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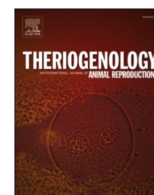

Original Research Article

Melatonin supplementation does not improve ovine pre- or post-hatching development *in vitro*

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ABSTRACT

Melatonin plays a crucial role in the regulation of reproductive seasonality in sheep. Melatonin supplementation has been widely used both *in vivo* to increase fertility and prolificacy, and *in vitro* during oocyte maturation, fertilization, and embryo culture. However, its effects have only been assessed *in vitro* up to the hatched blastocyst stage in conventional embryo culture systems. This study aimed to evaluate the effects of melatonin supplementation from oocyte *in vitro* maturation (IVM) through post-hatching embryonic stages *in vitro* on the development of the first three embryonic lineages. Supplementation with melatonin at 10^{-8} or 10^{-6} M during IVM, *in vitro* fertilization (IVF), and *in vitro* culture (IVC) did not affect cleavage and blastocyst rates at day (D) 8, nor embryo survival and growth at D12. While hypoblast development was not affected, epiblast survival was reduced in D12 embryos treated with 10^{-6} M melatonin from the blastocyst stage onward. In conclusion, melatonin supplementation did not show a clear beneficial effect on ovine *in vitro* embryo production or lineage development during post-hatching embryo culture.

1. Introduction

Melatonin is a hormone secreted by the pineal gland that regulates circadian rhythms by conveying photoperiod signals to the neuroendocrine axis [1]. Although it is present in all mammals, its role in the reproductive function is particularly relevant in sheep, where it controls the hypothalamic-pituitary-gonadal axis to regulate the species-specific reproductive seasonality, ensuring that births occur in the most favourable season [2–4].

Due to its importance, melatonin has been widely used in ewe reproductive management to synchronize oestrus cycles and improve prolificacy [5–7]. In particular, melatonin implants have been used to enhance the efficiency of superovulation and *in vivo* embryo production, with varying results [8–12]. Additionally, melatonin has been also employed as a supplement during *in vitro* embryo production, either during oocyte maturation, fertilization, or embryo culture.

Melatonin has been detected in follicular fluid [13–16], and melatonin receptors have been identified in ovarian cells from different species [17–20]. In line with these findings, a direct effect of melatonin on the oocyte has been reported in studies supplementing *in vitro* maturation (IVM) medium in various species, including mice [18,21], humans [22–24], sheep [25,26], goats [27], cows, and pigs [15]. In some of these studies, melatonin supplementation continued during *in vitro* fertilization (IVF), with reports of beneficial effects on subsequent embryo development [25]. Likewise, some authors have incorporated melatonin into the embryo culture medium in mice [21], sheep [28], cattle [29,30], and pigs [31,32], with variable outcomes. Many of these studies highlight the antioxidant properties of melatonin, acting as a free radical scavenger, which may explain its beneficial effects. Supporting this hypothesis, melatonin supplementation after the thawing of vitrified embryos, a critical period of high oxidative stress susceptibility, has been reported to improve embryo viability [33,34]. However, embryo culture after thawing has been limited to 24 or 48 h, up to the hatched blastocyst stage in conventional embryo culture systems.

In ungulate species, including some of the most relevant farm animals (cattle, sheep, goats, and pigs), complex cell differentiation and proliferation events occur from blastocyst formation until implantation, which is delayed compared to other species. At the late blastocyst stage,

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three distinct cell lineages are present: the hypoblast and trophectoderm, which contribute to the placenta, and the epiblast, which will form the foetus. During the subsequent days of development, the hypoblast proliferates and migrates to cover the entire inner trophectoderm surface, and both lineages undergo massive growth before implantation. Concomitantly, the epiblast develops into a flat embryonic disc (ED) [35]. The study of these developmental events was previously restricted to *in vivo*-developed embryos, limiting *in vitro* studies on the effect of melatonin supplementation to the hatched blastocyst stage. However, recent advances in *in vitro* culture systems now allow to achieve trophectoderm proliferation, complete hypoblast migration, and ED development from the epiblast in bovine and ovine embryos [36, 37]. Taking advantage of this *in vitro* system, this study aimed to analyze the effect of melatonin supplementation from IVM up to post-hatching stages *in vitro* on the development of the first three embryonic lineages during post-hatching development.

