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Melatonin enhances porcine embryo development via the Nrf2/ARE signaling pathway

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Abstract

Oxidative stress (OS) is a major problem during *in vitro* culture of embryos. Numerous studies have shown that melatonin, which is known to have antioxidant properties, prevents the occurrence of OS in embryos. However, the molecular mechanisms by which melatonin prevents OS in embryos are still unclear. The present study suggests a possible involvement of the nuclear factor erythroid 2-related factor 2/antioxidant-responsive element (Nrf2/ARE) signaling pathway, which is one of the prominent signals for OS prevention through Nrf2 activation, connecting melatonin, OS prevention and porcine embryonic development. The aim of this study was to investigate the effects of melatonin (10^{-7} M) on porcine embryonic development *via* the Nrf2/ARE signaling pathway; brusatol (50 nM; Nrf2 specific inhibitor) was used to validate the mechanism. Treatment of porcine embryo with melatonin significantly increased formation rates of blastocysts and their total cell numbers and also upregulated the expression of Nrf2/ARE signaling and apoptosis-related genes (*MT2*, *NRF2*, *UCLH*, *HO-1*, *SOD1* and *BCL-2*). Furthermore, the expression of proteins (NRF2 and MT2) was also upregulated in the melatonin-treated group. Concomitantly, brusatol significantly inhibited these effects, upregulating the expression of *KEAP1* and *BAX*, including the expression level of KEAP1 protein. These results provide evidences that melatonin prevents OS through Nrf2/ARE signaling pathway in porcine *in vitro* fertilization -derived embryos.

Key Words

- ▶ antioxidant responsive elements
- ▶ blastocyst
- ▶ melatonin
- ▶ NF-E2-related factor 2
- ▶ oxidative stress

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Introduction

Melatonin (N-acetyl-5-methoxytryptamine), a natural hormone, is synthesized by the mammalian pineal gland and peripheral reproductive organs, as well as being a byproduct from plants (Stehle *et al.* 2011). As is widely known, the functions of melatonin include regulating the circadian rhythm (Stehle *et al.* 2011), steroidogenesis (Macphee *et al.* 1975) and mammalian

reproduction (Tamarkin *et al.* 1985). In addition, Reiter *et al.* postulated that, because melatonin was found to be a direct free radical scavenger, it stimulates antioxidative enzymes, thereby quenching free radicals (Reiter *et al.* 2016). Moreover, melatonin acts as an antioxidant *via* antioxidant-responsive element activities (Nguyen *et al.* 2009). Numerous studies have already demonstrated that

treatment with an optimal concentration of melatonin during *in vitro* production (IVP) of embryos critically improves the quality of oocytes and determines subsequent embryonic development in various mammalian species, including mice (Tamura *et al.* 2008), cattle (Wang *et al.* 2014) and pigs (Rodriguez-Orsorio *et al.* 2007, Jin *et al.* 2017, Lee *et al.* 2018). Although some studies identified intracellular mechanisms of melatonin action by evaluating oocyte maturation and embryo development, thereby improving IVP outcomes (Amin *et al.* 2014, Lee *et al.* 2017), it is still uncertain how this hormone works. Furthermore, no study has studied the enhancement of porcine embryonic development by melatonin *via* the nuclear factor erythroid 2-related factor 2/antioxidant-responsive element (Nrf2/ARE) signaling pathway.

Nuclear factor erythroid 2-related factor 2, also known as NFE2L2 or Nrf2, is a potential transcription factor that has antioxidative functions in cellular defense mechanisms (Lee & Johnson 2004, Wells 2015). Moreover, there are numerous studies on Nrf2 signaling pathway-related antioxidation mechanisms in oocytes and embryos (Amin *et al.* 2014, Hahn *et al.* 2015, Akino *et al.* 2018, Lin *et al.* 2018). From these studies, it is generally accepted that Nrf2 translocates from the cytoplasm to the nucleus, binds to ARE sites, and then upregulates various enzymes responsible for producing cytoprotective compounds and proteins such as superoxide dismutase-1 (SOD1) and heme oxygenase-1 (HO-1) (Magesh *et al.* 2012). Such compounds that are activated by Nrf2 are also responsible for antioxidative functions related to cellular defense mechanisms (Lee & Johnson 2004, Magesh *et al.* 2012, Wells 2015). Some factors that are related to the Nrf2/ARE signaling pathway regulate Nrf2 under numerous stimuli. Among these, Kelch-like ECH-associated protein 1 (KEAP1) is one of the fundamental factors. As a natural inhibitor of Nrf2, Keap1 in part mediates Nrf2 activity by repressing it, through ubiquitination that leads to proteasomal degradation. However, under oxidative stress (OS) conditions, Keap1 releases Nrf2 by altering its conformation, thereby activating it and initiating transcription (Nguyen *et al.* 2009, Kansanen *et al.* 2013, Ma 2013). However, this mechanism can be ceased by brusatol, a specific inhibitor of Nrf2. It is a quassinoid isolated from the fruit of *Brucea javanica* and found to reduce burdens of tumor, mitigate chemoresistance in *in vitro* and *in vivo* cancer models (Ren *et al.* 2011, Tao *et al.* 2014, Wu *et al.* 2015), and most importantly, specifically inhibit Nrf2 *via* keap1-dependent ubiquitination and proteasomal degradation, thereby promotes a rapid depletion of Nrf2 (Olayanju *et al.* 2015).

