ENHANCING THE INNER CELL MASS CELL NUMBERS AND IMPLANTATION POTENTIAL OF BOVINE SOMATIC CELL NUCLEAR TRANSFER EMBRYOS BY USING MELATONIN DURING IN VITRO MATURATION OF OOCYTES

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SUMMARY

The success rate of establishing an embryonic stem cell line and achieving successful embryo transfer is directly proportional to the number of inner cell mass (ICM) cells in the blastocyst. However, the number of ICM in cloned bovine embryos is usually lower compared to in vitro fertilization embryos. Hence, melatonin, with its ability to reduce apoptosis, is considered a supplement to increase the number of ICM cells in cloned bovine embryos. Therefore, the objective of this study is to examine the effects of melatonin supplements on the developmental competence and number of ICM cells in bovine embryos. Additionally, this study investigates the ability of bovine cloned hatched blastocysts to implant on the feeder layer in groups with or without melatonin treatment. In the first experiment, the ICM cell number and the ratio of ICM to total cell number in parthenogenetic embryos treated with or without melatonin were recorded to determine the optimal concentration of melatonin. Following this, the effect of melatonin on the developmental competence and implantation potential of bovine cloned embryos was assessed. As a result, 10^{-7} M melatonin significantly increased the rate of late blastocyst formation, the number of ICM cells in the blastocyst, and the ratio of ICM to total cell number in the blastocyst. Moreover, 14% of blastocysts in the melatonin treatment group successfully implanted on the feeder layer, compared to only 8% of blastocysts in the group without melatonin treatment. In conclusion, melatonin ameliorates oocyte oxidative stress and improves the subsequent in vitro development of bovine cloned embryos.

Keywords: Bovine embryo, inner cell mass, melatonin, parthenogenetic, somatic cell nuclear transfer.

INTRODUCTION

Embryonic stem cells (ESCs), typically derived from the inner cell mass (ICM) of blastocysts, are a type of pluripotent cell with the remarkable ability to differentiate into all cell types and to continually self-renew. ESCs play a crucial role in advancing our understanding of developmental biology and serve as valuable research tools for genetic engineering and the development of disease models. On the other hand, somatic cell nuclear transfer (SCNT), or cloning, involves transferring a donor cell into an enucleated oocyte, enabling it to create offspring with identical genetics to the donor cell. SCNT technology was originally developed for the multiplication of genetically valuable animals, the rescue of endangered species, and the creation of genetically modified animals. Among the species used in SCNT experiments, cattle are the most commonly studied. Thus, ESCs derived from SCNT embryos can be a powerful tool in large animals for the conservation of endangered species, such as the gaur, and for the efficient production of transgenic animals capable of producing recombinant proteins in their milk. However, the establishment of ES cell lines in cattle remains challenging and controversial. Bovine ES/ES-like cell lines thus far lack compelling evidence of pluripotency both in vivo and in vitro, and further research is needed.

To establish an ES cell line, a critical factor is the embryo's ability to adhere to the feeder layer to form primary colonies. The high proportion of ESCs originates from the higher number of cells in the ICM (Kim *et al.*, 2012). The ICM cell number is vital for proper implantation, and a low count can decrease embryonic viability. A reduction of around 30% or more in the ICM cell number of a blastocyst significantly increases the risk of fetal loss or developmental injury (Shiao, Chan, 2009). Therefore, increasing the number of ICM cells in blastocysts is important for establishing bovine ESC lines. Apoptosis is a type of cell death in which a series of molecular steps in a cell lead to its death. Apoptosis has been observed in bovine embryos after the 8-cell stage when the bovine embryonic genome is activated. The majority of apoptotic nuclei in bovine blastocysts were located in the ICM, whereas in humans and mice, apoptotic cells were distributed randomly in the embryo. Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine produced in the pineal gland and other organs, such as the ovary, where it functions as a broad-spectrum antioxidant (Reiter *et al*., 2016). It has shown effectiveness in in vitro embryo

production across various species by enhancing oocyte developmental competence after in vitro maturation (IVM) and preventing apoptosis (Cruz *et al.*, 2014). This benefit is primarily due to its ability to reduce reactive oxygen species (ROS) (El-Raey *et al.*, 2011) and mitigate their damaging effects on oocytes (Tripathi *et al.*, 2011). Melatonin has been shown to improve ICM cell numbers in blastocysts of mice, pigs, and goats. There have been some reports that supplementing melatonin in IVM improves the developmental competence of cloned bovine embryos in vitro (An *et al.*, 2019). However, the potential of these embryos to implant in vitro and subsequently develop into embryonic stem cell lines has not yet been clarified. Therefore, this study will focus on using melatonin to enhance the implantation potential of cloned bovine embryos in vitro.

