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Effect of phytohemagglutinin (PHA) on nuclear transfer pig embryos *in vitro*

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Abstract: This study evaluated the effects of the phytohemagglutinin (PHA) treatment on the developmental competence of parthenotes and somatic cell nuclear-transferred (NT) porcine embryos. In the first part of the experiment, the effects of PHA treatment of different durations (1 mg/ml; 0, 5, or 10 min) on parthenogenetically produced porcine embryos was examined. Although, PHA treatment did not affect the percentage of embryos that cleaved and developed into blastocysts, and the total number of cells per blastocyst, mitochondrial activity was higher (P<0.05) in PHA-treated oocytes. In the second part of the experiment, porcine NT embryos were treated in PHA for 5 min before electrofusion. The cleavage and blastocyst rates of NT embryos treated with PHA were higher (P<0.05). The expression of mitochondrial-related genes in NT embryos was demonstrated for the first time. The expression of ATPase6 and ND1 was higher (P<0.05) in PHA-treated embryos than in the other groups. In conclusion, our experiments clearly demonstrate that pre-fusion treatment in PHA (1 mg/ml; 5 min) improved the development of porcine NT embryos in vitro. The results suggest that PHA enhances embryonic mitochondrial activity, and other energy-related target gene expressions.

Key words: Lectin, Nuclear transfer, Oocytes, Embryos development, Mitochondrial activity

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Introduction

The advent of the somatic cell nuclear transfer (NT) technique heralded the production of large numbers of genetically identical animals called clones using various somatic cell types as donor cells [1]. Although cloned animals of various species have been produced by this technique, the method is still inefficient and its outcomes are species dependent. In the pig, compared to other domestic animal species, a very low cloning efficiency has been reported [2, 3]. Even though the pig stands out as an important model of human disease and an ideal animal for the preclinical testing of cell transplantation [4], its use is fraught with because of the lack of genuine porcine embryonic stem cells (Esc) and induced pluripotent stem cells (iPSC). The establishment of these cell lines requires high-quality cloned porcine blastocyst embryos [5], production of which has met with limited success mainly due to the low cloning efficiency of embryo produced by NT. When compared with in vivo derived porcine blastocysts, there is a marked decrease in the quality of cloned blastocysts produced in vitro. This is evidenced by low total cell numbers and transformed inner cell mass ratios, and higher nuclear apoptosis and fragmentation [5–7]. Koo *et al.* [7] studied the structural integrity of cloned porcine blastocysts and highlighted their low total cell numbers and the low ratio of inner cell mass (ICM) to trophectoderm (TE) cells compared to their in vivo counterparts. Other associated causes of the low outcomes in SCNT embryos include differences in donor cell type, oocyte factors, incomplete reprogramming of the donor cell by the ovary, and inadequate support from the in vitro culture system [8–11]. Even though the above reasons have been highlighted the factors that

account for successful nuclear reprogramming are still undefined and obscure, highlighting the need for further study [11]. Therefore, in order to enhance blastocyst yield and quality, Gupta *et al.* [6] recommended an emphasis on improvements in porcine in vitro production regimens.

Phytohemagglutinin (PHA), an N-acetylgalactosamine/galactose sugar-specific lectin, has a wide variety of biological activities and is widely used in animal research involving mouse [12], bovine [13, 14], goat [15], porcine [6, 16] and human [17, 18]. The biological activities of PHA include strong agglutination of blood cells, mitogenesis, and mediation of cell recognition in both vertebrates and invertebrates [19, 20]. As lectin, it specifically binds to carbohydrate. Isolated from red kidney bean (*Phaseolus vulgaris*), PHA has been used to facilitate the aggregation of blastomeres [5, 14], the binding of donor cells to oocytes in handmade cloning [21, 22], and to improve the efficiency of porcine parthenotes [16] and the nuclear transfer of somatic cells for oocytes, due to its capacity to induce closer contacts between adjacent cell membranes [6, 15]. In this study, we assessed the effects of the phytohemagglutinin (PHA) treatment on the developmental competence of parthenotes and the somatic cell nuclear-transferred (NT) porcine embryos.

Material and Methods

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Collection and maturation of porcine oocyte

The pig ovaries were obtained from a local slaughterhouse. They were brought to the laboratory in 0.9% saline at 30°C within 3 h of slaughter. Immature oocytes surrounded by a compact cumulus cell mass were recovered from 3 to 6 mm diameter follicles with modified TL-HEPES polyvinyl alcohol according to our laboratory procedure [23]. Recovered oocytes with more than 2 to 3 layers of cumulus cells (50–70 COCs / 4-well dish) were cultured in modified NCSU37 (mNCSU37) medium [24] containing 10% porcine follicular fluid (PFF), 0.6 mM L-cysteine, 1.3 μg/ml follicle stimulating hormone (FSH), 0.6 μg/ml luteinizing hormone (LH) and 1 mM dibutyryl-cAMP for 20 h and in the same medium without hormones and dibutyryl-cAMP for another 20 h under an atmosphere of 5% CO_2 in air at 39°C. After 40 h maturation, the oocytes were denuded of cumulus cells in HEPES-buffered mNCSU37 (hNCSU37) medium containing 1% hyaluronidase by vortexing for 3 min. Matured oocytes were confirmed by the extrusion of the first polar body.

