographic and related medical data of the participants, including age, type and duration of infertility, and body mass index (BMI) were collected. Serum levels of baseline follicle-stimulating hormone (bFSH) before stimulation, baseline luteinizing hormone (bLH), E_2 and anti-Müllerian hormone (AMH) were also measured on the second to the fifth day of the previous menstrual cycle.

Stimulation protocols

Controlled ovarian hyperstimulation is a principal step of IVF therapy, and many stimulation protocols are available for controlled ovarian hyperstimulation in IVF, including the option of long agonist protocol, short agonist protocol, antagonist protocol or minimal stimulation protocol. The doses of gonadotropin mixtures given to each patient are usually adapted, depending on clinical experience. The decision is based on the benefits and shortcomings of each treatment option, and most importantly on the patients' response to ovarian stimulation. FSH (Fostimo, Genevrier, Sophia-Antipolis, France; Gonal-F, Merck Serono, Lyon, France; Puregon, MSD, Courbevoie, France) or human menopausal gonadotropin (Menopur, Ferring SAS, St Prex, Switzerland) were used for hyperstimulation. Follicle development was monitored by ultrasound scans from the day of stimulation and every two or three days until the day of human chorionic gonadotropin (hCG) application. The levels of serum E_2 , FSH, LH and progesterone (P) were also detected at the same time. When the leading follicle reached 20 mm in mean diameter with a serum E_2 level of 200–300 pg/mL per mature follicle (greater than 14 mm), the patients received hCG (Ovitrelle, Merck Serono, Lyon, France; Lizhu, Zhuhai, China) or triptorelin acetate (Decapeptyl, Ferring, Kiel, Germany; Decapeptyl, Ipsen, Paris, France). Ultrasound-guided oocyte collection was performed 33–36 h later. Type and dose of hCG or triptorelin acetate on each patient were selected by the physicians according to the plasma E_2 levels on the day of hCG administration and the number of mature oocytes.

IVF treatment and embryo assessment

Classical IVF or ICSI was performed according to the clinical indication. ICSI was performed only in the cases of a severe male factor such as oligoasthenoteratozoospermia, obstructive and non-obstructive azoospermia or previous fertilization failure. The day of oocyte retrieval was considered as day 0. Fertilization was confirmed at 16–18 h after insemination by the presence of two pronuclei. Cleavage-stage embryos were characterized by four grades depending on their morphological appearance (blastomere number, size and cytoplasmic fragmentation) following Balaban *et al.* (2001). According to our laboratory strategy, no more than four ‘top’ (excellent or good) quality cleavage-stage embryos were manipulated on D3, among them, two were transferred in a fresh cycle and the other two were cryopreserved; however, if the fresh embryo transfer was canceled, all the four were cryopreserved for future transfer. A maximum of two embryos were transferred each time. Supernumerary embryos were cultured to day 5 or day 6. Blastocyst assessment was evaluated according to the system of Gardener and Schoolcraft (Gardner *et al.* 2000). Only expanded blastocysts with at least several inner cell mass cells and some trophectoderm cells were vitrified.

Grouping criteria

In response to ovarian stimulation, all participants were sub-grouped as poor, normal and high ovarian response. Poor ovarian response is defined by the presence of two of the following three features: (1) advanced maternal age (≥ 40 years) or any other risk factor for poor response, (2) previous characterized poorly responsive cycles (≤ 3 oocytes retrieved with a conventional stimulation protocol), (3) an abnormal ovarian reserve test (i.e. antral follicle count < 5 – 7 follicles or AMH < 0.5 – 1.1 ng/mL), according to the ‘Bologna criteria’ (Ferraretti *et al.* 2011). The normal and high ovarian response definition is still debated. In our study, we took the agreement (Qiao *et al.* 2015) of ‘Society of Reproductive Medicine Study Groups of the Chinese Medical Association’ for reference. Normal ovarian response was defined as follows: (1) age < 35 years, (2) normal ovarian reserve test (bFSH < 10 IU/mL and 1 – 1.4 ng/mL $<$ AMH < 3.5 – 4.0 ng/mL) and (3) no history of cycle cancellation because of poor or high ovarian response. High ovarian response was defined as follows: (1) the number of oocytes retrieved is more than 15 or cycle cancellation is recommended when overabundance mature follicles are observed, (2) ovarian hyperstimulation syndrome observed after hormone treatment, (3) the number of follicles (> 12 – 14 mm) is more than 20 or (4) the serum E_2 level is higher than 5000 pg/mL in controlled ovarian hyperstimulation.

