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Effect of melatonin treatment on the developmental potential of parthenogenetic and somatic cell nuclear-transferred porcine oocytes *in vitro*

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Summary

Melatonin secreted from the mammalian pineal gland is a free-radical scavenger that protects tissues from cell damage. The present study examined the effects of addition of melatonin to the culture medium on the developmental potential of parthenogenetic and somatic cell nuclear-transferred (SCNT) porcine oocytes. Supplementation of the maturation medium with melatonin did not increase the maturation rate, the proportion of oocytes that cleaved and developed into blastocysts after parthenogenetic activation, or the blastocyst cell number compared to controls. When 10^{-7} M melatonin was added to the culture medium, the proportion of parthenogenetic oocytes that developed to the 2-cell and 4-cell stages was significantly higher than that of controls. The potential of melatonin-treated oocytes to develop into blastocysts was high but not significantly different from that of controls. The addition of 10^{-7} M melatonin to the culture medium did not increase the preimplantation development of SCNT oocytes. Melatonin treatment significantly reduced the levels of reactive oxygen species in 4-cell parthenogenetic and SCNT embryos, but did not reduce the proportion of apoptotic cells in parthenogenetic and SCNT blastocysts. Although the results indicated that parthenogenetic and SCNT melatonin -treated embryos had significantly lower levels of reactive oxygen species than controls, the potential of melatonin-treated embryos to develop into blastocysts was not significantly higher than that of controls, in contrast to previous reports. The beneficial effects of melatonin on the developmental potential of oocytes might depend on the culture conditions.

Keywords: Melatonin, Parthenogenesis, Somatic cell nuclear transfer

Introduction

The first successful production of porcine somatic cell clones was reported in 2000 (Betthauser *et al.*, 2000; Onishi *et al.*, 2000; Polejaeva *et al.*, 2000), and since that time a large number of cloned pigs have been produced in many laboratories (Campbell *et al.*, 2007). The somatic cell nuclear-transfer (SCNT) technique is a novel tool for improving domestic animals, rescuing endangered species and producing transgenic animals for medical use. Because pigs and humans have similar physiologic characteristics, genetically modified pigs, such as knockout pig have been produced for xenotransplantation studies (Fujimura *et al.*, 2008; Klymiuk *et al.*, 2010).

Animals of several species have been cloned using SCNT techniques, but the cloning success rate is low (Campbell *et al.*, 2007). As SCNT technology includes different processes and the oocytes have species-specific characteristics, there are various problems that need to be solved. Among them, an appropriate *in vitro* culture system for recipient and SCNT oocytes is a crucial factor.

Preimplantation mammalian embryos incur damage due to the actions of reactive oxygen species (ROS) during *in vitro* culture (Goto *et al.*, 1993), which results in damage to the organelles, particularly the mitochondria, and causes species-specific embryo developmental block and apoptosis (Yang *et al.*, 1998; Guerin *et al.*, 2001; Kamjoo *et al.*, 2002). The protective

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effects of many different free-radical scavengers, such as hypotaurine (Fujitani *et al.*, 1997), thioredoxin (Bing *et al.*, 2003), beta-mercaptoethanol (Takahashi *et al.*, 2002), glutathione (Luvoni *et al.*, 1996) and cysteine (Ali *et al.*, 2003), against oxidative stress have been tested in *in vitro*-cultured embryos. Melatonin, 5-methoxy-*N*- acetyltryptamine, a hormone secreted from the pineal gland, regulates circadian rhythm and seasonal breeding (Reiter *et al.*, 2009) and is a free radical scavenger that protects against cell damage in tissues such as porcine thyroid tissue (Karbownik & Lewinski, 2003). Supplementation of the culture medium with melatonin increases the development of *in vitro*-fertilized (IVF) mouse (Ishizuka *et al.*, 2000), porcine (Rodriguez-Osorio *et al.*, 2007) and bovine (Papis *et al.*, 2007) oocytes. Recently, Choi *et al.* (2008) reported that supplementation with melatonin significantly increased the development of parthenogenetic porcine oocytes to blastocysts as well as the blastocyst cell number. They also demonstrated that the potential of porcine SCNT oocytes to develop into blastocysts in melatonin-supplemented medium is significantly higher than that of controls, but the observed difference was small (12.2% vs. 10.1%; Choi *et al.*, 2008). Papis *et al.* (2007) reported that the effects of melatonin on the development of IVF bovine oocytes are influenced by the oxygen concentration during *in vitro* culture; the development of IVF oocytes is significantly increased compared with that of controls under a high oxygen concentration, but decreased under low oxygen concentration. This finding suggests that the effectiveness of melatonin supplementation of the culture medium differs depending on the culture conditions.