2. Materials and methods

2.1. In vitro production of ovine embryos

Cumulus oocyte complexes (COCs) were aspirated from 2 to 8 mm diameter follicles from ovine ovaries collected at a local slaughterhouse using a 21G needle connected to an aspiration pump (VMAR 5100, Cook Medical, Ireland) set to −25 mmHg. COCs were collected in a 50 ml tube containing Euroflush medium (IMV Technologies, Spain). Those with a compact cumulus and homogeneous cytoplasm were selected and matured for 24 h in IVM medium (Prod. No. 5.03.020, Stroebech Media, Denmark) at 38.5 °C under an atmosphere of 5 % CO_2 in air with maximum humidity. Following maturation, all COCs were partially denuded by gentle pipetting and fertilized with frozen-thawed BoviPure® (Nidacon, Sweden)-separated ram sperm at a final concentration of $2x10^6$ spermatozoa/ml. Gametes were co-incubated in 50 μl droplets of IVF medium (Prod. No. 5.06.020, Stroebech Media, Denmark) supplemented with 10 % (v/v) heat-inactivated oestrous sheep serum (made in-house) and 50 IU/ml heparin (37505, Calbiochem), covered by mineral oil (Nidacon, Sweden), at 38.5 °C in an atmosphere of 5 % CO_2 with maximum humidity. To avoid a possible confounding ram effect on developmental rates, semen from the same ram was used for all experiments. At 18–20 h post-insemination, fertilized oocytes were denuded and cultured in 50 μl droplets of IVC medium (Prod. No. 5.07.020, Stroebech Media, Denmark) under mineral oil at 38.5 °C in an atmosphere of 5 % CO_2, 5 % O_2, and 90 % N_2 with maximum humidity. Cleavage was assessed at 48 h (D2), and blastocyst rates were recorded at D8. Cleavage and blastocyst rates were referenced to the number of fertilized oocytes placed in IVC medium.

2.2. Post-hatching embryo culture

At D6 and D7 after fertilization, blastocysts were transferred to Nunclon Sphera low-attachment dishes (Thermo Scientific, Denmark) and cultured in N2B27 medium (1:1 Neurobasal and DMEM/F12 medium supplemented with penicillin/streptomycin, 2 mM glutamine, N2 and B27, Thermo Fisher Scientific) [36–39], supplemented or not with melatonin (M5250, Sigma-Aldrich) according to the experimental design. Embryos were cultured at 38.5 °C in a water-saturated atmosphere of 5 % CO_2, 5 % O_2, and 90 % N_2, with half of the culture medium replaced every other day until D12, when images were taken. Embryos were then fixed in 4 % paraformaldehyde (Electron Microscopy Services, USA) for 15 min at room temperature (RT), washed, and preserved in phosphate-buffered saline (PBS) with 1 % bovine serum albumin (BSA, A7906, Sigma-Aldrich) at 4 °C until immunofluorescence was performed.

