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RESEARCH ARTICLE

Melatonin Protects Bovine Embryos from Heat Stress and Oxygen Tension and Improves Embryo Production *In vitro* [1]

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ABSTRACT

The objective of this study was to determine melatonin's ameliorating effects against heat stress and oxygen tension in developing bovine embryos *in vitro*. The oocytes were collected from ovaries obtained from a local abattoir, followed by *in vitro* maturation, fertilization, and embryo culture. During *in vitro* culture, embryos were exposed to 5% (Group I) and 20% (Group II) oxygen tension with 10^{-3}, 10^{-6}, and 10^{-9} molar (M) melatonin, along with the control group without melatonin (Group III). Compared to the control group, melatonin at 10^{-6} and 10^{-9} concentrations increased *in vitro* development rates and decreased caspase 3/7 activity at 5% and 20% oxygen tension ($P<0.01$). One-half of the zygotes were cultured under normal temperature (38.5°C) during the culture period, and the other half of the zygotes were heat stressed at 41°C for six hours. Then they transferred into the normal culture conditions for the rest of the period using 0, 10^{-6}, and 10^{-9} M of melatonin (Group IV). Under normal temperature (38.5°C), melatonin at 10^{-9} M was beneficial for *in vitro* development and DNA integrity. Under heat stress at 41°C, melatonin at 10^{-6} and 10^{-9} M was useful for *in vitro* development and DNA integrity ($P<0.05$). Supplementation of melatonin to embryo culture medium did not alter the caspase 3 and 7 activities ($P>0.05$). In conclusion, melatonin prevents the adverse effects of heat stress and O_2 tension on preimplantation bovine embryos *in vitro*.

Keywords: DNA integrity embryo, Heat stress, Melatonin, Oxygen concentration

INTRODUCTION

Bovine embryos produced *in vitro* are vital in accelerating cattle genetics, and *in vitro* culture conditions are far from optimum. The continuing efforts to develop optimum culture conditions for *in vitro* embryo production and better protocols for improving *in vivo* survival could help advance cattle breeding and genetics for precision farming of the animals.

One of the main issues with *in vitro* embryo culture system is the oxygen tension (O_2). Tubal and uterine O_2 tension is lower than atmospheric O_2 tension routinely used in mammalian embryo culture [1]. Culturing embryos in an environment with high O_2 content (20%) *in vitro* can result in the production of reactive oxygen species (ROS) containing more free radicals [2]. Excessive physiological levels of ROS have harmful effects on embryos and can cause significant damage to cell structures [3,4]. Therefore, there is a need to pursue research on oxidative stress and its prevention using different oxygen concentrations and antioxidants to improve embryo culture systems.

Sensitivity of the embryo to temperature depends on the cleavage stages [5,6]. Embryos at the zygote to the eight-cell stage are more sensitive to high temperatures than blastocysts or morula [7]. Temperatures ranging between 40.0°C and 42.0°C reduce the *in vitro* development of embryos at the zygotic to eight-cell stage but do not significantly affect *in vitro* development to morula and

blastocyst stages. Exposure of early embryos to high temperatures can cause damage to microfilaments and microtubules and swelling of mitochondria [8]. With increasing temperature, apoptotic cell numbers increase in 2-cell embryos [9].

Melatonin (N-acetyl-5-methoxy tryptamine), an endogenously produced indole, is found throughout mammalian species, vertebrates, invertebrates, algae, bacteria, and a variety of plants. The metabolites produced when melatonin scavenges free radicals are also highly effective scavengers [10]. For example, melatonin and its metabolites can directly scavenge hydroxyl free radicals, organic oxygen free radicals, peroxy free radicals, peroxynitrite anions, nitric oxide, and singlet oxygen [11]. In addition, melatonin can repair some oxidized molecules [12].

This study was designed to test the hypothesis that melatonin improves bovine embryo production by protecting them against heat stress and oxygen tension *in vitro*.

