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Running Title: PHA agglutination improves membrane fusion during bovine somatic NT

Key Words: Phytohemagglutinin, somatic nuclear transfer, cell fusion, vitrification, development

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Abstract

One of the several factors that contribute to the low efficiency of mammalian somatic cloning is the poor fusion between the small somatic donor cell and the large recipient oocyte. This study was designed to test phytohemagglutinin (PHA) agglutination activity on fusion rate, and subsequent developmental potential of cloned bovine embryos. The toxicity of PHA was established by examining its effects on the development of parthenogenetic bovine oocytes treated with different dosages (Expt 1), and for different durations (Expt 2). The effective dosage and duration of PHA treatment (150 μg/mL, 20 min incubation) was selected and used to compare membrane fusion efficiency and embryo development following somatic cell nuclear transfer (Expt 3). Cloning with somatic donor fibroblasts vs. cumulus cells was also compared, both with and without PHA treatment (150 μg/mL, 20 min). Our results showed that the fusion rate of nuclear donor fibroblasts, after phytohemagglutinin treatment, was increased from 33 to 61 % ($P<0.05$), and from 59 to 88% ($P<0.05$) with cumulus cell nuclear donors. The nuclear transfer (NT) efficiency per oocyte used was improved following PHA treatment, for both fibroblast (13 vs. 22%), as well as cumulus cell (17 vs. 34%) ($P<0.05$). The cloned embryos, both with and without PHA treatment, were subjected to vitrification and embryo transfer testing, and resulted in similar survival (approximately 90% hatching) and pregnancy rates (17 to 25%). Three calves were born following vitrification and embryo transfer of these embryos; two from the PHA-treated group, and one from non-PHA control group. We conclude that PHA treatment can significantly improve the fusion efficiency of somatic NT in cattle, and therefore, increase the development of cloned blastocysts. Furthermore, within a determined range of dosage and duration, PHA

has no detrimental effect on embryo survival post vitrification, nor on pregnancy or calving rates following embryo transfer.

1. Introduction

Successful somatic cell nuclear transfer (NT) has resulted in live mammalian clones, including: sheep [1], cattle [2-5], mice [6], goats [7,8], pigs [9-12], cats [13], rabbits [14], rats [15], guars [16], mules [17] and horses [18]. For effective reprogramming of the genome of a differentiated somatic cell nucleus, the donor nucleus must be successfully introduced into the oocyte's cytoplasm, either by direct nuclear injection [6,11], whole cell injection [19], or most commonly, via membrane fusion by electrical stimulation [1,2,10,20,21]. Despite the successes of cloning, however, overall cloning efficiency [3,22,23] has remained low, caused to some extent by the low fusion efficiencies currently achieved between the small somatic donor cell and the recipient oocyte, following somatic NT [1,3,20]. In somatic nuclear transfer with induced fusion by electrical pulse(s), the area of membrane contact between a somatic donor cell and an oocyte is thought to be relatively limited, and smaller than that attained in conventional NT (early NT studies with an embryonic blastomere as the nuclear donor) [21,24-26]. Therefore, an apparent obstacle to efficient somatic NT is the difficulty of fusing a tiny somatic cell to a large recipient oocyte. In addition to the size disparity, the difference in membrane properties among the different somatic cell types considerably affects their fusion efficiency [27]. The development of cloned embryos is largely anomalous, and its inefficiency could be caused by other problems inherent to the oocyte's ability to completely reactivate and/or reprogram an introduced somatic genome during the cloning process [22,28-30].

It is well known, that a family of lectin proteins binds carbohydrates of glycoproteins in a reversible and specific manner [31,32]. The recognition of cellular

surface-specific carbohydrates by phytohemagglutinin (PHA) could have important implications for critical biological processes, as well as practical applications in cellular bioengineering. Phytohemagglutinins have been widely used in cell biology for enhancing cell agglutination and fusion in plant protoplasts [33], and in various types of mammalian cells including: erythrocytes [34], hybrid cell colonies [27,35], and bone marrow mesenchymal stem cells [36]. Moreover, PHA was used for nuclear transfer with human [37], goat [38] and bovine [39] oocytes, and for zona free NT in cattle [40-43]. However, the toxicity of PHA to oocytes, donor somatic cells, and its consequence to the developmental potential of NT embryos, has not been well established. It was reported that high doses of PHA were cytotoxic to Chinese Hamster ovary (CHO) and other mammalian cells [44].

In the present study, a series of experiments were designed to test the phytohemagglutinin (PHA) treatment (dosage and duration) optimal for bovine oocytes, and to determine its effect on the fusion rate and subsequent developmental potential of NT bovine embryos *in vitro*. In addition, the *in vivo* viability of PHA-treated somatic NT embryos was examined by transferring vitrified cloned blastocysts into synchronized recipients.

