pISSN 2466-1384 eISSN 2466-1392
大韓獸醫學會誌 (2017) 第 57 卷 第 2 號
Korean J Vet Res(2017) 57(2) : 89~95
https://doi.org/10.14405/kjvr.2017.57.2.89

<Original Article>

Oocyte maturation under a biophoton generator improves preimplantation development of pig embryos derived by parthenogenesis and somatic cell nuclear transfer

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(Received: January 24, 2017; Revised: March 24, 2017; Accepted: May 19, 2017)

Abstract: This study was conducted to determine the effects of biophoton treatment during *in vitro* maturation (IVM) and/or *in vitro* culture (IVC) on oocyte maturation and embryonic development in pigs. An apparatus capable of generating homogeneous biophoton energy emissions was placed in an incubator. Initially, immature pig oocytes were matured in the biophoton-equipped incubator in medium 199 supplemented with cysteine, epidermal growth factor, insulin, and gonadotrophic hormones for 22 h, after which they were matured in hormone-free medium for an additional 22 hr. Next, IVM oocytes were induced for parthenogenesis (PA) or provided as cytoplasts for somatic cell nuclear transfer (SCNT). Treatment of oocytes with biophoton energy during IVM did not improve cumulus cell expansion, nuclear maturation, intraoocyte glutathione content, or mitochondrial distribution of oocytes. However, biophoton-treated oocytes showed higher ($p < 0.05$) blastocyst formation after PA than that in untreated oocytes (50.7% *vs*. 42.7%). In an additional experiment, SCNT embryos produced from biophoton-treated oocytes showed a greater ($p < 0.05$) number of cells in blastocysts (52.6 *vs*. 43.9) than that in untreated oocytes. Taken together, our results demonstrate that biophoton treatment during IVM improves developmental competence of PA- and SCNT-derived embryos.

Keywords: biophoton, embryonic development, oocyte maturation, somatic cell nuclear transfer

Introduction

Alterations in embryo culture environments have been shown to influence embryonic development and the later outcomes of assisted reproductive technologies (ARTs) including *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) in livestock species. There has been an improvement in the developmental competence of embryos achieved from *in vitro* production (IVP) that can mostly be attributed to improved embryo culture systems based on improved knowledge of the *in vivo* environment, as well as the metabolic requirements of embryonic development. After extensive studies, many of the *in vitro* culture (IVC) conditions for IVP of mammalian embryos, such as composition of culture media, culture temperature, and atmospheric conditions, have been relatively standardized in both human and livestock species [1, 7, 12]. Nevertheless, developmental potential of IVC embryos is still much lower than that of those produced *in vivo*.

Biophotons are photons of light in the low visible and ultraviolet light range that are produced by a biological system. Several groups have investigated the effects or roles of biophoton emissions in live organisms, including mammalian cells in the last few decades. However, there is still little information available regarding the significance of the biophoton phenomenon. It is well known that organisms spontaneously emit ultraweak photons without any photoexcitation [4]. Typically, biological tissues produce a radiant emittance in the visible and ultraviolet frequencies [15]. Previous studies showed that photon emissions from human skin, mouse livers and fibroblasts are associated with the oxidative reaction of biomolecules [18]. In addition, leakage of a very small amount of photons from external sources has been shown to alter ultraweak photon emissions and cell-to-cell communica-

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tion [16]. However, no studies have investigated the relation between biophotons and mammalian germ cells to date.

In this study, an apparatus capable of generating energy homogeneous with a biophoton was placed in an incubator, after which immature pig cumulus-oocyte complexes (COCs) and embryos were cultured under this apparatus during IVM and/or IVC. The biophoton energy projector (BEP) was designed based on the effects of photons on live cells and their mutual relationship with resonance energy. The mechanism of the biophoton energy projector is based on biophoton theory. In contrast to conventional therapeutic methods employing light, the BEP is likely to have the potency of feasibility of *in vivo* effectiveness of ultraweak photons. In this study, we evaluated the effects of mechanical biophoton treatment on nuclear maturation and embryonic development after parthenogenesis (PA) and somatic cell nuclear transfer (SCNT) in pigs.