Materials and Methods

In vitro Maturation (IVM) and Fertilization (IVF)

The ovaries were obtained from a local abattoir within lactated ringer solution including 1000 IU Penicillin/mL + 1000 µg/mL streptomycin at 37°C were transferred to Parrish Lab (Department of Animal and Dairy Science, University of Wisconsin, Madison, WI, USA). Cumulus-oocyte complexes were aspirated from antral follicles sized 1-5mm using an 18-gauge needle. The standard IVM and IVF protocols were used according to the established methods as previously described in the Parrish lab [13]. For IVM, in all groups, oocytes were matured in TCM-199 with the following supplementations: 10% heat-treated FBS, 1 µg/mL of estradiol 17-ß, 0.2 mM Na-pyruvate, 5 µg/mL of LH (NIH-oLH-26), 0.5 µg/mL of FSH (NIH-FSH-S-17), and 50 µg/mL gentamycin. For the purpose of IVF, *in vitro* matured oocytes were fertilized with frozen-thawed semen after Percoll gradient separation.

In Vitro Embryo Culture (IVC)

Presumptive zygotes were cultured in KSOM medium with different melatonin concentrations (0, 10^{-3}, 10^{-6}, and 10^{-9} M) under 20% *(Group I)* and 5% *(Group II)* oxygen conditions in an adjustable three gas incubator with no FCS supplementation during the culture period. To compare the differences between 20 and 5% oxygen, zygotes from the same batch were cultured using the same melatonin concentrations (0, 10^{-3}, 10^{-6}, and 10^{-9} M) in *Group III*. While half of the zygotes were cultured under normal temperature (38.5°C) during the culture period, the other half of the zygotes were heat stressed with an adjustable incubator for 6 h at 41°C. Then they were transferred into the normal culture conditions for the rest of the period using 0, 10^{-6} and 10^{-9} M melatonin *(Group IV)*.

Nick End Labeling (TUNEL)

The percentage of DNA fragmented blastomeres and average cell numbers were determined using the TUNEL assay followed by the Fluorescein Apoptosis Detection System. Day 8 embryos with intact zona pellucida were fixed and permeabilized, according to Brison and Schultz [14]. For this purpose, embryos were kept in 2% TritonX-100 (Bio-Rad) solution for 180 minutes to ensure permeabilization, washed twice in PBS-polyvinylpyrrolidone (PVA) solution, and incubated in fluorescent-conjugated dUTP and TdT solution (Roche Diagnostics, Tokyo) in the dark at 37°C for 1 h. Next, they were kept in DNAse (1000 IU mL-1) solution as a positive control at 37°C for 20 min. Embryos in the negative control groups were incubated in a fluorescent-dUTP solution without TdT. Following the TUNEL, the embryos were washed three times, and propidium iodide (PI) (50 µg-1) was added to stain all nuclei after adding RNAse (50 µg RNAse for 60 min at room temperature). Day 8 blastocysts were collected and labeled with a TUNEL assay Fluorescein Apoptosis Detection kit. The total number of cells and TUNEL-positive blastomeres were counted for 0, 10^{-6}, and 10^{-9} M melatonin in both normal and heat-stressed culture conditions.

Caspase 3/7 Activity

Eight-cell embryos (10 embryos per assay) were incubated at room temperature for eight more hours. Then, the resulting luminescent signal representing the amount of caspase activity in the sample was measured using a luminometer. Briefly, embryos were subjected to Caspase-Glo® 3/7 Assay and Caspase-Glo® 8 Assay (Promega Corporation, Madison, WI, USA) kits. Again, 30 µL of the reagent included in the kit was used. After 30 minutes of incubation in the dark, values were measured with the GloMax®-96 Microplate Luminometer using the Caspase-Glo® Program.

