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Melatonin increases cleavage rate of porcine preimplantation embryos in vitro

Abstract: Melatonin has been used to promote in vitro embryo development in different species. This study determined the effects of melatonin on in vitro porcine embryo development; in particular, cleavage rate, blastocyst rate, and blastocyst cell number. Starting 5 hr after insemination, porcine zygotes were cultured in porcine zygote medium 3 (PZM-3) culture medium supplemented with melatonin at increasing concentrations (10^{-12} M, 10^{-9} M, 10^{-6} M, 10^{-3} M). Melatonin at a concentration of 10^{-9} M had a positive effect on cleavage rates, while the highest concentration of melatonin (10^{-3} M) significantly decreased cleavage rates. Although blastocyst rates were not increased by 10^{-9} M melatonin, blastocyst cell numbers were significantly higher for embryos subjected to 10^{-9} M melatonin. The expression levels of the pro-apoptotic gene BAX and anti-apoptotic gene BCL2L1 in blastocysts were not affected by the presence of melatonin in the culture medium. To further study the protective properties of 10^{-9} M melatonin against stressful conditions, hydrogen peroxide (0.01 mM) and heat (40°C) were used during embryo culture. The addition of melatonin to embryos subjected to 40°C for 3 hr increased cleavage rates, but had no protective effect for embryos subjected to 0.01 mM H_2O_2, probably because the physiological levels of melatonin could not counteract the pharmacological levels of H_2O_2. Our data indicate that 10^{-9} M melatonin has a positive effect on porcine embryo cleavage rates and blastocyst total cell numbers and it might have a protective effect against heat stress.

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Introduction

In vitro production (IVP) of porcine embryos is an important method for improving reproductive technologies and porcine genetics. However, the developmental potential of embryos produced by IVP is still low, and optimization of porcine embryo culture media would increase the production of developmentally competent embryos. A study by Pomar et al. [1] found remarkable differences in embryo viability among in vivo and in vitro produced embryos of equally-graded morphology. In addition, in various farm animal species significant differences in conception and birth rates have been demonstrated when comparing in vitro produced embryos to in vivo produced embryos. Studies by Abeydeera [2] and Peterson and Lee [3] showed significant differences in birth rates for in vivo and in vitro produced bovine embryos with 76% and 30% for in vivo and in vitro produced embryos respectively. Differences in farrowing rates were even higher with 60% for in vivo and 5% for in vitro produced embryos. Apoptosis during preimplantation development plays a critical role in eliminating defective cells; however, it must also be tightly regulated in order to prevent inappropriate loss of normal cells [4, 5]. In IVP embryos there is evidence that this regulation may be aberrant, due in part to the culture conditions [6]. The integration of apoptosis pathways depends on a complex balance in the function of the BCL-2 family of proteins, with anti-apoptotic (BCL-2, BCL-W, BCL-XL) and pro-apoptotic (BAX, BAK, BAD) members [7], which are expressed at different levels in the preimplantation embryo [8, 9].

Melatonin (N-aceyl-5-methoxytryptamine) is well known as a free radical scavenger, antioxidant, and anti-apoptotic [10]. The free radical scavenging activity of melatonin also extends to its metabolites, N1-acetyl-N2-formyl-5-methoxykynuramine and N-acetyl-5-methoxykynuramine, 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 [11].

Melatonin has been supplemented to embryo culture media as a protectant, in concentrations ranging from 10^{-3} to 10^{-13} M in different IVP systems. Ishizuka et al. [12] 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 rate of development of thawed blastocysts with higher hatching rates after 24 hr of culture [13]. Melatonin showed a potent free radical scavenging activity and a beneficial effect in bovine in vitro embryo development, especially at 20% oxygen tension. The potential toxicity of melatonin at concentrations ranging from 10^{-5} to 10^{-13} M was tested by McElhinny et al. in 1996, as melatonin was being evaluated in clinical

trials for its efficacy as an oral contraceptive. However, no negative effects of melatonin on embryo development were observed at any of the concentration levels tested [14].