In the present study, we examined the effect of melatonin addition to the culture medium on the *in vitro* maturation of porcine oocytes, and evaluated whether melatonin increased the *in vitro* development of parthenogenetic and SCNT porcine oocytes.

Materials and methods

All the chemicals used in the present study were purchased from Sigma-Aldrich Chemical Co. unless otherwise specified.

Preparation of recipient oocytes

Cumulus–oocyte complexes (COCs) were recovered from 3- to 6-mm ovarian follicles obtained from the slaughter house and washed with polyvinyl-alcohol (PVA)–phosphate-buffered saline (PBS)(–) or modified TL-HEPES-PVA according to previously reported procedures (Funahashi *et al.*, 1997; Kawakami *et al.*, 2005), COCs were cultured with modified NCSU37 (mNCSU37) medium (Petters & Wells, 1993) supplemented with 10% porcine follicular fluid, 1 mM dibutyryl-cAMP (dbcAMP) and hormones [1.3 μg/ml follicle stimulating hormone (FSH) and 0.6 μg/ml luteinizing hormone (LH)] for 20 h, and then cultured without dbcAMP and hormones for another 20 h under an atmosphere of 5% CO_2 in air at 39 °C. After 40 h of *in vitro* maturation, cumulus cells were removed in PBS containing 0.1% hyaluronidase. The number of matured metaphase II oocytes was determined by evaluating the presence of a polar body. In some experiments, COCs were maintained with mNCSU37 medium supplemented with 10% porcine follicular fluid and 1 mM dbcAMP for 24 h before use (Kawakami *et al.*, 2005), but the data were combined because there were no differences between fresh and preserved oocytes.

Parthenogenetic activation

Matured oocytes were activated with a direct current pulse (150 V/mm for 100 μs) in 0.28 M mannitol supplemented with 0.01% PVA, 0.1 mM $MgCl_2$, and 0.05 mM $CaCl_2$ (Kawakami *et al.*, 2005) and then cultured in porcine zygote medium III (PZM-3) (Yoshioka *et al.*, 2002) containing 3 mg/ml bovine serum albumin and 5 μg/ml cytochalasin B (CB) for 4 h (Hata *et al.*, 1996). The activated oocytes were cultured in PZM-3 medium under an atmosphere of 5% CO_2 in air at 39 °C for 6 days. Cleavage of the embryos was examined after 24 h and 48 h, and blastocyst formation was examined on day 6 and/or day 7.

Somatic cell nuclear transfer

Somatic cells from a fetus of unknown sex and age were prepared according to a previous report (Yin *et al.*, 2002) and passaged fewer than 17 times before use. Before nuclear transfer, somatic cells were cultured in Dulbecco's modified Eagle medium (Nissui Co.) supplemented with 10% fetal bovine serum (FBS) for 7 days. For donor cell preparation, the cells were scraped from the bottom of the culture dish, mixed with HEPES-buffered mNCSU37 (hNCSU37) medium, centrifuged, and then mixed with 10% clinical grade polyvinylpyrrolidone solution (MediCult) after removing the supernatant.

All procedures for nuclear transfer were performed using a piezo-actuated micromanipulator (PMA-CT150; Prime Tech Ltd) as previously reported (Wakayama *et al.*, 1998; Onishi *et al.*, 2000). The matured oocytes were incubated with 0.4 μg/ml demecolcine for 1 h according to a previous report (Yin *et al.*, 2002) and then chromosomes from matured oocytes were removed in hNCSU37 supplemented with 5 μg/ml CB, 0.4 μg/ml demecolcine, 0.05 M sucrose and 0.4% bovine serum albumin.