2.3. Immunofluorescence and lineage development analysis

If immunofluorescence was not performed immediately after fixation, embryos were incubated in 125 mM glycine (G7126, Sigma-Aldrich) in PBS with 1 % BSA for 2 h at 4 °C before immunostaining. Next, embryos were permeabilized in 1 % Triton X-100 (X100, Sigma-Aldrich) in PBS for 15 min at RT and blocked in 10 % Donkey Serum (D9663, Sigma-Aldrich) with 0.02 % Tween 20 (P1379, Sigma-Aldrich) in PBS for 1 h at RT. Embryos were then incubated overnight at 4 °C with primary antibodies to detect the hypoblast (SOX17; AF1924, R&D; 1:100 dilution), epiblast (SOX2; 14-9811-80, Invitrogen; 1:100 dilution), and trophectoderm (CDX2; MU392A-UC, Biogenex; 1:100 dilution). After four washes in PBS with 1 % BSA, embryos were incubated with the corresponding secondary antibodies (Donkey anti-Goat IgG Alexa 488, Donkey anti-Rat IgG Alexa 555, and Donkey anti-Mouse IgG Alexa 647 antibodies; Life Technologies;1:300 dilution) and counterstained with DAPI (10236276001, Roche) for 1 h at RT. Finally, embryos were washed 4 times in PBS - 1 % BSA. Then, embryos were mounted in an Invitrogen™ CoverWell™ Incubation Chamber Gasket (Life Technologies) and imaged each 5 μm along the Z-axis using a structured illumination equipment composed of a Zeiss Axio Observer microscope coupled to an ApoTome.2.

2.4. Experimental design and end-point analyses

In the first experiment (four experimental replicates), we aimed to analyze the effect of melatonin supplementation during *in vitro* embryo production. IVM, IVF and IVC media were supplemented with 10^{-8} (10 nM) or 10^{-6} M (1 μM) melatonin (M5250, Sigma-Aldrich), or with 1 μl/ml DMSO (D8418, Sigma-Aldrich) (equivalent to the concentration used in the 10^{-6} M melatonin group) as a control. Melatonin concentrations were selected according to previous studies [25–28]. COCs were randomly allocated to control (n = 233), 10^{-8} M melatonin (n = 235), or 10^{-6} M melatonin (n = 233) groups. Cleavage rates were assessed at D2 and blastocyst rates at D8.

In the second experiment (four experimental replicates), we aimed to analyze the effect of melatonin supplementation during post-hatching *in vitro* culture. D6 and D7 blastocysts produced with 10^{-8} M (10^{-8} MIV-D12; n = 105) or 10^{-6} M (10^{-6} MIV-D12; n = 88) melatonin supplementation from IVM, as well as those from the control group (with 1 μl/ml DMSO; n = 101) were transferred to post-hatching culture in N2B27 medium supplemented with 10^{-8} M, 10^{-6} M melatonin, or 1 μl/ml DMSO, respectively. Additionally, D6 and D7 blastocyst produced in regular IVM, IVF, and IVC media were randomly cultured in N2B27 medium supplemented with 10^{-8} M (10^{-8} Blast-D12; n = 113) or 10^{-6} melatonin (10^{-6} Blast-D12; n = 116) (Fig. 1).

Embryos were cultured until D12, when images were captured using a stereo microscope (Zeiss Stemi 305) and embryo area was measured using Fiji software [40]. Embryo survival was assessed (alive embryos maintained the blastocoel, whereas dead embryos collapsed), and surviving embryos were collected for lineage development analysis by immunofluorescence for SOX2 (epiblast), SOX17 (hypoblast) and CDX2 (trophectoderm). Epiblast survival was determined based on the presence of SOX2+ cells at the end of the culture period. The number of SOX2+ cells was manually counted using the multi-point counter plugin in ZEN 3.2 (Carl Zeiss Microscopy GmbH, Germany). Embryonic disc formation was identified by the presence of a compact structure of at least 30 SOX2+ cells. To quantify hypoblast migration, each spherical embryo was divided into two halves along the Z-axis, and orthogonal projections were generated. The total area and the area covered by SOX17+ hypoblast cells were quantified in each projection. The percentage of hypoblast migration was calculated as the area covered by hypoblast cells divided by the total area, with the final value for each embryo obtained as the mean of both projections.