The present study focused on determining the effects of melatonin on porcine embryo development. Porcine embryos obtained by in vitro fertilization were cultured in PZM-3 medium supplemented with melatonin at concentrations of 10^{-3}, 10^{-6}, 10^{-9}, and 10^{-12} M starting 5 hr post insemination (hpi). Cleavage rates, blastocyst rates, and blastocyst cell numbers were determined for all groups and the experiments were repeated eight times. Cleavage rates and blastocyst cell numbers were significantly higher in the groups cultured with 10^{-9} M melatonin when compared with embryos in the control groups and embryos with other melatonin concentrations, suggesting a positive effect of melatonin on porcine embryo culture at this concentration.

A complementary objective was to study the effects of melatonin on embryonic development after apoptosis induction by subjecting embryos to H_2O_2 or culturing them at 40°C for 3 hr in the presence of melatonin compared to embryos subjected to the same conditions but without melatonin supplementation.

Materials and methods

Experiment 1: Effect of melatonin on porcine embryo development

Porcine oocytes were aspirated from 2–8 mm follicles of abattoir-obtained ovaries and matured in Tissue Culture Medium (TCM-199, Gibco/Invitrogen, Grand Island, NY, USA) supplemented with 0.2 mM pyruvate, 0.5 μg/mL FSH (Sioux Biochemicals, Sioux City, IA, USA), 5 μg/mL LH (Sioux Biochemicals), 10% FCS (TCM-199, Gibco/Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco/Invitrogen) in 5% CO_2 in air at 39°C for 24 hr. Oocytes were then transferred into the same maturation medium without hormones and cultured for 20 hr. For fertilization, matured oocytes were transferred to fertilization medium and were fertilized using fresh sperm separated by percoll density gradient and further incubated for 5 hr. Presumptive zygotes were transferred to PZM-3 culture medium.

Starting 5 hr after insemination, embryos were randomly allocated to different groups and supplemented with 0 (control group) 10^{-3} M, 10^{-6} M 10^{-9} M, and 10^{-12} M melatonin (M-5250, Sigma, St. Louis, MO, USA). Embryo development among the groups was compared in terms of cleavage rates, blastocyst rates, and blastocyst cell numbers. Cleavage rates were recorded at 48 hpi and blastocyst rates were assessed on day 6 post fertilization.

Blastocyst cell numbers were determined by propidium iodide staining. Briefly, blastocysts from the control group and the melatonin concentration (10^{-9} M) with the highest cleavage and blastocyst rates were fixed in 4% v/v methanol free formaldehyde and stored at 4°C in 1% v/v polyvinylpyrrolidone (PVP) in phosphate-buffered saline (PBS), then washed three times in 100-μL drops of fresh 1% PVP in PBS, permeabilized in 50 μL of 0.5% w/v Triton X-100 for 15 min at room temperature in a humidified chamber and washed twice in PBS/PVP. Finally, embryos were stained with 1 μg/mL of propidium iodide for 15 min and washed three times with deionized water to eliminate extra stain. Embryos were mounted with a 1,4-diazabicyclo[2.2.2] octane (DABCO) anti-fade solution and covered with a coverslip for evaluation. Assessment of total cell numbers was determined by evaluating each embryo under an epifluorescent microscope (Nikon, Tokyo, Japan) equipped with a 450–490 nm excitation filter, a 520 nm dichroic mirror, and a 520 nm barrier emission filter, using a 40× objective.

Experiment 2: Expression of pro- and anti-apoptotic genes

Porcine oocytes were subjected to standard maturation and fertilization as described previously. The expression ratio of the pro-apoptotic gene BAX, and the anti-apoptotic gene BCL2L1 was determined for blastocysts in the control group and the group with 10^{-9} M melatonin. This melatonin concentration was chosen because of the consistent results from experiment 1.