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The donor cells were injected into enucleated oocytes in hNCSU37 medium supplemented with 0.05 M sucrose and 10% FBS. After injection, SCNT oocytes were cultured in NCSU37 supplemented with 10% FBS for 2.5 h, and activated using two direct current pulses of 120 V/mm for 30 μs at 0.1-s intervals in 0.28 M mannitol supplemented with 0.01% PVA, 0.1 mM $MgCl_2$ and 0.25 mM $CaCl_2$. The oocytes were cultured in PZM-3 containing 5 μg/ml CB, 10 μg/ml cycloheximide and 50 nM trichostatin A (TSA) for 2 h, and then cultured in PZM-3 containing 50 nM TSA for 22 h (Zhang *et al.*, 2007). The reconstructed oocytes were cultured in PZM-3 for 4 days and further cultured in PZM-3 with 10% FBS for 2 days under an atmosphere of 5% CO_2 in air at 39 °C (Okada *et al.*, 2006).

Blastocyst cell number

Blastocyst cell numbers were determined using a double-staining method according to previously reported procedures (Papaioannou & Ebert, 1988; Kawakami *et al.*, 2005). Briefly, the zonae pellucidae of blastocysts were removed by treatment with 0.5% pronase at 37 °C and incubated with 25% rabbit anti-pig whole serum for 1 h, and then cultured in 10% guinea pig complement, 12 μg/ml Hoechst 33342 stain and 12 μg/ml propidium iodide for 40 min at 37 °C. The blastocyst cell numbers were counted under UV light using a fluorescence microscope.

ROS measurement

The ROS levels in parthenogenetic and SCNT embryos at the 4-cell stage were measured using the dichlorohydrofluorescein diacetate method (Hashimoto *et al.*, 2000). Briefly, embryos were transferred into PBS containing 10 μM 2′,7′-dichlorodihydrofluorescein diacetate, and then placed in the dark for 15 min at 37 °C. After washing with PBS, images of the embryos were recorded through a fluorescein isothiocyanate filter, and then measured by converting the number of pixels to grayscale images for analysis using Image J (NIH).

Assessment of apoptosis

The proportions of apoptotic cells in day 7 parthenogenetic and SCNT blastocysts were determined using an *In Situ* Cell Death Detection Kit (TUNEL Kit; Roche Molecular Biochemicals) as previously reported (Neuber *et al.*, 2002).

Briefly, blastocysts were fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4 °C and then immersed in 0.5% Triton X-100 in PBS at room temperature for 1 h. Permeabilized embryos were incubated in a terminal deoxynucleotidyl transferase dUTP nick end labeling reaction medium for 1 h at 37 °C in the dark. After fragmented DNA were labelled with fluorescein isothiocyanate, the embryos were washed three times in PBS supplemented with 0.01% PVA.

Stained blastocysts were mounted on slides and the nuclei were stained with 4′,6-diamidino-2-phenylindole solution. The number of positive apoptotic nuclei per blastocyst was counted under UV light using a fluorescence microscope.

Statistical analysis

The cleavage and the blastocyst formation proportions, and the proportion of apoptotic cells were compared using the chi-squared test, and cell numbers were compared using Student's *t*-test. A *p*-value of less than 0.05 was considered to indicate statistical significance.

Experimental design

Experiment 1

To examine the effect of adding to the maturation medium on the maturation rate and the ability of oocytes to develop into blastocysts after parthenogenetic activation, 10^{-4} M, 10^{-7} M, 10^{-10} M or 10^{-13} M melatonin was added to the maturation medium. Melatonin was dissolved in DMSO, and therefore 1% DMSO, which was equivalent to the concentration of DMSO in the 10^{-4} M melatonin solution (the stock solution of melatonin was 10^{-2} M in DMSO), was added to the culture medium. Medium without DMSO was also used as a control.

Experiment 2

In this experiment, the effect of melatonin addition to the culture medium on the developmental potential of parthenogenetic oocytes was examined. Parthenogenetic oocytes were cultured in melatonin-supplemented medium at the same concentrations used in Experiment 1.