In this study, the effect of melatonin treatment during IVM culture on the ICM cell number of bovine blastocysts will be examined. Additionally, the primary colony formation ability of cloned bovine ESCs derived from oocytes treated with melatonin will also be tested.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA).

In vitro maturation of oocytes and production of parthenogenic embryo

OCGCs were aspirated from 4-8 mm follicles using an 18-gauge needle attached to a 10mL disposable syringe. The isolated OCGCs were washed several times with HEPES to remove any residual tissues and then cultured in floating drops of IVM medium. This medium consisted of tissue culture medium-199 supplemented with 10% fetal bovine serum (FBS), 10 µg/mL sodium pyruvate, 50 ng/mL epidermal growth factor (EGF), 1 µg/mL follicle-stimulating hormone (FSH), 0.0065 IU/mL luteinizing hormone (LH), and 1 µg/mL estradiol, with various concentrations of melatonin (0, 10^{-5}, 10^{-7}, and 10^{-9} M). The culture was maintained at 38.5°C under 5% CO_2. After 22 hours of IVM, hyaluronidase was added to the IVM floating drops. The oocytes were then gently pipetted to remove the surrounding cumulus cells in HEPES, selecting only matured oocytes with the first polar body for parthenogenesis activation. Matured oocytes were recovered in hormone-free TCM-199 supplemented with 10% FBS and 10 µg/mL sodium pyruvate for 30 minutes, followed by a 2-hour recovery in modified synthetic oviductal fluid (mSOF). Oocytes were activated using 5 µM ionomycin for 5 minutes, followed by exposure to 2 mM 6-DMAP in mSOF. The parthenogenetic embryos were then cultured at 38.5°C under 5% CO_2 for 7 days.

Immunostaining

After 7 days of culture, embryos were fixed in 4% paraformaldehyde for 45 minutes and then soaked in Triton X-100 at 4°C overnight. The embryos were subsequently immersed in the primary anti-CDX2 antibody for 2 hours. Following three washes, they were incubated with the secondary antibody, Alexa Fluor 568-labeled goat anti-mouse IgG, for 1 hour. After an additional three washes, the embryos were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 30 minutes to visualize DNA for cell counting.

Somatic cell nuclear transfer and cloned embryo culture

After optimizing melatonin concentration, the treatment resulting in the highest ICM count and the optimal ICM to total cell ratio of the blastocyst was selected for the SCNT experiment. After 19 hours of IVM, the cumulus removal process was performed as described in the parthenogenetic activation section. The oocytes were then recovered in hormone-free TCM-199 for 1 hour. The zona pellucida of the denuded and matured oocytes, identified by the extrusion of the polar body, was cut and removed using the XY laser system on the micromanipulator in HEPES drops containing 7.5 µg/mL cytochalasin B. The matured oocytes were enucleated by aspirating the nuclear material along with a small amount of surrounding cytoplasm using a glass pipette (15 µm inner diameter) attached to a micromanipulator. The oocytes were then subjected to a recovery process in hormone-free TCM-199 for 1 hour. Subsequently, the nuclei of cumulus cells were injected into the cytoplasm of the enucleated oocytes. The oocytes were recovered in hormone-free TCM-199 supplemented with 10% FBS and 10 µg/mL sodium pyruvate for 30 minutes, followed by a 2-hour recovery in mSOF to allow for nuclear remodeling. Oocytes were activated using 5 µM ionomycin for 5 minutes, followed by exposure to 2 mM 6-DMAP in mSOF. The cloned embryos were then cultured at 38.5°C under 5% CO_2 for 7 days.

Seeding cloned blastocyst on feeder layer

Hatched blastocysts were placed onto mitomycin-C inactivated mouse embryonic fibroblasts (MEFs) and cultured in DMEM/F12 medium containing 20% knockout serum (KO-serum), 0.1 mM β-mercaptoethanol, 1% nonessential amino acids, and 20 ng/mL basic fibroblast growth factor (bFGF). After 3 days, the embryos were checked to assess their ability to implant and form primary colonies.

Statistical analysis

Statistical analysis was performed by SPSS 20. The data labeling by single-factor analysis of variance (ANOVA) followed by the Turkey test. Each group of experiments was repeated 3 to 4 times to ensure the statistical power of the test. P values less than 0.05 were considered statistically significant. For quantitative analyses, the fluorescent images were subjected to densitometric analysis using NIS Elements BR. software.

