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Gene expression profiling of melatonin receptor subtypes in the ovarian hierarchical follicles of the Sichuan white goose

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ABSTRACT

Melatonin (*N*-acetyl-5-methoxytryptamine), an indole hormone, regulates a variety of important central and peripheral actions related to circadian rhythms and reproduction through its specific receptor subtypes (Mel-1a, Mel-1b and Mel-1c). However, the expression profile of melatonin receptor genes (*MTNR1A, MTNR1B* and *MTNR1C*) in ovarian hierarchical follicles of geese remains to be clarified. In this study, the expression level of melatonin receptors in small white follicle (SWF), small yellow follicle (SYF), the largest follicle (F1), second largest (F2), third largest (F3), fourth largest (F4), fifth largest (F5), and postovulatory follicle (POF) in the Sichuan white goose were examined using quantitative real-time PCR (qRT-PCR). The results showed that the expression levels of *MTNR1A*, *MTNR1B* and *MTNR1C* initially increased and later decreased. The highest levels of gene expression of these receptor subtypes were observed in F5 or F4 in all examined follicles. Furthermore, the expression of *MTNR1A* and *MTNR1B* mRNA was significantly greater in SYF compared with SWF ($P < 0.05$), but *MTNR1C* was absent in SWF. The expression of *MTNR1A*, *MTNR1B* and *MTNR1C* mRNA was significantly greater in F5 compared with SYF ($P < 0.05$), and the expression of *MTNR1A* and *MTNR1C* mRNA was higher in F1 compared with POF ($P < 0.05$). In addition, the oestrogen concentration in SWF, SYF, F4, F3, F2, F1 and POF was measured using ELISA. The oestrogen concentration and melatonin receptor expression both were initially observed to increase and subsequently decrease. The oestrogen concentration in F4 and F3 was highest in all examined samples and was 1318.2 pg/g and 1318.1 pg/g, respectively. These results suggest that the melatonin receptor may be involved in the activation of the SWF and SYF to allow the SWF and SYF to develop into the subsequent follicles. Furthermore, follicles and the expression of the melatonin receptors may be regulated by the secretion of the oestrogen.

1. Introduction

Melatonin, a multifunctional molecule is secreted in a pulsatile manner from the pineal gland and plays a major role in several circadian and seasonal processes including animal reproduction (Falcon et al., 2007; Renuka and Joshi, 2010; Ubuka et al., 2013). The biological action of melatonin is mediated via binding to G-protein-coupled receptors (Shiu and Pang, 1998; Carnevali et al., 2011). Depending on the molecular biology classification methodology that is employed, the melatonin receptors in birds can be classified into Mel-1a, Mel-1b, Mel-1c three subtypes (Yadav and Haldar, 2013), while mammalian melatonin receptors are grouped into MT1 and MT2. MT1 was known as Mel-1a or MTNR1A, and MT2 was known as Mel-1b or

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MTNR1B (Dubocovich et al., 2010; Slominski et al., 2012). Both belong to the family of G protein-coupled receptors. The existence of a third membrane-bound melatonin binding site (MT3 receptor) was theorised at one time (Dubocovich, 1995; Slominski et al., 2012). However, "MT3" was later characterised as the enzyme quinone reductase 2 (NQO2) and was not coupled to G proteins. Although the "MT3" receptor has not yet been found in humans, it is expressed in the hamster and the retina of rabbit (Nosjean et al., 2001; Pintor et al., 2001; Shi et al., 2013). In addition, there is a melatonin related receptor named G protein-coupled receptor 50 (GPR50). It is an orphan receptor with 45% amino acid homology to melatonin receptors (Batailler et al., 2012). Melatonin does not bind to GPR50, and the identity of its real ligand is unclear. However, GPR50 may heterodimerise with the MT1 receptor and inhibit its activity (Levoye et al., 2006). In chicken, three melatonin receptor subtypes (Mel-1a, Mel-1b and Mel-1c) with almost identical pharmacological profiles have been described in neural tissues (Natesan and Cassone, 2002). These receptor subtypes were shown to be differentially expressed in chicken ocular tissues and ovarian granulosa cells (Murayama et al., 1997; Adachi et al., 2002; Rada and Wiechmann, 2006). In humans, follicular melatonin is released from luteinising granular cells during late folliculogenesis and plays a positive role in oocyte maturation. Research shows that the administration of melatonin into medium in in vitro experiments improves cytoplasmic maturation of human immature oocytes (Kim et al., 2013). Melatonin can reduce the concentration of free radicals in the follicle, thereby reducing apoptosis in the follicular granular cell (Tamura et al., 2009; Rosen et al., 2012; Takada et al., 2012). In poultry, previous studies indicate that melatonin is a key regulator of GnIH synthesis and release. In the gonads of birds, melatonin and its receptors play an important role in regulating the viability and maturation of ovarian granular cells, gonadal steroidogenesis, folliculogenesis and maturation of oocytes (He et al., 2005; Chowdhury et al., 2013).