Several studies have demonstrated that melatonin plays a critical role in regulation of the Nrf2/ARE signaling pathway by modulating various mechanisms involved with OS *via* Nrf2 cascades (Tripathi & Jena 2010, Negi *et al.* 2011, Chen *et al.* 2017). Although some studies have associated the Nrf2/ARE signaling pathway with embryonic developments (Amin *et al.* 2014, Ma *et al.* 2017, Lin *et al.* 2018), further studies of their relationship are still needed including porcine species. Therefore, the aim of this study was to investigate if there is a direct impact and activation of Nrf2 transcripts by melatonin *via* the Nrf2/ARE signaling pathway in *in vitro* fertilization (IVF)-derived porcine preimplantation embryos.

Materials and methods

Animals and chemicals

The pig ovaries used in this study were obtained from a slaughter facility. They were not required to be screened by the Institutional Animal Care and Use Committee (IACUC) as the use of these ovaries are not regarded as an animal experiment in our university. All reagents and chemicals used in this study were obtained from Sigma-Aldrich Chemical Company, unless otherwise indicated.

Chemical preparations

Melatonin (Cat No. M5250) and brusatol (Cat No. SML1886) were purchased from Sigma-Aldrich and shipped as a powder form. DMSO was used as a solvent for melatonin and brusatol. Melatonin was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution at the concentration of 10^{-2} M, then diluted 10^{-3} , 10^{-5} , 10^{-7} , and 10^{-9} M and brusatol at the concentration of 800nM, and then diluted 50, 200, and 400nM. Each concentration was treated in porcine zygote medium-5 (PZM-5; Funakoshi Corporation, Tokyo, Japan) and for the exact comparison, the same amount of DMSO was treated in the control groups.

Oocyte recovery and *in vitro* maturation (IVM)

Porcine ovaries were obtained from prepubertal gilts at a local abattoir and sent to the laboratory. Cumulus-oocyte complexes (COCs) were retrieved by aspiration of 3–8mm sized follicles with an 18-gauge needle fitted on a disposable 10mL syringe, then washed two times in washing medium comprising 9.5g/L of medium-199 (M-199; Gibco), 2mM sodium bicarbonate, 0.3% polyvinyl alcohol (PVA), 5mM sodium hydroxide, 10mM N-piperazine-N'-[2-ethanesulfonic acid], and

1% penicillin-streptomycin (Invitrogen). COCs with homogenous cytoplasm and three or more layers of cumulus cells were carefully selected under a stereomicroscope. They were cultured in *in vitro* maturation (IVM) medium comprising medium (M-199; Invitrogen), 0.91 mM sodium pyruvate, 10 μ L/mL insulin-transferrin-selenium solution 100 $\times$ (Invitrogen), 10 ng/mL epidermal growth factor, 0.57 mM cysteine, 10 IU/mL human chorionic gonadotropin, 10 IU/mL equine chorionic gonadotropin, and 10% porcine follicular fluid (vol/vol). The COCs were incubated at 39°C under 5% CO₂ in 95% humidified air. After 20–22 h of maturation culture with hormones, the COCs were washed in hormone-free IVM medium, and then cultured again in IVM medium without hormones for additional 20–22 h.

In vitro fertilization (IVF)

After 44 h of IVM, matured oocytes were denuded with 0.1% hyaluronidase by gently pipetting and washed in Tyrode's albumin lactate pyruvate (TALP) medium. Then, oocytes that had homogeneous cytoplasm and clear membranes with polar bodies were selected and moved to 40 μ L of pre-incubated modified Tris-buffered medium (mTBM) drops, consisting of 113.10 mM NaCl, 3 mM KCl, 7.50 mM CaCl₂, 11 mM glucose, 1 mM caffeine, 0.57 mM L-cysteine, 20 mM Tris, and 8% bovine serum albumin (BSA) (w/v) in a 60 $\times$ 10 mm Petri dish (Falcon; Becton Dickinson Labware). Ejaculated spermatozoa were obtained from DARBY Pig Breeding Co. (Anseong, Korea). The semen was centrifuged at 1000g for 2 min, and the sperm pellet was resuspended with prewarmed porcine semen extender (Navibiotech, Cheonan, Korea). Then, the spermatozoa were centrifuged again at 1000g for 2 min. Immediately before the next process, sperm motility was evaluated and >90% motile spermatozoa were used in each replication. Swim-up procedures were performed at 39°C in Sperm- Tyrode's Albumin Lactate Pyruvate (Sp-TALP) medium, and then spermatozoa were injected into mTBM droplets at a final concentration of 5 $\times$ 10⁵ cells/mL. After a 6-h coincubation of oocytes and spermatozoa, zygotes with second polar bodies were washed in mTBM droplets by gently pipetting, then transferred to 40 μ L droplets of PZM-5, covered with prewarmed mineral oil, and cultured at 39°C in a humidified atmosphere of 5% O₂, 5% CO₂, and 90% N₂ for 7 days.