RESULTS

The effect of melatonin supplement on bovine embryo quality

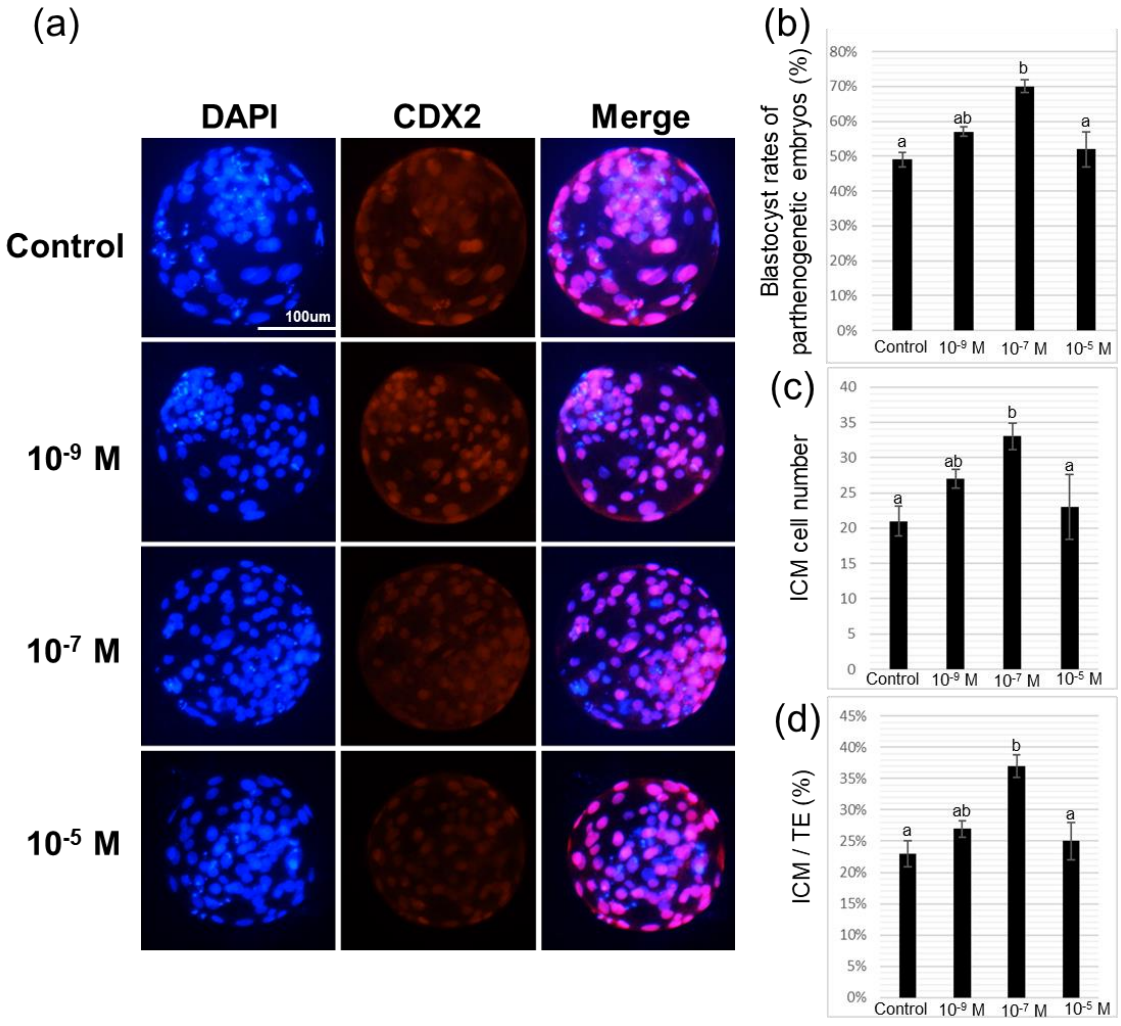

Figure 1. Effect of melatonin supplementation during in vitro maturation on the embryo quality of the parthenogenetic bovine blastocysts. (a) DAPI and CDX2 staining were performed on Day 7 blastocysts of four groups (scale bar = 100 µm). (b) Effects of different concentrations of melatonin on the blastocyst rates of parthenogenetic embryos. (c) The number of ICM on Day 7 blastocysts from the four groups. (d) The ratio of ICM to the TE cell number on Day 7 blastocysts from the four groups. Values with different superscripts are significantly different (^{a,b} < 0.05). The experiments were replicated three times