Pools of three blastocysts were frozen on day 6 post insemination for gene expression analysis and stored at −80°C on RLT lysis buffer (Qiagen, Valencia, CA, USA) until RNA isolation. RNA was isolated using the RNeasy MicroKit (Qiagen) according to the manufacturer's specifications. Briefly, embryos in lysis buffer at −80̊C were transferred to silica-gel membrane spin columns and washed with RW1 wash buffer and 80% ethanol. Final RNA elution was conducted using 14 μL of RNAse free water provided in the kit. RNA integrity and quality were assessed using a Bioanalyzer 2100 RNA 6000 picochip kit (Agilent Technologies, Palo Alto, CA, USA).

Complementary DNA was generated with the First-Strand cDNA Synthesis system for reverse transcriptase PCR (RT-PCR) using SuperScript III Platinum® Two-Step qRT-PCR Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The samples were incubated for 10 min at 25°C, 50 min at 42°C, and at 85°C for 5 min. Then 2U of *Escherichia coli* Rnase H was added to each tube and incubated at 37°C for 20 min. The cDNA was used for quantitative real-time PCR amplification with SYBR Green I chemistry (Roche Applied Sciences, Indianapolis, IN, USA). Starting amounts of cDNA were equalized for all of the samples. Real-time quantitative PCR was performed to assess expression of BAX and BCL2L1 genes on the LightCyclerTM instrument (Roche Applied Sciences). Real time PCR reactions were carried out in a total volume of 10 μL according to the manufacturer's manuals for DNA Master SYBR Green I mix (Roche Applied Sciences).

Primers were designed using Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA). Primer sequences used for real time PCR are shown in Table 1. The primer concentrations were adjusted to 0.5 μm for each gene. The cycling parameters were 30 s at 95°C for denaturation, 50 cycles of 2 s at 95°C, 10 s at 55°C for amplification and quantification and 12 s at 72°C for extension. The specificity of all individual amplification reactions was confirmed by melting curve analysis. The assays used Beta Actin as the endogenous internal house-

Table 1. Primers used for real time-PCR

Genes	Accession number	Primer sequences and positions (5′ → 3′)	Product size (bp)
BAX	AJ606301	TTTCTGACGGCAACTTCAACTG (299–321) AGCCACAAAGATGGTCACTGTCT (534–511)	236
BCL2L1	NM_214285	TGAATCAGAAGCGGAAACCC (166–186) GCTCTAGGTGGTCATTCAGGTAAG (581–605)	416
Beta Actin	U07786	ACTGGCATTGTCATGGACTCTG (79–101) AGTTGAAGGTGGTCTCGTGGAT (475–497)	397

keeping gene. The same initial amounts of target molecules were used, and the Cp values of Beta Actin mRNA were constant in all samples. Expression values were calculated using the relative standard curve method. Standard curves were generated using 10-fold serial dilutions for the endogenous control Beta Actin, and both of the target genes BAX and BCL2L1 by measuring the cycle number at which exponential amplification occurred. Values were normalized to the relative amounts of Beta Actin mRNA, which were obtained from a similar standard curve.

Experiment 3: Protective effects of melatonin on porcine embryos exposed to different stressors

In the first part of the study, supplementing culture medium with 10^{-9} M melatonin increased cleavage and blastocyst rate, therefore this concentration was used in order to determine its protective effect against two stressors. Porcine oocytes were subjected to standard maturation and fertilization as described previously. Presumptive zygotes were randomly transferred to six groups starting 5 hr after insemination. Group 1: Control group in PZM-3 culture medium without any supplementation. Group 2: PZM-3 culture medium supplemented with 10^{-9} M melatonin. Group 3: Culture for 3 hr in PZM-3 with 0.1 mM H_2O_2 (H_2O_2 concentration was determined after a dose-response study, data not shown), then embryos were returned to control culture conditions. Group 4: Culture for 3 hr in PZM-3 with 0.1 mM H_2O_2 with melatonin supplementation, then embryos were returned to 10^{-9} M melatonin culture conditions. Group 5: Culture for 3 hr at 40°C. Group 6: Culture for 3 hr at 40°C with 10^{-9} M melatonin supplementation, then embryos was returned to 10^{-9} M melatonin culture conditions. Cleavage rates were recorded 48 hpi and blastocyst rates were assessed 6 days post fertilization.

