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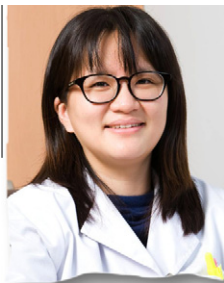
## ARTICLE

# Does supplementation of in-vitro culture medium with melatonin improve IVF outcome in PCOS?


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**Abstract** Human pre-ovulatory follicular fluid (FF) contains a higher concentration of melatonin than serum. The aim of this study was to evaluate the effect of melatonin supplementation of culture medium on the clinical outcomes of an in-vitro maturation (IVM) IVF-embryo transfer programme for patients with polycystic ovarian syndrome (PCOS). Melatonin concentrations in the culture media of granulosa cells (GC) or cumulus-oocyte-complexes (COC) were measured and the clinical outcomes after using IVM media with or without melatonin were analysed. In the culture media of GC or COC, melatonin concentrations gradually increased. When human chorionic gonadotrophin priming protocols were used, implantation rates in the melatonin-supplemented group were higher than those of the non-supplemented control group ( $P < 0.05$ ). Pregnancy rates were also higher, although not significantly. The findings suggest that the addition of melatonin to IVM media may improve the cytoplasmic maturation of human immature oocytes and subsequent clinical outcomes. It is speculated that follicular melatonin may be released from luteinizing GC during late folliculogenesis and that melatonin supplementation may be used to improve the clinical outcomes of IVM IVF-embryo transfer. 

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**KEYWORDS:** culture media, in-vitro maturation, IVF, melatonin, oocyte maturation, PCOS

## Introduction

Melatonin, which is primarily produced by the pineal gland, regulates a variety of important central and peripheral

actions related to circadian rhythms and reproduction (Reiter, 1980, 1991; Reiter et al., 2010). It is generally believed that melatonin functions in the regulation of gonadotrophin release in the hypothalamus/hypophysis and the

modification of ovarian function (Brzezinski et al., 1987; Ronnberg et al., 1990; Vanecek, 1995). Ovarian function depends on both circulating hormone concentrations and the appropriate expression of their receptors. High concentrations of melatonin exist in follicular fluid (FF) (Brzezinski et al., 1987), suggesting that it may play important roles in the growth and maturation of mammalian oocytes. Melatonin may regulate ovarian function through binding to its receptors expressed in human granulosa-lutein cells (Tamura et al., 2009; Yie et al., 1995). Moreover, the binding of melatonin to its receptor directly regulates progesterone production as well as LH receptor gene expression and gonadotrophin-releasing hormone receptor gene expression in human granulosa-lutein cells via the mitogen-activated protein kinase pathway and activation of Elk-1 (Woo et al., 2001).

The LH surge prompts follicular steroidogenesis to shift from oestradiol dominance to progesterone dominance through the inhibition of 17 $\alpha$ -hydroxylase-C<sub>17–20</sub> lyase activity (Roy and Greenwald, 1987), thereby inducing luteinization and ovulation. In humans, the concentrations of progesterone and oestradiol are significantly higher in larger follicles compared with smaller follicles. Interestingly, melatonin shows a similar pattern (Nakamura et al., 2003), and elevated melatonin in pre-ovulatory follicles is thought to be involved in progesterone production and subsequent luteinization and ovulation (Tamura et al., 2009). Moreover, the presence of high concentrations of melatonin in FF and specific melatonin receptors support the function of melatonin as a regulator of oocyte maturation. Consistent with this notion, Chatteraj et al. (2005) demonstrated that pre-incubation of carp oocytes with melatonin accelerated the maturation-inducing hormone-induced resumption of meiosis and early germinal vesicle breakdown. Furthermore, in studies on pig embryos (Kang et al., 2009; Shi et al., 2009), supplementation of the culture system with melatonin was found to enhance oocyte maturation and embryonic development *in vitro*.

Polycystic ovarian syndrome (PCOS), which is a common cause of female anovulatory infertility and menstrual irregularities, affects 6–8% of women of reproductive age (Azziz et al., 2004). The disease has been defined as a syndrome involving polycystic ovaries, hyperandrogenism, hyperinsulinaemia and chronic anovulation. Beneath the tunica albuginea, polycystic ovaries contain numerous small antral follicles (so-called cysts) that have stopped growing and developing. Generally, clomiphene citrate is the first line of treatment for anovulatory infertility associated with PCOS. For the subpopulation of clomiphene citrate-resistant patients, several options may be considered, including metformin, betrosols, ovarian drilling and gonadotrophin therapy. Ever since the first successful delivery of triplets after in-vitro maturation (IVM) IVF and transfer of immature oocytes retrieved via ovariectomy (Cha et al., 1991), this technique has been used for the infertility treatment of PCOS patients. It has become an important tool in human assisted reproduction treatment (Trounson et al., 1994). The advantages of IVM for treatment of PCOS patients include reduced costs, avoidance of ovarian hyperstimulation syndrome, and simpler treatment protocols than those of conventional IVF-embryo transfer. During the last decade, thousands of babies have been born following IVM