Collection of follicular fluid

Follicular fluid (without any diluting fluid or blood contamination) from the first leading dominant follicle (diameter 19–24 mm) per patient was collected by follicle puncture under transvaginal ultrasound guidance. All follicular aspirations were performed between 08:00 h and 11:00 h. After oocyte isolation, the fluid was centrifuged at 1000g for 20 min

at 0°C to remove cellular components. Then, the supernatants were collected and stored at -20°C for subsequent assay on the same day within 2 months. All procedures were performed under protection from light.

Melatonin radioimmunoassay (RIA)

Intrafollicular concentrations of melatonin were measured by ^{125}I -Radioimmunoassay using a RIA kit from DAsource ImmunoAssays S.A. (Belgium) followed by the fundamental principle of RIA, involving competition between a radioactive and a non-radioactive antigen for a fixed number of antibody-binding sites. The measuring range for melatonin was 2.3–1000 pg/mL. The intra-assay coefficient of variation was 9.7–13.4% and inter-assay coefficient of variation 8.0–13.3%. The melatonin levels in follicular fluid were calculated by multiplying the melatonin value in single sample of follicular fluid by the number of oocytes retrieved from each person.

Statistical analysis

All statistical analyses were performed using SPSS software (version 19.0, IBM Corporation, USA). Based on the Kolmogorov–Smirnov test and the Shapiro–Wilk test, normal distribution for all the parameters could not be assumed. Therefore, in descriptive statistics, median values and interquartile ranges (IQR) are given additionally to mean and s.d. Nonparametric test procedures were used for statistical evaluation of the data.

Kruskal–Wallis test was performed to analyze differences in parameter levels between patients with poor, normal and high ovarian response to ovarian stimulation. In the case of significance, pairwise comparisons between different groups were performed with the Mann–Whitney U test. Association between the melatonin levels in follicular fluid and other parameters was measured and tested by Spearman rank correlation coefficients (r_s). Qualitative data were compared between groups according to the chi-squared test. Statistical significance was set at $P < 0.05$.

Results

Baseline characteristics of patients and the melatonin levels in follicular fluid on the day of oocyte retrieval

In total, 61 women (23–45 years old) were enrolled in this study and divided into three groups: poor, normal and high ovarian response according to their response to ovarian stimulation. The characteristics of the patients in the subgroups were described in Table 1. The results showed that, as expected, age, bFSH and bE₂, which could predict ovarian reserve negatively, were the highest in poor ovarian response group, and the differences were quite marked. While AMH, another critical marker of ovarian reserve, showed an opposite tendency among the groups, the

Table 1 Baseline characteristics of patients and the melatonin levels in follicular fluid on the day of oocyte retrieval.

Variables	Poor response (n=21)		Normal response (n=21)		High response (n=19)		*P
	Mean $\pm$ S.D.	Median (IQR)	Mean $\pm$ S.D.	Median (IQR)	Mean $\pm$ S.D.	Median (IQR)	
Age (years)	40.57 $\pm$ 2.561	40 (39–43)	28 $\pm$ 2.864	27 (25.5–30.5)	27.2 $\pm$ 2.396	27 (26–28)	<0.001
bFSH (IU/L)	10.11 $\pm$ 3.136	8.87 (7.8–12.65)	6.825 $\pm$ 1.155	6.97 (5.96–7.405)	6.009 $\pm$ 1.578	5.45 (4.6–8.03)	<0.001
bE ₂ (pg/mL)	80.39 $\pm$ 83.61	45 (32.59–101)	36.45 $\pm$ 14.34	33.54 (28.19–46.07)	34.08 $\pm$ 10.07	30.64 (26.08–39.92)	<0.001
AMH (ng/mL)	0.7373 $\pm$ 0.7755	0.51 (0.25–0.8515)	2.671 $\pm$ 0.8956	2.74 (1.875–3.58)	10.18 $\pm$ 4.275	10.3 (5.57–12.3)	0.006 ^a , 0.011 ^b
Melatonin levels in FF (pg/mL)	23.26 $\pm$ 19.06	16.14 (8.69–40.4)	113.7 $\pm$ 102.4	82.17 (43.64–137.7)	237.4 $\pm$ 197.1	187.2 (64.8–382.4)	<0.001 ^{a,b,c}
BMI (kg/m ²)	22.37 $\pm$ 2.808	22.86 (19.73–24.95)	22.88 $\pm$ 4.168	23.03 (19.05–25.72)	21.51 $\pm$ 3.174	21.22 (18.59–24.03)	<0.001
bLH (IU/L)	6.986 $\pm$ 11.19	3.79 (2.7–6.845)	4.426 $\pm$ 1.752	4.08 (2.72–5.9)	5.071 $\pm$ 1.291	4.82 (4.19–6.03)	NS
Duration of infertility (years)	7.143 $\pm$ 6.421	5 (2.5–9)	3.667 $\pm$ 2.106	3 (2–5)	3.267 $\pm$ 2.492	2 (2–4)	NS
Primary infertility (% (n/n)) ^a	33.33 (7/21)		47.62 (10/21)		52.63 (10/19)		NS
Indications (% (n/n)) ^a							
Tubal	52.38 (11/21)		19.05 (4/21)		15.79 (3/19)		
Male	14.29 (3/21)		38.10 (8/21)		36.84 (7/19)		
Mixed	33.33 (7/21)		42.85 (9/21)		47.37 (9/19)		

