

EFFECT OF MELATONIN ON *In Vitro* DEVELOPMENT OF MORULA RABBIT EMBRYOS

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ABSTRACT

This study aimed to investigate the effect of melatonin supplementation at different levels, in culture medium, on rabbit embryo development. Embryos were recovered from nulliparous Red Baladi rabbit does by laparotomy technique 72 hours post-insemination. Normal morulae were cultured till the stage of hatched blastocysts in the following four culture media; (i) control (TCM-199 + 20% fetal bovine serum), (ii) control + 10⁻⁹M melatonin, (iii) control + 10⁻⁶M melatonin and (iv) control + 10⁻³M melatonin. The high level of melatonin at 10⁻³M significantly increased the *in vitro* development of embryos (92% for 10⁻³M vs. 84% for 10⁻⁶M and 76% for 10⁻⁹M and control, P<0.05). However, the addition of melatonin with different levels did not improve the hatchability rate of these embryos (16-30% vs. 52% for melatonin groups vs. control, P<0.05). Results show that adding melatonin at 10⁻³M to morula rabbit embryos enhances their development, while 10⁻⁶M melatonin slightly increased blastocyst rate and 10⁻⁹M had no effect on embryo development. This effect strongly suggests a physiological role of melatonin in embryo protection.

Key words: Melatonin, *In vitro* development, embryos, rabbits.

INTRODUCTION

The manipulation of embryos during *in vitro* culture at ambient oxygen concentrations, carries the risk of exposure to high levels of reactive oxygen species (ROS) and free radicals, that adversely affect early embryonic development (Burton and Hempstock, 2003; Kitagawa *et al.*, 2004; Agarwal *et al.*, 2006). Apoptosis during preimplantation embryo development plays a critical role in eliminating defective cells; however, inappropriate loss of normal cells may be induced (Jurisicova *et al.*, 1998; Byrne *et al.*, 1999), partially due to the culture conditions (Kamjoo and Brison Drkimber, 2002; Hao *et al.*, 2003).

Melatonin (N-aceyl-5-methoxytryptamine) is well known as a free radical scavenger, antioxidant, and anti-apoptotic production of developmentally competent embryos (Chen *et al.*, 2006). The free radical scavenging activity of melatonin also extends to its metabolites which up-regulate antioxidant enzymes and down-regulate the pro-oxidative and proinflammatory enzymes making melatonin highly effective, even at low concentrations, in protecting organisms from oxidative stress (Tan *et al.*, 2007). Melatonin has been supplemented to embryo culture media as a protectant, in concentrations ranging from 10⁻³ to 10⁻¹³ M. Ishizuka *et al.* (2000) found that melatonin at concentrations from 10⁻⁶ to 10⁻⁸ M supports fertilization and early *in vitro* development of mouse embryos. It was also found that melatonin in the culture medium improved the development rate of thawed ovine embryos with higher hatching rates after 24 hr of culture (Abecia *et al.*, 2002). Rodriguez-Osorio *et al.* (2007) reported that melatonin at a concentration of 10⁻⁹ M had a positive effect on cleavage rates and blastocyst cell numbers of porcine embryos, while the highest concentration of melatonin (10⁻³M) significantly decreased cleavage rates and blastocyst rate. Furthermore, it was reported that addition of melatonin to

culture medium of buffalo embryos increased the *in vitro* development rate and the number of transferable embryos (Manjunatha *et al.*, 2009).

To our knowledge, there is no report on the effect of melatonin as antioxidant in the culture medium on rabbit *in vitro* embryo development. Therefore, this study was carried out to investigate the effect of melatonin supplementation at different levels in culture medium on *in vitro* development of morula rabbit embryos.

MATERIALS AND METHODS

Source of embryos

A total number of 35 nulliparous donor does belonging to the Red Baladi breed (Khalil and Baselga, 2002) were used in this study. To synchronise the receptivity of females, a dose of 20 IU eCG per female (Folligon Intervet, Netherland) was intramuscularly injected 60 hours before insemination. The females were inseminated with semen from adult males of the same line and generation according to methods described by Lavara *et al.* (2000).

Embryos were surgically collected at 72 hours post insemination by ventral midline laparotomy as previously described by Forcada and López (2000) with some modifications. Anesthesia was induced by an intramuscular injection of 16 mg Xylazine (Xyla-Ject, ADWIA, Egypt), followed by an intravenous reinjection of ketamine hydrochloride (Ketam, E.I.P.I.CO, Egypt) in the marginal ear vein to maintain does under anesthesia during laparotomy. Embryos were recovered by separate perfusion of each oviduct from the fimbria to the utero-tubal junction with 5 ml of pre-warmed recovery medium followed by perfusion of each uterine horn with about 20 ml of recovery medium: DPBSCa [0.132 g calcium chloride/ 1 litre of Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich Chemicals S.A., Egypt)], supplemented with 2 g Albumin from Bovine Serum (BSA, Sigma) and 10 ml antibiotics (10,000 units penicillin-G and 10 mg streptomycin per ml, penicillin-streptomycin solution 100X, BioShop Canada Inc.).