Embryo evaluation and total cell count after IVF

The day of IVF, when presumptive zygotes were transferred to *in vitro* culture (IVC) medium, PZM-5, was considered

Day 0. Evenly cleaved embryos were monitored under a stereomicroscope on Day 2 (48 h). Blastocyst formation was evaluated on Day 7 (168 h) after IVF and total cell numbers were counted. Zona pellucida (ZP) digestion was performed with 0.5% pronase to remove remaining attached spermatozoa. After washing in TALP medium, zona-free blastocysts were stained with 5 μ g/mL of Hoechst 33342 for 8 min. After a final wash in TALP medium, stained blastocysts were mounted on glass slides in 100% glycerol drops, compressed with a cover slip, and observed under a fluorescence microscope (Nikon Corp.) at 400 $\times$ magnification.

Immunofluorescence staining

Indirect immunofluorescence staining was performed in order to evaluate and compare the expression levels of NRF2, KEAP1, and MT2 among treatment groups after IVF. IVF porcine blastocysts were selected and ZP was removed with 0.5% pronase in order to remove remaining attached spermatozoa. Then, washed in PBS containing 1% PVA, then, fixed with 4% paraformaldehyde (w/v) in phosphate buffered saline (PBS) for at least 2 h, permeabilized with 1% Triton X-100 (v/v) in distilled water (DW) for 1 h at 39°C, washed four times in 1% PVA in DW, and incubated in DW containing 2% BSA for 2 h in order to block non-specific sites. Then, blastocysts were directly transferred into 2% BSA containing primary antibody for Nrf2 (1:200; 70R-50116; Fitzgerald Industries International, Acton, MA, USA), Keap1 (4 μ L/mL; ab218815; Abcam), and MT2 (1:200; ARP64072_P050; Aviva Systems Biology, San Diego, CA, USA) and incubated at 4°C, overnight. Subsequently, they were washed three times in PBS with 1% PVA and incubated with a secondary fluorescein isothiocyanate-conjugated anti-rabbit polyclonal antibody (1:200, ab6717, Abcam) at 37°C for 1.5 h in darkness. The blastocysts were washed three times in PBS with 1% PVA, and then counterstained with 5 μ g/mL Hoechst-33342 for 8 min. They were mounted on glass slides and observed under a fluorescence microscope. The fluorescence measurements were performed using ImageJ software (version 1.46r; National Institute of Health, USA) and at least ten blastocysts from each group were used for the staining.

Analysis of gene expression by quantitative real-time PCR

IVF-derived blastocysts of each groups on Day 7 were stored at –80°C until RNA was extracted. A total of 30

blastocysts from each group were used for RNA extraction by the RNAqueous™ Micro Kit (Invitrogen). The total RNA was quantified on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific), and then immediately used for synthesizing cDNA using amfiRivert cDNA synthesis Platinum Master Mix 0 (genDEPOT, Houston, TX, USA) according to the manufacturer's protocol. For quantitative real-time PCR, each reaction mixture contained 1 µL cDNA, 0.4 µL (10 pmol/µL) forward primer, 0.4 µL (10 pmol/mL) reverse primer, 10 µL SYBR Premix Ex Taq (Takara), and 8.2 µL of nuclease-free water in a PCR plate (Micro-Amp Optical 96-Well Reaction Plate, Applied Biosystems). The mixture was amplified using the Applied Biosystems StepOne™ Real-Time PCR Systems (Applied Biosystems). For each sample, at least three replications were done in a plate. Forty cycles of reactions were performed with the following parameters: denaturation for 15 s at 95°C, annealing for 1 min at 60°C and extension for 1 min at 72°C. The sequences of primers used in this study are listed in Table 1. The expression of each target gene was quantified relative to that of the endogenous control gene (*GAPDH*) (Lee *et al.* 2017). Genes of interest were verified and considered as available data with three categories: (1) Efficiency (%) = $(10^{-1/\text{slope}} - 1) \times 100$, (2) C_T values ranged from >8 to <35, (3) R^2 value >0.98. The relative expression of each target gene was calculated using the following equation (Jin *et al.* 2017):

$$R = 2^{-[\Delta Ct \text{ sample} - \Delta Ct \text{ control}]}$$

Statistical analysis

Each experiment was performed at least in triplicates. Statistical analysis was performed using GraphPad Prism 5.01 (PRISM 5, GraphPad Software, Inc.). To determine the significant differences among experimental groups, data were expressed as the mean $\pm$ S.E.M. and analyzed using univariate analysis variance with Tukey's multiple

comparison test. P values <0.05 were considered to be significantly different among the treatments.