Statistical Analysis

The percentages of blastocysts were calculated from the number of presumptive zygotes. The arcsine square-root remodeled information was analyzed with a randomized complete block style exploitation SAS mixed procedure to see the numerous variations among the treatments. Significant differences in blastocyst cell numbers and the TUNEL-positive cells per blastocyst were determined by employing a unidirectional analysis of variance. Differences at $P<0.05$ were considered significant. In the study, three replicates were run for caspase 3/7 activity (10 eight-cell embryos/replicate), and five replicates

Table 1. Development of embryos cultured under 20% oxygen tension in the presence of different melatonin concentrations

Melatonin (M)*	Zygotes (n)	Oocytes Cleaved		Embryos Developed >		Blastocyst on Day 8 (%)
		at 32 h (%)	at 48 h (%)	8-cell (%)	16-cell (%)	
0	380	39.7	73.9[b]	39.9	22.0[a]	10.7[b]
10^{-3}	387	35.7	76.6[b]	25.1	7.5[b]	0.0[c]
10^{-6}	380	38.9	85.7[a]	38.2	22.5[a]	17.5[a]
10^{-9}	374	42.6	85.8[a]	47.3	26.1[a]	16.9[a]

**Presumptive zygotes were cultured in KSOM medium with different melatonin concentrations (0, 10^{-3} 10^{-6} and 10^{-9} M: molar) with no FCS supplementation during the culture period. Data represent the mean from six replicates. a-c: Values with different superscripts in the same column were significantly different (P<0.05)*

Table 2. Development of embryos cultured under 5% oxygen tension in the presence of different melatonin concentrations

Melatonin (M)*	Zygotes (n)	Oocytes to Blastocyst		Hatched Blastocyst Day 9 (%)
		Day 8 (%)	Day 9 (%)	
0	394	14.9 [b]	17.1 [b]	4.7 [b]
10^{-3}	408	1.6 [c]	2.3 [c]	0.0 [c]
10^{-6}	400	21.4 [a]	24.9 [a]	6.6 [a]
10^{-9}	414	21.8 [a]	23.3 [a]	5.1 [a]

** Presumptive zygotes were cultured in KSOM medium with different melatonin concentrations (0, 10^{-3}, 10^{-6} and 10^{-9} M: molar) with no FCS supplementation during the culture period. Data represent the mean from six replicates. a-c: Values with different superscripts in the same column were significantly different (P<0.05)*

were performed (50-75 zygotes for group/replicate) for blastocyst development, DNA fragmentation, and blastocyst cell number.

Results

Melatonin improved embryo development in 20% oxygen tension in vitro

We found that melatonin supplementation at 10^{-6} and 10^{-9} M concentrations increased embryo cleavage rate in 20% oxygen tension compared to the control group at 48 h, in 16-cell embryo rates, and in blastocyst rate on day 8 (P<0.05). But, melatonin supplementation at 10^{-3} M concentration decreased embryo cleavage according to the control group in 16-cell embryo rates and blastocyst rate on day 8 (P<0.05) *(Table 1)*.

Melatonin improved cleavage rates in 5% oxygen tension in vitro

We found in Experiment II that melatonin supplementation at 10^{-6} and 10^{-9} M concentrations increased embryo cleavage rate as compared to the control group on day 8 and day 9 embryos cultured under 5% oxygen tension (P<0.05). Conversely, melatonin supplementation at 10^{-3} M concentration decreased embryo cleavage rate according to the control group on day 8 and day 9 embryos cultured under 5% oxygen tension (P<0.05) *(Table 2)*.