IVF-embryo transfer. However, the pregnancy and implantation rates are lower than those obtained with conventional IVF-embryo transfer, perhaps because, although more oocytes are recovered from PCOS patients, the derived oocytes and generated embryos tend to be of poorer quality (Plachot et al., 2003). Alternatively, the high oxidative stress resulting from increased lipid peroxidation and the chromosome aneuploidy that arises during extended culture for maturation *in vitro* could explain the lower pregnancy and implantation rates (Combelles et al., 2009; Harris et al., 2010; Requena et al., 2009). Interestingly, Tamura et al. (2009) showed that intrafollicular melatonin concentration was significantly lower in PCOS patients than those in women undergoing IVF-embryo transfer, possibly accounting for the anovulation and poor oocyte quality seen in PCOS (Tamura et al., 2009).

To date, the majority of studies on melatonin and ovarian follicles have been conducted in animals, and the direct role of melatonin on oocyte maturation in the human system has not yet been established. The present study evaluated the effects of melatonin supplementation on IVM of human immature oocytes and the clinical outcomes of PCOS patients undergoing a IVM IVF-embryo transfer programme.

## Materials and methods

### Patients

From July 2004 to December 2010, infertile couples with PCOS awaiting IVM IVF-embryo transfer were invited to participate in the present study. All patients provided written informed consent after counselling. The study was approved by the Institutional Review Board of CHA Gangnam Medical Centre, Seoul, Korea (IRB reference number 2003-01, approved 6 January 2003). PCOS was defined according to the consensus criteria (Rotterdam ESHRE/ASRM-sponsored PCOS Consensus Workshop Group, 2004). The enrolled individuals comprised PCOS patients displaying multiple small follicular cysts (2–8 mm in diameter) arranged around a dense stroma or scattered throughout an increased amount of stroma on ultrasonography, as well as those with clinical findings of hyperandrogenism and/or chronic oligomenorrhoea or amenorrhoea. All enrolled patients had failed to respond adequately to clomiphene citrate and had not conceived after several cycles of ovulation induction with clomiphene citrate/gonadotrophins or gonadotrophins (Cha et al., 2005).

### Determination of melatonin in FF

The concentrations of melatonin in FF from small antral follicles of PCOS patients ( $n = 13$ ) were measured using a competitive enzyme-linked immunosorbent assay (Melatonin ELISA; Immuno Biological Laboratories, Hamburg, Germany) and compared with those of FF from large mature follicles of non-PCOS patients subjected to conventional IVF-embryo transfer ( $n = 24$ ) and also the results of Tamura et al. (2009). For clinical and ethical reasons, this study was unable to directly compare FF from similarly sized follicles of PCOS and non-PCOS patients. The clinical profiles of the female patients are summarized in Table 1. The sampled follicles

**Table 1** Clinical profiles of non-PCOS patients and PCOS patients.

	Non-PCOS (n = 24)	PCOS (n = 13)
Age (years)	36.6 ± 0.8	31.1 ± 0.8
Body mass index (kg/m <sup>2</sup> )	21.1 ± 0.5	22.2 ± 1.1
Duration of infertility (years)	3.1 ± 0.4	2.7 ± 0.6
Cycle day of oocyte retrieval	12.9 ± 0.3	14.3 ± 0.6
Follicle size (mm)	≥18	≤10
Follicular fluid melatonin (pg/ml) <sup>a</sup>	136.8 ± 26.1	20.9 ± 3.6

Values are mean ± SEM unless otherwise stated.

PCOS = polycystic ovarian syndrome.

<sup>a</sup>P < 0.05.

(≤10 mm in PCOS patients and ≥18 mm in non-PCOS patients) were selected using ultrasonography, and FF was collected during transvaginal aspiration of immature and mature oocytes and stored at −20°C until use. Melatonin was extracted from 0.5 ml FF (90–100% recovery yield) using C18 reversed-phase columns (Immuno Biological Laboratories) and methanol elution. The methanol was evaporated and the extracts were dried and then reconstituted with water. Samples (50 l each of blank reagents, extracted calibrators, extracted samples and extracted standard solutions containing 0, 3, 10, 30, 100 or 300 pg/ml melatonin) were loaded onto a 96-well microtitre plate coated with captured goat anti-rabbit immunoglobulin. Next, 50 µl melatonin biotin and 50 µl melatonin antiserum were added into each well. The plate was shaken carefully, sealed with adhesive foil and incubated overnight (14–20 h) at 2–8°C. After three washes with 250 µl diluted assay buffer, 150 µl of enzyme conjugate was added to each well and the plate was incubated for 2 h at room temperature. The reaction was then developed using a *p*-nitrophenyl phosphate substrate solution, and optical densities (which were inversely proportional to the melatonin concentration) were determined at 405 nm using a photometer. Melatonin standards were used to construct a calibration curve for calculating the concentrations of the unknown samples. The sensitivity of the melatonin assay was 1.6 pg/ml. Both the intra- and inter-assay coefficients of variation were less than 20%.

