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Melatonin regulates the development and function of bovine Sertoli cells via its receptors MT1 and MT2

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ABSTRACT

Melatonin and its receptors are found in the testis of many species, where they mediate testicular functions. The present study aimed to investigate the expression of melatonin receptors (MT1 and MT2) in bovine Sertoli cells (SCs), using reverse transcription polymerase chain reaction (RT-PCR) and western blot. In addition, we assessed the mRNA levels of spermatogenesis-related genes (real-time PCR) and secretion of inhibin B after treatment with various concentrations (0, 80, 160, and 320 pg/mL) of melatonin at different time points (24, 48, or 72 h). We found that bovine SCs express MT1 and MT2 receptors, which were regulated by melatonin in time- and dose-dependent manners after treatment with melatonin. Exogenous melatonin up-regulated the expression of spermatogenesis-related genes, including *Cyclin D1*, *Cyclin E*, *Pdgfa*, *Dhh*, *Occludin*, and *Claudin*, and decreased the mRNA levels of *P21* and *Kit1* in a time or dose-dependent manner. Meanwhile, melatonin supplementation significantly affected *Inhba*, *Inhbb* and *Inha mRNA* expression. These findings were consistent with inhibin B levels detected in the culture medium. In conclusion, exogenous melatonin acts via its receptors and appears to play regulatory roles in the development and function of bovine SCs.

1. Introduction

Sertoli cells play a pivotal role in the regulation and maintenance of spermatogenesis, provide physical support to germ cells, form the blood–testis barrier, and secrete protein products thought to be essential for spermatogenesis (Maran et al., 1999; Holsberger and Cooke, 2005; Johnson et al., 2008). In addition, these cells constitute the site of action of all hormonal influences modulating testis development (Jannini et al., 1995; Alves et al., 2013).

Melatonin is a derivative of tryptophan, an amino acid primarily generated in the pineal gland and secreted in a circadian and seasonal rhythmic manner (Brainard et al., 1982; Cozzi et al., 1991). Melatonin mediates its physiological functions through melatonin receptor-mediated G protein signal transduction pathways. To date, three subtypes of high-affinity melatonin receptors have been identified in vertebrates (Barrett et al., 2003); MT1 and MT2 subtypes are present in all vertebrates, whereas, the Mel1c subtype is only found in non-mammalian species. Both melatonin receptors MT1 and MT2 are G protein

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coupled receptors and were reported in rat, mouse, and human testis (Liu et al., 2009; Vargas et al., 2011; Zhang et al., 2012; Chabra et al., 2013).

In females, previous studies have shown that melatonin is involved in multiple physiological processes, including ovarian maturation (Adriaens et al., 2006; Berlinguer et al., 2009) and embryonic development (Papis et al., 2007; Tian et al., 2010; Vázquez et al., 2013). Melatonin acts directly on the ovary (Berlinguer et al., 2009), with direct effects on granulosa cell (GC) steroidogenesis and regulation of follicular functions in several species (Misztal et al., 2002; Adriaens et al., 2006; DeNicolo et al., 2008; Kang et al., 2009). In males, Frungieri et al. (2005) demonstrated that melatonin acts as a local inhibitor of human chorionic gonadotropin-stimulated cAMP and androgen production. In mice, melatonin attenuates the testicular damage and germ cell apoptosis induced by hyperlipidaemia (Zhang et al., 2012), cyclophosphamide (Chabra et al., 2013), or carbon ions (Liu et al., 2009). Furthermore, Heindel et al. (1984) found that melatonin injection resulted in the restoration of SC responsiveness to FSH, and this effect was reversed by pinealectomy in hamsters. Taken together, these findings describe the important role of melatonin in testis, in particular its involvement in the physiological regulation of SCs.

In the present study, we used an in-vitro model of bovine SCs to determine the role of melatonin in the regulation of bovine SC development and expression of spermatogenesis-related genes. We found that exogenous melatonin acts via its receptors to modulate development and function of bovine SCs. Our findings provide a basis for studying the mechanisms underlying SC development and spermatogenesis, in order to improve current methods of sperm production.