COCs were cultured in oocyte maturation media with different concentrations of melatonin (0, 10^{-5}, 10^{-7}, and 10^{-9} M) to assess whether melatonin positively affects the developmental competence of bovine embryos produced by parthenogenesis. Parthenogenesis involves the activation of an oocyte without fertilization, leading to a more uniform and controlled experimental environment. Testing supplements using parthenogenetic embryos instead of SCNT embryos is often preferred since parthenogenetic embryos are simpler to produce and provide a more consistent model system compared to SCNT embryos. The results showed that 10^{-7} M melatonin supplemented in the M199-based medium significantly increased the blastocyst formation rate of the oocytes to 70% compared with 49% in the control group ($p < 0.05$; Figure 1b). DAPI is a fluorescent stain that binds strongly to DNA, allowing us to observe and count the cell number of the embryo. CDX2 is a transcription factor specifically expressed in the trophectoderm cells of the blastocyst stage. Immune-fluorescent staining with the CDX2 antibody was used to confirm the identity of the trophectoderm cells. By merging DAPI (blue) and CDX2 (red) signals, the ICM cells, which do not express CDX2, will appear blue, while the trophectoderm cells will appear purple, enabling the counting of ICM cells. The addition of 10^{-7} M melatonin during IVM also significantly improved the number of ICM cells in the parthenogenetic embryos ($p< 0.05$, Figure 1c). Notably, 10^{-7} M melatonin supplemented during IVM significantly increased the ratio of ICM to total cell number at the Day 7 blastocyst stage compared to oocytes cultured without melatonin ($p < 0.05$, Figure 1c).

The effect of melatonin supplement on the implantation potential of cloned bovine embryo

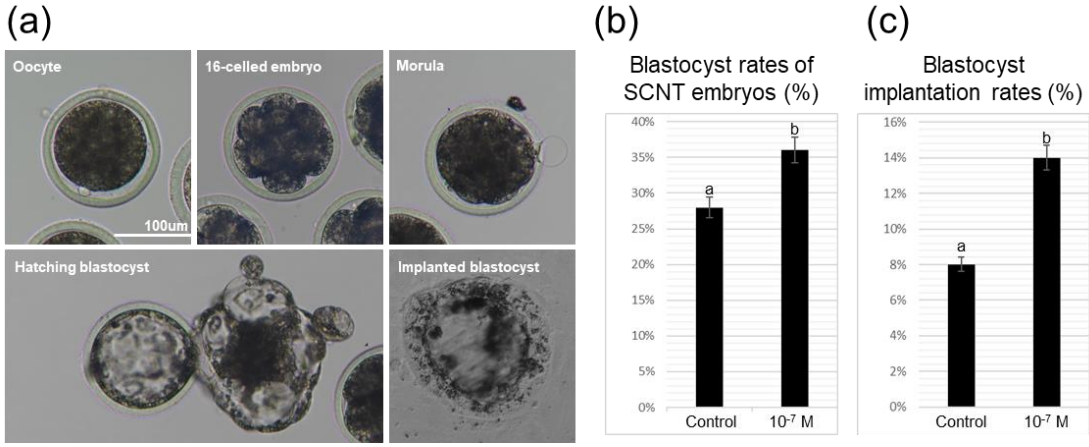

Figure 2. Effect of melatonin supplementation during in vitro maturation on the embryo quality of the cloned bovine blastocysts. (a) Morphology of developmental stage of cloned bovine embryos (b) Effects of melatonin on the blastocyst rates of cloned embryos. (c) Effects of melatonin on the implantation potential of cloned embryos. Values with different superscripts are significantly different (^{a,b} < 0.05). The experiments were replicated three times

After optimizing the melatonin concentration, 10^{-7} M was selected to test on cloned bovine embryos. As shown in Figure 2b, 10^{-7} M melatonin significantly improved oocyte developmental competence and the subsequent development of cloned bovine embryos compared with the oocytes cultured without melatonin ($p < 0.05$). Once the blastocysts hatched, they were pressed onto an MEF feeder layer to assess their attachment capability, a crucial step in the attempt to establish a bovine ES cell line. Melatonin increased the implantation ability from 8% in the control group to 14% in the melatonin-supplemented group.

