Animal Reproduction Science 172 (2016) 164–172

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Melatonin modulates the functions of porcine granulosa cells via its membrane receptor MT2 *in vitro*

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ARTICLE INFO

Article history:
Received 25 February 2016
Received in revised form 11 July 2016
Accepted 24 July 2016
Available online 25 July 2016

Keywords:
Melatonin
Porcine
Granulosa cells
Apoptosis
Steroidogenesis

ABSTRACT

Melatonin (*N*-acetyl-5-methoxytryptamine) is documented as a hormone involved in the circadian regulation of physiological and neuroendocrine function in mammals. Herein, the effects of melatonin on the functions of porcine granulosa cells *in vitro* were investigated. Porcine granulosa cells were cultivated with variable concentrations of melatonin (0, 0.001, 0.01, 0.1, 1.0, and 10 ng/mL) for 48 h. Melatonin receptor agonist (IIK7) and antagonist (Luzindole, 4P-PDOT) were used to further examine the action of melatonin. The results showed optimum cell viability and colony-forming efficiency of porcine granulosa cells at 0.01 ng/mL melatonin for 48-h incubation period. The percentage of apoptotic granulosa cells was significantly reduced by 0.01 and 0.1 ng/mL melatonin within the 48-h incubation period as compared with the rest of the treatments. Estradiol biosynthesis was significantly stimulated by melatonin supplementation and suppressed for the progesterone secretion; the minimum ratio of progesterone to estradiol was 1.82 in 0.01 ng/mL melatonin treatment after 48 h of cultivation. Moreover, the expression of *BCL-2, CYP17A1*, *CYP19A1*, *SOD1*, and *GPX4* were up-regulated by 0.01 ng/mL melatonin or combined with IIK7, but decreased for the mRNA levels of *BAX*, *P53*, and *CASPASE-3*, as compared with control or groups treated with Luzindole or 4P-PDOT in the presence of melatonin. In conclusion, the study demonstrated that melatonin mediated proliferation, apoptosis, and steroidogenesis in porcine granulosa cells predominantly through the activation of melatonin receptor MT2 *in vitro*, which provided evidence of the beneficial role of melatonin as well as its functional mechanism in porcine granulosa cells *in vitro*.

1. Introduction

The follicle is the structural and functional unit of the mammalian ovary; it consists of an oocyte surrounded by cumulus cells, antral and mural granulosa cell layers adjoined to the follicular wall which is composed of basement membrane, and the theca interna and theca externa. Granulosa cells were demonstrated to play a crucial role in follicular steroidogenesis and, thus, are involved in establishing an essential microenvironment for the follicle-enclosed oocyte (Manabe et al., 2004).

Melatonin, a serotonin-derived hormone mainly secreted by the pineal gland, was demonstrated to possess strong free radical scavenging properties and could alter reproductive processes in a variety of species, including human, porcine, bovine, sheep, mouse, and rat (Arendt,

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http://dx.doi.org/10.1016/j.anireprosci.2016.07.015
0378-4320/

1986; Tamura et al., 2013; Reiter et al., 2014). Evidences indicated that melatonin was present in follicular fluid, and that the melatonin in follicles was synthesized by the oocytes, except that taken from blood circulation (Shi et al., 2009; El-Raey et al., 2011; Sakaguchi et al., 2013). In women, melatonin was suggested to be an efficient predictor of positive *in vitro* fertilization outcomes, melatonin and myo-inositol have been shown to synergistically enhance oocyte and embryo quality in patients with polycystic ovarian syndrome (Pacchiarotti et al., 2016). In mice, the fertilization rate and the development of *in vitro* fertilized embryos were significantly improved by melatonin in a dose-dependent pattern (Ishizuka et al., 2000). Melatonin acted as an antioxidant to protect the integrity of mouse granulosa cells in the pre-ovulatory follicle by reducing oxidative stress in nuclei, mitochondria, and plasma membranes (Tanabe et al., 2015). The development of vitrified-warmed mouse preantral follicles was promoted by melatonin supplementation, correlating with increased surviving follicles (Ganji et al., 2015). In caprid, the development of preantral follicles was promoted by melatonin in combination with follicle-stimulating hormone (FSH) (Rocha et al., 2013). Melatonin remarkably prevented the reduction of proliferation and increase in apoptosis rate caused by thermal stress in sheep granulosa cells (Fu et al., 2014). Melatonin interacted with maternal nutrient restriction could alter placental vascularity during late pregnancy and cell proliferation in ovine placenta (Eifert et al., 2015). In bovine, the *in vitro* development of embryo was also improved by the addition of melatonin through eliminating free radicals (Papis et al., 2007). In porcine, the *in vitro* maturation of immature oocytes and the development of parthenogenetic embryos were stimulated by melatonin supplementation (Shi et al., 2009). Melatonin not only improved the polar body rate of oocytes and the blastocyte rate of porcine parthenogenetic activation embryos, but also preserved the normal levels of steroid hormone, which is disrupted by heat stress (Li et al., 2015).