#### Determination of melatonin in granulosa cells and cumulus-oocyte-complexes by ELISA

Proliferating GC were collected from FF during the non-stimulated IVM IVF-embryo transfer programme, washed three times and cultured in GC medium (M199 medium; Gibco, Grand Island, NY) containing 75 mIU/ml recombinant FSH (Gonal F; Merck-Serono, Modugno Bari, Italy) and 10% fetal bovine serum. After two or three passages, the GC were transferred to GC medium supplemented with 0.5 IU/ml human chorionic gonadotrophin (HCG, Pregnyl; Organon Pharmaceuticals, Korea). Culture media were collected at 0, 24, 48 and 72 h after HCG treatment, and melatonin secretion was assessed. The experiment was replicated three times.

Five COC obtained were cultured for 24 h in IVM medium (G2; Vitrolife, Goteborg, Sweden) containing 20% human FF, 75 mIU/ml recombinant FSH, 0.5 IU/ml HCG and 1 µg/ml oestradiol (Sigma–Aldrich, St Louis, MO). After maturation, the oocytes were fertilized for clinical use by intracytoplasmic sperm injection (ICSI), and media collected before and after IVM were analysed for the secretion of melatonin. The experiment was replicated three times.

#### Real-time reverse-transcription PCR analysis of ASMT and AANAT expression

To investigate the presence of the enzymic pathways for melatonin synthesis in cultured GC, acetylserotonin O-methyltransferase (ASMT or HIOMT) and aralkylamine N-acetyltransferase (AANAT) expression was analysed in cultured GC with/without 50 µg/ml tryptophan supplementation. Cultured GC were collected at 0, 24, 48 and 72 h after HCG treatment, and total RNA was extracted from these cells using a RNeasy Mini kit (Qiagen, Hilden, Germany) and quantified using a Qubit fluorometer and a Quant-iT RNA BR assay kit (Invitrogen). First-strand cDNA was synthesized using a PrimeScript 1st-strand cDNA Synthesis kit (TaKaRa Bio, Shiga, Japan) according to the manufacturer's instructions. For quantification of expression levels, real-time PCR was performed using a DyNAmo HS SYBR Green qPCR kit (Finnzymes, Espoo, Finland) using a Bio-Rad iQ5 real-time PCR machine. The  $\Delta\Delta C_t$  method was used to normalize the mRNA expression levels of ASMT (forward 5'-ATGCTTGTGCAGACGGAAG-3'; reverse 5'-TTCCTGGCTAAAATGGCATC-3'; 128 bp, NM\_004043.2) and AANAT (forward 5'-AGTGGAGTGGCCACCGAGGAA-3'; reverse 5'-GGGCTAGGC TAGGACGCCAAGG-3'; 107 bp, NM\_001166579.1) with respect to that of  $\beta$ -actin (Livak and Schmittgen, 2001). The experiment was replicated 10 times.

#### Effect of melatonin in IVM for PCOS patients (phase I)

At the start of the clinical study, which ran from July 2004 to December 2008 (phase I), PCOS patients (*n* = 111) were randomized to the IVM medium supplemented with melatonin (MEL+) group or no melatonin (control) group. Patients were subjected to two different IVM IVF-embryo transfer protocols performed by one physician. Protocol 1 consisted of non-stimulated cycles (Cha et al., 2000) and protocol 2 consisted of non-stimulated cycles, except for HCG priming, which was intended to improve the maturation rate of immature oocytes (Cha and Chian, 1998). All cycles were monitored via transvaginal ultrasonography on cycle days 3 and 9 and thereafter as necessary. For HCG priming, 5000 IU HCG was administered at 35–36 h before immature oocyte retrieval.

Transvaginal ultrasound-guided oocyte collection was performed using a 20-gauge single-lumen aspiration needle (Cook, Queensland, Australia). Patients were sedated with diazepam (10 mg; Roche, Zurich, Switzerland) and midazolam (5 mg; Roche). Immature oocytes were collected via careful aspiration of each follicle. All retrieved oocytes were washed three or four times in P1 medium (Irvine Scientific, Irvine, CA, USA). Immature oocytes surrounded by

compact cumulus cells were selected, rinsed once with basic IVM medium and placed in organ culture dishes containing 800  $\mu$ l IVM medium with or without 10  $\mu$ mol/l melatonin (M5250-1G; Sigma–Aldrich). This concentration of melatonin was selected based on a preliminary study using human oocytes, in which better maturation and embryonic development was observed with 10  $\mu$ mol/l melatonin compared with 0–1  $\mu$ mol/l melatonin (data not shown). All IVM procedures were performed at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. After the oocytes were cultured for 24–48 h, they were denuded with hyaluronidase solution (ICSI Cumulase; Origio, Malov, Denmark) and their maturity was microscopically determined by the presence of the first polar body. Mature oocytes were inseminated with ICSI, and the fertilization and embryonic development in the two groups (MEL+ versus control) was assessed. At 66–70 h post ICSI, 2–3 embryos were selected and transferred into the patient's uterus.