High doses of melatonin increased caspase 3/7 activity in 20% tension

We showed that 10^{-3} M concentrations of melatonin

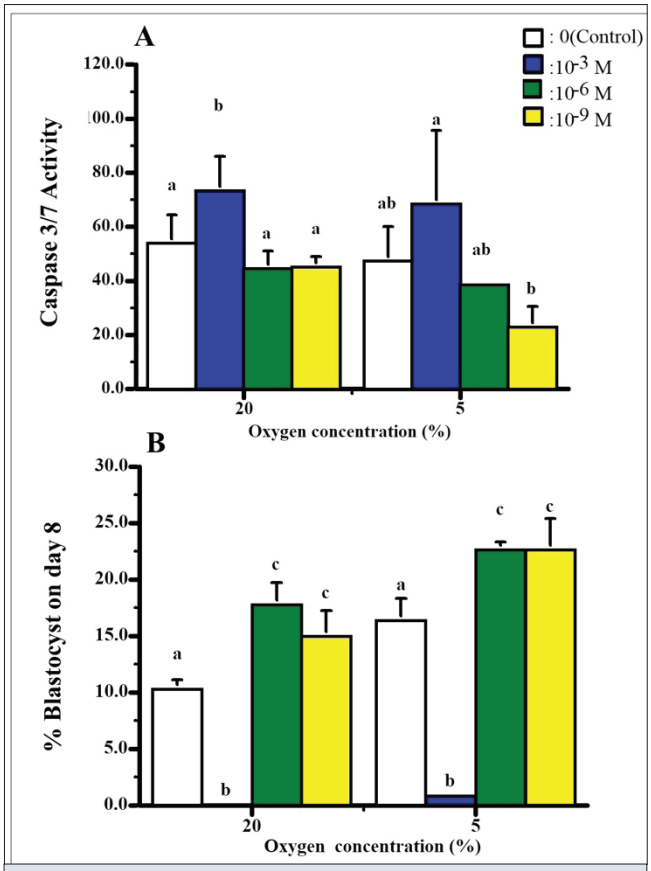

Fig 1. Effect of melatonin on caspase 3/7 activity (A) and blastocyst development (B) of preimplantation bovine embryo cultured under different oxygen concentrations (20% vs. 5%). a-b: Values with different superscripts indicate significant difference within the same culture temperature (P<0.05)

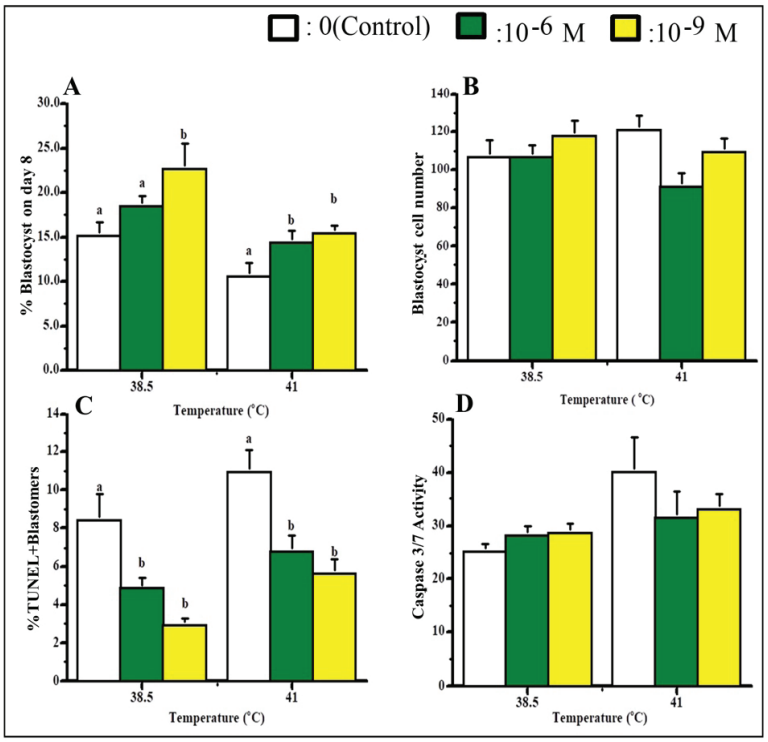

Fig 2. The effects of melatonin on blastocyst development (A), total cell number (B), DNA fragmentation (C) and caspase 3/7 activity (D) of bovine embryos cultured under normal (38.5°C) and heat stressed (41°C) conditions. a-b: Values with different superscripts indicate significant difference within the same culture temperature (P<0.05)

increased caspase 3/7 activity at 20% oxygen concentration in *Fig. 1-A* (P<0.05) but did not change at 5% oxygen tension (P>0.05). In *Fig. 1-B*, 10^{-6} and 10^{-9} M melatonin concentrations increased blastocyst rates at 20% and 5% oxygen tension (P<0.05).

Melatonin decreased DNA fragmentation and increased blastocyst rates in vitro in high temperatures

We found that supplementation of 10^{-9} doses of melatonin increased the blastocyst rates at both 38.5°C and 41°C compared to the control *Fig. 2-A*, while blastocyst rates were also increased with 10^{-6} at 41°C *Fig. 2-A* (P<0.05). In addition, both doses of melatonin decreased DNA fragmentation at the temperature of 38.5°C and 41°C *Fig. 2-B* (P<0.05). However, it was determined that blastocyst cell numbers and caspase 3/7 activity rates did not change *Fig. 2-B* (P>0.05).