^aPoor/high, ^bpoor/normal, ^chigh/normal, ^dChi-squared test.

FF, follicular fluid; IQR, interquartile ranges; NS, not statistically significantly different; *P, Kruskal–Wallis test; *P, pairwise comparisons between sub-groups.

Table 2 Comparisons of IVF outcomes among patients with poor, normal, and high ovarian response.

Variables	Poor response (n=21)		Normal response (n=21)		High response (n=19)		*P
	Mean $\pm$ S.D.	Median (IQR)	Mean $\pm$ S.D.	Median (IQR)	Mean $\pm$ S.D.	Median (IQR)	
Cycles	21		21		19		
No. of oocytes retrieved	2.095 $\pm$ 0.9952	2 (1–3)	10 $\pm$ 2.864	11 (8–12)	22 $\pm$ 5.438	20 (18–24)	<0.001 ^{a,b,c}
No. of oocytes fertilized	1.476 $\pm$ 0.8136	1 (1–2)	6.19 $\pm$ 2.804	7 (4.5–7)	14.2 $\pm$ 4.539	15 (10–19)	<0.001 ^{a,b,c}
No. of zygotes cleaved	1.667 $\pm$ 0.7958	2 (1–2)	7.095 $\pm$ 3.048	8 (4.5–9.5)	16.67 $\pm$ 4.655	18 (13–21)	<0.001 ^{a,b,c}
No. of top quality embryos on D3	1.048 $\pm$ 0.8646	1 (0–2)	2.762 $\pm$ 1.7	3 (1.5–4)	7.067 $\pm$ 4.367	6 (4–9)	<0.001 ^a , 0.001 ^{b,c}
No. of blastocysts obtained	0.1905 $\pm$ 0.5118	0 (0–0)	2.143 $\pm$ 2.175	1 (0.5–3.5)	6.533 $\pm$ 3.182	7 (4–9)	<0.001 ^{a,b,c}
No. of embryos suitable for transplantation	1.19 $\pm$ 0.9284	1 (0–2)	3.571 $\pm$ 2.461	4 (2–5.5)	6.733 $\pm$ 2.685	7 (4–10)	<0.001 ^a , 0.001 ^b , 0.002 ^c
Blastocyst rate (%)	13 $\pm$ 32	0 (0–0)	26 $\pm$ 22	25 (5–42)	40 $\pm$ 17	33 (29–50)	<0.001 ^a , 0.002 ^b
Percentage of top quality embryos on D3 (%)	58 $\pm$ 44	67 (0–100)	40 $\pm$ 23	38 (27–53)	41 $\pm$ 20	40 (24–61)	NS

Blastocyst rate was based on the number of blastocysts obtained/the number of zygotes cleaved. Percentage of top quality embryos on D3 was based on the number of top quality embryos on D3/the number of zygotes cleaved.

^aPoor/high, ^bpoor/normal, ^chigh/normal.

IQR, interquartile ranges; NS, not statistically significantly different; *P, Kruskal–Wallis test; *P, pairwise comparisons between sub-groups.

high response group had the maximum AMH levels, whereas the poor ovarian response group had the lowest ($P=0.000$). Interestingly, the melatonin levels in follicular fluid on the day of oocyte retrieval also showed a statistically significant difference between groups ($P=0.000$), the values were 23.26 ± 19.06 (pg/mL) for the poor response group, 113.7 ± 102.4 (pg/mL) for the normal response group and 237.4 ± 197.1 (pg/mL) for the high response group. Other baseline characteristics such as BMI, bLH, melatonin in single sample of follicular fluid, duration of infertility, primary infertility and indications for infertility including tubal, male and mixed showed no significant differences among the three groups.