2. Materials and methods

All the chemicals were manufactured in the USA. Most chemicals were obtained from Sigma Chemicals Co (St. Louis, MO, USA) unless otherwise indicated. All embryo cultures were maintained at 39 °C in 5% CO_2 and humidified air unless otherwise specified.

2.1. Bovine oocyte recovery, culture, and maturation in vitro

Bovine cumulus oocyte complexes (COCs) were aspirated from antral follicles of slaughterhouse ovaries as described previously [45]. COCs with at least 4 intact layers of tight cumulus cells were selected, washed three times in Dulbecco's phosphate buffered saline (D-PBS; Gibco, Grand Island, NY, 15240-013) containing 0.1% polyvinyl alcohol (PVA; P-8136) (D-PBS+PVA). COCs, in groups of 20 to 25, were matured in 75 μL drops of maturation medium for18 to 20 h. Maturation medium was a basal Medium 199 (M199) with Earle's salts, L-glutamine, 2.2 g/L sodium bicarbonate, and 25 mM HEPES (Gibco, Grand Island, NY, 12340-014) containing 7.5% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, UT, SH0070.03), and supplemented with 0.5 μg/mL ovine FSH (NIDDK), 5.0 μg/mL ovine LH (NIDDK), 1.0 μg/mL estradiol (E-8875) and antibiotics (Gibco, Grand Island, NY, 15240-013). The matured oocytes with well-expanded cumulus layers were selected for proceeding of cumulus cell denuding for either parthenogenetic activation or enucleation and NT.

2.2. Culture of bovine skin fibroblast and cumulus donor cells

Bovine cumulus oocyte complexes (COCs), and ear tissue biopsies were collected from one adult female dairy cattle of high merit, from the University of Connecticut's Kellogg Dairy center (KDC), by standard oocyte ultrasound-guided retrieval (OPU) [46] and ear notching, respectively. Briefly, COCs were recovered using an Aloka 5005 scanner fitted with a human vaginal probe (5MHz), and sterile hypodermic needle. With the aid of vacuum pressure, follicular fluid is aspirated along with the cumulus oocyte complexes. COCs, in groups of 3 to 5, were cultured in Dulbecco's Minimum Eagle's medium (DMEM; Gibco, Grand Island, NY, 31600), containing 20% FBS (Hyclone, Logan, UT, SH0070.03) and antibiotics (Gibco, Grand Island, NY, 15240-013) in Falcon

35x10 mm culture dishes (Becton Dickinson, Franklin Lakes, NJ, 3001). Ear skin explants were cultured in 10% FBS DMEM. Both COCs and skin tissues were cultured at 37°C in 5% CO_2 humidified air until confluency. Cumulus cells formed a monolayer around ova, and fibroblast monolayers formed from the tissue explants, in about two weeks. For passaging, cells were washed with 1 mL of Dulbecco's PBS, then gently digested by a three-minute incubation in 100 μl 0.05% trypsin (ICN, Aurora, OH, 103140) and 0.5 mM EDTA (Baker, Phillipsburg, NJ, 8991) at 37°C. The reaction was terminated by adding 5% FBS in DMEM. Subsequently, the cell suspension was centrifuged at 800 x g for 5 min, and cells were resuspended and divided into three new dishes and maintained for 6 to 7 d. Cells cultured to different numbers of passages were collected and frozen in 7% dimethylsulfoxide (DMSO, D-5879) and 7% glycerol (G–2025) at –80°C for 1 d, then stored in liquid nitrogen.

Donor cells at passage 5 to 10, derived either from cattle cumulus or fibroblast cells were used for nuclear transfer. After reaching confluency, bovine fibroblast cells were serum starved in 0.5% FBS DMEM for 4 to 5 d. The bovine cumulus cells were not starved, but were continuously cultured for an additional 5 to 6 d after confluency, in 20% FBS DMEM . Nuclear donor cells were then disassociated by 2 to 3 min of trypsinization at 37°C, and resuspended in 1 mL 5% FBS in DMEM. Prior to NT, cell suspensions were allowed to recover for about 20 to 30 min at 37°C.

2.3. Nuclear transfer, phytohemagglutinin treatment, and cell fusion

Recipient oocyte enucleation was conducted in D-PBS containing 20% FBS by squashing and compressing out the first polar body and its surrounding cytoplasm with an enucleation needle (Fig. 2A); approximately 1/8 of the oocyte's total cytoplasmic volume

was extruded through a slit made on the zona pellucida [17]. Successful enucleation was confirmed by fluorescent microscopy after staining with 10μg/mL Hoechst 33342. For nuclear transfer, a small, round somatic donor cell (Fig. 2B), usually with compacted nucleus, was inserted into the perivitelline space of the enucleated ova (Fig. 2C).