Results

Effect of melatonin treatment during IVC

In the first experiment, several concentrations (0, 10^{-3} , 10^{-5} , 10^{-7} and 10^{-9} M) of melatonin were included during IVC to determine the optimal concentration. Melatonin treatment showed no significant differences on cleavage rate among the groups. However, there were significant differences in blastocyst formation rate and total cell numbers. Treatment with 10^{-7} M melatonin significantly increased the porcine blastocyst formation rate compared to the control, 10^{-3} , 10^{-5} and 10^{-9} M melatonin concentrations (29.5 vs 15.7%, 11.0, 18.3, and 16.9%, respectively, $P < 0.05$, Table 2). Total cell numbers of blastocysts were also significantly increased when embryos were treated with 10^{-5} , 10^{-7} and 10^{-9} M melatonin compared to control and the 10^{-3} M melatonin (84.3, 83.3, 87.0 vs 65.0 and 44.0, $P < 0.05$, Table 2). However, 10^{-3} M melatonin group showed the lowest number of cells in blastocysts among all groups ($P < 0.05$). Consequently, 10^{-7} M melatonin was chosen as the optimal concentration for subsequent experiments because it showed the highest blastocyst formation rate among the groups (Table 2).

The inhibitory effect of brusatol treatment during IVC

In the second experiment, the concentrations of brusatol used were 0, 50, 200 and 400 nM according to a previous study on brusatol treatment of mouse embryos (Lin *et al.* 2018). Treatment with brusatol significantly decreased every steps of the porcine embryo development – cleavage rate, blastocyst formation rate, and total cell number of

Table 1 Information on primer sequences for real-time PCR.

Genes	Primer sequences (5'–3')		Product size (bp)	Accession No.
	Forward	Reverse		
<i>GAPDH</i>	GTCGGTTGTGGATCTGACCT	TTGACGAAGTGGTCGTTGAG	207	NM_001206359
<i>MT2</i>	AGCTGCCTTAACGCCATCAT	ATTGTCGCCAGTCAGTGAG	219	XM_021063941.1
<i>Nrf2</i>	GCCAGTCTTCATTGCTCCT	AGCTCCTCCCAACTTGCTC	115	XM_013984303
<i>Keap1</i>	ACCCAATTCTGCCCTGAG	ACTTGACCTGCAGCGTAACA	214	NM_001114671
<i>UCHL1</i>	CCCTTCGCTTTATCCCCGTT	CGCTTATCTGCAGACCCAA	117	NM_213763
<i>SOD1</i>	TGACTGCTGGCAAAGATGGT	TTTCCACCTCTGCCAAGTC	133	NM_001190422
<i>HO-1</i>	ACCCAGGACACTAAGGACCA	CGGTTGCATTACAGGGTTG	227	NM_001004027
<i>Bax</i>	CATGAAGACAGGGCCCTTT	CATCCTCTGCAGCTCCATGT	181	XM_003127290
<i>Bcl-2</i>	AGGGCATTCACTGACCTGAC	CGATCCGACTCACCAATACC	193	NM_214285

Table 2 Effect of melatonin during IVC on embryonic development after IVF.

Chemicals (M)	No. of embryos cultured	No. of embryos developed to (mean $\pm$ S.E.M.*, %)		Total blastocyst cell number (mean $\pm$ S.E.M.*)
		≥ 2 cells	Blastocyst	
Control (0)	185	145 (76.67 $\pm$ 5.30)	22 (15.73 $\pm$ 1.39) ^a	65.00 $\pm$ 4.79 ^a
10 ⁻⁹	190	158 (83.07 $\pm$ 5.28)	32 (16.87 $\pm$ 2.28) ^a	87.00 $\pm$ 3.08 ^c
10 ⁻⁷	188	161 (84.03 $\pm$ 3.92)	56 (29.48 $\pm$ 1.66) ^b	83.25 $\pm$ 3.68 ^c
10 ⁻⁵	184	139 (76.33 $\pm$ 4.69)	31 (18.25 $\pm$ 3.72) ^a	84.25 $\pm$ 2.93 ^c
10 ⁻³	185	139 (74.40 $\pm$ 3.21)	20 (11.00 $\pm$ 0.80) ^a	44.00 $\pm$ 2.48 ^b