An epidural needle (1 mm of inner diameter, Vigor Epidural G17) was introduced at the base of uterine horn receiving the recovery medium with embryos in a falcon tube. After recovery of does, the reproductive tract was washed with 0.1% ethylenediaminetetraacetic acid (EDTA, Sigma) solution in PBS in order to diminish possible abdominal adhesions post-laparotomy and does were intramuscularly injected with 0.5 ml antibiotics (0.123 mg streptomycin, ADWIA, Egypt) in order to prevent any bacterial infection.

Treatments and embryo culture

Melatonin was obtained from Sigma-Aldrich Chemicals S.A., Egypt and stored at -20°C till use immediately at culture time. The culture media consists of Medium-199 + 20% fetal bovine serum (Sigma, Egypt) supplemented with 1% antibiotics (10,000 units penicillin-G and 10 mg streptomycin per ml, penicillin-streptomycin solution 100X, BioShop Canada Inc.). Only normal recovered embryos of each donor (compact morula with intact mucin coat and zona pellucida) were randomly allocated into the following four culture media; (i) control (culture media without melatonin), (ii) control + 10^{-9}M melatonin, (iii) control + 10^{-6}M melatonin and (iv) control + 10^{-3}M melatonin. Embryos were cultured for 48 hours at 38.5°C , 5% CO_2 and saturated humidity, and the number of total blastocysts and hatched blastocysts were recorded at the end of culture.

Statistical analysis

A probit link with binomial error distribution was used to analyze the effect of melatonin level on blastocyst and hatching rates. General linear model was performed with SPSS 16.0 Soft-Ware Package (SPSS Inc., Chicago, Illinois, USA, 2002).

RESULTS AND DISCUSSION

The receptivity rate of females was 85.7% (30/35) and the average of ovulation sites was 10.4 on both ovaries. The recovery rate (oocytes + normal embryos + abnormal embryos/number of ovulation sites) was 73% and the embryo donor rate (number of donor does with at least one recovered normal embryo/number of ovulated does) was 90% (data not shown in Tables).

A total of 206 normal embryos were recovered at 72 h post-insemination in morula stage and results of culture with melatonin were summarized in Table 1. Results indicated that the *in vitro* development rate of embryos at morula stage was higher in melatonin groups 10⁻⁶M and 10⁻³M than in control group but this difference was significant only with 10⁻³M level (92% vs. 76% blastocyst rate for 10⁻³M vs. control, P<0.05). However, the addition of melatonin with different levels did not improve the hatchability rate of these embryos (16-30% vs. 52% for melatonin groups vs. control, P<0.05).

Table 1: Effect of melatonin on *in vitro* development and hatchability rates of rabbit embryos recovered at 72 h post-insemination

Treatment	Number of cultured embryos	¹ Blastocyst rate (Means ± SE)	² Hatchability rate (Means ± SE)
Control	46	0.76 ± 0.06 ^b	0.52 ± 0.07 ^a
10 ⁻⁹ M	62	0.76 ± 0.05 ^b	0.16 ± 0.05 ^b
10 ⁻⁶ M	50	0.84 ± 0.05 ^{ab}	0.30 ± 0.06 ^b
10 ⁻³ M	48	0.92 ± 0.04 ^a	0.27 ± 0.06 ^b
Total	206	0.83 ± 0.03	0.30 ± 0.03

Values with different letters in the same column are significantly different (P<0.05).

¹Developed embryos as a percentage of cultured embryos.

²Hatched embryos as a percentage of cultured embryos.

Our results indicated that blastocyst rate of embryos at 10⁻⁹M melatonin was similar to that in control group; maybe due to that this level is very close to the physiological concentrations of melatonin in plasma of rabbits (Noguchi *et al.*, 2003). With higher melatonin concentrations (10⁻⁶M and 10⁻³M), the blastocyst rate increased compared to the control group but this difference was significant only with 10⁻³M level. Nazzaro *et al.* (2011) reported that, in women with low oocyte quality history, melatonin increased the uptake of essential amino acids and vitamins such as myo-inositol and folic acid by embryo and ameliorates its activity by improving growth and development of embryos. Abecia *et al.* (2002) studied the development rate of thawed ovine embryos in compact morula or early blastocyst stages after culture in medium supplemented with 1 µg/ml melatonin (equivalent to 10⁻⁶M). They reported that melatonin improved the development rate of blastocysts with high hatching and low degeneration rates after culture, but seemed to exert no effect on the development of embryos at morula stage. In our study, we randomly subjected embryos recovered at 72 h for culture with melatonin at different levels irrespective of its specific stage either being compact morulae or early blastocysts. This may be the reason of the different response of embryos at these stages to seem not effective on hatchability rates at all levels of melatonin.

CONCLUSION

The present study demonstrates that adding melatonin at 10⁻³M to morula rabbit embryos enhances development. This effect strongly suggests a physiological role of melatonin in embryo protection. Lower concentrations of melatonin at 10⁻⁹M to *in vitro* culture embryo media had no effect on embryo development, although 10⁻⁶M melatonin slightly increased blastocyst rate. More studies are needed to optimize the use of melatonin in *in vitro* embryo culture procedures in rabbits.

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