Somatic cell nuclear transfer

Before NT, matured oocytes were cultured in hNCSU37 medium supplemented with 0.05 M sucrose and 0.4 μg/ml demecolcine for 60 min at 39°C, and then the resulting protrusion containing the maternal chromosome was removed in medium supplemented with 7.5 μg/ml cytochalasin B, 0.05 M sucrose, and 0.4 μg/ml demecolcine using a piezo-actuated micromanipulator (PMA-CT150; Prime Teck Ltd.) as previously reported [25–27]. Porcine embryonic fibroblasts (PEFs) cells at 4 to 6 passages were used as donor cells [23]. Nuclear transfer was achieved by introducing donor cells into the perivitelline space of a recipient MII oocyte through the opening in the *zona pellucida* made during the enucleation using a piezo-actuated micromanipulator (PMA-CT150; Prime Teck Ltd.). The donor cells were wedged between the zona and the cytoplast membrane to facilitate membrane contact before electro-fusion. The nuclear-transferred oocytes were fused and activated simultaneously using two direct current pulses of 120 V/mm for 30 μs at 0.1-s intervals in 0.28 M mannitol supplemented with 0.01%PVA, 0.1 mM $MgSO_4$, and 0.25 mM $CaCl_2$. The oocytes were cultured in modified PZM-5 (mPZM5) containing 5 μg/ml CB, 10 μg/ml cycloheximide and 50 nM trichostatin A (TSA) for 2 h, and then cultured in mPZM 5 containing 50 nM TSA for 22 h. After 22 h, the reconstructed oocytes were cultured in mPZM 5 for 4 days and further cultured in mPZM5 with 10% FBS for 2 days without changing the mPZM5 medium, under an atmosphere of 5% CO_2 in air at 39°C. The nuclear-transferred oocytes were observed and data collected at the 2- to 4- cell stages (48 h) and blastocyst stage (168 h) to assess embryo development in vitro.

In vitro fertilization

Liquid-stored semen, which was commercially prepared and diluted for artificial insemination (Swine Genetic Co., Ltd., Shizuoka, Japan) and delivered to the laboratory on the day after semen collection, was used for in vitro fertilization. The liquid-stored semen was stored at 15°C until use. In vitro fertilization (IVF) was performed using the method described by Yoshioka *et al.* [28]. Liquid stored semen was centrifuged at 400 × g for 5 min and resuspended in 1x- strength Modena extender (SEM-5x; Research Institute for the Functional Peptides–IFP1050P). The spermatozoa pellets were resuspended in 1 ml of SEM and washed once by centrifugation at 500 × g for 5 min. Then, the liquid stored spermatozoa pellets were resuspended in Modena again and motile spermatozoa separated using Percoll gradient (50% and 80%; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) by

centrifugation at 700 × g for 20 min. The separated motile spermatozoa were washed twice with porcine fertilization medium (PFM; Research Institute for the Functional Peptides–IFP1020P) by centrifugation at 500 × g for 5 min. The COCs were co-incubated for 20 h with spermatozoa at a concentration of 2 × 10^6 spermatozoa/ml in 100-*μ*L droplets of PFM. Each droplet contained 15 to 20 COCs.

After IVF, the presumptive zygotes were stripped of cumulus cells by vortexing for 4 min in porcine oocyte embryo-collection medium (POE-CM; Research Institute for the Functional Peptides–IFP1040P). The presumptive zygotes were washed twice with POE-CM and PZM-5 (Research Institute for the Functional Peptides–IFP0410P) and cultured in 40 *μ*l PZM-5 (20 to 25 presumptive zygotes) for 5 days. The rate of cleavage (>2-cell stage) and the rate of blastocyst formation were evaluated at 48 h and 168 h after IVF, respectively.

Parthenogenetic activation (PA)

Activation of metaphase II (MII) stage oocytes was performed by the method described by Kawakami *et al*. [23] and Nakano *et al*. [25] with slight modification. The MII oocytes were oriented in an electro-chamber (Iwaki, Japan) filled with 25 ml 0.28 M mannitol solution supplemented with 0.01% PVA, 0.1 mM $MgSO_4$ and 0.25 mM $CaCl_2$, and then pulsed once with 150 V/mm DC for 99 *μ*s in a 0.1 s interval using a LF101 Electro Fusion Generator (Nepagene, Japan). Thereafter, activated oocytes were cultured in mPZM 5 medium containing 5 *μ*g/ml CB for 4 h [29]. At the end of 4 h, the oocytes were washed three times in mPZM5, and cultured in sets of 10 in 30-*μ*L droplets (10 gametes per drop) of mPZM5 covered with pre-warmed mineral oil for up to 7 days at 39°C in a humidified atmosphere of 5% CO_2. The rate of cleavage and blastocyst formation were assessed at 48 and 168 h, respectively.