Statistical analysis

All statistical analyses were performed using SAS software. ANOVA and Orthogonal Lineal Contrast were used to determine differences in cleavage and blastocyst rates with a significance level of 0.05. Results are expressed as rate means ± S.E.M. Student's *t*-test was used to determine statistical differences in the mean cell number per blastocyst and the significance level used was 0.01. Statistical analysis of gene expression patterns was performed using a relative expression software tool (REST©, 384-beta version May 2005, Technical University of Munich, Munich, Germany), which is based on an efficiency corrected mathematical model for data analysis. It calculates the relative expression ratio on the basis of the PCR efficiency (E) and crossing point deviation (ΔCP) of the investigated transcripts and on a newly developed randomization test macro. The mathematical model used is based on the PCR efficiencies and the crossing point deviation between the samples [15–18]. Differences at $P < 0.01$ were considered significant.

Results

In experiment 1, a total of 1738, 1301, 1666, 1641, and 1576 oocytes (from eight repeats) were used for control, 10^{-12}, 10^{-9}, 10^{-6}, and 10^{-3} M melatonin groups, respectively. Cleavage rates were significantly higher ($P < 0.05$) in the group supplemented with 10^{-9} M (45.08% ± 1.94) (mean rate ± S.E.M.) when compared with the control group (38.67% ± 2.01) and other melatonin concentrations. There was no difference in cleavage rates for the groups supplemented with melatonin at concentrations of 10^{-12} M and 10^{-6} M. However, cleavage rates were significantly lower for the group supplemented with 10^{-3} M melatonin with a cleavage rate of 32.17% ± 2.59.

There was no difference in blastocyst rates, at the 0.05 level of significance, for any of the groups except for the one supplemented with the highest melatonin concentration (10^{-3} M) where the blastocyst rate only reached 19 ± 0.87 against 35.25 ± 1.85 in the control group. Number of oocytes, cleavage and blastocyst rates for all groups are summarized in Table 2.

A total of 11 blastocysts from each control and melatonin 10^{-9} M groups were stained and evaluated in order to determine the mean cell number per blastocyst. The blastocyst cell number (mean ± S.E.M.) of 10^{-9} M melatonin supplemented group (65 ± 3.22) was higher than control group (50 ± 2.07) ($P < 0.01$). These findings are summarized in Fig. 1.

In experiment 2, RNA was isolated from four pools of three blastocysts from the control group and the 10^{-9} M

Table 2. In vitro development of porcine embryos in PZM-3 medium supplemented with different concentrations of melatonin. Blastocyst rates refer to the percentage of cleaved embryos that developed into blastocysts

Group	Number of oocytes	Cleavage rates (% ±S.E.M.)	Blastocyst rates (% ±S.E.M.)
Control	1738	38.67 ± 2.01^{b}	35.25 ± 1.85^{ab}
Melatonin 10^{-12} M	1301	36.90 ± 1.65^{bc}	38.50 ± 2.40^{ab}
Melatonin 10^{-9} M	1666	45.08 ± 1.94^{a}	43.00 ± 2.46^{a}
Melatonin 10^{-6} M	1641	36.50 ± 1.36^{bc}	33.75 ± 2.06^{b}
Melatonin 10^{-3} M	1576	32.17 ± 2.59^{c}	19.00 ± 0.87^{c}

[a,b,c]Different letters indicate statistically significant differences. $P < 0.05$.

