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The effect of melatonin on in vitro fertilization and embryo development in mice

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Abstract: To examine the effect of melatonin on in vitro fertilization and embryonic development, mouse embryos after insemination in vitro were cultured in a physiological medium with or without melatonin. Melatonin increased the fertilization rate significantly at a concentration between 10^{-6} and 10^{-4} M (27.6 vs. 43.9 or 40.4%, $P < 0.01$). Furthermore, a significant increase in the rate of embryos reaching the four-cell stage (16.0 vs. 26.7%, $P < 0.01$), the eight-cell stage (12.1 vs. 25.8 or 23.5%, $P < 0.01$), and blastulation (8.9 vs. 23.5 or 17.5%, $P < 0.01$) was observed when the embryos were cultured in a medium containing 10^{-8} or 10^{-6} M melatonin. These results demonstrate that melatonin supports fertilization and early embryo development after in vitro fertilization.

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Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine), a pineal secretory product, modulates ovarian function and reproduction in mammals. Female rats given melatonin had advanced vaginal opening, blocked ovulation, and a disturbed estrous cycle [Wurtman et al., 1963; Ying and Greep, 1973; Villanua et al., 1989]. On the other hand, melatonin given to female hamsters and mice decreased the number of Graafian follicles and corpora lutea and slowed the proliferation of interstitial tissue in the ovary [Ooi and Ng, 1989; Chan and Ng, 1995]. All the reported effects of melatonin on reproduction in rodents were essentially negative. Melatonin is present in human preovulatory follicular fluid at concentrations 3-fold higher than in peripheral serum [Brzezinski et al., 1987; Rönnberg et al., 1990; Yie et al., 1995]. The ampullar ends of mammalian fallopian tubes, where fertilization occurs, are bathed by follicular fluid [Blandau, 1968; Odar and Blandau 1973]. Thus, melatonin in follicular fluid may play a physiological role in fertilization and early embryo development.

Mammalian embryo development before preimplantation in vitro is blocked at the species-specific stage of development [Wright and Bondioli, 1981; Fisher, 1987]. The proliferation in vitro of fertilized rodent eggs is arrested at the two-cell stage [Whitten, 1957; Yanagimachi and Chang, 1964; Whittingham, 1975]. Superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are thought to be involved in the in vitro developmental block of two-cell embryos [Nasr-Esfahani et al., 1990; Noda et al., 1991].

Evidence suggests that melatonin is a reactive oxygen species (ROS) scavenger [Poeggeler et al., 1993; Reiter et al., 1993, 1995; Reiter, 1998; Okatani et al., 1997]. However, whether or not melatonin exerts its effects on embryo development in rodents has not been clarified. The present study examined the effects of melatonin on the development of mouse pre-implantation embryos fertilized in vitro.

Materials and methods

Chemicals

Melatonin, Percoll, and hyaluronidase (type IV-S) were purchased from Sigma Chemical Co. (St Louis, MO). Dimethyl sulfoxide (DMSO) was

purchased from Wako Pure Chemical Industries, Ltd. (Oosaka, Japan), and pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were obtained from Teikoku Zoki Co. (Tokyo, Japan). All other chemicals were at least of reagent grade.

Animals

Sexually mature ICR female mice (SLC:ICR, Japan SLC Inc., Hamamatsu, Japan), aged 7–8 wk, were acclimated to a 14L:10D cycle for 2 wk or longer in air-conditioned quarters with free access to food and water.

Oocyte collection and insemination

Female mice were superovulated with i.p. injections of 5 IU of PMSG followed 48 hr later by 5 IU of hCG; they were then killed 14 hr later by cervical dislocation, at which point cumulus-enclosed oocytes were collected in dishes from excised swollen ampullae of oviducts under mineral oil at 37°C. The cumulus masses from each oviduct were washed twice in Brinster's BMOC-3 medium (GibcoBRL Co., Grand Island, NY) and pooled prior to use. Spermatozoa were prepared as described by Kuribayashi and Gagnon (1996). Briefly, they were collected from two 10-wk-old ICR mice killed by cervical dislocation. The cauda epididymis were excised and placed in 2 mL of BMOC-3 under mineral oil for 15 min to release spermatozoa from the tissue. Spermatozoa were placed on a discontinuous two-layer Percoll gradient consisting of 0.4 mL of 40% and 0.2 mL of 80% Percoll in BMOC-3 in a 1.5 mL microfuge tube, overlayered with mineral oil, and sedimented by centrifugation at 2,000 *g* for 3 min. Spermatozoa at the bottom of the tube were collected, counted, and stored under mineral oil until they were added to the oocytes. Sperm suspension was added to an insemination medium containing 15–20 oocytes for a final concentration of 1×10^5 spermatozoa/mL.

Melatonin treatment of embryos

Gametes were cultured in a humidified atmosphere of 5% CO_2 in air. After 4 hr, oocytes enclosed in cumulus masses were transferred into insemination medium containing 0.05% hyaluronidase to remove the cumulus cells. To assess its effect on in vitro fertilization and embryo development, 15–20 embryos were cultured in 50 μL of the medium containing 10^{-8}, 10^{-6}, or 10^{-4} M melatonin. Melatonin was dissolved in DMSO, which was added to the medium to yield a final volume of 50 μL. We used two controls; one containing 500 mM DMSO only and another containing no DMSO. The fertilization rate was first evaluated by the presence of two-cell embryos 24 hr after insemination. Embryos were then placed in fresh medium without melatonin and the number of four-, eight-cell embryos and blastocysts were recorded 2, 3 and 5 days later, respectively. Preliminary experiments indicated that the rate of parthenogenic activation, namely, the rate at which oocytes develop to the two-cell stage without insemination, was negligible ($< 5\%$) and that $> 90\%$ of pronuclear stage embryos developed normally to the two-cell stage.