Determination of total cell numbers in porcine blastocysts

On day 7, blastocysts were washed three times with Ca^{2+} and Mg^{2+} free PBS (DPBS) containing 0.01% polyvinylalcohol, stained with the same solution containing 10 *μ*g/ml bisbenzimide solution (Hoechst 33342) for 5 min at 39°C. Blastocysts were washed twice and mounted onto a precleaned glass slide, and the nuclei of blastocyst were then counted using a Nikon fluorescence microscope.

TUNEL assay

Quantification of apoptosis at the single cell level was performed on day-7 blastocysts based on labeling of DNA strand breaks (TUNEL Technology) using an In Situ Cell Death Detection Kit, POD (Roche) according to the manufacturer's instruction. Briefly, day-7 blastocysts were washed twice in DPBS solution containing 0.1% (w/v) PVA. The blastocyst embryos were fixed in 4% paraformaldehyde solution in DPBS for 1 h at room temperature. Thereafter, the embryos were permeabilized in PBT (DPBS containing 0.2% triton X-100 and 10% FBS) for 30 min at 39°C and rinsed in DPBS solution containing 10% FBS. The blastocysts were incubated for 1 h in the dark at 39°C in TUNEL reaction mixture containing TdT and fluorescein-dUTP (1:9), rinsed three times at 5 min intervals in DPBS solution with 0.1% PVA, counterstained in DPBS containing Hoechst solution (10 *μ*g/ml) for 5 min and finally mounted on a glass slide in glycerol. For negative controls, the TdT was omitted from the reaction. The blastocyst embryos were observed under a fluorescence microscope (Nikon, Japan) to count the number of cells and TUNEL-marked cells at an excitation wavelength in the range of 450–490 nm. For each blastocyst, the TUNEL index was calculated as the number of apoptotic cells per total cell number of blastocysts.

Determination of mitochondrial activity

PA oocytes incubated in PZM-5 supplemented with 5 *μ*g/ml CB for 4 h after activation were stained for mitochondria separately using MitoTracker Deep Red FM (Thermo Fisher Scientific Molecular Probes™, M-22426) and MitoTracker Green FM (Thermo Fisher Scientific Molecular Probes™, M-7514). A stock solution of both fluorescence dyes (1 mM) was prepared by reconstituting the solid in dimethyl sulfoxide and stored at −20°C. Oocytes were incubated in DPBS supplemented with 0.5 *μ*mol/L MitoTracker Red or Green in a dark environment under humidified air with 5% CO_2, for 30 min at 39°C, followed by three washes with DPBS for 15 min. The oocytes were placed in 5-*μ*L drops of DPBS and then observed under a fluorescent microscope using red or green filters. The fluorescence intensity of the images was analyzed using Image J software.

Quantitative real-time polymerase chain reaction

Quantification of expression levels of mitochondria-related genes (ATPASE6, DPPA3, ND1 and CYTOCHROME B) was accomplished by real-time quantitative reverse transcription polymerase chain reaction (qPCR). Briefly, complementary DNA (cDNA) was produced from 10 blastocysts of good to excellent quality per experiment group, without RNA isolation, using Cells-to-cDNA II kit (Ambion®, California, USA) according to the manufacturer's instruction. All blastocysts were washed three times in DPBS and stored at −80°C in less than 1 *μ*l of the same

Table 1. Details of the primers used for quantitative real-time PCR analysis

Gene	Primers	Sequence (5'-3')	Product size, bp	Accession Number
ATPase6	Forward	TGCCTCTTTCATTGCCCCTA	121	KF888634.1
	Reverse	TTGGATCGAGATTGTGCGGT		
DPPA3	Forward	ATGCCAGCTGCCTCTCAACCC	245	XM_005655688.2
	Reverse	CGCCCCCTAGTTGCTTGCGA		
ND1	Forward	CTCAACCCTAGCAGAAACCA	274	KJ782448.1
	Reverse	TTAGTTGGTCGTATCGGAATC		
CYTOCHROMEb	Forward	ACCTACTAGGAGACCCAGACAACT	136	KF888634.1
	Reverse	TGAACGTAGGATAGCGTAGGCGAA		
YWHAG	Forward	TCCATCACTGAGGAAAACTGCTAA	28	XM_003124396.5
	Reverse	TTTTTCCAACTCCGTGTTTCTCTA		

medium until used for analysis. Quantitative RT-PCR was performed with 1 μL of cDNA template added to 10 μL of Thunderbird SYBR qPCR mix (TOYOBO Company, Japan) and 5 μL of specific primers at a concentration of 3 pM each. A total reaction volume of 10 μL was used for each analysis and the reactions were carried out in 40 cycles using an ABI Prism 7000 Sequence Detection System (Applied Biosystems®). The cycling parameters were as follows: pre-denaturing at 95°C for 60 min, denaturing at 95°C for 10 s and extension at 60°C for 45 min. All qPCRs were conducted at least three times for every gene of interest. The sequences and Gene Bank accession numbers of the primers used for the amplification of target genes are shown in Table 1.