Experiment 3

To examine the effect of melatonin addition to the culture medium on the developmental potential of SCNT oocytes, we used 10^{-7} M melatonin based on the results obtained in Experiments 1 and 2. For the control, 0.0001% DMSO, which was equivalent to the concentration of DMSO in the 10^{-7} M melatonin solution (the stock solution of melatonin was 10^{-1} M in DMSO), was added to the culture medium.

Experiment 4

The ROS levels were measured in parthenogenetic and SCNT embryos at the 4-cell stage developed from oocytes that were cultured for 2 days in PZM-3 supplemented with 10^{-7} M melatonin or 0.0001%

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Table 1 Effect of melatonin addition to the maturation medium on the maturation of porcine oocytes

Melatonin concentration	No. of oocytes matured	No. of oocytes with first polar body (%)
0	231	159 (65)^{a,c}
0 (+ DMSO)	221	153 (69)^{a}
10^{-4} M	276	155 (56)^{b}
10^{-7} M	280	169 (60)^{b,c}
10^{-10} M	260	138 (53)^{b}
10^{-13} M	263	158 (60)^{b,c}

$^{a-c}$Values with different superscripts in the same colum differ significantly ($p < 0.05$).

DMSO. Parthenogenetic oocytes were produced by two different electric conditions: (1) a single direct current pulse of 150V/mm for 100 μs followed by treatment with CB for 4 h; or (2) two direct current pulses of 120 V/mm for 30 μs at 0.1-s intervals followed by treatment with CB, cycloheximide and TSA for 2 h.

Experiment 5

In this experiment, the apoptotic cell number and apoptotic cell rate were examined in blastocysts developed from parthenogenetic and SCNT oocytes that were cultured for 6 days in PZM-3 supplemented with 10^{-7} M melatonin or 0.0001% DMSO. The apoptotic cell number denotes the average number of apoptotic cells in each blastocyst, and apoptosis proportion denotes the number of apoptotic cells divided by the total cell number of all blastocysts examined.

Results

The effects of melatonin addition to the maturation medium on the maturation of porcine oocytes are shown in Table 1. The proportion of matured oocytes based on the presence of the first polar body in the 10^{-4} and 10^{-10} M melatonin-supplemented groups was significantly lower than that of the controls. The proportion of oocytes matured in the presence of melatonin that developed to the 2-cell, 4-cell and blastocyst stages after parthenogenetic activation was not different from that in the controls. The blastocyst cell number also did not differ among melatonin-treated and control groups (Table 2).

The effects of melatonin addition to the culture medium on the potential of oocytes to develop *in vitro* after parthenogenetic activation are shown in Table 3. The proportion of activated oocytes that developed into blastocysts in the 1% DMSO and 10^{-4} M melatonin-supplemented groups was significantly lower than that of control without DMSO (4% and 4% vs. 22%). This finding implies that the presence of 1% DMSO during 6 days of *in vitro* culture was detrimental to the development of parthenogenetic porcine oocytes. When 10^{-7} M melatonin was added to the medium, the proportion of oocytes that developed to the 2-cell and 4-cell stages was significantly higher than that of the control without DMSO (65% vs. 45% for the 2-cell stage, 61% vs. 35% for the 4-cell stage), and the proportion of oocytes that developed into blastocysts was slightly higher (31% vs. 22%). The blastocyst cell number, however, did not differ among the groups.

The effects of melatonin on the developmental potential of SCNT oocytes are shown in Table 4. The proportion of SCNT oocytes that developed into the 2-cell and 4-cell stages did not differ between the 10^{-7} M melatonin- and 0.0001% DMSO-supplemented groups. The proportion of oocytes that developed into blastocysts on days 6 and 7 did not differ between groups. The blastocyst cell number also did not differ between the two groups.