Melatonin plays an important role in regulation of avian reproduction. However, to the best of our knowledge, no studies have addressed the expression profile of melatonin receptors in geese ovarian follicles. The relationship between oestrogen concentration and melatonin receptors in the course of follicle development was unclear. Therefore, the experiment was conducted to determine the expression levels of *MTNR1A*, *MTNR1B* and *MTNR1C* in the ovarian hierarchical follicles of the geese during the egg-laying stage, as well as the oestrogen concentration in each follicle. These will provide the better understanding of melatonin action with respect to the process of geese follicular development.

2. Material and methods

2.1. Experimental geese and collection of samples

Three female Sichuan white geese were selected from a local breeding geese farm and raised according to the standard programme used at the farm. All of the geese were fed under uniform standard management in conditions of natural light and provided with free access to feed and water. The geese were sacrificed by electrical stunning followed by exsanguination. The ovaries along with the ovarian follicles were removed from experimental geese and immediately placed on ice. The follicles were separated from the ovary and weighed to identify/verify the largest (F1), second largest (F2), third largest (F3), fourth largest (F4), fifth largest (F5), small yellow follicles (SYF), small white follicles (SWF) and postovulatory follicles (POF). All of the preovulatory follicles were cut transversely along the stigma to completely eliminate the yolk material. The follicular membranes were washed with ice-cold sterile saline, ensuring that there was no adherent yolk material.

2.2. Determination of oestrogen concentration in each follicle

The oestrogen concentration was measured using the Goose Estradiol ELISA Kit (beyotiom, Shanghai, China). Following manufacturers' instructions, 0.1 g follicular samples were added to 0.5 mL normal saline and homogenated and then the supernatant was extracted. All standards and samples were added to the Microelisa Stripplate in three replicates for each sample. For the set standard wells and the testing sample wells, 50 μL standard was added to the standard well and 10 μL testing sample was added to the testing sample wells. Next, 40 μL Sample Diluent was added to the testing ample wells; nothing was added to the Blank wells. Then, 100 μL of HRP-conjugate reagent was added to each well, which was covered with an adhesive strip and incubated for 60 min at 37 °C. Each well was aspirated and washed, repeating the process four times for a total of five washes. Then, 50 μL of chromogen solution A and 50 μL of chromogen solution B were added to each well; the wells were gently mixed and incubated for 15 min at 37 °C, protected from light. Then 50 μL Stop Solution was added to each well, and the Optical Density (O.D.) at 450 nm was read within 15 min using a microtitre plate reader.

2.3. Total RNA extraction and reverse transcription PCR

Following manufacturers' instructions, total RNA was prepared from the geese hierarchical follicles with Trizol reagent (Takara Bio Inc, Dalian, China). Approximately, 20 mg of tissue samples were used for RNA isolation. The concentrations and purities of the RNA preparations were determined spectrophotometrically at OD_{260} versus OD_{280}. cDNA was synthesised using PrimeScript® RT reagent Kit (Takara Bio Inc, Dalian, China), according to the manufacturer's instructions. Briefly, the 20 μL reaction consisted of 4.0 μL of total RNA, 4.0 μL of 5 × PrimeScript® Buffer, 1.0 μL of PrimeScript® RT Enzyme Mix, 1.0 μL of Random 6 mers, 1.0 μL of oligo dT Primer and 9.0 μL of RNase Free H_2O. Thermal cycling was performed for 15 min at 37 °C, and then 5 s at 85 °C.

2.4. Construction of MTNR1A, MTNR1B, MTNR1C and GAPDH cDNA plasmid

The recombinant plasmids containing *MTNR1A*, *MTNR1B*, *MTNR1C* and glyceraldehyde-3-phosphate