DISCUSSION

During natural development in living organisms, oocytes are exposed to a low-oxygen (hypoxic) environment and are protected from oxidative damage by free-radical scavengers and antioxidant enzymes present in the follicular fluids. However, in the case of IVM, oocytes are obtained from slaughtered cattle and matured in a laboratory setting, creating a relatively high-oxygen (hyperoxic) environment not found in vivo. Consequently, in vitro-cultured oocytes experience elevated levels of oxidative stress and lack the complex protection provided by natural antioxidant mechanisms. The overproduction of ROS leads to oxidative stress, which can disrupt cell membranes and induce apoptosis, thereby impairing the quality and developmental potential of oocytes. Increased ROS levels are often associated with higher apoptosis rates, and the majority of apoptotic nuclei in bovine blastocysts are located in the ICM), leading to a lower number of ICM cells in in vitro-produced embryos. Melatonin, an effective free radical scavenger and broad-spectrum antioxidant, along with its metabolites, directly scavenges ROS and reduces cellular oxidative damage. Thus, melatonin significantly enhances embryo development and increases the number of ICM cells in parthenogenetic blastocysts.

The low efficiency of animal cloning is commonly attributed to incomplete and aberrant epigenetic reprogramming of cloned embryos, which involves various epigenetic modifications such as DNA methylation and histone modification. Both histone acetylation and histone methylation are crucial in the nuclear reprogramming process during SCNT, ultimately influencing the development of cloned embryos (Liu *et al.*, 2018). Melatonin can modulate the expression of genes involved in cell cycle regulation, differentiation, and development (Yang *et al.*, 2020), thereby enhancing the developmental competence of SCNT embryos. This improvement in embryo quality leads to better implantation success. These findings are consistent with the reports by Kim *et al.* (2012).

CONCLUSION

In conclusion, melatonin supplementation during IVM significantly enhances the developmental competence of bovine embryos. The addition of melatonin not only increases the ICM cells but also improves the ratio of ICM cells to the total cell number in blastocysts. These improvements are particularly evident in SCNT embryos, where melatonin treatment leads to enhanced developmental competence and a higher implantation success rate. By mitigating oxidative stress and supporting favorable gene expression profiles, melatonin serves as a vital supplement in bovine embryo production, ultimately advancing the efficiency and success of SCNT procedures. These findings underscore the potential of melatonin to improve outcomes in animal cloning and embryonic stem cell research.

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CẢI THIỆN SỐ LƯỢNG TẾ BÀO CỦA KHỐI NỘI PHÔI BÀO VÀ KHẢ NĂNG LÀM TỔ CỦA PHÔI BÒ NHÂN BẢN BẰNG SỬ DỤNG MELATONIN TRONG QUÁ TRÌNH NUÔI TẾ BÀO TRỨNG CHÍN TRONG ỐNG NGHIỆM

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TÓM TẮT

Tỷ lệ thành công trong việc thiết lập dòng tế bào gốc phôi và chuyển phôi thành công phụ thuộc vào số lượng tế bào của khối nội phôi bào (ICM-Inner cell mass) trong phôi nang. Tuy nhiên, số lượng tế bào ICM trong phôi bò nhân bản thường thấp hơn so với phôi thụ tinh trong ống nghiệm. Do đó, melatonin, với khả năng giảm thiểu tế bào chết theo chu trình, được xem là một chất bổ sung để tăng số lượng tế bào ICM trong phôi bò nhân bản. Mục tiêu của nghiên cứu này là đánh giá tác động của việc bổ sung melatonin lên khả năng phát triển và số lượng tế bào ICM trong phôi bò. Ngoài ra, nghiên cứu này cũng khảo sát khả năng làm tổ của phôi bò nhân bản đã thoát màng trong các nhóm bổ sung hoặc không bổ sung melatonin. Trong thí nghiệm đầu tiên, số lượng tế bào ICM và tỷ lệ ICM so với tổng số tế bào trong phôi trinh sản nuôi từ trứng trưởng thành trong ống nghiệm có hoặc không có bổ sung melatonin được ghi nhận để xác định nồng độ tối ưu cho qui trình nuôi cấy. Tiếp đến, ảnh hưởng của melatonin đối với khả năng phát triển và làm tổ của phôi bò nhân bản sẽ được đánh giá. Kết quả cho thấy, melatonin với nồng độ 10^{-7} M làm tăng đáng kể tỷ lệ hình thành phôi nang cũng như số lượng tế bào ICM và tỷ lệ ICM so với tổng tế bào trong phôi trinh sản. Hơn nữa, 14% phôi nang bò nhân bản đã làm tổ thành công vào lớp các tế bào bám vào đáy dĩa (feeder layer), trong khi chỉ có 8% phôi nang trong nhóm không bổ sung melatonin có khả năng làm tổ. Tóm lại, melatonin cải thiện khả năng chịu stress oxy hóa của trứng và cải thiện khả năng phát triển của phôi bò nhân bản trong ống nghiệm.

Từ khóa: Khối nội phôi bào, melatonin, nhân bản, phôi bò, trinh sản

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