Discussion

As an effective free radical scavenger and antioxidant, melatonin and its metabolites (Cyclic 3-hydroxy melatonin, N^1-acetyl-5-methoxykynuramine, and N^1-acetyl-N^2-formyl-5-methoxykynuramine) are widely used to protect embryos cultured *in vitro*. Adding melatonin to the culture medium can promote early embryonic development in mice [15]. The beneficial effects of melatonin on the development of *in vitro* production (IVP) embryos include increased blastocyst formation rate, average cell number, and hatching rate [16]. Melatonin improves the quality and survival rate of sheep embryos *in vitro* and *in vivo* [17,18] and increases the division rate of embryos and the total cell number of blastocysts [19]. In particular, its hydrophilic and hydrophobic structure allows melatonin to pass through membranes and disperse into tissues quickly [20]. Although it is known that melatonin has antioxidant properties, plays an antiapoptotic role and protects DNA integrity; it is unknown how it affects *in vitro* developmental rates, DNA integrity, apoptotic effect, and cell numbers in blastocysts exposed to high oxygen tension or temperature. The goal of this research was to pursue the intriguing idea that melatonin can protect embryos from damage that may occur at high oxygen tension and temperatures by taking advantage of the mentioned effects.

It was shown in this study that *in vitro* culture with a low dose of melatonin (10^{-6} M, 10^{-9} M) on heat stress and oxygen tension may have significant effects on the final development rate and cell number of bovine embryos. Incubation with 10^{-6} and 10^{-9} M melatonin concentrations improved bovine embryo development cultured in the KSOM media at both oxygen levels regarding blastocyst development and cleaved embryos at 48 h compared to

no melatonin supplementation. However, when 10^{-3} M melatonin was added to the KSOM media with no serum during embryo culture, melatonin decreased embryo development beyond 16-cell stages regardless of oxygen concentration. Moreover, the higher doses of melatonin (10^{-3} M) increased the caspase 3/7 activity, a sign of apoptosis at 8-cell stage embryos. Furthermore, the blastocyst development was higher when cultured under 5% oxygen tension compared to 20% with no melatonin supplementation. Additionally, 10^{-6} and 10^{-9} M melatonin concentrations improved blastocyst development when embryos were cultured under 5% oxygen ($P<0.05$). Although the blastocyst cell numbers did not differ, heat stress caused a decrease not only in blastocyst development but also increased DNA damage, both of which were improved by culturing embryos with melatonin.

Low concentration (5%) of oxygen tension has been shown to have a positive effect on *in vitro* growth rates in buffalo [21] and cattle [22]. In addition, low-concentration oxygen plays a stimulatory role but does not change blastocyst rates. However, other researchers [24,25] claim thatneither high nor low oxygen concentrations affects *in vitro* growth rates. On the other hand, adding melatonin to the culture medium has been shown to increase the *in vitro* growth rate [26]. In fact, supplementing melatonin under high oxygen concentration promoted the development of *in vitro* bovine embryos [27]. Similarly, in this study, we demonstrated that adding 10^{-6} and 10^{-9} M melatonin increased *in vitro* growth rates in both low (5%) and high oxygen concentrations (20%) compared to the control group. However, addition of 10^{-3} M melatonin decreased the blastocyst rates. It is evident that H_2O_2, which may result from a high concentration of O_2 ratio, can be removed by melatonin.