Comparisons of IVF outcomes among patients with poor, normal and high ovarian response

When comparing the IVF outcomes among groups, we observed that the patients in poor ovarian response group who had the lowest melatonin levels in follicular fluid had poorest outcomes of IVF. As showed in Table 2, the criteria of IVF outcomes such as the number of oocytes retrieved, oocytes fertilized, zygotes cleaved, top quality embryos on D3, blastocysts obtained and embryos suitable for transplantation, as well as the rate of development to blastocyst increased significantly from patients with the poor ovarian response. All parameters were greater in the normal response group and reached a maximum in patients with elevated response. The melatonin's concentration increased at the same time among the groups.

Additionally, we did the paired comparisons among the three groups. We observed there were differences in the number of oocytes retrieved, oocytes fertilized, zygotes cleaved, top quality embryos on D3, blastocysts obtained and embryos suitable for transplantation among the three groups (Table 2). As for the blastocyst rate, there was a difference between poor and normal and poor and high ovarian response groups (Table 2). Although the percentage of top quality embryos on D3 showed no significant difference among the three groups (Table 2).

Melatonin levels in follicular fluid on the day of oocyte retrieval are highly relevant markers of ovarian reserve

Analysis of non-normal distributed values of melatonin and AMH levels, showed a positive correlation (Spearman-Rho $r_s=0.593$, $P<0.001$) (Fig. 1A). Additionally, we found a negative correlation between the melatonin levels and other ovarian reserve parameters, including age ($r_s=-0.632$, $P<0.001$) (Fig. 1B) and bFSH ($r_s=-0.507$, $P<0.001$) (Fig. 1C). Moreover, we analyzed the correlation of the melatonin levels and ovarian stimulation parameters to explore the influence of stimulation protocols on the melatonin levels in follicular fluid. We identified the relationship between the melatonin levels and stimulation characteristics in patients ($n=41$) who used the standard long agonist protocol, short agonist protocol and antagonist protocol, as summarized in Table 3. We observed that the melatonin levels had a positive correlation with plasma E_2 and progesterone levels on the day of hCG administration (dhCG), their correlation coefficients were $r_s=0.636$ and $P<0.001$ for E_2 -dhCG and $r_s=0.473$ and $P<0.001$ for P-dhCG; at the same time, the melatonin levels had a negative correlation with LH-dhCG ($r_s=-0.382$, $P=0.002$). However, there were no significant relationships between the melatonin levels and serum FSH levels on the day of hCG administration (FSH-dhCG), duration of stimulation, the initial dose and total dose of gonadotropin and endometrial thickness (mm) (Table 3).

Melatonin levels in follicular fluid on the day of oocyte retrieval can predict IVF outcomes

To further explore the function of melatonin in the follicular fluid on the day of oocyte retrieval in predicting IVF outcomes, we identified the correlation of the melatonin levels in follicular fluid and IVF outcomes. We observed that the melatonin levels had a significant positive correlation with IVF outcomes parameters (Table 4), including number of oocytes retrieved ($r_s=0.67$, $P<0.001$), oocytes fertilized ($r_s=0.653$, $P<0.001$), zygotes cleaved ($r_s=0.646$, $P<0.001$),

Figure 1 Correlation between the melatonin levels in FF on the day of oocyte retrieval and AMH, age and bFSH levels ($n=61$). (A) A significant positive correlation between the melatonin levels in follicular fluid and AMH level (Spearman-Rho $r_s=0.593$, $P<0.001$). (B) A significant negative correlation between the melatonin levels in follicular fluid and female age (Spearman-Rho $r_s=-0.632$, $P<0.001$). (C) A significant negative correlation between the melatonin levels in follicular fluid and bFSH level (Spearman-Rho $r_s=-0.507$, $P<0.001$).

Table 3 Correlation between the melatonin levels in follicular fluid (pg/mL) and ovarian stimulation parameters in patients ($n=41$) who used the standard long agonist protocol, short agonist protocol and antagonist protocol.