For endometrial preparation, patients received 4 mg of oral oestradiol valerate (Progynova, Schering, Berlin, Germany) early on the day of oocyte retrieval. Luteal support was provided with daily intramuscular injection of 100 mg progesterone (Taiyu Progesterone; Taiyu Chemical and Pharmaceutical, Shing Ju, Taiwan) in oil, beginning on the day of fertilization. Once the pregnancy was confirmed by serial serum  $\beta$ -HCG measurements and ultrasonography, oestradiol valerate and progesterone administration were continued until 12 weeks of gestation. Clinical pregnancy was defined as a positive  $\beta$ -HCG with the confirmation of an intrauterine pregnancy by ultrasound. Ongoing pregnancy was defined as the presence of at least one viable fetus beyond week 20 of pregnancy on ultrasound.

### Clinical trial of melatonin supplementation in IVM IVF-embryo transfer for PCOS patients (phase II)

At the start of the clinical trial, which ran from January 2009 to December 2010 (phase II), PCOS patients ( $n = 132$ ) were subjected to IVM IVF-embryo transfer using protocol 2, and aspirated oocytes matured *in vitro* in IVM medium containing melatonin (MEL+). Oocytes were cultured, maturity was determined and insemination was performed using ICSI. Fertilization and embryonic development were assessed, and two or three embryos were selected for transfer. The clinical outcomes are monitored.

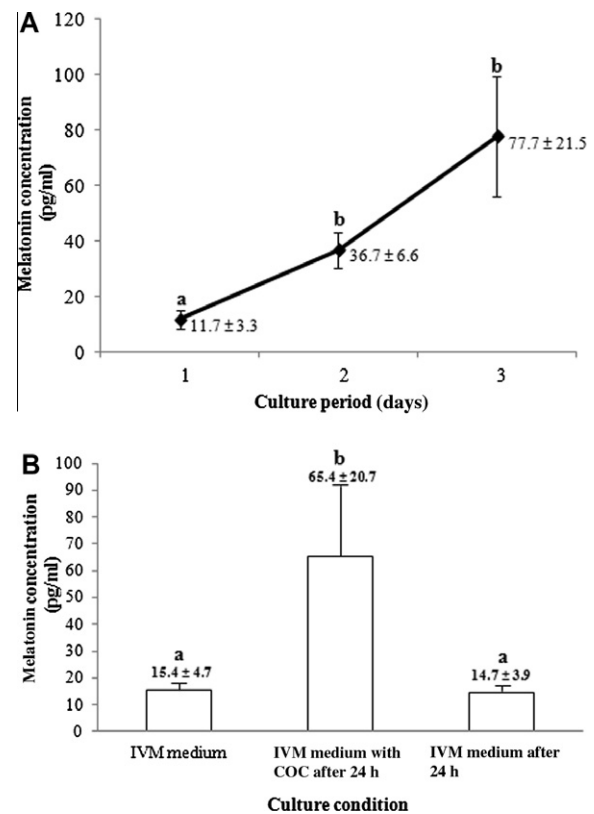
### Statistical analysis

Unless otherwise specified, the data are expressed as mean  $\pm$  standard error of the mean (SEM). For statistical comparisons, clinical outcomes were analysed using the Chi-squared test and the t-test. Analyses were using SAS (SAS Institute, Cary, NC, USA).  $P < 0.05$  was considered statistically significant.

## Results

### Analysis of melatonin concentrations in FF and culture media

Because clinical limitations made it very difficult to obtain FF from large follicles ( $\geq 10$  mm) of PCOS patients and small