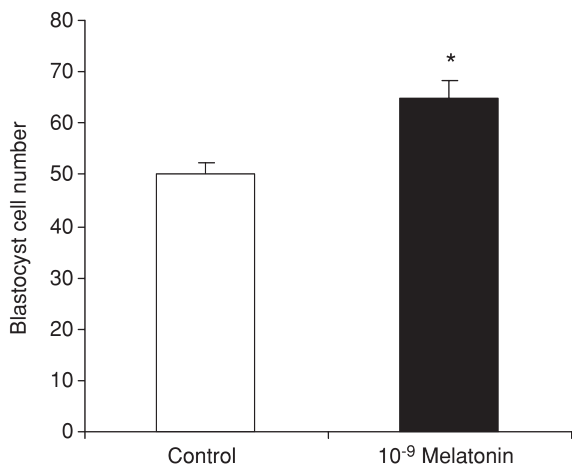

Fig. 1. Average cell numbers per blastocyst (mean ± S.E.M.). Control group 50 ± 2.07 (n = 11). Blastocysts supplemented with 10^{-9} M melatonin 65 ± 3.22 (n = 11). Asterisk indicates statistical significance ($P < 0.01$). Open bar: control group, solid bar with 10^{-9} M melatonin supplementation.

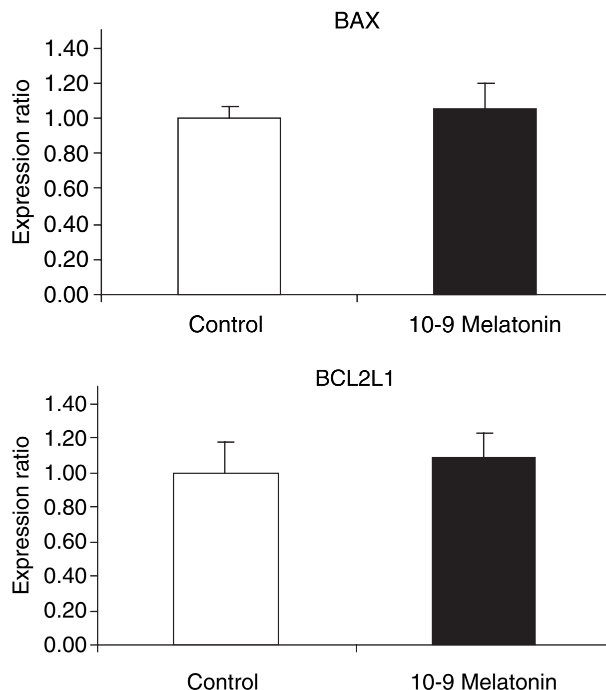

Fig. 3. Relative gene expression ratios. Expression ratios of BAX and BCL2L1 genes in blastocysts cultured in medium supplemented with 10^{-9} M melatonin compared to blastocysts in the control group. There were no significant differences. Open bars without melatonin, solid bars with melatonin supplementation

melatonin group. Quality of total RNA can be observed in Fig. 2. The 28S to 18S ribosomal RNA bands ratio was 1.80–1.94 for all isolated samples.

Expression values were calculated for BAX and BCL2L1 using the relative standard curve method and normalizing their values to those of the reference gene Beta actin. A standard curve was established for each gene and the cycle threshold values were used to determine the concentrations. These values were normalized to the reference gene. After using REST©, 384-beta version for data analysis, no significant differences in the transcript abundances were found for both genes in total RNA isolated from blastocysts supplemented with melatonin as compared to control group (Fig. 3). The BAX:BCL2L1 ratio was 1.73 for embryos in the control group and 1.69 for embryos cultured in 10^{-9} M melatonin.

In experiment 3, 10^{-9} M melatonin increased cleavage rate (39.7 ± 3.48) as compared to control (32.3 ± 1.45) (P value < 0.05). The presence of 0.1 mM H_2O_2 significantly reduced cleavage rates (22.3% ± 1.20) as compared to