The comparative C_T method was used for relative quantification of target gene expression levels (ABI Prism Sequence Detection System, Applied Biosystems). The quantification was normalized using the internal control YWHAG gene. Within the log-linear phase region of the amplification curve, a doubling of the amplified PCR product was taken to be equivalent to the difference between one cycle. The ΔC_T value was determined by subtracting the YWHAG C_T value of each sample from the target gene C_T value of the sample. For the calculation of $\Delta\Delta C_T$, the highest sample ΔC_T value was used as an arbitrary constant which was to subtracted from all other ΔC_T sample values. The formula $2^{-\Delta\Delta CT}$ was used to determine the fold-change in the relative mRNA expression of the target gene.

Experimental design

Experiment 1. Effect of PHA treatment on PA embryos and mitochondrial activity: In the first part of the experiment, we investigated the developmental competence of PA embryos treated with 1 mg/ml PHA for 0 (control), 5 and 10 min. before activation. Porcine MI oocytes were incubated in 30 μL droplets of PZM-5 supplemented with 1 mg/ml PHA at 39°C under 5% CO_2 before activation by an electric pulse. As a control, non-treated PA oocytes were used (0 min).

Because the activity of PHA is speculated to be linked to its possible effect on the mitochondrial pathway in cells [30, 31], we conducted a follow-up experiment to investigate the mitochondrial activity within PA oocytes pre-treated with (+) or without (−) PHA for 5 min before activation. The PHA-treated and control groups were stained with fluorescent dyes 4 h after activation. A 5-min pre-treatment with PHA was chosen based on the results of the first experiment.

Experiment 2. Effect of PHA treatment on porcine NT embryos and apoptotic index: This experiment compared the effect of pre-fusion treatment with PHA on the developmental ability of somatic cell nuclear transfer porcine embryos *in vitro*. The PHA treated and non-treated samples were analyzed as independent samples using an appropriate statistical tool (independent sample *t*-test). Nuclear-transferred porcine oocyte-cell construct were incubated in mPZM5 medium containing 1 mg/ml PHA for 5 min at 39°C under 5% CO_2 in air, and were simultaneously fused and activated by a DC pulse immediately after PHA incubation, as described for nuclear transfer embryos. The developmental competence, i.e. the cleavage and blastocyst rates and total nuclei number was compared between the treated and untreated groups of NT embryos on days 2 and 7. To further examine the quality of PHA-treated NT embryos, we carried out a TUNEL assay to assess apoptosis.

Experiment 3. *Mitochondrial-related gene expression levels of PHA-treated porcine NT embryos:* In order to gather additional information on the effect of PHA treatment on the mitochondrial activity of NT embryos, we investigated the relative expression levels of the mitochondrial-related genes, ATPASE 6, DPPA3, ND1, and CYTOCHROME B in porcine NT embryos treated with

Table 2. Effect of PHA treatment on the developmental ability of in vitro produced parthenotes

PHA [1] treatment, min	No. of PA[2] embryos	Cleavage, %	Blastocysts, % of No. of PA embryos	No. of nuclei
0	50	35 (68.5 ± 8.0)	27 (54.0 ± 3.2)	37.3 ± 2.8[a]
5	59	44 (75.7 ± 8.3)	25 (43.4 ± 13.3)	32.0 ± 3.3[a, b]
10	66	45 (71.4 ± 7.5)	37 (59.0 ± 11.3)	27.4 ± 1.9[b]

[1]PHA = phytohemagglutinin. [2]PA = parthenogenetic activation. [a, b]Data without parentheses represent numbers, whereas data inside parentheses represent means ± SEM. Within a column, means without a common superscript differ significantly, $P<0.05$.

(PHA +) or without (PHA -) PHA by qPCR. As a control, normal IVF embryos were used.

Statistical analysis

The effect of PHA treatment was analyzed and compared with non-treated PHA embryos. In Exp. 1, the durations of PHA treatment (0, 5 and 10 min) were considered experimental treatment groups, whereas for Exp. 2 and 3, porcine embryos were grouped into 3 treatments: PHA-treated (PHA +), PHA non-treated (PHA -) and IVF embryos. The PHA non-treated and IVF treatment groups were considered experimental control groups. Analysis of variance of the data was performed using Fit Y by X platform in JMP 12.2 (SAS Institute Inc, Cary, NC) following a completely randomized design (CRD). Probability levels of $P<0.05$ were considered significant and means were compared using the Tukey-Kramer HSD. All data are expressed as the mean ± SEM. Differences between the experimental groups were considered significant at $P<0.05$. Data represents at least three independent experiments.

Results

Effect of PHA treatment on PA embryo development

After maturation, MII oocytes were exposed to PHA at various time points before PA. Pretreatment of MII oocytes with PHA for 10 min showed a significant ($P<0.05$) negative effect on the number of nuclei in porcine blastocysts (Table 2). When compared with the non-treated group (0 min), anapproximately 10% decrease in the number of nuclei in porcine blastocysts was observed after 10 min incubation of MII oocytes in PHA compared to the control (37 vs. 27.4).

Pre-fusion exposure of PHA for 5 or 10 min had no detrimental effects ($P>0.05$) on the cleavage and blastocyst rates evaluated at 48 and 168 h after activation, respectively. Generally, the high concentration of PHA (1 mg/ml) and short incubation time (5 to 10 min) of MII oocytes before PA, did not hamper the development of porcine parthenotes to blastocysts.