Statistical analysis

The significance of differences between experimental groups was determined by the χ^2 test. $P < 0.01$ was considered statistically significant.

Results

When treated with 10^{-4} M of melatonin at 4 hr after insemination, the rates of embryos at the two-cell were significantly increased 24 hr later (27.6 vs. 40.4%, $P < 0.01$), but further develop-

Table 1. Effect of melatonin on mouse embryo development after in vitro fertilization

	Embryo development to			
Culture conditions	Two-cell stage	Four-cell stage	Eight-cell stage	Blastocysts
Control	27.6% (71/257)	16.0% (41/257)	12.1% (31/257)	8.9% (23/257)
DMSO	20.5% (52/254)	14.6% (37/254)	11.0% (28/254)	7.5% (19/254)
10^{-8} M	31.9% (80/251)	26.7% (67/251)*	23.5% (59/251)*	17.5% (44/251)*
10^{-6} M	43.9% (97/221)*	26.7% (59/221)*	25.8% (57/221)*	23.5% (52/221)*
10^{-4} M	40.4% (103/255)*	17.3% (44/255)	14.5% (37/255)	12.5% (32/255)

The mouse embryos were cultured with melatonin at the concentrations of 10^{-8}, 10^{-6}, and 10^{-4} M, or 500 mM dimethyl sulfoxide (DMSO).
*$P < 0.01$, compared to control.

ment to the blastocyst was not increased (Table 1). Melatonin (10^{-8} M) increased development rates from four-cell to blastocyst (four-cell stage, 16.0 vs. 26.7%, $P < 0.01$; eight-cell stage, 12.1 vs. 23.5%, $P < 0.01$; blastocyst, 8.9 vs. 17.5%, $P < 0.01$), but not that of two-cell stage embryos. Moreover, a promoting effect in all stages from two-cell to blastocyst was observed at 10^{-6} M of melatonin (two-cell stage, 27.6 vs. 43.9%, $P < 0.01$, four-cell stage, 16.0 vs. 26.7%, $P < 0.01$; eight-cell stage, 12.1 vs. 25.8%, $P < 0.01$; blastocyst, 8.9 vs. 23.5%, $P < 0.01$).

When the embryos were cultured with BMOC-3 medium in the presence of 500 mM DMSO, the rate of development to each stage between two-cell stage and blastocyst formation was not significantly different from that of controls.

Discussion

We found that melatonin improved the development rate of mouse two-cell embryos when added 4 hr after insemination. The rate of development to blastocysts was also significantly higher when embryos were cultured in BMOC-3 medium containing melatonin. These findings demonstrate that melatonin stimulates fertilization and early embryo development after in vitro fertilization.

Melatonin may influence cell division and cell cycle in embryos, as it is present in a number of cell culture systems in vitro [Slominski and Prunski, 1993; Roth et al., 1997]. However, recent reports have shown that melatonin does not affect mouse embryo development after in vivo fertilization [Chan and Ng, 1994; McElhinny et al., 1996]. Our results differ from those reported as follows. We dissolved melatonin in DMSO instead of ethanol, which induces oocyte fragmentation and parthenogenesis [Mann et al., 1993]. We added melatonin to the medium 4 hr after insemination, when spermatozoa should have completely penetrated the oocyte cytoplasm [Yanagimachi, 1994], instead of at the two-cell or eight-cell stage. Furthermore, embryo development was assessed after in vitro rather than in vivo fertilization.

Embryo metabolism after fertilization depends upon species-specific timing [Domon, 1982; Howlett and Bolton, 1985] and mouse embryo development in vitro is blocked at the two-cell stage [Whitten, 1957; Yanagimachi and Chang, 1964; Whittingham, 1975]. The present data demonstrate that melatonin decreases the two-cell block and increases the blastulation rate. Thus, melatonin may be involved in metabolism at certain stages during early embryogenesis to stimulate the formation of blastocysts.

ROS are involved in the two-cell block phenomenon in the mouse. Superoxide dismutase decreases the mouse two-cell block and increases the blastulation rate [Noda et al., 1991]. Moreover, the increased H_2O_2 production at the G_2/M phase of the second cell cycle has been associated with two-cell block [Nasr-Esfahani et al., 1990], and embryo development to the four-cell stage is reportedly increased by catalase [Natsuyama et al., 1993]. It has been reported that melatonin is an effective ROS scavenger [Poeggeler et al., 1993; Reiter et al., 1993, 1995; Okatani et al., 1997]. Thus, melatonin may support fertilization and early embryo development through its ROS scavenging action. It is also possible that melatonin receptor may be expressed in the early embryos and may mediate its effects on embryos. To confirm these hypotheses, further studies are required.

In various in vivo and in vitro experiments, pharmacological levels of melatonin have been shown to be non- or low-toxic [Reiter, 1998]. Also, in the present study, pharmacological concentrations of melatonin seem to be non-toxic to mouse early embryos.

In conclusion, the present study demonstrates that adding melatonin to early pre-implantation mouse embryos enhances development. Although our data from mouse embryo development can not be directly applied to human fertilization and embryo transfer in vitro, melatonin might improve culture conditions for such programs.

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