*Six replicates were carried out. ANOVA was used as the statistical method. ^{a,b,c}Values with different superscripts in the same column are significantly different ($P < 0.05$).

blastocyst. The 400 nM brusatol-treated group showed the lowest cleavage rate compared to control, 50 and 200 nM (73.7 vs 93.4%, 84.6, and 81.4%, respectively, $P < 0.05$, Table 3). Moreover, the effect of brusatol was evaluated on blastocyst formation rate, and all treatment groups (50, 200 and 400 nM) showed a significant decrease compared to control (5.6, 1.5 and 1.8 vs 14.8%, respectively, $P < 0.05$). Lastly, total cell numbers of blastocysts were significantly decreased in all brusatol-treated groups (50, 200 and 400 nM) compared to control (38.3, 29.3 and 28.7 vs 56.5, respectively, $P < 0.05$). Therefore, 50 nM brusatol was considered as the optimal concentration of inhibiting Nrf2 transcription factor for the subsequent experiment.

Inverse effects of melatonin and brusatol treatment during IVC

To our knowledge, melatonin and brusatol have opposite functions when regulating NRF2, therefore, to investigate the inverse effects of melatonin and brusatol, they were co-treated during IVC on subsequent embryonic development after IVF. The melatonin-treated group showed a significant increase in blastocyst formation rate compared to the control and brusatol groups (24.65 vs 15.55% and 10.13%, respectively, $P < 0.05$, Table 4). Moreover, embryos treated with melatonin showed a significant difference in blastocyst total cell number compared to the control, brusatol and co-treated groups (78.80 vs 52.60, 48.00 and 57.40, respectively, $P < 0.05$). However, there were no differences in cleavage rate. This

result demonstrates the inverse effects of melatonin and brusatol on blastocyst formation rate and a possible effect of restoration in the co-treated group (Table 4).

Effect of melatonin and brusatol treatment during IVC on gene expression in IVF-derived porcine blastocysts

The expression of genes shown in Table 1 was investigated in IVF-derived porcine blastocysts. Figure 1A shows gene expression levels related to the melatonin receptor and the Nrf2/ARE signaling pathway and melatonin significantly increased mRNA transcript levels of *MT2*, *Nrf2* and *UCHL1* with no expression difference of *Keap1* in porcine blastocysts compared to control ($P < 0.05$). Unlike in other groups, the co-treatment group showed the same increase of *UCHL1* as the melatonin-treated group and the highest increase in *MT2* transcript level. In the brusatol-treated group, expression of *Nrf2* gene was significantly downregulated and expression of *Keap1* upregulated compared to control. Additionally, mRNA transcript levels related to ARE were examined (Fig. 1A). The expression levels of *HO-1* and *SOD1* were significantly increased in the melatonin-treated group, and *SOD1* was decreased in the brusatol-treated group compared to control; however, no difference was observed in *HO-1* expression. Lastly, as shown in Fig. 1, the expression levels of *Bax* and *Bcl-2*, the apoptosis-related gene, were evaluated in porcine blastocysts. The mRNA transcript level of *Bax* was significantly increased in the brusatol-treated and

Table 3 Effect of brusatol during IVC on embryonic development after IVF.

Concentrations (nM)	No. of embryos cultured	No. of embryos developed to (mean $\pm$ S.E.M.*, %)		Total blastocyst cell number (mean $\pm$ S.E.M.*)
		≥ 2 cells	Blastocyst	
Control	177	165 (93.40 $\pm$ 1.29) ^a	24 (14.86 $\pm$ 1.59) ^a	56.50 $\pm$ 1.56 ^a
50	180	155 (84.58 $\pm$ 3.39) ^a	10 (5.56 $\pm$ 1.07) ^b	38.25 $\pm$ 2.18 ^b
200	181	149 (81.42 $\pm$ 3.72) ^a	3 (1.48 $\pm$ 0.92) ^b	29.33 $\pm$ 3.76 ^b
400	185	138 (73.70 $\pm$ 5.92) ^a	3 (1.80 $\pm$ 1.30) ^b	28.67 $\pm$ 1.45 ^b

*Five replicates were carried out. ANOVA was used as the statistical method. ^{a,b}Values with different superscripts in the same column are significantly different ($P < 0.05$).

Table 4 Co-treatment effects of melatonin and brusatol during IVC on embryonic development after IVF.