Materials and Methods

Culture media

All chemicals were obtained from Sigma-Aldrich (USA) unless specified otherwise. The base medium for IVM of oocytes in this study was medium-199 (M-199) added with 10% (v/v) pig follicular fluid, 0.91 mM pyruvate, 0.6 mM cysteine, 10 ng/mL epidermal growth factor, 1 μg/mL insulin, and 75 μg/mL kanamycin. The IVC medium for embryonic development was porcine zygote medium (PZM)-3 containing 0.3% (w/v) bovine serum albumin (BSA) [23], which was further supplemented with 0.34 mM tri-sodium citrate, 2.77 mM myo-inositol, and 10 μM β-mercaptoethanol [24].

Oocyte collection and IVM

Pig ovaries were collected at a local abattoir. Ovaries were washed two times in a warm physiological saline and transported to the laboratory. Follicular contents were aspirated from antral follicles (3–8 mm in diameter) in the ovaries. COCs that had more than three layers of compact cumulus cells were selected. COCs were washed three times in HEPES-buffered Tyrode's medium (TLH) containing 0.05% (w/v) polyvinyl alcohol (PVA) (TLH-PVA), placed into each well of a four-well multi-dish (Nunc, Denmark), and cultured in IVM medium with 80 μg/mL follicle-stimulating hormone (Antrin R-10; Kyoritsu Seiyaku, Japan) and 10 IU/mL human chorionic gonadotropin (Intervet International, the Nederlands) at 39°C under a humidified atmosphere of 5% CO_2 and 95% air as previously reported [11]. After 22 h of maturation culture, the COCs were washed in fresh hormone-free IVM medium, then further cultured for 22 h in a hormone-free medium.

Preparation of donor cells

Fetal fibroblasts were used as nuclei donors for SCNT. Cells were seeded into four-well dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) with the nutrient mixture F-12 (Invitrogen, USA) and 15% (v/v) fetal bovine serum until a complete monolayer of cells had formed. Donor cells were induced to synchronize at the G0/G1 stage of the cell cycle by contact inhibition for 72–96 h. On the day of SCNT, confluent cells were trypsinized to prepare a single-cell suspension, washed, and resuspended in TLH containing 0.4% (w/v) BSA (TLH-BSA).

SCNT and PA

After IVM, oocytes were freed from cumulus cells and incubated for 10–15 min in a calcium-free TLH-BSA (manipulation medium) supplemented with 5 μg/mL bisbenzimide (Hoechst 33342). Then, the oocytes were washed several times in a fresh manipulation medium and transferred into a droplet of manipulation medium containing 5 μg/mL cytochalasin B (CB) under warm mineral oil. For enucleation, the polar body and metaphase II (MII) chromosomes were removed using a 17-μm beveled glass pipette (ORIGO Humagen Pipets, USA). Enucleation was checked under an epifluorescence microscope (IX73; Olympus, Japan). A single cell was placed into the perivitelline space of each enucleated oocyte. Cell-cytoplast couplets were placed on a fusion electrode chamber (NepaGene) overlaid with 280 mM mannitol solution supplemented with 1 μM $CaCl_2$ and 50 μM $MgCl_2$, as previously described [21]. To induce fusion of cell-cytoplast couplets, an alternating-current field of 2 V cycling at 1 MHz for 2 sec and then two pulses of 170 V/mm direct current (DC) for 30 μsec were applied using a cell fusion generator (LF101; NepaGene, Japan). Then, oocytes were washed several times and incubated for 30 min in TLH-BSA, after which they were examined for fusion. Reconstructed oocytes were activated by applying two DC pulses of 120 V/mm for 60 μsec in a 280 mM mannitol solution with 100 μM $CaCl_2$ and 50 μM $MgCl_2$. For PA, the MII oocytes having polar bodies were electrically activated using a pulse sequence identical to that used for activation of SCNT oocytes.