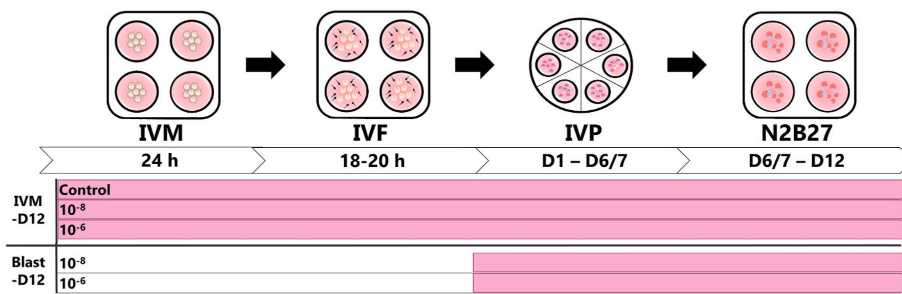

Fig. 1. Experimental design. In the first experiment, the effect of melatonin supplementation from IVM to D12 of post-hatching culture was analyzed. COCs were randomly allocated to the control (media supplemented with 1 μl/ml DMSO; equivalent to the concentration used in the 10^{-6} M melatonin group), 10^{-8} M or 10^{-6} M groups. In the second experiment, the effect of 10^{-8} M or 10^{-6} M melatonin supplementation was assessed only during post-hatching culture.

2.5. Statistical analysis

Data were analyzed using the GraphPad Prism software package (GraphPad Software, San Diego, CA, USA), and a P-value of <0.05 was considered significant. The chi-square test was used to analyze differences in epiblast survival and embryonic disc formation rates between groups. Differences in cleavage (D2) and blastocyst (D8) rates, embryo survival and area, percentage of hypoblast migration, and SOX2+ cell number (D12) were analyzed using one-way ANOVA when the data followed a normal distribution. When the normality test failed, statistical differences were assessed using a non-parametric one-way ANOVA (Kruskal-Wallis test).

3. Results and discussion

3.1. Effect of melatonin supplementation during in vitro embryo production

In vitro ovine embryos were produced in the presence of 10^{-8} or 10^{-6} M melatonin during IVM, IVF and IVC (Fig. 1). These concentrations were selected based on previous studies reporting increased development rates following melatonin supplementation in IVM or IVC media at doses ranging from 10^{-6} to 10^{-8} M in sheep [25,26,28] and goats [27], which exhibit similarities to sheep in the process of *in vitro* embryo production. However, no significant differences were detected in cleavage rates at D2 or in blastocyst rates at D8 between the 10^{-8} or 10^{-6} M melatonin groups and the control group (Fig. 2). Consistent with our findings, other studies have reported no effects of melatonin supplementation during IVM, IVF, or IVC on developmental rates. A previous study in sheep reported that melatonin supplementation at 10^{-5} or 10^{-6} M during IVM, IVF and IVC did not affect blastocyst rates [25]. Similarly, in bovine, supplementation with 10^{-9} M melatonin during IVM had no impact on cleavage or blastocyst rates [41]. Likewise, the addition of 10^{-9} M melatonin during porcine IVM and/or IVF did not influence developmental parameters. However, while 10^{-9} M melatonin supplementation during IVC increased cleavage but not blastocyst rates in *in vitro*-produced embryos, it had no effect on *in vivo*-produced and subsequently cultured porcine embryos [31,42]. *In vitro*-produced embryos are more exposed to stress factors, such as temperature variations, light exposure, pipetting, and culture media composition (reviewed in Ref. [43]), compared to their *in vivo*-produced counterparts. This could explain why melatonin may have a greater potential for improving developmental rates when applied to *in vitro*-produced embryos, particularly in pigs, where oocyte IVM presents more challenges than in other species [44].