Chemicals	No. of embryos cultured	No. of embryos developed to (mean $\pm$ S.E.M. ^a , %)		Total blastocyst cell number (mean $\pm$ S.E.M.)
		≥ 2 cells	Blastocyst	
Control	187	163 (86.99 $\pm$ 2.52)	29 (15.55 $\pm$ 1.13) ^a	52.60 $\pm$ 4.73 ^a
Melatonin	183	162 (88.35 $\pm$ 2.83)	45 (24.65 $\pm$ 1.65) ^b	78.80 $\pm$ 3.04 ^b
Brusatol	197	157 (79.41 $\pm$ 3.25)	20 (10.13 $\pm$ 1.26) ^c	48.00 $\pm$ 2.21 ^a
Mtn + Bru	193	167 (86.42 $\pm$ 1.22)	29 (14.98 $\pm$ 1.09) ^{a,c}	57.40 $\pm$ 1.57 ^a

^aFive replicates were carried out. ANOVA was used as the statistical method. ^{a,b,c}Values with different superscripts in the same column are significantly different ($P < 0.05$). Mtn, 10^{-7} M melatonin; Bru, 50 nM brusatol. ^aANOVA.

co-treated groups compared to control ($P < 0.05$), although the co-treated group was significantly lower than the brusatol-treated group. Lastly, the expression level of *Bcl-2* was observed to be the highest in the melatonin and co-treated groups.

Assessment of MT2, Nrf2, and Keap1 levels by immunofluorescence staining

To elucidate the effects of these agents at the protein levels, we analyzed the presence of specific proteins related to the Nrf2/Keap1 signaling pathway in porcine blastocysts. In Fig. 2, MT2 was detected in all experimental groups and the highest intensity was observed in the melatonin-treated group compared to the control, brusatol and co-treated groups ($P < 0.05$). As shown in Fig. 3, there was a significant increase of Nrf2 protein intensity in melatonin-treated blastocysts compared to other groups ($P < 0.05$). Protein expression level of Keap1 was also examined by

immunocytochemistry (Fig. 4A). The intensity of the brusatol-treated and co-treated groups were significantly increased compared to the other groups ($P < 0.05$, Fig. 4B).

Discussion

For the first time, our study examined the role of the Nrf2/ARE signaling pathway in development of porcine preimplantation embryos by treating them with melatonin. Our results provide evidence that communications between melatonin and the Nrf2/ARE signaling pathway improved porcine embryo IVC, regulation of the Nrf2 cascade-related gene transcript levels, and subsequent protein expression in the blastocysts. Moreover, increased mRNA transcript levels were prevented by the Nrf2-specific inhibitor, brusatol. This implies that melatonin improves embryonic development and increases the expression of Nrf2/ARE signaling genes and protein expression levels in accordance with our hypothesis.

Figure 1

Effect of 10^{-7} M melatonin on expression of Nrf2/ARE signaling pathway-related genes in IVF-derived porcine blastocysts. (A) Nrf2/ARE signaling pathway-related genes (*MT2*, *Nrf2*, *Keap1*, *UCHL1*, *SOD1*, and *HO-1*). (B) Apoptosis-related genes (*Bax* and *Bcl-2*). Within the same mRNA, bars with different alphabetical letters are significantly different among the groups ($P < 0.05$). The experiment was replicated at least three times.

Figure 2

Immunocytochemical analysis of MT2 in porcine blastocysts. (A) Images of blastocysts were obtained by fluorescence microscopy. Representative *in vitro*-fertilized porcine blastocysts in each group were stained with MT2 and counterstained with Hoechst 33342. (B) Statistical analysis of 10^{-7} M melatonin, 50 nM brusatol, and co-treatment effects on MT2 protein expression in porcine blastocysts. Five replicates were carried out for this experiment. Data are shown as the means $\pm$ S.E.M. Groups marked with different alphabetical letters are significantly different ($P < 0.05$). Mtn, 10^{-7} M melatonin; Bru, 50 nM brusatol. Original magnification 200 $\times$. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0093>.

Previous studies suggesting that the role of melatonin during porcine embryo development is pivotal and optimized concentrations of melatonin were treated during IVC in order to prove its effects (Rodriguez-Osorio *et al.* 2007, Choi *et al.* 2008, Nakano *et al.* 2012, Do *et al.* 2015). Possible reasons for the inconsistencies might be different experimental methods, gas conditions during incubation or culture conditions such as media compounds and even differences within species (Rodriguez-Osorio *et al.* 2007, Nakano *et al.* 2012, Do *et al.* 2015). Therefore, we optimized the melatonin concentration to establish the appropriate concentration in our experimental environment. In our results, 10^{-7} M melatonin was considered to be the optimal concentration for subsequent experiments. This is consistent with the fact that melatonin is effective at antioxidation during porcine IVC (Rodriguez-Osorio *et al.*

Figure 3

Immunocytochemical analysis of nuclear factor erythroid 2-related factor 2 in porcine blastocysts. (A) Representative *in vitro*-fertilized porcine blastocysts in each group were stained with nuclear factor erythroid 2-related factor 2 and counterstained with Hoechst 33342. (B) Statistical analysis of 10^{-7} M melatonin, 50 nM brusatol, and co-treatment effects on nuclear factor erythroid 2-related factor 2 protein expression in porcine blastocysts. Six replicates were carried out for this experiment. Data are shown as the means $\pm$ S.E.M. Groups marked with different alphabetical letters are significantly different ($P < 0.05$). Mtn, 10^{-7} M melatonin; Bru, 50 nM brusatol. Original magnification 200 $\times$. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0093>.

