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Synergistic effects of resveratrol and melatonin on *in vitro* maturation of porcine oocytes and subsequent embryo development

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ABSTRACT

Resveratrol and melatonin are known for their antioxidant properties and have various biological activities. The fact that they exhibit possible synergistic effects in phytomedicine researches suggests the use of a combination of these agents to promote porcine *in vitro* maturation (IVM) of oocytes. Therefore, we investigated the effects of resveratrol and/or melatonin on this process; cumulus-oocyte complexes underwent IVM culture with four different conditions (control, resveratrol, melatonin or their combination). Cumulus expansion, oocyte nuclear maturation and subsequent embryo development after parthenogenetic activation (PA) and somatic cell nuclear transfer (SCNT) were evaluated. In experiment 1, all treatment groups significantly increased the proportion of complete cumulus expansion (degree 4) compared to the control, showing no difference among the treatment groups ($P = 0.30$). In experiment 2, oocytes matured with resveratrol and the combination had significantly higher metaphase-II (MII) rates than the control and melatonin groups, showing the highest ($P < 0.05$) MII rates in the combination group. In experiment 3, all treatment groups significantly increased blastocyst formation rates and total blastocyst cell numbers after PA compared to the control, but especially the combination showed the highest ($P < 0.05$) total cell numbers. In experiment 4, we selected the combination as the optimal condition and used this IVM system prior to SCNT. The combination treatment showed a significant ($P < 0.05$) increase in blastocyst formation rate and total cell numbers after SCNT. In conclusion, our results suggest that the combination of resveratrol and melatonin supported a synergistic increase in oocyte nuclear maturation and total cell numbers of PA blastocysts and improved the development of SCNT embryos.

1. Introduction

Transgenic pigs are important animal models in biomedical research for studying xenotransplantation [1] and human diseases [2], since pigs shows several similarities to humans with regard to physiology, metabolism and genome organization [3]. Successful *in vitro* maturation (IVM) of oocytes is an essential step for production of transgenic animals through assisted reproductive techniques including somatic cell nuclear transfer (SCNT) [4]. However, the developmental ability of porcine oocytes matured *in vitro* still remains low compared with oocytes matured *in vivo* [5]. To overcome this limitation, many studies have been conducted to improve porcine IVM by supplementing antioxidants such as resveratrol [6,7] or melatonin [8–10].

Resveratrol (a naturally existing phytoalexin) and melatonin (a natural hormone synthesized by the mammalian pineal gland as well as a plant-derived product) are well-studied antioxidants [11] and exhibit many similar beneficial properties, including free radical scavenging [12], sirtuin 1 activation [13], anti-aging and stem cell protection [14]. Both resveratrol and melatonin are present in the Mediterranean diets such as red wine and olive oil, and they would have synergistic preventive effects compared to the diets that contain only a single compound [15]. This could contribute to potential benefits associated with Mediterranean dietary patterns in humans [16]. For these reasons, there have been several studies investigating possible synergistic effects of resveratrol and melatonin. These studies reported that co-treatment of resveratrol and melatonin synergistically exerts neuroprotective

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effects against neuronal injuries [16,17] and chemopreventive effects on rat mammary carcinogenesis [18].

Recently, we demonstrated that resveratrol [19] or melatonin [20] has a relationship with the sonic hedgehog (Shh) signaling pathway during porcine IVM. As expansion of the granulosa and cumulus cell layers, which is regulated by hedgehog signaling [21], is an important process for oocyte maturation [22], the fact that resveratrol or melatonin increases porcine cumulus expansion through the activation of Shh signaling suggests a potential benefit of these agents on porcine IVM and their possible synergism. Therefore, we hypothesized that resveratrol and melatonin could also have synergistic effects on porcine IVM.

Although some studies revealed that resveratrol and melatonin have synergistic effects in phytomedicine researches and each showed beneficial effects on IVM, the effects of combination of these agents on porcine IVM have not been evaluated. The aim of this study, therefore, was to investigate possible synergistic effects of resveratrol and melatonin on porcine IVM. In this study, we compared the effects of resveratrol, melatonin or their combination on cumulus expansion, oocyte nuclear maturation and subsequent embryonic development.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise stated.