As shown in Table 5, ROS levels in 4-cell parthenogenetic embryos were influenced by the activation procedures. When oocytes were activated by a single

Table 2 Effect of melatonin addition to the maturation medium on the developmental potential of oocytes after parthenogenetic activation

Melatonin concentration	No. of oocytes cultured	No. of oocytes developed to (%)			Cell numbers of blastocysts (mean ± SD)
		2-cell	4-cell	Blastocyst	
0	145	70 (48)	51 (35)^{a}	24 (17)	26.6 ± 12.9 (n = 21)
0 (with DMSO)	137	76 (55)	65 (47)^{b}	30 (22)	23.9 ± 9.1 (n = 23)
10-4M	152	68 (45)	60 (39)^{a,b}	34 (22)	24.2 ± 13.3 (n = 25)
10^{-7} M	145	73 (50)	64 (44)^{a,b}	25 (17)	25.3 ± 10.1 (n = 20)
10^{-10} M	131	61 (47)	52 (40)^{a,b}	27 (21)	24.6 ± 7.6 (n = 21)
10^{-13} M	140	65 (46)	60 (43)^{a,b}	26 (19)	30.4 ± 14.4 (n = 23)

^{a,b}Values with different superscripts in the same colum differ significantly ($p < 0.05$).

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Table 3 Effect of melatonin addition to the culture medium on the developmental potential of parthenogenetic oocytes

Melatonin concentration	No. of oocytes cultured	No. of oocytes developed to (%)			Cell numbers of blastocysts (mean ± SD)
		2-cell	4-cell	Blastocyst	
0	88	40 (45)[a]	31 (35)[a]	19 (22)[a,b]	25.8 ± 13.9 (n = 14)
0 (+ DMSO)	97	26 (27)[c]	21 (22)[c]	4 (4)[c]	28.3 ± 9.5 (n = 3)
10-4M	83	28 (34)[a,c]	27 (33)[a,c]	3 (4)[c]	19.5 ± 6.4 (n = 2)
10^{-7} M	84	55 (65)[b]	51 (61)[b]	26 (31)[a]	29.4 ± 12.5 (n = 19)
10^{-10} M	84	40 (48)[a]	33 (39)[a]	20 (24)[a,b]	30.0 ± 11.5 (n = 16)
10^{-13} M	88	38 (43)[a]	34 (39)[a]	15 (17)[b]	29.4 ± 10.4 (n = 10)

[a–c]Values with different superscripts in the same colum differ significantly ($p < 0.05$).

Table 4 Effect of melatonin addition to the culture medium on the developmental potential of SCNT oocytes

Melatonin concentration	No. of oocytes cultured	No. of oocytes developed to (%)				Cell numbers of blastocysts (mean ± SD)
		2-cell	4-cell	Day 6 blastocyst	Day 7 blastocyst	
0	123	53 (43)	41 (33)	19 (15)	21 (17)	39.1 ± 22.6 (n = 15)
10^{-7} M	123	55 (45)	40 (33)	24 (20)	24 (20)	32.3 ± 18.2 (n = 20)

Table 5 Effect of melatonin addition to the culture medium on ROS levels of parthenogenetic and SCNT embryos

Group	Treatment	Melatonin concentration	No.of oocytes cultured	No.of oocytes developed to (%)		ROS levels (mean ± SD)
				2-cell	4-cell	
Parthenogenone	1	0	114	53 (46)	46 (40)[a,b]	37.61 ± 13.14[a] (n = 46)
		10^{-7} M	154	71 (46)	52 (34)[a]	24.43 ± 9.48[b] (n = 52)
	2	0	52	32 (62)	28 (54)	24.89 ± 7.47 (n = 28)
		10^{-7} M	53	30 (57)	29 (55)	25.13 ± 10.88 (n = 29)
Nuclear transfer	2	0	93	52 (56)	35 (38)	44.09 ± 17.23[a] (n = 33)
		10^{-7} M	103	53 (51)	30 (29)	25.33 ± 10.46[b] (n = 27)

[a,b]Values with different superscripts in the same group, treatment and colum differ significantly ($p < 0.05$).

current pulse of 150 V/mm for 100 μs (the same method used for the results shown in Tables 2 and 3), ROS levels in the 10^{-7} M melatonin-supplemented group were significantly lower than those in the 0.0001% DMSO-supplemented group. When oocytes were activated by two direct current pulses of 120 V/mm for 30 μs at 0.1-s intervals following treatment with cycloheximide and TSA treatment (the same method used for nuclear transfer), however, the ROS levels did not differ between the melatonin- and DMSO-supplemented groups. The ROS levels in embryos developed from SCNT oocytes in melatonin-supplemented medium were also significantly lower than that in controls (Table 5).