Using similar melatonin concentrations during IVM or IVC in bovine *in vitro* embryo production, ranging from 10^{-7} to 10^{-9} M, other authors have reported increased blastocyst rates [30,45,46]. Curiously, Soto-Heras et al. demonstrated that the beneficial effect of 10^{-7} M melatonin was only observed when the antioxidant cysteamine was removed from the IVM medium, indicating that its positive effects were due to its antioxidant properties [27]. Consistently, several studies have shown that melatonin can regulate the expression of genes involved in antioxidant responses, such as *SOD1, GPX1,* and *GPX4* [30,47].

Thus, the positive effects of melatonin supplementation on *in vitro* embryo production and culture appear to be linked to its antioxidant properties and may therefore vary depending on the specific culture conditions used in each study, which can result in a different levels of oxidative stress status in embryos. Supporting this idea, different studies using the same melatonin concentrations during bovine IVM have reported contrasting results in blastocyst rates [41,46]. Most of the aforementioned studies used homemade culture media, whereas we used commercial media, which may provide a more stable environment that minimizes embryo exposure to oxidative stress. This could explain

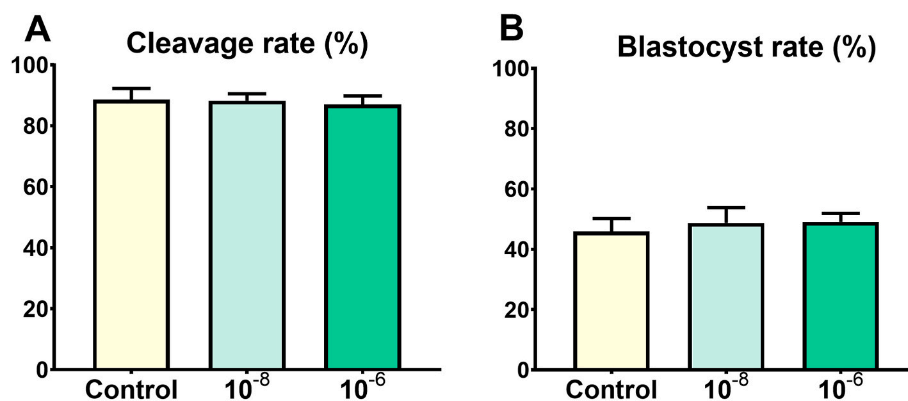

Fig. 2. A) Cleavage and B) blastocyst rates in control (supplemented with 1 μl/ml DMSO, equivalent to the concentration used in the 10^{-6} M melatonin group), 10^{-8} M, and 10^{-6} M melatonin groups. Graphs represent mean ± s.e.m from four experimental replicates. No statistically significant differences were detected by one-way ANOVA ($p > 0.05$).

why cleavage and blastocyst rates did not improve following melatonin supplementation in our study.

3.2. *Effect of melatonin supplementation during post-hatching in vitro culture*

No significant differences were found in embryo survival, embryo area, or the percentage of hypoblast migration at D12, regardless of whether melatonin (10^{-8} or 10^{-6} M) was added from IVM or from the blastocyst stage (Fig. 3,4). However, the percentage of embryos with SOX2+ surviving epiblast cells was significantly lower in D12 embryos treated with 10^{-6} M melatonin from the blastocyst stage (Table 1). The number of SOX2+ cells was also significantly lower in embryos treated with 10^{-6} M melatonin from the blastocyst stage compared to those treated with 10^{-8} M melatonin from the blastocyst stage or embryos produced and cultured in 10^{-6} M melatonin from IVM (Fig. 3). However, no significant differences were detected in the percentage of D12 embryos forming an embryonic disc (ED) (Table 1).