Bovine NT treatment groups were incubated for 20 min in M199-FCS supplemented with 0 or 150 μg /mL phytohemagglutinin-L (PHA-L, L-4144). PHA-L was classified as a lecuoagglutinin, derived from *Phaseolus vulgaris* (red kidney bean). Donor-cytoplast complexes were incubated for 5 min in Zimmerman cell fusion medium [47], then manually oriented by fine electrical rods (Fig. 2D,E) under light microscopy. Electric fusion of the donor cells to the recipient oocytes was accomplished by a double electrical pulse of 2.3 kV/cm for 10 μsec. After the electric stimulation, oocyte-donor cell complexes were incubated for 15 to 30 min in 20% FBS PBS at room temperature before being subjected to further activation procedures. Fusion rates were determined 90 min after electrical pulse.

2.4 Activation and culture of cloned bovine embryos in vitro

After electrical stimulation, cloned bovine embryos were cultured in M199+FCS supplemented with 2.5 μg/mL cytochalasin D (CD) (C-8273) and 10 μg/mL cycloheximide (CHX) (C-6255) for 1 h, and then for an additional 4 h in M199+FCS with 10 μg/mL CHX. The culture medium for bovine NT embryos was a defined CR1 medium (referred to as CR1aa [48]) consisting of: 114.7 mM NaCl, 3.1 mM KCl, 26.2 mM $NaHCO_3$, 1 mM L-Glutamine, 0.4 mM sodium pyruvate (P-2256) and 5.5 mM hemicalcium lactate (L-4388), and was supplemented with 1X MEM (M-7145) and 1X BME amino acid (B-6766). Activated bovine NT embryos were cultured in CR1aa

containing 6 mg/mL BSA for 2 d (initiation of activation = day 0), under 5% CO_2, 5% O_2 and 90% N_2 with high humidity. Embryos were then switched to 10% FBS CR1aa co-culture with a bovine cumulus monolayer for an additional 5 d. For somatic cell co-culture of NT embryos, the medium in the maturation droplets that contained a layer of 80 to 90% confluent cumulus cells was changed to the appropriate CR1aa culture medium.

2.5. Vitrification of cloned embryos

Cloned bovine blastocysts on day 7, usually at the expanding stage comparable to that of normally fertilized embryos, but beginning to hatch through the cuts made on the zona pellucida during NT, were selected for cryopreservation. Embryos were cryopreserved by a modified vitrification protocol, liquid nitrogen surface vitrification (LNSV) described previously [49,50]. Briefly, bovine NT blastocysts were pre-incubated in serially increased concentrations of dimethyl sulfoxide (DMSO), ethylene glycol (EG) and 20% FBS PBS medium for 3 min, two embryos per group were then vitrified in a micro-droplet containing a high concentration of DMSO, EG, and sucrose (Vitrification and Warming Kit, Evergen Biotechnologies Inc, Storrs, CT) by directly dropping into a thin layer of liquid nitrogen on the solid surface of a metal plate that generated a super cold surface for vitrification [49]. The vitrified embryo-containing droplets were then transferred into a small freezing vial and stored in the vapor phase of liquid nitrogen (-150^{o}C). The liquid nitrogen used throughout the LNSV procedure was passed through a ceramic filter to remove microorganisms, the filtered liquid nitrogen was then stored in a tank tested free of viral contamination prior to use.

To test the viability of vitrified NT embryos *in vitro*, frozen embryos were sequentially warmed, rehydrated in 20% FBS M199 with different concentrations of sucrose, and washed in 20% FBS M199 (Vitrification and Warming Kit, provided by Evergen Biotechnologies Inc, Storrs, CT) for 5 min. Cell counts and nuclear evaluations of embryos were performed under fluorescent microscopy following staining with 10μg/mL Hoechst 33342.

2.6. Evaluation of fresh and vitrified NT embryos in vitro

The different stages of bovine embryo development were recorded as cleavage (2 to 8 cell), morula, and blastocyst, on day 2, day 4, and day 7, respectively, according to the standard of the International Embryo Transfer Society (IETS manual, 1998) [51]. Vitrified bovine NT embryos were cultured for three days in M199 supplemented with 7.5%FBS. Survival and development to hatching were recorded at 0h, 24h, 48h, and 72h of culture (warming = 0 h).