To examine whether PHA treatment of PA oocytes impacts on their mitochondrial activity, embryos were evaluated by the intensity of red and green fluorescence dyes. Mitochondrial activity stimulated by PHA was identified by the significantly ($P<0.05$) increased fluorescence intensity of the PHA-treated group, compared to the control, when evaluated using both fluorescence dyes (Fig. 1).

Effect of PHA treatment on the development and quality of porcine NT embryos

Following the results of Experiment 1, nuclear transfer porcine embryos were pre-treated with PHA for 5 min before electro-fusion and activation. Pre-treatment of NT porcine embryos enhanced *in vitro* development of reconstructed porcine oocytes to the blastocyst stage. A significant ($P<0.05$) increase in cleavage number and development of cleaved embryos into blastocysts was evident at 48 and 168 h after activation of NT oocytes with PHA (Table 3). A two-fold increase in the development of PHA-treated NT embryos that cleaved into blastocysts was found in comparison with the non-treated group ($P<0.05$). However, in the PHA-treated and non-treated groups, no significant differences were observed in the number of nuclei in blastocysts between the groups.

To further evaluate the quality of PHA-treated embryos, we also examined the effect of PHA treatment on the numbers of apoptotic cells in a TUNNEL assay performed 168 h after activation. PHA treatment slightly increased the numbers of apoptotic cells in NT embryos, but the value was similar ($P>0.05$) to those of the non-treated and IVF groups (Table 4). The TUNEL index was higher in normal IVF embryos.

PHA treatment enhanced the relative expression of mitochondrial-related genes

To confirm whether increased developmental ability of PHA-treated NT embryos could be linked with increased expression of mitochondrial genes, we evaluated the ex-

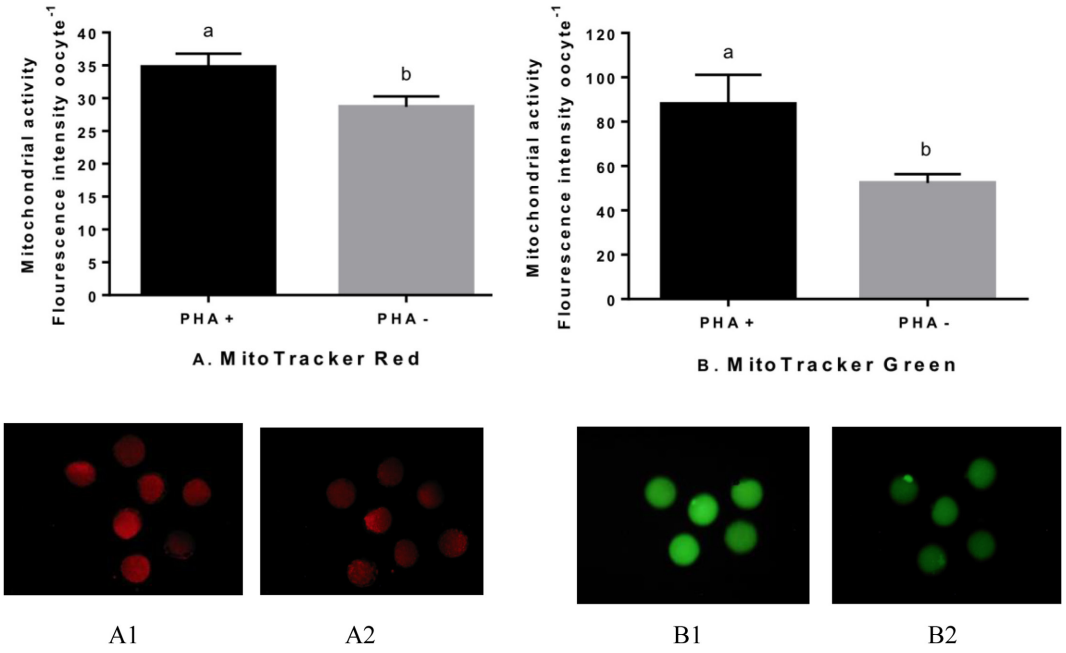

Fig. 1. Mitochondrial activity within PA embryos pre-treated with (+, A-1, B-1) or without (–, A-2, B-2) PHA before activation. Oocytes were stained with fluorescent dyes: (A) MitoTracker Deep Red FM (Thermo Fisher Scientific Molecular Probes™ M-22426 and (B). MitoTracker Green FM (Thermo Fisher Scientific Molecular Probes™ M-22426). Data are mean ± SEM. The values of bars without a common superscript differ significantly, $P<0.05$. At least three replicate trials were performed for each group.

Table 3. Effect of PHA treatment on the development of in vitro-produced porcine SCNT[1] embryos

Embryos	No. of SCNT[1] embryos	Cleavage, %	Blastocyst, % of No. of SCNT embryos	No. of nuclei
PHA+[2]	136	92 (68) [a]	38 (28) [a]	34.0 ± 2.9
PHA–[3]	142	77 (54) [b]	19 (13) [b]	33.9 ± 7.0
IVF[4]	145	86 (59) [b]	19 (13) [b]	30.8 ± 3.4

[1]SCNT = somatic cell nuclei transfer. [2]PHA+ = phytohemagglutinin treated embryos. [3]PHA− = phytohemagglutinin non-treated embryos. [4]IVF = *in vitro* fertilization. [a, b]Within a column, means without a common superscript differ significantly, $P<0.05$.