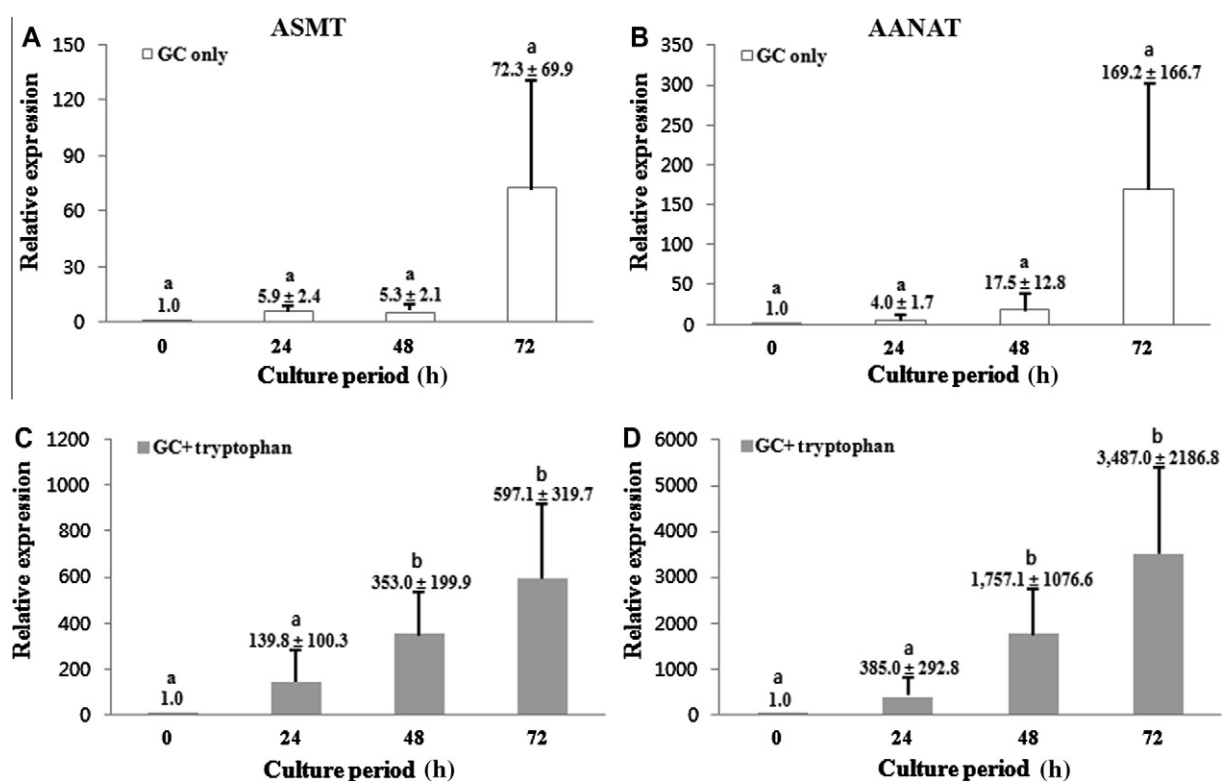


**Figure 1** Melatonin concentrations in culture media measured by ELISA. (A) Media were collected from granulosa cell (GC) culture at day 1 (24 h), day 2 (48 h) and day 3 (72 h) after human chorionic gonadotrophin treatment. (B) Media of cultures with/without five cumulus-oocyte-complexes (COC) were collected before and after in-vitro maturation (IVM). Data are mean  $\pm$  SEM. All experiments were replicated three times. Different lower-case letters indicate a significant difference ( $P < 0.05$ ).

follicles of non-PCOS patients, this study collected and pooled FF obtained from large follicles of non-PCOS patients and small follicles of PCOS patients for determination of melatonin and compared the results with a previous report on melatonin concentrations in small and large follicles of human ovaries (Tamura et al., 2009).

The clinical profiles of the participants are summarized in **Table 1**. Consistent with the previous report, the average melatonin concentration of mature FF obtained from the conventional IVF-embryo transfer programme was significantly higher than that of FF obtained during IVM IVF-embryo transfer treatment of PCOS patients ( $136.8 \pm 26.1$  pg/ml versus  $20.9 \pm 3.6$  pg/ml;  $P < 0.05$ ). Furthermore, in GC cultures obtained from the non-stimulated group, the melatonin concentration gradually increased ( $11.7 \pm 3.3$  pg/ml at 24 h,  $36.7 \pm 6.6$  pg/ml at 48 h,  $77.7 \pm 21.5$  pg/ml at 72 h) following the addition of HCG to the medium (**Figure 1A**). This study additionally measured the melatonin content in media during IVM of COC. The concentration of melatonin in basal IVM medium containing 20% FF before culture was  $15.4 \pm 4.7$  pg/ml. After 24 h of culture, the melatonin concentration in IVM medium containing five COC was higher than that in medium from the COC-free culture ( $65.4 \pm 20.7$  pg/ml versus  $14.7 \pm 3.9$  pg/ml, respectively;  $P < 0.05$ ; **Figure 1B**).





**Figure 2** Quantification of aralkylamine acetylserotonin O-methyltransferase (ASMT; A and C) and N-acetyltransferase (AANAT; B and D) mRNA expression in cultured granulosa cells (GC) with (C and D) and without (A and B) 50 µg/ml tryptophan supplementation. Cultured GC were collected at 0, 24, 48 and 72 h after HCG treatment, total RNA was extracted and real-time reverse-transcription PCR analysis assessed gene expression. Data are mean ± SEM. All experiments were replicated 10 times. Different lower-case letters indicate a significant difference ( $P < 0.05$ ).