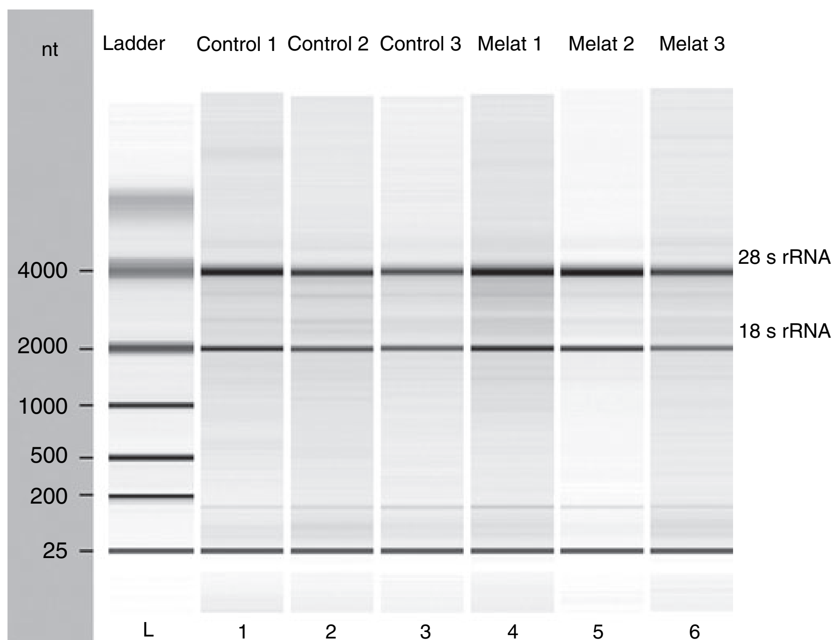

Fig. 2. Bioanalyzer gel image of total RNA isolated from pools of three blastocysts. nt: size in nucleotides or bases; L: ladder; Control 1, Control 2, Control 3: Control group repeats; Melat 1, Melat 2, Melat 3: 10^{-9} M melatonin repeats. The 28s to 18s ribosomal RNA bands ratios ranged from 1.80 to 1.94.

Table 3. In vitro development of porcine embryos in PZM-3 medium with H_2O_2 for 3 hr with or without supplementation of 10^{-9} M melatonin, and cultured at 40°C for 3 hr with or without supplementation of 10^{-9} M melatonin. Blastocyst rates refer to the percentage of cleaved embryos that developed into blastocysts

Group	Number of oocytes	Cleavage rates (% ±S.E.M.)	Blastocyst rates (% ±S.E.M.)
Control	297	32.3 ± 1.45[b]	10.67 ± 4.18[a]
Melatonin 10^{-9} M	299	39. 7 ± 3.48[a]	17.0 ± 6.12[a]
H_2O_2	292	22.3 ± 1.20[c]	4.3 ± 2.61[a]
H_2O_2 with 10^{-9} M melatonin	299	28.3 ± 3.18[bc]	6.7 ± 3.39[a]
40°C	295	31.3 ± 0.33.[b]	9.7 ± 5.49[a]
40°C with 10^{-9} M melatonin	295	41.7 ± 2.41[a]	9.3 ± 4.85[a]

[a,b,c]Different letters indicate statistically significant differences. $P < 0.05$.

control group ($P < 0.05$). When 10^{-9} M melatonin was added to embryos in 0.1 mM H_2O_2, cleavage rates numerically increased to 28.3% ± 3.18, without statistical significance ($P = 0.0896$). The culture of zygotes at 40°C for 3 hr had no significant effect, on cleavage rate (31.3% ± 0.33). Surprisingly, supplementation with 10^{-9} M melatonin under this temperature, significantly increased cleavage rates (41.7% ± 2.41) as compared to the control group and to embryos cultured at 40°C without melatonin. No significant effect was detected for blastocyst rates among any of the groups. These results are summarized in Table 3.

Discussion

Melatonin and its metabolites are effective antioxidants, which scavenges free oxygen radicals and up-regulate several antioxidant enzymes [11, 19, 20]. Melatonin has potent anti-apoptotic effects on different cell types [21–23], and it particularly acts at the mitochondrial level to reduce mitochondrial oxidative stress-induced damage [24].