Previous reports have shown that supplementation with high melatonin concentrations during IVM or IVC might have detrimental effects. For example, 10^{-3} M melatonin during IVC reduced blastocyst rates and cell numbers in mice [48,49], and cleavage and blastocyst rates in sheep [26]. Similarly, one study reported that 10^{-3} M melatonin during IVM significantly reduced blastocyst cell numbers [32], while another study found that 10^{-3} M melatonin during IVM and/or IVC reduced blastocyst rates in pigs [15]. Although our study used lower concentrations, to the best of our knowledge, this is the first study to evaluate the effect of melatonin supplementation on post-hatching embryonic stages *in vitro*. This developmental period involves complex cell differentiation and proliferation processes, during which excessive melatonin concentrations could exert negative effects. In fact, several studies have reported a wide range of melatonin-induced signalling responses in diverse cell types [50]. Thus, it is possible that 10^{-6} M melatonin negatively affected the epiblast, which has been reported to be the most sensitive lineage during post-hatching development [35]. This observation, together with the divergent effects reported in the literature regarding melatonin supplementation during *in vitro* embryo production at similar concentrations, suggests that melatonin may have a very narrow range of concentrations that provide positive effects.

Previous *in vitro* studies were limited to the hatched blastocyst stage, the endpoint of conventional embryo culture systems. However, some studies have analyzed the effects of melatonin supplementation in IVC medium on later stages *in vivo*, reporting enhanced implantation, pregnancy rates, and litter size in mice [49,51,52]. Additionally, supplementation with 10^{-9} M melatonin during IVC of human embryos improved implantation rates, as well as biochemical and clinical pregnancy rates, in patients undergoing repeated assisted reproductive treatments after failed IVF and intracytoplasmatic sperm injection (ICSI) cycles [53]. In sheep, positive effects of melatonin supplementation in ewes, before or during pregnancy, have been reported. Placentas from melatonin-supplemented ewes had more binucleate trophectoderm cells and higher placental lactogen expression, as well as higher expression of genes involved in angiogenesis in the caruncular endometrium [54]. Later in gestation, maternal melatonin supplementation increased uterine blood flow and fetal oxygenation, also showing neuroprotective effects in fetuses exposed to acute hypoxia in utero [55].

Our study was limited to earlier developmental studies, but we did not observe beneficial effects of melatonin supplementation during post-hatching embryo culture in N2B27 medium. As mentioned above, the effects of melatonin supplementation during *in vitro* embryo production and culture may be influenced by concentration used and the oxidative stress status of the embryos, which varies across studies depending on the culture conditions. Our post-hatching *in vitro* embryo culture system is based on N2B27, a complex medium containing amino acids, lipids, hormones, vitamins, and growth factors [36]. Some of its components have intrinsic antioxidant properties, such as L-Alanine [56], L-Arginine