Table 4. Effect of PHA treatment on the apoptotic cell index[1]

Embryos	No. of blastocysts	No. of nuclei in blastocysts	No. of Apoptotic cells	TUNEL Index[5]
PHA+[2]	12	29.4 ± 3.9	1.8 ± 0.4	6.7 ± 1.5
PHA–[3]	12	34.6 ± 3.9	1.7 ± 0.4	5.4 ± 1.6
IVF[4]	12	39.8 ± 4.1	2.9 ± 0.5	7.8 ± 1.7

[1]Values in the table are means ± SEM. [2]PHA+ = phytohemagglutinin treated embryos. [3]PHA− = phytohemagglutinin non-treated embryos. [4]IVF = *in vitro* fertilization. [5]TUNEL index = no. of apoptotic cells / total no. of nuclei in blastocyst.

pression of the ATPase 6, DPPA3, ND1 and Cytochrome B genes in PHA-treated embryos. PHA treatment significantly increased the expression of ATPase6 and ND1 genes in porcine NT embryos (Fig. 2). When compared with normal IVF embryos, the expression of Cytochrome B gene was significantly lower in all NT embryos, however, no significant difference in the expression of the DPPA3 gene was shown between IVF and NT embryos.

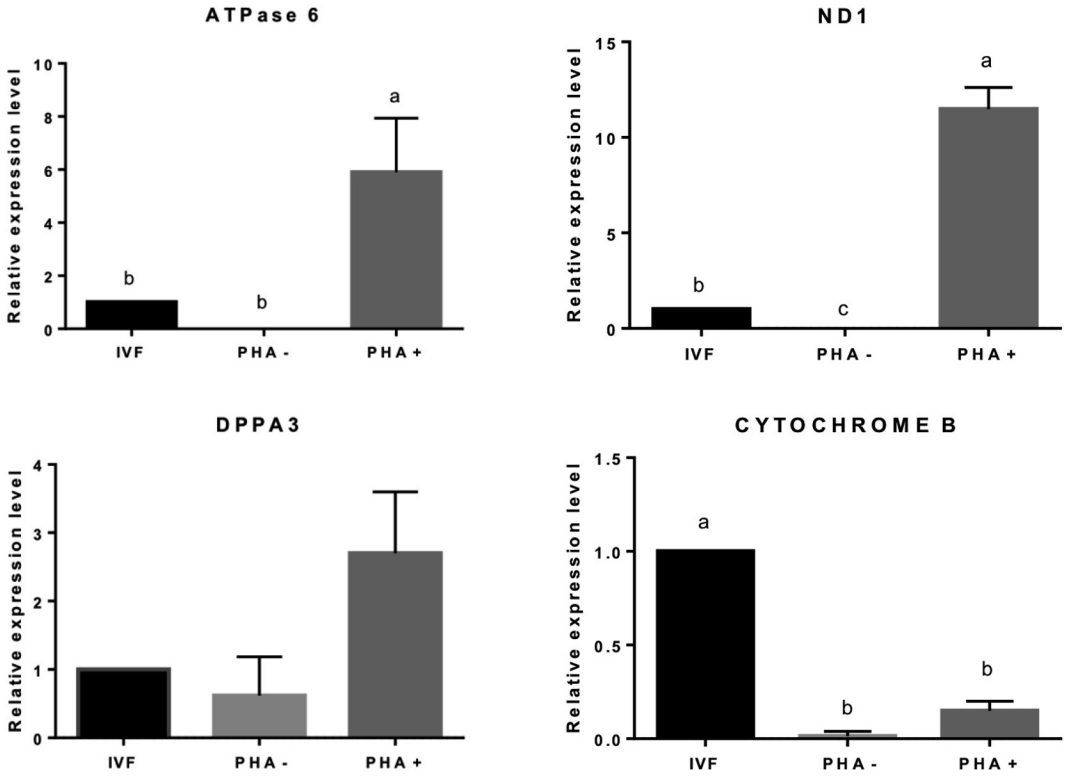

Fig. 2. Relative expression of mitochondria-related genes (ATPASE6, DPPA3, ND1 and CYTOCHROME B) in porcine SCNT embryos pretreated with PHA (PHA+) or without PHA (PHA−), and IVF embryos. Data are means ± SEM. The values of bars without a common superscript differ significantly, $P<0.05$.