2.7. Transfer of vitrified embryos and pregnancy monitoring

Recipients cattle used for embryo transfer were pooled from the breeds of Chinese native yellow cattle and Holstein cattle at several Chinese and Taiwanese farms. Recipients were pre-screened in the farms based on the criteria of the age, health status and history, size and weight, and nutritional management. The sign of onset estrus of recipients was monitored closely and identified as the standing heat estrus. On day 7 after standing estrus, qualified recipients were selected by palpation per rectum to verify the presence and quality of functional Corpus Luteum (CL). Vitrified bovine NT embryos were allocated for embryo transfer to examine their developmental potential *in vivo*. As a control, fresh NT blastocysts, derived from fibroblasts, were loaded in straws following

5% CO_2 gassing of 20% FBS M199 culture medium, and transferred to a farm for direct embryo transfer. Frozen NT embryos were thawed by transferring the vitrified spheres containing embryos through a series of steps as described above. Two embryos were transferred into each synchronous recipients by non-surgical means. Pregnancy was determined by palpation per rectum on day 70 after transfer.

2.8. Specific experiments

Experiment 1: Toxicity effect of phytohemagglutinin dosage on the development of parthenogenetic embryos. Parthenogenetic activation was used to examine the potential toxicity of phytohemagglutinin (PHA) on embryo development *in vitro*. Denuded metaphase II oocytes, after 24 hr of maturation, were randomly allocated to treatments with PHA for 20 min at a concentration of 0, 150, 300, or 600 μg/mL in 7.5% FBS M199 medium. Oocytes were subsequently activated by the regime used for NT embryos. Treated oocytes were cultured in CR1aa BSA/co-culture system as described above.

Experiment 2: Toxicity effect of phytohemagglutinin treatment duration on the development of parthenogenetic embryos. After establishing the least toxic effective concentration of PHA, in Expt 1, the durational toxicity of PHA was determined by its effect on the development of parthenogenetically activated oocytes. Matured oocytes were treated with the minimum concentration of 150 μg/mL PHA for 0, 10, 20, or 60 min, before activation, and cultured under the same condition as in Expt 1.

Experiment 3: Fusion rates, and subsequent development of nuclear transfer bovine embryos incubated with phytohemagglutinin. As described for Expt 3a - determining the best fusion rate, somatic donor cell-cytoplast pairs were incubated with 150 μg/mL phytohemagglutinin, in 7.5% FBS M199 for 0, 10, 20 or 60 min, before being subjected

to an electrical pulse, and the fusion rates were examined 90 min later. After determining the best fusion parameters (150 μg/mL for 20 min) (Expt 3a), somatic donor cell-oocyte cytoplast pairs were randomly allocated to a 2 X 2 factorial design, with phytohemagglutinin treatment for 20 min at a concentration of either 0 or 150 μg/mL in 7.5% FBS M199, and with either cumulus or fibroblast cells as nuclear donors; subsequently, the NT embryos were allocated to *in vitro* tests to determine their developmental potentials (Expt 3b).

2.9. Statistical analyses

Each experiment was repeated four times. For each replicate, the proportions of embryos from various treatments reaching cleavage and developing to 8-cell, morula, or blastocyst stage, within each experiment, were determined and transformed by an arc sine transformation. The transformed data then were analyzed by ANOVA (General Linear Model, SPSS 11.0, Chicago, IL). For Expt 3, a two-way ANOVA (General Linear Model, SPSS 11.0, Chicago, IL) with main effects and an interaction was used to analyze the 2 X 2 experimental designed data [52]. A P value of less than 0.05 is considered to show statistical significance.

3. Results

3.1. Experiment 1

This experiment was designed to test the effect of phytohemagglutinin (PHA) dosage on oocyte survival and subsequent development. As shown in Table 1, the survival rates of oocytes were not different when treated with PHA at a dose from 0 to 300 μg/mL. In contrast, a PHA concentration of 600 μg/mL proved to be highly toxic to oocyte development. Likewise, the total efficiency of blastocyst development was

similar among groups treated with 0, 150, or 300 μg/mL (28, 21, and 20% respectively, P>0.05), whereas, the blastocyst development rate for oocytes treated with 600 μg/mL PHA went as low as 8% (P<0.05).

3.2. Experiment 2

To further elucidate the effects of the duration of phytohemagglutinin (PHA) treatment on parthenogenetic development, oocytes were treated with PHA at a dose of 150 μg/mL (determined in Expt 1) for 0, 10, 20 or 60 min, prior to activation . Data (Table 2) showed, following PHA treatment of oocytes for 0 to 20 min, the survival (91 to 94%), cleavage (67 to 85%), and blastocyst development rates (23 to 38%) were not affected; neither was there an adverse effect on total efficiency of blastocyst development, calculated as a percentage of the total number of oocytes treated (21 to 35%). However, incubation with 150 μg/mL PHA for 60 min not only reduced the cleavage rates (56%), as well as 8-cell (30%), morula (29%), and blastocyst development (21%), but also reduced the total efficiency (19%), compared to those observed with shorter treatments (P<0.05). Therefore, we conclude that a prolonged incubation with phytohemagglutinin is detrimental to oocyte development, similar to the adverse effect observed with a high dosage.