In most mammals, melatonin exerts its activities principally via two high-affinity, G-protein-coupled receptors, MT1 and MT2 (Dubocovich and Markowska, 2005). Previous research has shown the expression of MT1 and MT2 in follicular cumulus and mural granulosa cells and oocytes (Kang et al., 2009; Tian et al., 2014). The activation or inhibition of MT1 and MT2 is achievable by the use of potent and subtype-selective melatonin receptor agonists and antagonists, which consequently helps in the identification of several functional responses to melatonin. Specifically, IIK7 was widely used in various species including bovine oocytes (Tian et al., 2014) and rat cerebellar granule cells (Liu et al., 2014) to activate melatonin receptor MT1 and MT2; Luzindole (*N*-acetyl-2-benzyltryptamine) was used in rat ovaries (Soares et al., 2003), sheep granulosa cells (Fu et al., 2014), porcine coronary arteries (Tunstall et al., 2011), and human colostral mononuclear cells (Pires-Lapa et al., 2013) as a melatonin receptor antagonist; 4P-PDOT (4-phenyl-2-propionamidotetralin) was used in rat ovaries (Soares et al., 2003), human colostral mononuclear cells (Pires-Lapa et al., 2013), porcine coronary arteries (Tunstall et al., 2011) and various species as a selective antagonist of MT2 (Hardeland, 2010; Zlotos, 2012).

Considering the beneficial effect of melatonin on oocyte maturation, embryonic development, and the development of growing follicles as stated earlier (Ishizuka et al., 2000; Papis et al., 2007; Ganji et al., 2015; Li et al., 2015), there is still little evidence specifically reporting the effects of melatonin supplementation on porcine granulosa cells. Because of this, the present study was carried out using an *in vitro* model of porcine granulosa cells, so as to assess the potential effects of melatonin on porcine granulosa cells in cell viability, colony-forming efficiency, apoptosis, steroidogenesis, and antioxidant activities, and to investigate which subtype is responsible for these actions on porcine granulosa cells *in vitro*. The current study may provide new information to understand the beneficial role of melatonin in porcine granulosa cells.

2. Materials and methods

2.1. Chemicals

All reagents and chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2. Preparation of porcine granulosa cells

Porcine ovaries were collected from the local slaughterhouse and taken to the laboratory in PBS at 37 °C within 1 h. Healthy follicles of medium size (3–5 mm in diameter), which were identified as having an intact and well-vascularized follicular wall, clear follicular fluid, and neatly arranged granulosa cell layers, were dissected from the ovaries using scissors, according to the morphological criteria (Jolly et al., 1997; Lin and Rui, 2010).

After three washes, follicles were hemisected in pre-warmed DMEM/F12. Theca halves were agitated back and forth with a medicine dropper, and granulosa cells were gently scraped from the theca with a micro-spatula under a dissecting microscope, so that most of the antral, mural, and cumulus granulosa cells were scattered by this mechanical isolation. Consequently, the suspension, which contained porcine antral, mural, and cumulus granulosa cells, cumulus-oocyte complexes, and oocytes, was aspirated and filtered through a 400 mesh (38-μm) cell strainer; as a result, the cumulus-oocyte complexes and oocytes were filtered out, and the filtrate which containing granulosa cells was collected. Dispersed granulosa cells were pelleted by centrifugation at 300*g* for 8 min, and then resuspended with DMEM/F12 before centrifugation again at 300*g* for 8 min.

2.3. Culture of granulosa cells and experimental design

Porcine granulosa cells were incubated in a basic medium consisting of DMEM/F12 (Gibco BRL, Carlsbad, CA, USA) with 0.3% bovine serum albumin (BSA; Roche, USA), 0.23 mmol/L pyruvic acid, 3 mmol/L-glutamine, 10 mmol/L HEPES, 25 mmol/L sodium bicarbonate, 10 μg/mL transferrin, 100 μg/mL L-ascorbic acid, 2 mM hypoxanthine, 5 ng/mL selenium, 50 ng/mL insulin, 0.1 IU/mL FSH, and