Parameters	The melatonin levels in follicular fluid (pg/mL)*	P value
E ₂ -dhCG (pg/mL)	0.636	<0.001
P-dhCG (ng/mL)	0.473	<0.001
LH-dhCG (IU/L)	-0.382	0.002
FSH-dhCG (IU/L)	-0.007	NS
Duration of stimulation (days)	0.143	NS
Total dose of Gn (IU)	-0.077	NS
Initial dose of Gn (IU)	-0.297	NS
Endometrial thickness (mm)	0.103	NS

*Spearman rank correlation coefficients (r_s).

E₂-dhCG, serum E₂ levels on the day of hCG administration; FSH-dhCG, serum FSH levels on the day of hCG administration; LH-dhCG, serum LH levels on the day of hCG administration; NS, not statistically significantly different; P-dhCG, serum P levels on the day of hCG administration.

top quality embryos on D3 ($r_s=0.587$, $P<0.001$), embryos cultured ($r_s=0.559$, $P<0.001$), blastocysts obtained ($r_s=0.533$, $P<0.001$), embryos suitable for transplantation ($r_s=0.607$, $P<0.001$) and blastocyst rate ($r_s=0.36$, $P=0.005$). However, we found the melatonin levels in follicular fluid had no significant correlation with percentage of top quality embryos on D3. Given the results described previously, the melatonin levels in follicular fluid have a significant positive correlation with IVF outcomes.

Discussion

In this study, we determined the melatonin levels in follicular fluid and investigated their relationships with the clinical parameters and the outcomes of IVF. The results demonstrated that the melatonin levels in the follicular fluid displayed good correlations with

Table 4 Correlation of the melatonin levels in follicular fluid on the day of oocyte retrieval and IVF outcomes parameters ($n=61$).

Parameters	The melatonin levels in follicular fluid*	P value
No. of oocytes retrieved	0.67	<0.001
No. of oocytes fertilized	0.653	<0.001
No. of zygotes cleaved	0.646	<0.001
No. of top quality embryos on D3	0.587	<0.001
No. of embryos cultured	0.559	<0.001
No. of blastocysts obtained	0.533	<0.001
No. of embryos suitable for transplantation	0.607	<0.001
Blastocyst rate (%)	0.36	0.005
Percentage of top quality embryos on D3 (%)	0.028	NS

*Spearman rank correlation coefficients (r_s).

Blastocyst rate was based on the number of blastocysts obtained/the number of zygotes cleaved. Percentage of top quality embryos on D3 was based on the number of top quality embryos on D3/the number of zygotes cleaved.

NS, not statistically significantly different.

age, AMH and bFSH, which are reliable predictors of ovarian reserve. Furthermore, melatonin levels were crucial to IVF outcomes, patients with higher melatonin levels retrieved more oocytes, and the number of oocytes fertilized, zygotes cleaved, top quality embryos on D3, blastocysts obtained and embryos suitable for transplantation as well as blastocyst rate increased significantly together with the melatonin levels from the poor response group to high response group. Thus, melatonin levels in the follicular fluid can act as a good predictor of ovarian reserve as well as IVF outcomes.

The ovarian reserve associated with both quantity and quality of oocytes indicates female's reproductive potential and reflects the outcomes of IVF as a result. Although IVF treatment outcomes are highly variable and difficult to predict, the ovarian reserve is now routinely tested in women undergoing IVF cycles. This pretreatment assessment of ovarian reserve allows individual prediction of ovarian responsiveness and modification of individual treatment protocol in an attempt to maximize IVF success rates. Both spontaneous and treatment-induced pregnancy rates decline with women aging as a result of both a quantitative and a qualitative reduction of the primordial follicle pool (Bancsi *et al.* 2002). Unfortunately, there is no marker of ovarian reserve available that can accurately predict oocyte quality (Aydin *et al.* 2015). AMH as well as bFSH is by now presented as a good serum marker of ovarian reserve. Nevertheless, AMH is not related to embryo quality; its predictive capacity is too limited to be of clinical value (Smeenk *et al.* 2007). An elevated bFSH reflects a quantitative rather than qualitative decline of the ovarian reserve (Abdalla & Thum 2004). So neither of them can be used to predict oocyte quality.