Although the direct effects of melatonin on porcine embryo development are not known, melatonin's influence on pig fertility has two main roles: first the endocrinological communication of photoperiodic information to the pituitary-gonadal axis that leads to follicle maturation and ovulation [25, 26] and second the reduction of uterine contractility, as melatonin stimulates the secretion of progesterone and inhibits the synthesis of prostaglandins [26].

The manipulation of gametes and embryos during IVP of embryos carries the risk of exposure to high levels of reactive oxygen species (ROS), as oocyte aspiration, fertilization and embryo culture done at ambient oxygen concentrations could generate higher amounts of free radicals that adversely affect early embryonic development [27, 28]. Different systems of free radical scavengers have been studied during the last few years for in vitro culture of mammalian embryos [29], and as a ROS scavenger, melatonin has been tested in the promotion of mouse, sheep, and cattle embryo development in vitro. However, the direct mechanism of action of melatonin on embryonic development remains to be elucidated.

To our knowledge, the present study is the first report on the use of melatonin in the culture of porcine embryos. We observed that melatonin, supplemented to the embryo culture medium at a concentration of 10^{-9} M, had a marked positive effect on cleavage rate and blastocyst cell numbers (Table 2 and Fig. 1). These results agree with previous studies in that melatonin at concentrations of 10^{-4} to 10^{-9} M increased embryo development in murine and bovine embryos [12, 29, 30]. However, no effects were observed for blastocyst stage probably because, at this moment, melatonin was depleted in the culture medium. Supplementation of the culture medium with 10^{-12} M melatonin failed to elicit the same effect probably because the concentration was lower than the amount required to protect embryos from the free radicals.

Contrary to McElhinny's study, the present results suggest a negative effect of melatonin at higher concentrations (the 10^{-3} M), with a marked decline in both cleavage and blastocyst rates (Table 2). This finding could suggest some degree of toxic activity. However, it is necessary to point out that the highest concentration used in the present study was 100 times higher than the one used by McElhinny et al. [14].

Although Ishizuka et al. [12] reported a marked positive effect of melatonin at 10^{-6} M on mouse embryo development; our results did not show any statistically significant effects on either cleavage or blastocyst rates at this concentration. This could suggest a higher anti-apoptotic activity of melatonin on their in vitro fertilization and culture system. The positive effect of melatonin on the total cell numbers per blastocyst in the present study was evident, which could be explained by its anti-apoptotic effect on individual blastomeres.

According to Iwata et al. [31], the use of some antioxidants such as superoxide dismutase and dimethylthiourea during oocyte aspiration had beneficial effects for embryo production. The results of the present study allowed us to recommend the use of melatonin at concentrations of 10^{-9} M in the culture medium for porcine IVP of embryos, as an effective antioxidant with free radical scavenging potency and anti-apoptotic activity abilities. Testing the effects of using melatonin from aspiration through embryo culture should provide us with more information regarding the effectiveness of its anti-apoptotic activity depending on the time of exposure.

We determined the effects of melatonin on the expression of the pro-apoptotic gene BAX, and the anti-apoptotic gene BCL2L1. However, no significant change in the mRNA levels of either gene was detected, although there was a slight decrease in the BAX:BCL2L1 ratio. These could lead us to conclude that the anti-apoptotic effect of melatonin does not induce down-regulation or up-regulation of these particular genes. It is possible that melatonin is targeting other genes in its anti-apoptotic activity.

Hydrogen peroxide had marked detrimental effects on both cleavage and blastocyst rates. Although the use of 10^{-9} M melatonin slightly increased both rates, the fact that there was no statistical significance could suggest that at physiological concentrations, melatonin cannot protect embryos from apoptosis induced by pharmacological concentrations of H_2O_2. An unexpected result was the high cleavage rate in the embryos cultured for 3 hr at 40°C with melatonin supplementation. Although this temperature on

its own did not seem to affect cleavage rates, the addition of melatonin provided the higher cleavages observed for this particular experiment. This suggests exploration of the molecular effects of melatonin on other apoptosis and temperature-related genes.

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