Discussion

In the present investigation, we evaluated the toxicity of PHA by testing its effect on the development of PA oocytes in vitro, and then compared the mitochondrial activity within PHA-treated embryos. Since PHA treatment for 5 min enhanced PA embryos' development, we examined further the effect of pre-fusion PHA treatment of NT embryos on the in vitro developmental ability of porcine embryos. The quality of PHA-treated NT blastocysts was also screened for apoptotic cell damage in a TUNEL assay. Finally, we evaluated the expression of mitochondrial-related genes of PHA-treated NT embryos to explore the possible role of PHA on the activities of mitochondrial genes in porcine NT embryos. Our data confirmed the findings of Gupta *et al.* [6, 16], that pre-treatment of porcine parthenotes and nuclear transfer oocytes with PHA prior to electrical pulsing improved their development into blastocyst embryos. The mitochondrial activity of PHA-treated embryo was also evaluated and our results revealed there was higher mitochondrial activity in PHA embryos. In addition, when PHA blastocysts were assessed for quality by examination of the rate of programmed cell death, or apoptosis, our results endorsed those an earlier study [6] indicating there was no loss of quality in PHA-treated embryos, compared with the control, as evidenced by the similar cell numbers and the apoptotic cell index. Surprisingly, a comparison of mitochondrial-related gene expressions in porcine NT blastocyst stage embryos using qPCR revealed a significant increase in the relative expressions of ATPase6 and ND1 genes in PHA-treated porcine embryos compared to control and normal IVF embryos. Furthermore, the relative expression level of CYTOCHROME B was low in PHA-treated embryos. Our results show that the expression of the DPPA3 gene in PHA-treated embryos was relatively high compared with non-treated and IVF embryos. However, the expression of the DPPA3 gene in the treated embryos didn't show a significant variation from those of non-treated and IVF embryos. Even though no significant differences were observed, it may be inferred that PHA may activate mitochondria, facilitate metabolism, and signaling of pluripotency in the blastocyst. To our knowledge, this is the first study linking the

positive effect of PHA on embryo development to mitochondrial activities in porcine NT embryos.

Since the discovery of lectins more than 100 years ago [20], they have been reported to have numerous uses in animal research due to their important biological activities. The lectin of red kidney bean (PHA) is known to be mitogenic, and thus possesses the ability to stimulate lymphocytes to undergo mitosis [31]. Additionally, it has a strong affinity to carbohydrates and may bind to a soluble carbohydrate, or to a part of a glycoprotein or glycolipid. PHA is known to agglutinate certain animal cells and / or precipitate glycoconjugates [20, 32], and it has been used effectively in animal research to bind donor cells to oocytes in handmade cloning [6, 16, 21], and in the aggregation of embryos in nuclear transfer studies [33]. Lectins are cytotoxic and may induce apoptosis or necrosis depending on their concentrations and time in contact withthe cells in the medium [20]. The developmental of porcine parthenotes to the cleavage stage and their transformation into blastocysts was not influenced by the concentration and time of PHA (5 and 10 min) treatment. Gupta *et al.*[16] reported significant variations in blastocyst rate and total nuclei number when porcine parthenotes were incubated in vitro in medium containing various concentrations of PHA (0, 5, 10, 15, 20 *μ*g/ml). Similar to our present findings, no variation in cleavage rate was observed. In summary, Gupta and co-workers suggested concentrations below 15 *μ*g/ml are suboptimal, while 20 *μ*g/ml is inappropriate as some embryos may suffer toxicity. The differences in their reported blastocyst rate and ours may be accounted for by the obvious differences in experimental methods. In the present study, porcine parthenotes were incubated in medium containing 1 mg/ml PHA for 0, 5 or 10 min before electric pulsing, and then cultured in vitro in PHA-free medium. Our results suggest that long exposure of parthenotes in PHA, up to 10 min, had a profound negative effect on the quality of embryos (Table 2). After 10 min treatment, the numbers of nuclei in blastocyst embryos declined by about 26% compared with the non-treated control group (no PHA treatment). This difference may be ascribed to the toxic effect of PHA on the cells during the period of incubation [13]. In the mouse, the addition of PHA concentrations >1,000 mg/L blocked embryo development from the 1-cell to blastocyst stage, and cells showed apoptosic morphology or death [34]. In the goat, Zhang *et al.* [1] tested the toxicity of PHA by evaluating its effect on the development of parthenogenetic caprine oocytes and found PHA treatment did not affect the development of parthenogenetic embryos. The mechanism of action of PHA on porcine embryos may be linked to its effect on the ultrastructure of the *zona pellucida,* which is constantly being modified, and possibly to its specific interaction with *zona pellucida* glycoprotein and integrin. Specific culture conditions and media supplements may considerably upset the ultrastructure of porcine *zona pellucida,* changing it from a porous, net-like structure into a nearly smooth and compact surface [35, 36]. We found that the mitochondrial activity of porcine PA embryos was increased by PHA treatment. To date, however, there have been no reports on the effect of PHA treatment on the mitochondrial activity of porcine NT oocytes. Reports in the literature have stopped at the use of PHA for porcine embryo aggregation, production and quality evaluations [5, 16, 33]. In a normal mammalian cell, mitochondria play a significant role in the determination of normal pre-implantation embryonic development due to their active participation in diverse cellular processes (oogenesis, fertilization, metabolic activities, etc.) and are the primary energy-generating system [37–39]. Based on these results, we propose that PHA-treated embryos may have increased cell metabolic activity due to the enhanced mitochondrial activity triggered by PHA activity.