Post-activation treatment and embryo culture

After electrical activation, the PA embryos were treated for 4 h with 5 μg/mL CB in IVC medium and the SCNT embryos were treated for 4 h in IVC medium with 0.4 μg/mL demecolcine and 1.9 mM 6-dimethylaminopurine. Then, SCNT and PA embryos were then washed properly in a fresh IVC medium, placed to 30-μL IVC droplets under mineral oil, and cultured for 7 days at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Embryonic development to the cleavage and the blastocyst stages were observed on Days 2 and 7, respectively (Day 0 was the day of SCNT or PA). Blastocysts developed on Day 7 were stained with bisbenzimide and then stained nuclei were counted under an epifluorescence microscope [11].

Measurement of oocyte diameter after IVM

To measure the size of IVM oocytes, images of MII oocytes were recorded using a digital camera (DS-L3; Nikon,

Japan) attached to an inverted microscope (TE-300; Nikon). The size of each part of oocytes was taken using image analysis software (ImageJ 1.46r; National Institutes of Health, USA) as reported previously [11].

Measurement of intraoocyte glutathione (GSH) content in IVM oocytes

GSH content in IVM oocytes was measured as previously described [8, 20]. Briefly, a total of 7–10 oocytes per replicate were treated for 30 min in TLH-PVA supplemented with 10 μM Cell Tracker CMF2HC (Invitrogen) in the dark. Then, treated oocytes were incubated for 30 min in PZM-3 at 39ºC. After incubation, oocytes were washed with D-PBS (Invitrogen) containing 0.1% (w/v) PVA, then placed into 2-μL droplets and observed for fluorescence using an epifluorescence microscope with a UV filter (370 nm). The fluorescence intensities of oocytes were analyzed with the ImageJ software and normalized to untreated control oocytes.

Observation of mitochondrial distribution

For the examination of mitochondrial distribution, MII-stage oocytes that had been untreated or treated with BEP during IVM were incubated for 15 min in PBS containing 0.1% (w/v) PVA supplemented with 10 μg/mL rhodamine-123 at 39ºC. After wash three times in TLH-PVA, 3-5 oocytes were placed into 2-μL droplets of D-PBS (Invitrogen) containing 0.1% (w/v) PVA, and the stained oocytes were observed under an epifluorescent microscope.

Blastocyst differential staining

Zona-intact blastocysts were stained with 5 μg/mL Hoechst 33342 for 1 h after rinsing in PZM-3 medium, the blastocysts were treated with 0.04% (v/v) Triton X-100 for 3 min and then with 0.005% (w/v) propidium iodide for 10 min. Stained blastocysts were mounted on glass slides and observed for fluorescence. The propidium iodide and Hoechst 33342-labeled trophectoderm nuclei appeared pink or red and bisbenzimide-labeled inner cell mass (ICM) nuclei appeared blue.

Experimental design

In Experiments 1 and 2, we investigated the effects of mechanical biophoton treatment during IVM of oocytes on nuclear maturation and embryonic development after PA (Experiment 1) and SCNT (Experiment 2). The effects of IVM culture under BEP (BEP-AN15; Biolight, Korea) on cumulus cell expansion, diameter of ooplasm, and intraoocyte GSH content in IVM oocytes were examined in Experiment 3. The effects of biophoton treatment during IVM on mitochondrial distribution in MII oocytes were examined in Experiment 4. In Experiments 5 and 6, oocytes were cultured under BEP during the IVM and/or IVC, and the effects of biophoton treatment on embryonic development and quality of PA blastocyst in terms of ICM and trophectoderm cell number were examined.

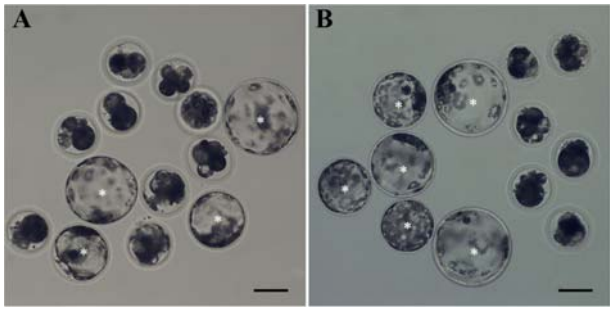

Fig. 1. Photomicrographs of blastocysts (asterisks) produced by parthenogenetic activation using oocytes that were untreated (A) and treated with biophoton (B) during *in vitro* maturation (IVM). Scale bars = 100 μm.