2. Materials and methods

2.1. Primary Sertoli cell isolation and culture

The differential adherent selection method was used to isolate bovine primary Sertoli cells as described by Teng et al. (2005) and Zheng et al. (2013), with minor modifications. Briefly, Sertoli cells were prepared from testis of 6 week-old Holstein calves ($n = 3$) washed three times in PBS. After tunica albuginea removal, organs were minced with a razor blade into small pieces. Then, the seminiferous tubules were transferred into new 60-mm petri dishes containing 4–5 mL of 1.0 mg/mL collagenaseIV/DNase solution (Sigma, USA), and incubated at 37 °C in a humid environment containing 5% CO_2 until the tubules were separated (about 45 min). After a washing step with PBS, the tubules were resuspended in PBS and further digested with 2.5 mg/mL trypsin for 15 to 20 min at 37 °C. After digestion, the mixture was passed through a 120 μm stainless mesh and washed with PBS. After decantation of the enzyme solution by centrifugation at 200 × *g* for 10 min, the cell pellet obtained was resuspended in DMEM/F12 containing 15% FBS and seeded onto plates pre-coated with 1% gelatin. Floating cells were slowly and gently aspirated after 2–4 h culture, fresh medium was added for culture at 37 °C in a humid environment containing 5% CO_2.

2.2. Sertoli cell treatment

For treatment, 1×10^6 SCs were cultured per well in 1 mL medium for 24 h. Afterwards, the culture medium was replaced with serum-free DMEM/F12 supplemented with penicillin (50 IU/mL), streptomycin (50 μg/mL), and fungizone (2.5 mg/mL) for 8 h. Then, cells were treated with melatonin (Sigma-Aldrich, Corp., St. Louis, MO, USA) at 0, 80, 160, and 320 pg/mL for 24, 48, and 72 h, respectively. At each time point, culture medium was collected and stored at −20 °C for further analysis. Treatments were carried out in triplicate in a total of three independent experiments.

2.3. Total RNA isolation and real-time PCR

Total RNA was isolated from uncultured and cultured cells using the RNAprep pure Cell/Bacteria Kit (Tiangen Biotech, Beijing, China) according to manufacturer's instructions, and treated with DNase I for 30 min to digest genomic DNA. First strand cDNA was synthesized using reverse transcription kit (Fermentas). Quantitative real-time PCR (ABI7500) was carried out using SYBR Green (Takara). For evaluation of relative gene expression, *β-actin* was used as internal control. Primers were designed with the Primer 5.0 software (Table 1). Each experiment was performed at least three times in duplicate. The relative mRNA expression levels from real-time PCR were calculated using

Table 1
Sequences of primer pairs and amplification conditions for real time PCR.

Gene	Primer sequences (5′–3′)	Annealing temperature (°C)
Dhh	GGAGGGTGACAAGAGGTTC GGAGGGTGACAAGAGGTTC	59
Kit1	GATAAGCGAGATGGTGGAAC CCTGGGTTCTGGGCTCTTAG	61
Pdgfa	AAGACCAGGACTGTCATTTACG TTGACGCTGCTCGTGTTGC	61
Cyclin D1	GCCCTCGGTGTCCTACTTCAA ACAGGAAGCGGTCCAGGTAGT	61
Cyclin E	GCTTATGTCACTGATGGTGCTTG GCCAGGAGATGACCGTTACAGGA	60
P21	CGTCTCAGGAGGACCACTT TCAGTCTGCGTTTGGAGTG	61
MT1	CAGCCTCAGATACGACAAGC GACGGACTGCGTGAAGGTA	61
MT2	GCAACCTCCTGGTCATCCT TGGCGGTGATGTTGAAGAC	61
Inha	GCACCCTCCCAGTTTCATCT GGTTGGGCACCATCTCATACT	61
Inhba	GCAGTCGCACAGACCTTTCCT CTCACAGTAGTTGGCGTGGTAGC	61
Inhbb	TCCAACGAAGGCAACCAGA TGGATGGGTTCGGTGAGTG	61
Occludin	GGTAACTTGGAGACGCTTTC GTTCTGCTTGTAGGCTCTTAT	60
Claudin	CTTCATCGGCAGCAGCATC CAGTAGGATGGCGATGACGA	60
β-actin	CACCAACTGGGACGACAT ATACAGGGACAGCACAGC	61