Our NT data showed improved cleavage and blastocyst formation rates in the PHA-treated group of cloned embryos (Table 3). The cleavage rate and blastocyst yield of PHA-treated cloned embryos increased by about 1.3 and 2.1 folds, respectively. In another study, Gupta *et al.*[6] reported almost 1.5- and 2-fold increases in parthenogenetic and PHA-treated porcine cloned embryos. Little is known about the mechanism underlying the improved efficiency and quality of PHA-treated NT embryos. However, our results indicate that PHA-treatment triggered a faster rate of division and subsequent development into blastocysts in porcine cloned embryos. Even though we did not report the hatching rate in the present study, an earlier study reported that co-culture with PHA enhanced the expansion and hatching of porcine blastocysts from their *zona pellucidae* and shortened the blastocyst formation time [6]. Possible reasons for our results may be that PHA, due to its agglutinating factor, may have assisted the adhesion of donor cells to be recipient cytoplasm, and facilitated close cell contact by binding to the carbohydrate core structure of glycoprotein or glycolipid on the cell membrane [1, 31]. In porcine, the morphologically good quality mature oocytes indicate 3- to 4-fold higher mRNA levels of porcine zona pellucida glycoprotein 3 (ZP3) and integrin beta 2 protein [35]. It has previously been suggested that various important physiological processes in germ cells are mediated by surface glycoconjugates [12]. Besides, the increased cleavage and developmental rates, our results confirmed

the concentration of the PHA used and the short time of exposure were adequate for the normal development of porcine cloned embryos. The total cell number of the blastocyst is a good indicator of the quality and viability of an in vitro-produced blastocyst [6, 28, 40]. The number of nuclei in blastocysts derived from the PHA-treated and untreated groups of cloned embryos was evaluated using Hoechst 33342 staining. The mean total nuclei number in PHA-treated blastocysts was similar to that of blastocysts derived without PHA treatment (Table 3). Thus, we consider that thePHA used in our study had a limited effect on the total nuclei number of cloned blastocysts. However, Gupta *et al.* [6] found significantly higher cell numbers in PHA-supplemented embryos compared to the control group (45.5% vs. 34.3%). In some ways, the porcine cloned embryosobtained in the present study were considered to be of good quality and viable. This is because the number of nuclei of both experimental groups of blastocysts was within the range of average values reported for porcine blastocysts derived from somatic cell nuclear transfer [6].

Another index of the quality of in vitro-produced blastocysts is apoptosis, or programmed cell death. It is now commonly accepted that the apoptotic mechanism contributes to the elimination of defective embryos [41]. Therefore, the present study also assessed the quality of cloned embryos using the number of TUNEL-positive nuclei in PHA-treated and untreated cloned blastocysts. The total incidence of apoptosis in NT embryos was not significantly affected by PHA incubation (Table 4). Although the molecular mechanism by which PHA affects embryo yield and quality in mammalian embryos is not clear [6], it is speculated that a cytotoxic agent may induce either apoptosis or necrosis depending on the concentration and time of contact with the substance [20]. Moreover, its action may be linked to its positive influence on mitochondrial activity as observed in this study in PHA-treated oocytes. In the literature, Faheina-Martins *et al.*[30] documented that other lectins (e.g. Concanavalin-A and Concanavalin-Br) induce apoptosis by triggering an intrinsic mitochondrial pathway in leukemic cells or by interacting with the cell surface, being first endocytosed, and then reaching the mitochondria [20, 42]. Supporting the previous authors report, it has been stated that PHA modulates apoptosis pathways to prevent blastomeric death resulting in net enhancement of porcine embryo viability [6]. Taken together, our data show that PHA caused an increase in mitochondrial activity in PA oocytes and also enhanced the expression of PHA-related genes (ATPase and ND1) of NT porcine embryos. In mammalian cells, ATP for all energy-requiring events is provided by the mitochondria through the oxidative phosphorylation pathway [43] and any dysfunction of mitochondria in oocytes and early embryos impacts ATP generation and can cause anomalous chromosomal isolation or abnormal development [44]. The ND1 and ATPase 6 genes are closely related to mitochondrial function and ATP synthesis. Whereas the ND1 gene (one of the seven NADH dehydrogenase subunits) is the 'entry enzyme' of oxidative phosphorylation in mitochondria, a decrease in ATPase6 gene expression may impact the generation of ATP [44, 45]. Also important, and closely related to mitochondrial function, are the Cytochrome b and DPPA3 gene; however, our data show that both these genes were expressed similarly in PHA-treated and untreated porcine NT embryos. Surprisingly, there has been no indication of association between the PHA treatment of embryos and the expression of the above genes in porcine NT embryos until now. Therefore, our present results provide persuasive evidence that the increase in developmental competence of PHA-treated NT embryos in vitro is associated with higher mitochondrial activity and ATP synthesis.

In conclusion, we found that the pre-treatment of porcine embryos with PHA had no detrimental effect on the developmental competence of PA embryos; rather it increased the mitochondrial activity of treated embryos. Pre-fusion treatment with PHA improved the development of porcine NT embryos in vitro by enhancing embryonic mitochondrial activity, and other energy-related target gene expressions.

Conflict of Interest

None

Authors' contribution

NSM and YK contributed to the conception, design of the study, and did the experiments. TT supplied the PEF cells. SS, MH, and KO supplied the ovaries and other materials. NSM and YK wrote the paper.

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