The effects of melatonin on apoptosis in parthenogenetic and SCNT blastocysts are shown in Table 6. The proportion of parthenogenetic and SCNT oocytes that developed to the 2-cell and 4-cell stages was not different between the 10^{-7} M melatonin- and 0.0001% DMSO-supplemented groups. There were also no significant difference between these groups in the proportion of oocytes that developed into blastocysts on days 6 and 7; the cell numbers of parthenogenetic and SCNT blastocysts; or the mean number of apoptotic cells or proportion of apoptotic cells in parthenogenetic blastocysts. When 10^{-7} M melatonin was added to the culture medium during *in vitro* culture of SCNT oocytes, the mean number of apoptotic cells and the proportion of apoptotic cells in SCNT blastocysts were both slightly lower than, but not significantly different from, those in the 0.0001% DMSO-supplemented group.

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Table 6 Effect of melatonin addition to the culture medium on apoptosis of parthenogenetic and SCNT embryos

Group	Melatonin concentration	No.of oocytes cultured	No.of oocytes developed to (%)			Cell numbers of blastocysts (mean ± SD)	No.of apoptotic cells/blastocyst (mean ± SD)	Apoptosis proportion
			2-cell	4-cell	Day 6 blastocyst	Day 7 blastocyst		
Parthenogenone	0	181	110 (61)	97 (54)	25 (14)	50 (28)	37.75 ± 21.18	0.98 ± 1.23	2.6
	10^{-7} M	180	109 (61)	94 (52)	28 (16)	48 (27)	38.81 ± 22.28	1.06 ± 1.52	2.7
Nuclear transfer	0	203	95 (47)	72 (35)	21 (10)	37 (18)	20.37 ± 12.68	0.71 ± 1.15	3.5
	10^{-7} M	213	101 (47)	67 (31)	23 (11)	28 (13)	17.85 ± 9.90	0.46 ± 0.76	2.6

Discussion

Recent studies have demonstrated that melatonin, which is mainly secreted from the mammalian pineal grand, enhances *in vitro* oocyte maturation and embryo development in mice (Ishizuka *et al.*, 2000), sheep (Abecia *et al.*, 2002), heifers (Papis *et al.*, 2007), sows (Rodriguez-Osorio *et al.*, 2007) and goats (Berlinguer *et al.*, 2009). The present study demonstrated that addition of melatonin to the maturation medium of porcine oocytes for 40 h, however, did not increase the oocyte maturation rates (53–60% vs. 65% and 69%) or the potential of oocytes to develop into blastocysts after parthenogenetic activation compared with the levels in the controls (17–22% vs. 17% and 22%). The total blastocyst cell number was not significantly different among groups. Kang *et al.*(2009) reported that supplementation of the culture medium with 10 ng/ml (4.4×10^{-8} M) melatonin significantly increased the proportion of porcine oocytes that matured, and supplementation with 50 ng/ml (2.2×10^{-7} M) melatonin also significantly increased the proportion of oocytes that developed into blastocysts after parthenogenetic activation (21.4% vs. 13.3%). The main reason for these discrepancies might be the differences in the culture media used. NCSU37 medium supplemented with 10% porcine follicular fluid was used in the present study because the addition of follicular fluid to the maturation medium promotes oocyte quality (Naito *et al.*, 1988), but Kang *et al.* (2009) used TCM 199 without follicular fluid for oocyte maturation. Because porcine follicular fluid contains approximately 10^{-11} M melatonin (Shi *et al.*, 2009), the beneficial effect of supplementation with melatonin, if these is one, might not have been observed in the present study. Shi *et al.* (2009) reported that TCM199 with follicular fluid supplemented with 10^{-9} M melatonin, however, enhanced cleavage and blastocyst rates after parthenogenetic activation. The difference in the atmosphere during *in vitro* culture of activated oocytes –5% CO_2 and 5% O_2 in the report of Shi *et al.* (2009) and 5% CO_2 in air in the present study – is a possible reason for the difference in the proportion of oocytes cleaving and developing into blastocysts.