To confirm that the enzymic pathways for melatonin synthesis are present in GC, this study used quantitative real-time reverse-transcription PCR to examine mRNA expression of the ASMT and AANAT genes in GC with or without tryptophan supplementation. The gene expression levels showed no significant differences in cultured GC without tryptophan supplementation during in-vitro culture (Figure 2A and B). However, the relative mRNA expression levels of these two genes were significantly and time-dependently enhanced in cultured GC supplemented with tryptophan for 48 h and 72 h compared with non-cultured GC (Figure 2C and D).

#### Clinical outcomes of melatonin supplementation in IVF-embryo transfer for PCOS patients (phases I and II)

The clinical profiles and outcomes of the enrolled PCOS patients are summarized in Table 2. Patients were randomly divided into a MEL+ group ( $n = 62$ ) and a control group ( $n = 49$ ). Both groups were further divided into a non-stimulated group and a non-stimulated but HCG-primed group and their clinical profiles and outcomes were analysed according to the protocols. In the non-stimulated and HCG-primed groups, there were no differences in mean age, body mass index, duration of infertility and cycle day of oocyte retrieval between PCOS patients in the MEL+ and control groups. Notably, the oocyte numbers differed significantly

with the protocols (non-stimulated versus HCG-primed,  $P < 0.05$ ), but were comparable between the MEL+ and control groups.

With the non-stimulation protocol, a total of 373 ( $9.1 \pm 0.7$ ) and 305 ( $12.2 \pm 1.3$ ) immature oocytes were retrieved in the MEL+ group ( $n = 41$ ) and the control group ( $n = 25$ ), respectively (Table 2). The maturation, fertilization, cleavage and embryonic development in the MEL+ group were similar to those in the control group (Figure 3A). However, the implantation rate in the MEL+ group was higher than that of controls ( $P < 0.05$ ). The pregnancy rates in the MEL+ and control groups were 45.7% (16/35) and 33.3% (8/24), respectively (Figure 3A).

With the HCG-primed protocol, 341 ( $16.2 \pm 1.9$ ) and 374 ( $17.0 \pm 2.1$ ) immature oocytes were retrieved in the MEL+ ( $n = 21$ ) and control ( $n = 24$ ) groups, respectively (Table 2). The maturation rate at 24 h in the MEL+ group (51.3%, 175/341) was slightly, although not significantly, higher than that in the control group (44.9%, 168/374), but the two were comparable at 48 h (Figure 3B). Fertilization, cleavage and embryonic development were similar between the MEL+ and control groups. However, the implantation rate in the MEL+ group was higher than that of the control group ( $P < 0.05$ ), and the pregnancy rate was slightly increased in the MEL+ group; however, the difference failed to reach statistical significance (Figure 3B).

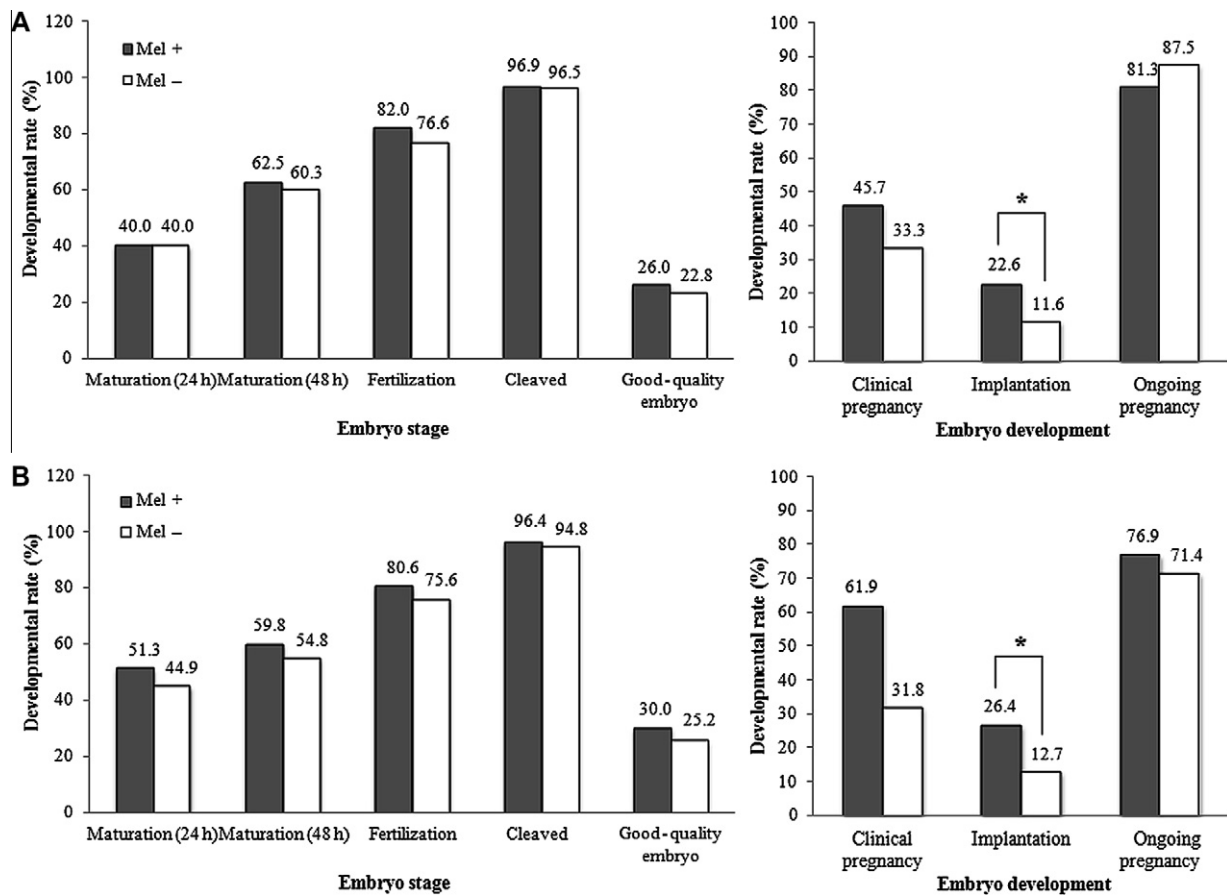
At phase II, only protocol 2 (HCG priming) was used for IVF-embryo transfer treatment of PCOS patients