2.2. Oocyte recovery and in vitro maturation

Porcine ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 3 h in physiological saline at 32–35 °C. Cumulus oocyte complexes (COCs) were aspirated from superficial ovarian follicles (3–6 mm in diameter) using an 18-gauge needle attached to a 10 mL disposable syringe and allowed to sediment in 50 mL conical tubes at 37 °C for 5 min. After the supernatant was discarded, the sediment was washed three times in washing medium comprising tissue culture medium (TCM)-199 (Invitrogen, Carlsbad, CA, USA) containing 5 mM sodium hydroxide, 2 mM sodium bicarbonate, 10 mM HEPES, 0.3% polyvinyl alcohol, and 1% Pen-Strep (Invitrogen). In each experimental group, approximately 50 COCs were transferred to IVM medium comprising TCM-199 supplemented with 10 ng/mL epidermal growth factor, 0.57 mM cysteine, 0.91 mM sodium pyruvate, 10 μL/mL insulin transferrin selenium solution (ITS-A) 100X (Invitrogen), 10% porcine follicular fluid, 10 IU/mL eCG, and 10 IU/mL hCG. The selected COCs were cultured at 39 °C in an incubator containing 5% CO_2. For the first 21–22 h, the COCs were cultured with the hormones and then switched to medium without hormones for another 21–22 h.

2.3. Cumulus expansion assessment

The degree of cumulus expansion was assessed by microscopic examination as described previously [23]. Briefly, a degree of 0 indicates no expansion, characterized by detachment of cumulus cells from the oocyte, leaving a partially or fully denuded oocyte. A degree of 1 indicates the minimum observable response with spherical and compacted cumulus cells around the oocyte. A degree of 2 indicates only the outermost layers of cumulus cells have expanded. A degree of 3 indicates all cell layers except the corona radiata expanded. A degree of 4 indicates the maximum degree of expansion including the corona radiata.

2.4. Assessment of nuclear maturation

After 42–44 h of IVM, COCs were denuded by gently pipetting with 0.1% hyaluronidase in Tyrode's albumin lactate pyruvate (TALP) medium and washed three times in TALP medium. The denuded oocytes were evaluated under a microscope (TE2000-S, Nikon Corp., Tokyo, Japan) and classified as immature (without first polar body extrusion), degenerate, or at metaphase-II (MII, with polar body extrusion).

2.5. Parthenogenetic activation (PA) of oocytes

Denuded oocytes were gradually equilibrated in activation medium consisting of 0.28 M mannitol, 0.5 mM HEPES, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$ and transferred into a chamber connected to a BTX Electro-Cell Manipulator 2001 (BTX Inc., San Diego, CA, USA). Oocytes were activated by a single direct current (DC) pulse of 1.5 kV/cm for 60 μs Then, electrically-activated oocytes were washed three times in fresh Porcine Zygote Medium-5 (PZM-5) (Funakoshi Corporation, Tokyo, Japan), transferred into wells containing 500 μL PZM-5 and cultured at 39 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 7 days.

2.6. Somatic cell nuclear transfer (SCNT)

SCNT was performed as described in a previous report [7]. In brief, *in vitro* matured oocytes were enucleated by aspirating the first polar body and the adjacent cytoplasm containing chromosomes with an aspiration pipette. Then, using a fine pipette, a trypsinized porcine fetal fibroblast was transferred into the perivitelline space of each enucleated oocyte. These couplets were electrically fused with a single DC pulse of 200 V/mm for 30 μs using an electro cell fusion generator (LF101; Nepa Gene Co., Japan). Then, 30 min after fusion, fused couplets were activated with a single DC pulse of 1.5 kV/cm for 60 μs using a BTX Electro-Cell Manipulator 2001 (BTX Inc.). The resulting activated embryos were cultured in PZM-5 at 39 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 7 days.

2.7. Embryo evaluation and total cell count

The day of PA and SCNT was considered Day 0. Cleavage and blastocyst formation were evaluated on Day 2 (48 h) and on Day 7 (168 h), respectively. To count the total cell numbers of blastocysts, Day 7 blastocysts were washed in TALP medium and then stained with 5 μg/mL Hoechst-33342 for 10 min. After a final wash in TALP medium, stained blastocysts were mounted on glass slides in a drop of 100% glycerol, gently flattened with a cover glass, and observed under a fluorescence microscope (Nikon Corp.) at 400 × magnification.

2.8. Gene expression analysis by real-time PCR

Each real-time PCR was performed with isolated mature oocytes and with PA- and SCNT-derived blastocysts. A total number of 200 oocytes or 10 blastocysts were used in each batch for total RNA extraction. Total RNA was extracted using the easy-spin Total RNA Extraction Kit (iNtRON, Seoul, Korea) and complementary DNA (cDNA) was synthesized using the RNA to cDNA EcoDry Premix, cDNA synthesis kit (Clontech Laboratories Inc., Mountain View, CA, USA). The following were placed in a MicroAmp optical 96-well reaction plate (Applied Biosystems, Singapore): 1 μL cDNA, 0.4 μL (10 pmol/μL) forward primer, 0.4 μL (10 pmol/μL) reverse primer, 10 μL SYBR Premix Ex Taq (Takara, Otsu, Japan), and 8.2 μL of Nuclease-free water (Ambion, Austin, TX, USA). For each sample, at