The present study demonstrated that supplementation of the culture medium with 10^{-7} M melatonin significantly increased the proportion of parthenogenetically activated oocytes that developed to the 2-cell and 4-cell stages compared with controls (65% vs. 45% for 2-cell and 61% vs. 35% for 4-cell). Preimplantation mammalian embryos incur some level of oxidative stress during *in vitro* culture, which leads to a reduced developmental potential of embryos. Supplementation with melatonin, a scavenger of nitric oxide radicals (Noda *et al.*, 1999), reduces the oxidative stress and

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improves the developmental potential of embryos in mice (Ishizuka *et al.*, 2000), sheep (Abecia *et al.*, 2002), heifers (Papis *et al.*, 2007) and sows (Rodriguez-Osorio *et al.*, 2007). The improved development of porcine parthenogenetic oocytes to the 2-cell and 4-cell stages in the present study might also have been due to the low ROS levels, as shown in Table 5. The difference in the parthenogenetic activation procedures, however, affected the ROS levels in the embryos.

The potential of parthenogenetic oocytes to develop into blastocysts in 10^{-7} M melatonin-supplemented medium was slightly higher than, but not significantly different from, that of the controls (31% vs. 22%). The potential of porcine oocytes to develop into blastocysts after parthenogenetic activation has been reported to be significantly higher than that of controls when oocytes are cultured in the presence of melatonin under a low oxygen atmosphere (Choi *et al.*, 2008: Kang *et al.*, 2009; Shi *et al.*, 2009). In contrast, however, Rodriguez-Osorio (2007) reported that the potential for IVF porcine oocytes to cleave was significantly higher than that of controls, but the potential to develop into blastocysts was not different from that of controls when oocytes were cultured in medium supplemented with 10^{-9} M melatonin under 5% CO_2 in air. One possible reason for this disagreement regarding the effectiveness of melatonin to improve the development into blastocysts is the difference in the gas atmosphere during *in vitro* culture of the embryos. Papis *et al.* (2007) demonstrated that the potential of IVF bovine oocytes to develop into blastocysts in the presence of melatonin was significantly higher than that of controls if the oocytes were cultured under a high oxygen atmosphere, but lower than that of controls when the oocytes were cultured under low oxygen atmosphere.

The present study demonstrated that supplementation of the maturation medium with 1% DMSO, which was used to dissolve the melatonin, did not inhibit the oocyte maturation or the developmental potential of oocytes after parthenogenetic activation, but supplementation of the culture medium with 1% DMSO significantly inhibited the development of activated oocytes. Because inhibitory effects of ethanol and DMSO on nuclear and cytoplasmic maturation have been reported (Avery & Greve, 2000), the effect of the solvent on the developmental potential of oocytes should be taken into account.

The ROS levels in 4-cell embryos developed from SCNT oocytes in the presence of 10^{-7} M melatonin were significantly lower than that in controls. The mean number of apoptotic cells of blastocysts developed from SCNT oocytes in the presence of melatonin was low but not significantly different from that of controls. The beneficial effects of melatonin supplementation on the ROS levels, however, were not reflected by the potential of SCNT oocytes to develop to the 2-cell, 4-cell and blastocyst stages, the developmental rate, or the total blastocyst cell numbers. The results of the present study did not confirm the report of Choi *et al.*(2008), which is the only report that has demonstrated that the supplementation of the culture medium with 10^{-10} M melatonin slightly but significantly improved the potential of SCNT porcine oocytes to develop into blastocysts (12.2% vs. 10.1%). In the present study, SCNT oocytes were treated with a histone deacetylase inhibitor, TSA, for 24 h to enhance the developmental potential (Kishigami, 2006; Rybouchkin *et al.*, 2006; Zhao *et al.*, 2010). Because TSA induces apoptosis in bovine 2-cell embryos (Carambula *et al.*, 2009), it is possible that TSA treatment offset the anti-apoptotic effects of melatonin on the embryos (Choi *et al.*, 2008).

Although the present study revealed that parthenogenetic and SCNT melatonin-treated embryos had significantly lower ROS levels than controls, the potential of melatonin-treated embryos to develop into blastocysts was not significantly improved, in contrast to previous reports. The effectiveness of supplementation with melatonin on the developmental potential of oocytes might depend on the culture conditions.

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