**Table 2** Clinical profiles of patients according to the IVM IVF-embryo transfer protocol used at phase I (from July 2004 to December 2008).

	Melatonin supplementation		No melatonin (control)	
	Non-stimulation	HCG priming	Non-stimulation	HCG priming
Cycles	41	21	25	24
Transfer cycles	35	21	24	22
Age (years)	32.5 ± 0.5 (22.5–31.6)	33.0 ± 0.8 (31.3–34.6)	31.4 ± 0.5 (30.5–32.3)	31.3 ± 0.6 (30.0–32.5)
Oocytes retrieved	373 (9.1 ± 0.7; 7.6–10.5) <sup>a</sup>	341 (16.2 ± 1.9; 12.6–19.9) <sup>b</sup>	305 (12.2 ± 1.3; 9.6–14.8) <sup>c</sup>	374 (17.0 ± 2.1; 12.8–21.1) <sup>b</sup>
Body mass index (kg/m <sup>2</sup> )	22.2 ± 0.6 (21.1–23.4)	22.0 ± 1.0 (20.1–23.9)	23.2 ± 0.9 (21.4–25.0)	21.7 ± 0.7 (20.3–23.0)
Duration of infertility (years)	3.4 ± 0.3 (2.8–4.0)	5.6 ± 0.70 (4.2–6.9)	4.2 ± 0.5 (3.1–5.2)	4.0 ± 0.5 (2.9–5.0)
Cycle day of oocyte retrieval	14.3 ± 0.6 (13.2–15.5)	12.2 ± 0.7 (10.9–13.6)	13.7 ± 0.8 (12.2–15.2)	14.5 ± 0.7 (13.2–15.8)
Embryos transferred	115 (2.8 ± 0.2; 2.3–3.3)	72 (3.4 ± 0.1; 3.2–3.7)	86 (3.4 ± 0.7; 2.1–4.8)	79 (3.3 ± 0.3; 2.7–3.9)

Values are *n* (mean ± SEM; range).

<sup>a–c</sup>Comparisons between different superscript letters are statistically significant ( $P < 0.05$ ).



**Figure 3** Effect of melatonin on the clinical outcomes from IVM IVF-embryo transfer using non-stimulation (A) and human chorionic gonadotrophin priming (B) in patients with polycystic ovary syndrome. MEL+ = IVM medium with 10  $\mu\text{mol/l}$  melatonin; MEL- = IVM medium without 10  $\mu\text{mol/l}$  melatonin (control). Asterisks indicate significant differences ( $P < 0.05$ ).

(Table 3). In total, 1853 immature oocytes were retrieved from 132 patients, and 743 (40.1%) and 1043 (56.3%) oocytes

had matured after 24 and 48 h of culture, respectively. The fertilization, cleavage and good embryonic development

**Table 3** Clinical outcomes of IVM IVF-embryo transfer using HCG priming with melatonin supplementation in polycystic ovary syndrome patients in the phase-II clinical trial (from January 2009 to December 2010).