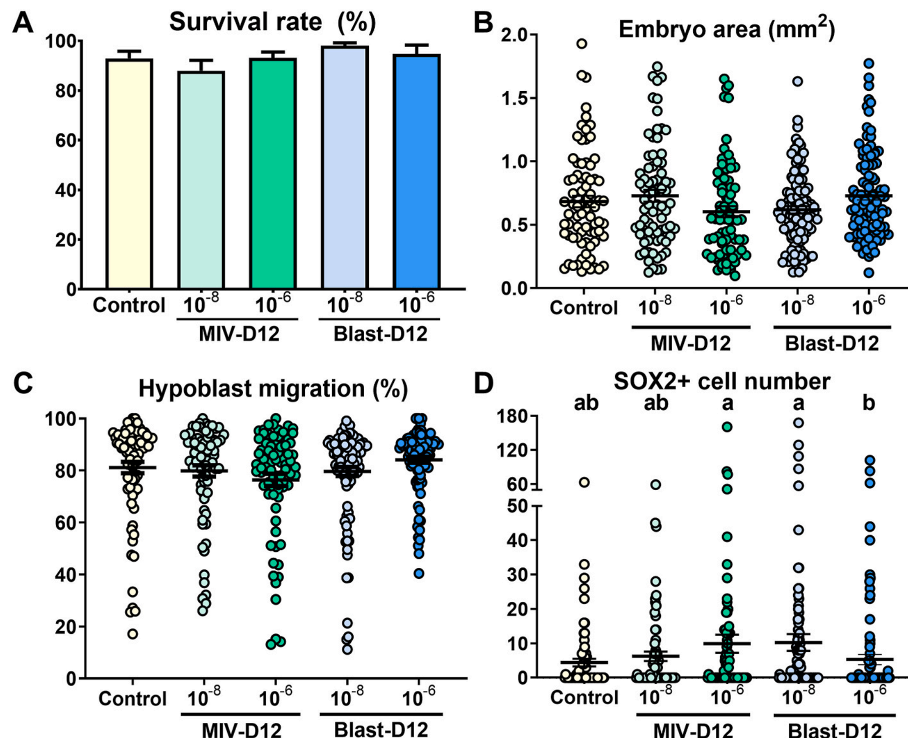

Fig. 3. A) Column graph indicating embryo survival. Scatter plots indicating B) embryo area, C) the percentage of hypoblast migration and D) SOX2+ cell number in D12 control embryos (n = 77; N2B27 medium supplemented with 1 μl/ml DMSO, equivalent to the concentration used in the 10^{-6} M melatonin group), embryos treated with 10^{-8} M (n = 75) or 10^{-6} M (n = 77) melatonin from IVM, and embryos treated with 10^{-8} M (n = 108) or 10^{-6} M (n = 107) melatonin from the blastocyst stage. Graphs represent mean ± s.e.m from four experimental replicates. Different letters indicate statistically significant differences (Kruskal-Wallis test; $P < 0.05$).