3.3. Experiment 3

To determine whether phytohemagglutinin had an effect on the fusion rate of donor-oocyte complexes, we conducted NT followed by incubation in PHA, at a concentration of 150 μg/mL, for various durations: 0, 10, 20 and 60 min (Expt 3a; same as in Expt 2). The data in Fig. 1 showed that the fusion rate was not improved after a 10 min PHA incubation, compared to the 0 min control. In contrast, the fusion rates of

donor-oocyte complexes were significantly increased following a 20 min PHA treatment. However, the donor-cytoplast fusion rate was not significantly improved by a prolonged incubation (60 min).

Utilizing the optimized fusion protocol, established from the previous experiments (150 μg/mL PHA for 20 min), in Exp 3b, we carried out a series of NTs to examine PHA's effect on the developmental potential of NT embryos. As a 2 X2 factorial design, the donor cell-cytoplast pairs, after transfer with either fibroblast or cumulus cells as nuclear donors (Fig. 2B) into the oocyte's subzonal spaces (Fig. 2C), were incubated for 20 min with 0 vs. 150 μg/mL phytohemagglutinin. With PHA treatment a high rate (91%, n=530) of adhesion between the nuclear donor and the oocyte membrane was apparently achieved (Fig. 2D), whereas, a large number (67%,n=465) of the donor-cytoplast complexes not undergoing PHA incubation showed the attachment of donor cells to zona pellucida (Fig. 2E). The results of Expt 3b (Table 3) showed that the fusion rate of nuclear donors to oocyte recipients was increased after PHA treatment; from 33 to 61% for skin fibroblasts, and from 59 to 88% for cumulus cells, respectively ($P<0.05$). There were significant differences in the subsequent cleavage rates and morula development among the fused embryos with phytohemagglutinin treatments. Furthermore, the NT efficiency judged by the blastocyst development (Fig. 2F) over the number of oocytes used, was significantly improved from 13 to 22% for skin fibroblasts, and from 17 to 34% for cumulus cells when PHA was used. However, the percentage of blastocyst development (29 to 38%) over the number of fused oocytes was not significantly different, regardless of phytohemagglutinin treatments or donor cell type (Table 3). The quality of NT blastocysts, estimated by their mean cell numbers (Fig. 2G)

was not statistically different between those PHA treated, 175 ± 5 (n=16), and the control group without PHA (168 ± 7, n=21).

3.4. Developmental potentials of phytohemagglutinin treated NT embryos in vitro and in vivo after vitrification

Viability of phytohemagglutinin (PHA) treated NT embryos following vitrification, thawing, and embryo transfer was tested. Cloned embryos derived from NT with cumulus donor cells were used for the *in vitro* study. After thawing and culturing *in vitro* 2 h, the survival rates of vitrified embryos were similar (96%, P>0.05) between PHA treated (n=50) and control (non-PHA treated, n=46) groups. Likewise, there were no differences (P>0.05) in the hatching rates after 24, 48, 72 h culture; the hatching rates were as high as 92% and 86%, from PHA-treated and control groups, respectively.

Embryos vitrified after NT were thawed and cultured for 2 h, to evaluate their post cryopreservation viability prior to ET. After thawing, survival rates achieved with PHA-treated embryos, with either cumulus (94%, n=16), or fibroblast cells (96%, n=44) as nuclear donors, was similar to that of the non- treated controls (n=40) (P>0.05). Total 62 recipients were used for embryo transfer. Viable pregnancies, on day 70 of gestation, indicated no statistically significant difference among ETs with blastocysts from: fresh NT (25%, n=12), vitrified NT without PHA treatment (20%, n=20), or vitrified NT with PHA treatment (17%, n=30) (P>0.05). Two female calves were born alive and healthy, from fresh-NT embryos. One healthy calf was delivered from vitrified NT embryo, without PHA mediated fusion. Two live, healthy calves resulted from vitrified NT embryos, with PHA mediated fusion (Fig. 2H). All cloned calves were delivered by Caesarean section (C-section) on day 270 to 290 of gestation.