The follicular fluid microenvironment is crucial for oocytes quality as oocytes are protected and nourished by follicular fluid *in vivo*. Excess ROS in follicular fluid caused by aging or IVF procedures will deteriorate oocyte quality (Goud *et al.* 2008); therefore, it is vital to get a balance between ROS production and their detoxification for the quality of oocytes. As a broad-spectrum antioxidant and a potent free-radical scavenger, melatonin can collaborate against excess ROS within cells by causing a cascade reaction (Tan *et al.* 2007) and modulating antioxidant enzymes gene transcription (Tomás-Zapico & Coto-Montes 2005). Granulosa cells play a crucial role in follicular steroidogenesis and are involved in establishing an essential microenvironment for the oocyte. Evidence indicated that melatonin acted as an antioxidant to protect the integrity of granulosa cells and influences the production of progesterone and estradiol in porcine (He *et al.* 2016). So we assume that melatonin as the primary antioxidant in follicular fluid could protect oocytes from being oxidatively abused by free-radical damage through quenching ROS (Wang *et al.* 2013) and has a positive impact on oocyte quality as a consequence.

Our results showed a good correlation between the melatonin levels in follicular fluid and AMH. This factor is synthesized exclusively by granulosa cells of secondary, preantral and small antral follicles (Weenee *et al.* 2004) – the same cells where melatonin is produced in the ovary. The amount of melatonin produced by human pineal gland diminishes with advancing age (Tan *et al.* 2007). Although the mechanism of decreasing melatonin levels with aging is not yet clearly understood, the explanation appears to be (1) increased calcareous deposits in the pineal gland; (2) decreased β -adrenergic receptors in the pinealocyte membranes or the poor response to norepinephrine; (3) the decreased N-acetyltransferase in the pineal gland with age; (4) progressive alterations in the concentration of melatonin α 1-receptors in hypothalamus and (5) increased clearance of melatonin (Touitou 2001). The same tendency was found in the concentration of melatonin in the follicular fluid according to our findings. This finding of an age-related decrease of melatonin is in general agreement with the research on serum melatonin (Waldhauser *et al.* 1988) and on urinary melatonin (Vakkuri *et al.* 1996). Furthermore, our results showed a negative correlation between the melatonin levels in follicular fluid and serum bFSH level, which was in agreement with previous studies (Walecka-Kapica *et al.* 2015). As age, AMH and bFSH reflect the ovarian reserve, we conclude that the melatonin levels in the follicular fluid are markers of ovarian reserve and diminish with advancing age, possibly because of the dysfunction of granulosa cells and pineal gland.

Melatonin levels are highly related to IVF outcomes, patients with higher melatonin levels yielded more oocytes, and the number of oocytes fertilized, zygotes cleaved, top quality embryos on D3, blastocysts obtained, embryos suitable for transplantation as well as blastocyst rate, increased significantly from the poor response group to high response group. The blastocyst rate is an indicator of oocyte developmental competence and of oocyte quality. Therefore, the melatonin levels in the follicular fluid as highly relevant biochemical markers of both oocyte quantity and quality can act as a good predictor of ovarian reserve as well as IVF outcomes.

In contrast with other markers of ovarian reserve, melatonin is a stable compound produced endogenously and also occurs naturally in some food. Both acute and chronic toxicity of melatonin is reported to be extremely low; no adverse effects were found when 1–300 mg or one gram melatonin was administered to human volunteers daily for 30 days (Jahnke *et al.* 1999, Vijayalaxmi *et al.* 2004). Melatonin has frequently been given to promote sleep as a dietary supplement (Jahnke *et al.* 1999). Despite great advances in IVF procedures, poor ovarian reserve remains a profound problem for female infertility. So the efficacy of administration of melatonin to the patients with poor ovarian reserve by which the intrafollicular melatonin levels appear to raise at the

same time (Tamura *et al.* 2008) and supplementation of melatonin to their embryo culture medium or oocyte mature medium, as a new strategy, can be validated by more clinical trials.

In addition, we did not analyze the pregnancy rate because the fresh embryo transfer in poor ovarian response group was canceled due to the thin endometrium or insufficient embryos. We did not calculate cleavage rate either because of the mixture of IVF and ICSI procedures in our study. Other limitations of our study cannot be ignored. For example, the melatonin levels in follicular fluid were calculated by multiplying the melatonin value in single follicle by the number of oocytes retrieved. This calculation may have contributed to some bias if the concentration of melatonin in each preovulatory follicle was different. We attempted to minimize such bias by extracting fluids from follicles more than 19 mm in diameter and excluding blood-tinged or diluted fluids according to other investigators' strategy where the correlation coefficient between preovulatory follicular melatonin and follicular volume was not statistically significant (Brzezinski *et al.* 1987).

In conclusion, we show for the first time that the melatonin levels in follicular fluid are associated with both quantity and quality of oocytes and can predict IVF outcomes and can act as highly relevant biochemical markers of ovarian reserve. However, further large-scale, prospective studies are needed to validate our results.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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