Statistical analysis

Statistical significance of biophoton treatment was examined using the Statistical Analysis System (ver. 9.4; SAS Institute, USA). Data were analyzed using a general linear model procedure, followed by the least-significant-difference mean separation procedure when the treatments differed at $p < 0.05$. The results are expressed as the means ± SEM.

Results

Experiment 1. Effects of biophoton treatment during IVM on embryonic development after PA

Biophoton-treated oocytes showed higher ($p < 0.05$) rates of blastocyst formation after PA (50.7% *vs*. 42.7%) than untreated oocytes (Fig. 1). However, no significant differences in the nuclear maturation of oocytes (88.7–90.9%), embryonic cleavage (97.1–97.2%) or mean cell number in blastocysts (34.6–35.5 cells/blastocyst) were observed between the untreated control and biophoton treatment groups (Table 1).

Experiment 2. Effects of biophoton treatment during IVM on embryonic development after SCNT

Biophoton-treated oocytes showed higher ($p < 0.05$) mean cell numbers in blastocysts after SCNT (52.6 *vs*. 43.9%) than untreated oocytes. However, embryonic development to the cleavage stage (82.3–87.5%) and blastocyst formation (29.2–37.4%) were influenced by biophoton treatment during IVM (Table 2).

Experiment 3. Cumulus cell expansion, diameter of ooplasm, and intraoocyte GSH content after biophoton treatment

As listed in Table 3, the cumulus cell expansion and diameter of ooplasm were not altered by the IVM culture under biophoton treatment (3.42–3.47; 114.7–115.6 μm, respectively). In addition, biophoton treatment did not influence intraoocyte GSH contents in MII oocytes compared to untreated oocytes (1.00–1.05 pixels/oocyte).

Table 1. Effects of biophoton treatment during IVM of oocytes on embryonic development after parthenogenesis (PA)

Biophoton treatment	% of oocytes that reached metaphase II	Number of PA oocytes cultured*	% of embryos developed to		Number of cells in blastocyst
			≥ 2-cells	Blastocyst	
No	88.7 ± 2.1	141	97.1 ± 0.1	42.7 ± 2.8[a]	34.6 ± 1.5
Yes	90.9 ± 1.5	142	97.2 ± 1.2	50.7 ± 1.4[b]	35.5 ± 1.3

*Four replicates. [a,b]Values in the same column with different superscript letters are different ($p < 0.05$).

Table 2. Effects of biophoton treatment during IVM of oocytes on embryonic development after somatic cell nuclear transfer (SCNT)

Biophoton treatment	% of oocytes that reached metaphase II	Membrane fusion (%)	Number of SCNT oocytes cultured*	% of embryos developed to		Number of cells in blastocyst
				≥ 2-cells	Blastocyst	
No	91.7 ± 1.8	77.4 ± 2.1	166	82.3 ± 2.1	29.2 ± 3.6	43.9 ± 3.3[a]
Yes	93.2 ± 1.8	77.4 ± 2.2	155	87.5 ± 5.6	37.4 ± 5.3	52.6 ± 2.8[b]

*Four replicates. [a,b]Values in the same column with different superscript letters are different ($p < 0.05$).

Table 3. Effects of biophoton treatment during IVM on cumulus cell expansion, diameter of ooplasm and intraoocyte glutathione (GSH) content

Biophoton treatment	Number of oocytes examined for cumulus cell expansion	Cumulus cell expansion score*	Number of oocytes examined for diameter and GSH	Diameter (μm) of ooplasm	Relative level (pixels/oocyte) of GSH
No	43	3.42	34	114.7 ± 0.5	1.00 ± 0.09
Yes	36	3.47	34	115.6 ± 0.7	1.05 ± 0.10

*Cumulus cell expansion was scored as 0 (no response), 1 (minimum observable response with the cells in the outermost layer of the cumulus becoming round and glistening), 2 (expansion of outer cumulus cell layers), 3 (expansion of all cumulus cell layers except the corona radiata), and 4 (expansion of all cumulus cell layers).