4. Discussion

In this study, we have demonstrated that the efficiency of somatic cloning can be markedly increased by the use of phytohemagglutinin (PHA), presumably due to its agglutinating factors that assist in the adhesion between a nuclear donor cell and the recipient cytoplast. Obtaining adequate contact between a donor cell and a recipient oocyte, and thereby, achieving a higher rate of fusion has been one of the challenges of somatic nuclear transfer (NT). This modification of the NT procedure will help to increase its overall efficiency, particularly for the cloning novices. Our study provides a systematic investigation of effects of PHA dosage and duration on the efficiency of bovine somatic cloning. Following PHA treatment, the fusion rates of NT donor-oocyte complexes were increased from 33% to 61% for skin fibroblasts, and from 59% to 88% for cumulus cells. Embryonic development of the fused clone embryos to the blastocyst stage (from 29 to 38%) was not significantly affected by PHA treatment. However, the overall blastocyst developmental efficiency was significantly improved following pre-incubation of donor-cytoplast complexes in PHA. In our study, using cumulus cells as donor nuclei, nuclear transfer into metaphase II oocytes resulted in the highest percentage (34%) of cloned blastocysts. Incubation with PHA has been shown to increase the efficiency of cell fusion, as well as the number of viable hybrid cells, depending on the cell types [53]. It is believed that PHA may have great potential in tissue engineering and cell therapy [36]. Furthermore, phytohemagglutinin has been successfully used for membrane induced fusion in human oocytes [37], nuclear transfer with inner cell mass as nuclear donors [35], and hand-made somatic cell cloning- a zona free somatic cloning method in cattle [39,40,43,54]. Since human oocytes are easily activated by

electrofusion, enucleated oocytes were first treated with PHA, then induced to fuse with the aid of polyethylene glycol (PEG) [37]. A period of exposure of the nuclear donors and enucleated human oocytes, which were previously agglutinated by PHA, into PEG lead to a high yield of fused embryos, without causing oocyte activation [37]. In cattle somatic hand-made cloning, donor cell and oocyte recipient were briefly treated with PHA before electrical fusion, and a healthy, apparently normal calf was born after the resultant cloned embryos had been vitrified by opened pulled straw vitrification (OPS), thawed, and transferred into recipients [42].

The fusion rates in cattle, between the somatic donor cell and the recipient cytoplasm vary to a great extent, depending upon the donor cell types and their origins, and the techniques used among different laboratories [2,5,20,55]. The different membrane properties present in various cell types affect the fusion efficiency considerably [27]. In our study, for the control treatment – without PHA mediation, nuclear transfer with cultured cumulus cells as nuclear donors showed a higher fusion rate (59%), as compared to that of the skin fibroblasts (33%). The difference in membrane surface properties between cumulus cells and skin fibroblasts may contribute to this disparity in successful fusion with oocytes [50]. During the development and maturation of follicle and ovum, cumulus cells disperse outside of the oocyte; their processes and extrusions penetrate the zona pellucida and interdigitate with the microvilli arising from an oocyte that may provide it with nutrients and maternal proteins [56]. It is evident that the cumulus cells and the oocyte possess similar membrane surface properties, such as the types of glycoproteins present [29,48], thus, their membrane fusion can be easily facilitated by electrical stimulation. We achieved membrane fusion

rates as high as 59% between cumulus donor cells and oocytes under control conditions, without PHA treatment, which was higher than that reported by Tsunoda's group (47%) [2]. A fusion rate of up to 77.4% was generated with cultured adult mural granulose cells, by the Wells and Tervit group [55], and 63% with oviductal epithelial cells, by Tsunoda's group [2]. In NT using skin fibroblast as nuclear donors, Kubota et al [20] achieved a 36 to 43% fusion rate with adult fibroblast cells of different passage number (comparable to our 33%). In another study of fibroblast NT, Hill et al [5] reported as high as 59% fusion rate with adult skin cells derived from a Brahman bull. The method of enucleation used in most laboratories is aspiration, using a beveled glass pipette to remove both the polar body and the metaphase spindles [3,5,39,55,57]. By contrast, in our study we accomplish enucleation by the compressing or squashing method (Fig 2), utilizing a glass needle similar to that of Kubota et al [20,58]. About 1/8 of the total cytoplasm is extruded out through a slit on the zona pellucida to ensure a complete enucleation. We speculate that the lower fusion rates reported by different laboratories are likely attributable to the excess removal of cytoplasm. In addition, the high osmotic pressure of the fusion medium will result in cytoplasm shrinkage, this may subsequently lead to poor cell-oocyte contact, and thus, insufficient fusion during electrical stimulation.

Our study also demonstrated that, within the appropriate dosage range and incubation duration (150μg/mL for 20 min), phytohemagglutinin (PHA) showed no detrimental effects on pre-implantation nor term development of NT embryos. Phytohemagglutinins have powerful effects on proliferation and differentiation of various animal cells, including: lymphocytes, osteoblasts, and chondrocytes [59,60]. Although, a brief treatment of bone marrow mesenchymal stem cells with phytohemagglutinin had