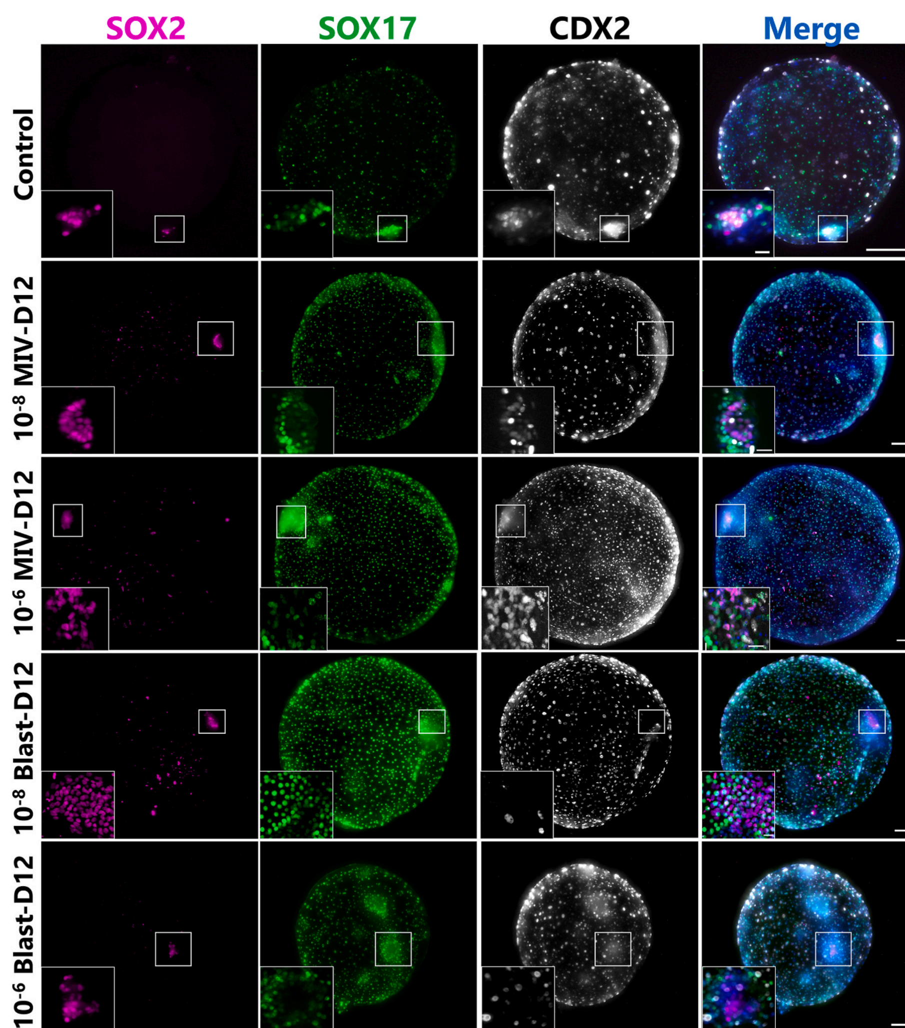

Fig. 4. Representative immunofluorescence images of D12 embryos after culture in the indicated treatments: Control supplemented with 1 μl/ml DMSO, equivalent to the concentration used in the 10^{-6} M melatonin group, embryos treated with 10^{-8} or 10^{-6} M melatonin from IVM, and embryos treated with 10^{-8} or 10^{-6} M melatonin from the blastocyst stage. Embryos were stained for SOX2 (magenta), SOX17 (green), CDX2 (white) and DAPI (blue). Scale bars: 200 μm for whole embryos, and 20 μm for magnifications.

Table 1
Effect of melatonin supplementation on post-hatching development. Epiblast survival and ED formation rate of D12 embryos treated with 10^{-8} or 10^{-6} M melatonin from IVM, and embryos treated with 10^{-8} M or 10^{-6} M melatonin from the blastocyst stage. Control group was supplemented with 1 μl/ml DMSO, equivalent to the concentration used in the 10^{-6} M melatonin group.

	Epiblast survival (%)	ED formation (%)
Control	29/77 (37.66)[a]	6/29 (20.69)
10^{-8} M MIV-D12	31/73 (42.47)[a]	3/31 (9.68)
10^{-6} M MIV-D12	35/77 (45.45)[a]	6/35 (17.14)
10^{-8} M Blast-D12	49/106 (46.23)[a]	8/49 (6.33)
10^{-6} M Blast-D12	25/108 (23.15)[b]	6/25 (24)

Different letters indicate statistically significant differences between groups (Chi-square test; $P < 0.05$).

[57,58], and other amino acids [59]; lipids such as L-Carnitine [60]; and vitamins, including folic acid [61], vitamins A, C and E [62], as well as the potent antioxidant molecule glutathione [63] These components likely provided stable conditions where the potential antioxidant effects of melatonin were not detectable.

4. Conclusions

Melatonin supplementation at 10^{-8} or 10^{-6} M during IVM, IVF, and IVC did not affect *in vitro* ovine embryo production in our study. When the effect of melatonin supplementation was assessed on the development of the first three embryonic lineages during post-hatching stages *in vitro*, no beneficial effects were detected.

The literature presents conflicting results regarding the positive effects of melatonin on *in vitro* embryo production up to the blastocyst stage. However, variations in melatonin dosage and culture media across studies may contribute to these discrepancies. These differences are critical, as the specific melatonin concentration to which gametes and embryos are exposed, as well as the presence of other antioxidant agents in the culture media, play a key role. Given that the beneficial effects of melatonin have been linked to its antioxidant properties, these factors must be carefully considered when evaluating its impact.

CRediT authorship contribution statement

M. Carvajal-Serna: Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **N. Martínez de los Reyes:** Writing – review & editing, Investigation, Formal analysis. **P. Marigorta:** Writing – review & editing, Investigation, Formal analysis. **P.**

Bermejo-Álvarez: Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **P. Ramos-Ibeas:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Priscila Ramos-Ibeas reports financial support was provided by the Spanish Ministry of Science and Innovation. Pablo Bermejo-Alvarez reports financial support was provided by the Spanish Ministry of Science and Innovation. Melissa Carvajal-Serna reports financial support was provided by the Spanish Ministry of Universities. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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