Table 4. Effects of biophoton treatment during IVM on mitochondrial distribution of pig oocytes

Biophoton treatment	Number of oocytes examined	Density of mitochondria	Number of oocytes showing normal distribution of mitochondria (%)*
No	60	1.00 ± 0.04	50 (83.3)
Yes	60	1.01 ± 0.05	48 (80.0)

*Normal distribution, intact mitochondria evenly distributed in the ooplasm of MII oocytes; abnormal distribution, mitochondrial reduced uneven distribution or mitochondrial broken distribution.

Experiment 4. Effects of biophoton treatment on mitochondrial distribution of MII-stage oocytes

As previously described [5], mitochondrial distribution was classified into two categories, normal and abnormal distribution. Intact mitochondria evenly distributed in the ooplasm of MII oocytes was considered normal distribution, while mitochondrial reduced uneven distribution or mitochondrial broken distribution was considered abnormal distribution. As shown in Table 4, the proportion of oocytes showing normal distribution of mitochondria in ooplasm (80.0–83.3%) did not differ significantly between the control and biophoton treatment groups.

Experiment 5. Effects of biophoton treatment during IVM and/or IVC on embryo development after PA

Biophoton-treated oocytes during IVM showed a higher ($p < 0.05$) blastocyst rate after PA compared to untreated oocytes (52.7 *vs*. 40.9%), but no significant differences in the rates of blastocyst formation (47.8–49.5%) were observed among biophoton-treated oocytes during IVC (Table 5). Biophoton-treated oocytes during IVM and IVC showed a higher ($p < 0.05$) number of cells in blastocysts than biophoton-treated oocytes during only IVM or only IVC (45.5 *vs*. 40.7–40.9).

Experiment 6. Effects of biophoton treatment during IVM and/or IVC on the number of ICM and trophectoderm cells in PA blastocysts

Incubation of PA-derived embryos with biophotons during IVM and/or IVC did not affect the ICM ratio. Additionally, biophoton-treated oocytes during IVM and/or IVC showed no significant differences in the number of inner cell mass nuclei (10.2–12.6), the number of TE nuclei (28.7–33.4), or

Table 5. Effect of biophoton treatment during IVM and *in vitro* culture (IVC) on embryonic development after PA

Biophoton treatment during		% of oocytes that reached MII	Number of PA oocytes cultured*	% of embryos developed to		Number of cells in blastocyst
IVM	IVC			≥ 2-cells	Blastocyst	
No	No	90.6 ± 2.2	161	91.8 ± 2.2	40.9 ± 1.9[a]	41.9 ± 1.9[ab]
No	Yes	91.1 ± 1.6	197	93.4 ± 1.7	49.5 ± 3.7[ab]	40.7 ± 1.7[a]
Yes	No	91.2 ± 2.9	195	91.8 ± 1.6	52.7 ± 2.9[b]	40.9 ± 1.4[a]
Yes	Yes	91.2 ± 2.9	196	95.2 ± 1.6	47.8 ± 3.5[ab]	45.5 ± 1.9[b]

*Five replicates. [a,b]Values in the same column with different superscript letters are different ($p < 0.05$).

Table 6. Number of inner cell mass and trophectoderm cells in PA blastocysts derived after treatment with biophotons during IVM and/or IVC

Biophoton treatment		Number of blastocysts examined	Cell number of blastocysts		Proportion of inner cell mass
IVM	IVC		Inner cell mass	Trophectoderm	
No	No	28	10.7 ± 1.2	29.9 ± 2.4	26.0 ± 1.7
No	Yes	30	11.7 ± 1.5	30.2 ± 2.3	28.2 ± 2.0
Yes	No	37	10.2 ± 0.9	28.7 ± 1.4	25.7 ± 1.5
Yes	Yes	34	12.6 ± 1.2	33.4 ± 3.0	27.6 ± 1.6

the proportion of ICM nuclei (25.7–28.2%) compared to the untreated control (Table 6).