2007, Choi *et al.* 2008, Nakano *et al.* 2012, Do *et al.* 2015). In contrast, 10^{-3} M melatonin decreased the total cell number of blastocysts compared to control (Table 2) and this, being consistent with a previous study, supports the idea that 10^{-3} M melatonin treatment during IVC of IVF-derived porcine embryos may be detrimental for embryo development partially due to high concentration-induced toxicity (Rodriguez-Osorio *et al.* 2007).

Positive effects of melatonin treatment during IVM have also been reported: 10^{-9} M melatonin was the most effective concentration for oocyte maturation, cumulus cell expansion, lipid content and even subsequent embryonic development *via* several signaling pathways such as sonic hedgehog signaling and the pentose phosphate pathway (Shi *et al.* 2009, Alvarez *et al.* 2013, Jin *et al.* 2017, Lee *et al.* 2017, 2018). On the other hand,

Figure 4

Immunocytochemical analysis of Kelch-like ECH-associated protein 1 in porcine blastocysts. (A) Representative *in vitro* fertilized porcine blastocysts in each group were stained with Kelch-like ECH-associated protein 1 and counterstained with Hoechst 33342. (B) Statistical analysis of 10^{-7} M melatonin, 50 nM brusatol, and co-treatment effects on Kelch-like ECH-associated protein expression in porcine blastocysts. Five replicates were carried out for this experiment. Data are shown as the means $\pm$ S.E.M. Groups marked with different alphabetical letters are significantly different ($P < 0.05$). Mtn, 10^{-7} M melatonin; Bru, 50 nM brusatol. Original magnification 200 $\times$. A full colour version of this figure is available at <https://doi.org/10.1530/JME-19-0093>.

investigations on modulation of specific pathways by melatonin during porcine IVC still need further details and clarification. Nrf2 is generally accepted as having crucial roles in upregulating the expressions of cytoprotective enzymes, cellular antioxidant defense system, and reproduction processes. (Leung *et al.* 2003, Lee & Johnson 2004, Hu *et al.* 2006, Wells 2015, Akino *et al.* 2018). When activated, it in turn activates AREs to prevent OS (Nguyen *et al.* 2009, Wells 2015, Akino *et al.* 2018). In addition, blastocyst formation is a critical indicator for the efficiency of embryo development and culture conditions (Nomura *et al.* 2007) and the total cell number of blastocysts is a standard criterion for evaluating the quality of embryos (Knijn *et al.* 2003). Consequently, our results showed that melatonin was effective on increasing porcine embryonic

development and regulated Nrf2 and its related genes (Keap1, UCHL1, SOD1, and HO-1) in porcine blastocysts in our experiment. Our results may support the fact that melatonin activates the Nrf2/ARE signaling pathway as demonstrated in previous studies (Tripathi & Jena 2010, Negi *et al.* 2011, Chen *et al.* 2017, Guo *et al.* 2017).

Here, brusatol, the Nrf2-specific inhibitor, was applied to our study to specify whether melatonin truly functions as an antioxidant by regulating the Nrf2/ARE signaling pathway. Brusatol inhibits Nrf2 directly and specifically in many types of cells and oocytes by inducing a rapid depletion of Nrf2 (Olayanju *et al.* 2015, Ma *et al.* 2017). Moreover, it is proved that brusatol with micromolar concentrations inhibits protein synthesis of Nrf2, and also it inhibits Nrf2 transcription specifically with nanomolar concentrations (Ren *et al.* 2011). In Table 3, the negative effect is clearly shown and also the result is supported by the study of Lin *et al.* which showed that 50 nM brusatol also had negative effects during IVC of mouse embryos (Lin *et al.* 2018). This supports our results shown in Fig. 1A and Table 3, and these results indicate that treatment with brusatol is detrimental for embryonic development in a dose-dependent manner. Therefore, our study demonstrated that brusatol inhibited the actions of Nrf2.