Outcomes	Phase II population
No. of transfer cycles/cycles started	126/132
No. of oocytes retrieved (mean $\pm$ SEM)	1853 (14.2 $\pm$ 0.6)
No. of oocytes matured at 24 h	743 (40.1)
No. of oocytes matured at 48 h	1043 (56.3)
No. of fertilized oocytes (% of mature oocytes at 48 h)	888 (85.1)
No. of cleaved embryos (% of fertilized oocytes)	805 (90.7)
No. of good-quality embryos (% of cleaved embryos) <sup>a</sup>	320 (39.8)
No. of transferred embryos (mean $\pm$ SEM)	306 (2.5 $\pm$ 0.6)
No. of implanted embryos (% of embryos transferred)	79 (25.8)
No. of clinical pregnancies (% of transfer cycles)	55 (43.7)
No. of miscarriages (% of clinical pregnancies)	5 (9.1)
No. of ongoing pregnancies (% of clinical pregnancies)	50 (90.9)

<sup>a</sup>Good-quality embryos were defined as having  $\geq 8$  cells and  $\leq 10\%$  fragmentation on day 3.

rates were 85.1% (888/1043), 90.7% (805/888) and 39.8% (320/805), respectively. The implantation rate per embryos transferred and the clinical pregnancy rate per transfer cycle were 25.8% (79/306) and 43.7% (55/126), respectively. Five pregnancies were miscarried and the ongoing pregnancy rate per clinical pregnancy was 90.9% (50/55).

## Discussion

Following successful pregnancies from in-vitro matured human oocytes of donor or PCOS patients, the IVM IVF-embryo transfer programme has become an integral part of human assisted reproduction treatment (Cha and Chian, 1998; Cha et al., 1991; Trounson et al., 1994). Despite its evident success, however, there have been delays in the development of adequate media and culture systems for IVM, as well as appropriate protocols for hormone priming. The present study confirmed that high concentrations of melatonin are present in FF obtained from mature follicles (Table 1) and are secreted from luteinizing GC (Figure 1). Also, enhanced mRNA expression of the ASMT and AANAT genes in GC supplemented with tryptophan during in-vitro culture was observed (Figure 2C and D). These data may suggest that ASMT and AANAT are present in the GC and actively synthesizing the hormone. Although melatonin concentrations were not directly measured in this system, this study may suggest that melatonin would be synthesized in luteinizing GC and would increase the melatonin concentration in FF and IVM medium. These finding also suggest that insufficient exposure to melatonin during

IVM may lead to poor cytoplasmic maturation and unsatisfactory clinical outcomes. In the IVM IVF-embryo transfer programme for PCOS patients, immature oocytes are aspirated 2–5 days earlier and cultured *in vitro* for 2–5 days longer than in the IVF-embryo transfer programme of non-PCOS patients. Here it is shown that the addition of melatonin to the maturation medium appears to have positive effects on the clinical outcomes of human immature oocytes derived from antral follicles via specific protocols using either non-stimulation or HCG priming (Figure 3).

In the early period of IVM treatment, successful pregnancies were achieved in non-stimulated PCOS patients, but at relatively lower rates than those obtained with conventional IVF-embryo transfer using ovarian stimulation. The lower pregnancy rate may be due to insufficient cytoplasmic maturation. Chian et al. (1999, 2000) introduced priming with 10,000 IU HCG at 36 h before immature oocyte aspiration and achieved an impressively high pregnancy rate. The phase-I trial of the present study analysed the effect of melatonin using non-stimulation and HCG priming protocols prior to aspiration. Interestingly, melatonin affected the oocytes and subsequent clinical outcomes of patients in both the non-stimulated and HCG-primed groups (Figure 3). Following the phase-I study, HCG priming and melatonin supplementation in the IVM medium were specifically employed in the IVM IVF-embryo transfer programme (phase-II study), which successfully led to a clinical pregnancy rate of  $>40\%$  in the PCOS patients (Table 3).

In contrast to data obtained from animal models (Kang et al., 2009; Shi et al., 2009), the current study did not find that supplementation with melatonin during IVM improved oocyte maturation, fertilization or early embryonic development rates. However, melatonin affected pregnancy and implantation in both the non-stimulated and HCG-primed groups, suggesting that melatonin may enhance cytoplasmic maturation or further embryonic development by improving IVM conditions. Several limitations should be addressed, however, when discussing the beneficial effects of melatonin on human oocyte maturation. For example, it would be beneficial to study development over 5–6 days, because the 2–3 days of culture used in the present study did not provide sufficient time to analyse embryonic development. Also, difficulty in accessing human materials is an obstacle in this type of work. Recently, several researchers have reported that melatonin supplementation of the culture medium increased maturation rate, increased receptor expression and reduced reactive oxygen species in porcine and cattle (El-Raey et al., 2011; Kang et al., 2009). Other studies in animal models have suggested that the beneficial effects of melatonin occur through its antioxidant actions against oxidative stress and related damage (Acuna-Castroviejo et al., 2001; Combelles et al., 2009; Harris et al., 2010; Requena et al., 2009). This may suggest that melatonin promotes oocyte maturation by binding to its receptor and/or improving culture conditions by direct scavenging of free radicals. Therefore, future studies are needed to more precisely define the action mechanism of melatonin in the human oocytes.

In conclusion, follicular melatonin secreted from luteinizing GC and supplied from the circulation has a positive effect on oocyte maturation. Therefore, supplementation of IVM medium with melatonin may facilitate

the cytoplasmic maturation of human immature oocytes and improve subsequent clinical outcomes.

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