little effect on their migration, proliferation, or differentiation [36]. However, a high dosage of PHA was cytotoxic to Chinese hamster ovary (CHO) cell mutants [44]. In the present study, we found high dosages (up to 600 μg/mL for 20 min) and/or prolonged treatment (150 μg/mL for 60 min) of phytohemagglutinin-L (PHA-L) was detrimental to the survival and subsequent development of parthenogenetically activated bovine oocytes *in vitro*. In human oocyte nuclear transfer, 300 μg/mL PHA has been supplemented in the medium, and an incubation of up to 60 min used to induce chemical fusion [37], however, the subsequent developmental potential of those fused oocytes was not examined. We expect that the developmental potential of those NT embryos would be adversely affected by such a prolonged incubation with PHA. A very short exposure, 3 sec, of the cytoplast to PHA, at concentrations of 200 μg/mL [42] or 500 μg/mL [54], was carried out during nuclear transfer/fusion to zona-free oocytes. This indicated that PHA attachment to the cell membrane could be completed within an extremely short period of time when zona-free oocytes directly contact the PHA, thus, reducing the possibility of PHA toxicity. However, we believe that this hand-made cloning protocol requires intensified technical skills for rapid hand-manipulations, in order to handle the small donor and cytoplast pairs and to ensure their correct alignment. Our experiment demonstrated that PHA could penetrate the zona pellucida, however, it required at least 20 min, probably to allow for absorption into the sub-zonal space, and to facilitate the agglutinating process. The results of Expt 1 showed that treatments with two doses of PHA (150 and 300 μg/mL) for 20 min were equally harmless to the oocytes (Table 1). It is possible that, similarly, the fusion rate could be increased by a dose of 300 μg/mL PHA (for 20 min) without affecting oocyte viability. As a precaution, however, we

deemed it more reasonable and safer to select a regime utilizing a lower PHA dosage (150 μg/mL). The development of fused NT embryos generated from PHA mediated fusion was not different from those without PHA treatment *in vitro*, suggesting the toxic effect of PHA was minimized in our system. Through a series of experiments, a concentration of 150 μg/mL was found safe and effective, among all PHA treatments tested, and the acceptable duration for a non-toxic, optimal fusion efficiency was determined to be 20 min.

We used phytohemagglutinin-L (PHA-L), extracted from red kidney bean (*Phaseolus vulgaris*) [53,61]. It possesses a specific binding to N-linked carbohydrate core structure (beta 1-6 branching) of glycoproteins on the cell membrane [32,62], and leucoagglutinating properties [32,53,63]. Therefore, because the cell membranes of bovine oocytes and fibroblast/cumulus donor cells distribute a variety of glycoproteins containing beta 1-6 branching, we concluded that they would be recognized and adhesion/fusion mediated by PHA-L [63].

In this study, we reported three full-term and apparently healthy female NT calves, produced with embryos frozen by our modified solid surface vitrification method, liquid nitrogen surface vitrification (LNSV). The newborns were generated from embryos either phytohemagglutinin treated (two calves), or untreated (one calf). To our knowledge, this is only the second full report of the success of cloned cattle from vitrified embryos, similar to that of Tecirliogue et al [42], who reported the first male calf from vitrified embryos constructed by hand-made cloning. Recent data indicate that a rapid, rather than slow cooling method, namely vitrification, might be beneficial for the survivability of IVF embryos [64,65], such as Open Pulled Straw (OPS) vitrification [66-

68] and Liquid Nitrogen Surface Vitrification (LNSV), recently developed by our group [50]. In the present study, we used the same LNSV vitrification protocol for the cryopreservation of PHA treated or un-treated NT embryos. Because most of the embryos were half-hatched, through the slit in the zona made during the nuclear transfer process, the developmental potential of cloned embryos *in vitro* was determined by completed hatching from the zona. We observed an extremely high, up to 96%, survival rate of vitrified embryos from both PHA treated and non-PHA control cloning groups, and subsequent complete hatching rates of up to 86 to 92%. These results demonstrated that bovine vitrified cloned embryos derived from PHA mediated fusion have equal ability to survive and subsequently develop as do non-PHA treated embryos *in vitro*. The full developmental potential and health status of newborns derived from PHA cloned embryos were also shown to be similar to those from non-PHA treated embryos, indicating that PHA, at the optimal dosage and incubation duration, did not have a toxic effect on *in vivo* development of cloned embryos In conclusion, an optimized procedure of somatic cloning using phytohemagglutinin (PHA) was shown to be effective by significantly increasing the fusion rate, and thus, the overall efficiency of somatic cloning in cattle. *In vitro* and *in vivo* development of NT embryos was not harmed by PHA assisted membrane fusion treatment. This efficient cloning technology should be applicable to improve nuclear transfer efficiency in other domestic animals, such as pigs, sheep, rabbits, goats and horses, where electrical fusion is used. The combination of NT and PHA mediated cell fusion could also be applied to the efforts to preserve endangered species.