We hypothesized that melatonin would activate this pathway through melatonin receptor 2 (MT2), subsequently preventing OS during porcine IVP. At present, including MT2, other melatonin receptors are known such as MT1, in mammals, and MT3 in amphibians and birds (Reppert *et al.* 1996, Sugden *et al.* 2004). Moreover, they appear to be part of the superfamily of guanine nucleotide binding protein (G protein)-coupled receptors (GPCR) (Reppert 1997). It was suggested that the mechanism and abilities of melatonin to enhance the expansion of cumulus cells in COCs and oocytes independently and to enhance subsequent embryonic development is mediated by MT2 (Lee *et al.* 2018) which implies that functions of MT2 is pivotal for melatonin mechanism during embryonic development. In Figs 1A and 2, MT2 was also expressed in the control and brusatol-treated groups. This can be explained by the study of Pala *et al.* that MT2 bindings can be achieved through non-specific hydrophobic interactions (Pala *et al.* 2013); moreover, brusatol is suitable for binding with MT2 because it is also known to have poor aqueous affinity (Zhou *et al.* 2017). To sum up, we presume that IVF-derived porcine blastocysts took up melatonin *via* MT2, and a result the blastocyst formation rate and total cell number of blastocysts were increased.

In Fig. 3, our results may support the idea that melatonin is infused through MT2 into developing embryos, thereby activating AREs *via* the Nrf2/ARE signaling pathway. In particular, *HO-1* and *SOD1* are genes that are responsible for antioxidative mechanisms related to the Nrf2/ARE signaling pathway (Ma 2013, Akino *et al.* 2018, Lin *et al.* 2018). According to Fig. 1A, brusatol is closely related to *SOD1* and downregulates its function. Although HO-1 has a close relationship with the Nrf2/ARE signaling pathway, further studies of the possible cross-talk between brusatol and HO-1 are needed because opposite effects on HO-1 by brusatol have been reported in some studies (Xu *et al.* 2015, Liu *et al.* 2019). Therefore, we speculate that brusatol has no effect on HO-1 at least in porcine preimplantation blastocysts, but affects *Nrf2* and *SOD1* regulation. Connections between melatonin and the Nrf2/ARE pathway may partially be explained by the expression of *Nrf2*, because its expression level was inversely regulated in each treatments.

According to our results, because melatonin upregulated mRNA and protein expression level of NRF2, we next assumed that melatonin would also affect ubiquitination in Nrf2 and Keap1 interaction. Therefore, along with the proteasomal degradation, *UCHL1* can be a potential marker for deubiquitylation of enzymes from the proteasome system using ubiquitin because its decrease is related to decreased cell proliferation (Sanchez-Diaz *et al.* 2017). It is known that OS and apoptosis have mutual interactions (Kannan & Jain 2000) and also the antioxidative mechanism of melatonin is closely involved in apoptosis of cells and embryos (Zhao *et al.* 2016, Lan *et al.* 2018). Therefore, *Bcl-2* and *Bax*, apoptosis-related genes, were selected for analysis. Figure 1B demonstrates that the apoptosis-related genes are regulated by both melatonin and brusatol.

Ren and colleagues have stated that cell lines had different statuses of *Keap1*, and in part, brusatol promoted Nrf2 degradation in a Keap1-dependent manner depending on the types of cells (Ren *et al.* 2011). However, there are no studies on brusatol-Keap1 interaction in gametes or zygotes. Interestingly, as shown in Figs 1A and 4, the results may support the partial dependence of brusatol on *Keap1*. In brief, the actions of brusatol may depend directly on *Keap1* at least in porcine blastocysts in accordance with our result. However, the mechanism by which brusatol depends on *Keap1* warrants further investigation.

In this experiment, we hypothesized that through the uptake of melatonin by porcine embryos, melatonin directly activates and translocates *Nrf2*, and then AREs are produced. However, we applied brusatol (the *Nrf2* specific inhibitor) in order to verify that the activation

of *Nrf2* is truly affected by melatonin. We found that melatonin upregulated mRNA and protein expression level of MT2 in the porcine embryos (Figs 1A and 2) and also upregulated mRNA and protein expression level of Nrf2. Additionally, the inhibitory works of brusatol on *Nrf2* was also observed in the porcine embryos after the uptake of melatonin. Altogether, we suggest a plausible interaction between a direct impact of melatonin through the Nrf2/ARE signaling pathway on porcine preimplantation embryos during IVC. The MT2 receptor, as one of the GPCRs, communicates with numerous pathways that activate antioxidative responses (Han *et al.* 2017, Lee *et al.* 2017) and among them, the Nrf2/ARE signaling pathway could be one of the potential pathways for embryo researches. As this is the first research into the mechanism of the Nrf2/ARE signaling pathway in porcine IVF-derived preimplantation embryos, further studies should be initiated in order to elucidate this mechanism.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Eui Hyun Kim conceived the study, carried out the experiments, then performed the statistical analysis and drafted the manuscript. Geon A Kim, Curie Ahn, and Byeong Chun Lee assisted with designing the experiment, statistical analysis of the data and manuscript drafting. Anukul Taweechaipaisankul, Seok Hee Lee, and Muhammad Qasim assisted with implementation of the experiments. All co-authors revised the manuscript. Byeong Chun Lee supervised the research and supplied the funding. All authors read, revised, and approved the final manuscript.

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