Several studies have demonstrated that melatonin can protect embryos and oocytes exposed to heat stress in different animal species [28-30]. In particular, embryonic deaths have been reported to occur with increased summer temperatures, particularly in the northern hemisphere [31]. The primary issue caused by heat stress is the production of ROS [32]. In this study, adding 10^{-6} M and 10^{-9} M melatonin to the culture medium increased the blastocyst rates. Heat stress has been reported to increase ROS production in embryos, increase the number of apoptotic cells, and decrease *in vitro* development [33]. Moreover, ROS generated by the increase in temperature can cause DNA damage in cells [34]. In cattle, heat shock protein 70 begins to be produced during early embryonic development, and its expression continues to increase until the morula stage [35]. Thus, embryos are highly sensitive to temperature changes during the early stages of embryonic development in cattle [36]. As shown in *Fig. 2*, blastocyst rates decreased with an increase in the temperature (41°C); however, adding 10^{-6} and 10^{-9} M melatonin preserved the *in vitro* growth rates compared to the control group. The cleavage data might include cytoplasmic divisions stemming cytoplasmic fragmentation as well.

During preimplantation embryo development, apoptosis plays an essential role in removing defective cells and proceeds in a coordinated manner. This process continues to develop in a certain harmony with the expression of antiapoptotic (BCL-W, BCL-2, BCL-XL) and proapoptotic (BAK, BAX, BAD) members of the BCL-2 family proteins expressed at different levels [37,38]. However, there are reports that this coordination progresses abnormally under the culture conditions [39,40]. Phosphorylated BECLIN1 can bind to BCL2 in the process of apoptosis, increase free BAX in cells, and cause mitochondrial-dependent apoptosis [41]. Melatonin ameliorates reactive oxygen species by promoting the expression of MnSOD and SIRT1.

Melatonin's function is dependent on melatonin receptors 1 and 2 (MT1 and MT2), G protein-coupled membrane receptors that can heterodimerize with the G protein receptor, affecting cellular functions [42]. Caspase 3/7 activity is also a method used to determine apoptosis [43]. In our study, caspase 3/7 activity was evaluated under 5% and 20% oxygen tensions and heat stress. The group that used 10^{-9} M melatonin at 5% oxygen tension showed the lowest caspase 3/7 activity ($P<0.05$), while in control, 10^{-6} M, and 10^{-9} M melatonin groups at 20% oxygen tension, the lowest caspase 3/7 activity was observed ($P<0.05$). The results showed that the use of 10^{-3} M melatonin increased caspase 3/7 activity under both oxygen tensions. Researchers state that 10^{-3} and higher doses of melatonin are insoluble, hydrophobic, and cannot penetrate cells and that high-dose melatonin has an apoptotic effect [44,45]. In our study, we demonostrated that high-dose melatonin at both 5% and 20% oxygen tensions increased caspase 3/7 *(Fig. 1),* consistent with other studies. It was determined that the activity of caspase 3/7 did not change at 38.5°C and 41°C in both the control and the groups containing melatonin. Although it does not alter caspase 3/7 activity, melatonin can modulate the up- or down-regulation of different genes related to temperature and apoptosis.

Researchers have stated that melatonin protects DNA integrity by preventing ROS in embryos and oocytes [46,47] in many animal species [48]. In this study, when DNA damage was examined, it was revealed that melatonin protects DNA at high temperatures compared to the control group ($P<0.05$). The resulting DNA damage is thought to be due to the failure of HSP70 proteins to be produced at the beginning of the embryonic development period, resulting in the formation of ROS and damage to the cell DNA.

Studies show that melatonin positively affects both oocyte maturation [49] and *in vitro* embryo development rates. Similarly, in this study, as in other studies [26,50], melatonin allaviated oxygen tension and heat stress, improving blastocyst development and decreasing the number of TUNEL-positive nuclei by protecting embryos from damage caused by oxidative and heat stress. The result of this study suggest that the adding melatonin in culture media at concentrations of 10^{-6} M or 10^{-9} M can positively impact embryo development.

Data Availability

All data in this article can be obtained through the corresponding authors.

Ethical Approval

Ethics committee approval is not required for this study.

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Conflict of Interest

The authors declared no interest.

Authors' Contributions

AK and EM conceived the research idea, and AK conducted the research. MH, AK, and EM searched literatures. AK, JJP and JS performed quality assessment. AK and JJP completed data analysis and MH, AK, EM, MB, and EM drafted the manuscript. EM, AK, JJP, JS, and MH revised the article. All authors discussed and contributed to the final manuscript.

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