Discussion

A variety of exogenous factors affecting oocyte maturation and embryonic development have been evaluated to obtain information necessary for improving an IVP system in mammalian species. However, the developmental competence of *in vitro*-produced embryos still remains low relative to their *in vivo* counterparts [3, 14]. Accordingly, improving the quality of IVM oocytes makes it possible to produce embryos with higher developmental competence and will greatly increase the efficiency of ARTs in terms of offspring production in livestock species.

In this study, we found that IVM of pig oocytes under the biophoton energy generator improved blastocyst formation of PA embryos and increased the embryonic cell number of SCNT blastocysts. Although the physiological mechanism of action was not clarified in this study, this improved developmental competence was most likely due to the altered IVM culture environments by an artificial treatment with ultraweak biophotons, which might be beneficial for later embryonic development. Several previous studies [4, 16] have demonstrated that a system exists for controlling cell-to-cell communication in various living organisms via electromagnetic or ultraweak photon emission, through which cell division and neutrophil activation has been stimulated and development of loach embryos has been altered. As previously reported [17], living cells or tissues of plants and animals radiate ultraweak biophotons, and it is possible to detect these photons using special equipment. Although biophotons are associated with the physiological status of cells and organs, the origin of the photon emissions remains unclear.

Over decades of research in this field, several studies in humans have led to progress in determining biophoton emissions in both basic and applied research. Boveris *et al.* [2] first demonstrated the potential usefulness of ultraweak photon detection as a non-invasive tool to monitor the oxidative processes in living cells. Furthermore, several studies have repeatedly illustrated that the intensity of photon emission changes in a state of a certain disease [9], and that diseased cells emit significantly more biophotons than healthy cells. Changes in biophotonic activity have also been shown to be indicative of the changes in mitochondrial ATP energy production that manifested under physiological and pathological conditions [22]. To the best of our knowledge, this study is the first study to apply a biophoton energy generator to the IVM and IVC of mammalian oocytes. In this study, several indicators of oocyte maturation were used to evaluate how artificially generated biophotons influence the developmental competence of oocytes and embryos in pigs. Evaluation of oocyte quality is traditionally based on morphological characteristics such as cumulus cell expansion, size of the perivitelline space, and size of oocytes after IVM [6, 11]. We evaluated the morphological features of oocytes as a non-invasive method to predict maturational and developmental competence of pig oocytes matured *in vitro*. Although artificial biophoton treatment during IVM of immature COCs did not alter cumulus cell expansion or ooplasm diameter after IVM, embryonic development after PA was significantly improved. It is not known why or how embryonic development was increased by the biophoton treatment despite the unaltered morphological criteria.

It is believed that the origin of photon emissions is related to cellular metabolic processes such as respiratory activity or

oxidative stress [10, 13], although the biochemical processes or associated molecules are not fully understood. Accordingly, mitochondrial status and distribution have been reported as useful indicators of oocyte competence and embryo quality. Thus, we investigated the possibility that biophotons might induce changes in biochemical properties such as intraoocyte GSH content and mitochondria distribution in IVM oocytes. However, treatment of oocytes with biophotons during IVM did not alter intraoocyte GSH contents in MII stage oocytes. In addition, no significant differences were observed in the mitochondrial distribution patterns of the non-treatment and biophoton groups in this study. Additional studies should be conducted to detect probable cellular or molecular changes that were not identified in this study.

Biophoton emission has been found to be associated with oxidative metabolic processes in the mitochondria such as free radical reactions with biomolecules, proteins, and DNA. These emissions have also been shown to be related to the generation of reactive oxygen species [19]. Although we could not find clear evidence of the effects of biophoton treatment on redox state in oocytes, biophoton emissions may be used as a tool for the control of oxidative stress on animal cells. In summary, our results demonstrate that biophoton treatment during IVM improves developmental competence of PA and SCNT embryos in pigs. To enable practical application of the IVC system using this biophoton energy generator, further studies are needed to identify the physiological mechanism by which biophoton treatment influenced the developmental ability of pig oocytes and embryos.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (grant No. 2015R1A2A2A01005490).

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