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Table 1. Effect of phytohemagglutinin (PHA) dosage on parthenogenetic development of activated bovine oocytes

Treatment (μg/mL PHA)	No. Total	% Oocytes Survived*	% Survived oocytes developed				Total Efficiency (%)§
			Cleaved	8-cell	Morula	D7 BLs	
A. 0	227	96 ± 0.7[a]	84 ± 2.3	35 ± 3.9	39 ± 4.9	29 ± 1.5[a]	28 ± 1.6[a]
B. 150	229	92 ± 1.9[a]	67 ± 8.2	30 ± 8.2	35 ± 4.8	23 ± 2.0[a,b]	21 ± 2.0[a]
C. 300	221	82 ± 4.2[a]	72 ± 2.7	33 ± 3.2	28 ± 1.7	24 ± 1.5[a,b]	20 ± 1.7[a]
D. 600	225	54 ± 9.8[b]	67 ± 5.2	27 ± 3.7	23 ± 3.4	13 ± 4.3[b]	8 ± 3.2[b]

[a,b]Values with different superscripts within columns differ, P<0.05. BL, blastocyst, D7, day 7. Oocytes were treated with different concentrations of PHA for 20 min. Oocyte survival was evaluated morphologically under stereomicroscope. Oocyte development to cleaved (2 to 8 cell), 8-cell stage and morula was evaluated on day 2 and day 4, respectively, according to the standard of the International Embryo Transfer Society [51].

*Oocytes that survived following PHA treatment were selected from each group for further activation and culture. §The overall blastocyst rate was calculated using the total number of oocytes in each treatment.

Table 2. Effect of phytohemagglutinin (PHA, 150 μg/mL) treatment duration on parthenogenetic development of activated bovine oocytes

Treatment (min)	No. Total	% Oocytes Survived*	% Survived oocytes developed				Total Efficiency (%)§
			Cleaved	8-cell	Morula	D7 BLs	
A. 0	166	94 ± 2.3	85 ± 3.1[a]	42 ± 6.2[a]	38 ± 6.5[a]	34 ± 4.7[a]	32 ± 4.1[a]
B. 10	157	91 ± 3.1	76 ± 7.7[a]	45 ± 10[a]	45 ± 4.5[a]	38 ± 3.9[a]	35 ± 2.9[a]
C. 20	189	94 ± 1.9	67 ± 11[a]	33 ± 9.4[a,b]	36 ± 5.9[a]	23 ± 2.6[a,b]	21 ± 2.6[a,b]
D. 60	172	91 ± 3.7	56 ± 15[b]	30 ± 11[b]	29 ± 7.8[b]	21 ± 7.1[b]	19 ± 5.9[b]

[a,b]Values with different superscripts within columns differ, P<0.05. BL, blastocyst, D7, day 7. Oocytes were treated with a concentration of PHA at 150 μg/mL for different durations. Oocyte survival was evaluated morphologically under stereomicroscope. Oocyte development to cleaved (2 to 8 cell), 8-cell stage and morula was evaluated on day 2 and day 4, respectively, according to the standard of the International Embryo Transfer Society [51]. *Oocytes that survived following PHA treatment were selected from each group for further activation and culture. §The overall blastocyst rate was calculated using the total number of oocytes in each treatment.

Table 3. Effects of phytohemagglutinin (PHA) treatment and donor cell type on the development of cloned bovine embryos (Expt 3b)

Treatment		Total	% Oocytes	% Embryos developed to			% BL/fused oocytes*
Donor Cell	PHA (μg/mL)	No	Fused	2-8 cell	Morula	D7 BLs	
Skin fibroblast	0	232	33 ± 3.9[a]	27 ± 2.1[a]	18 ± 3.9[a]	13 ± 4.4[a]	36 ± 11[a]
Skin fibroblast	150	288	61 ± 4.5[b]	57 ± 5.0[b]	35 ± 9.4[b]	22 ± 5.5[b,c]	35 ± 6.7[a]
Cumulus cells	0	233	59 ± 5.6[b]	52 ± 5.8[b]	30 ± 8.8[b]	17 ± 3.7[a,b]	29 ± 4.9[a]
Cumulus cells	150	242	88 ± 3.5[c]	72 ± 7.1[c]	52 ± 8.8[c]	34 ± 8.5[c]	38 ± 8.6[a]

[a,b,c]Values with different superscripts within columns differ, P<0.05. 2-8 cell, 2 to 8 cell, BL, blastocyst, D7, day 7. NT embryo development to cleaved (2 to 8 cell), and morula stage was evaluated on day 2 and day 4, respectively, according to the standard of the International Embryo Transfer Society [51]. The over all developmental rates to cleavage, morula, and blastocyst, in NT embryos, were calculated from the total number of oocytes used for NT. [*]The percentage of blastocyst development was calculated based upon the number of fused oocytes in each treatment.

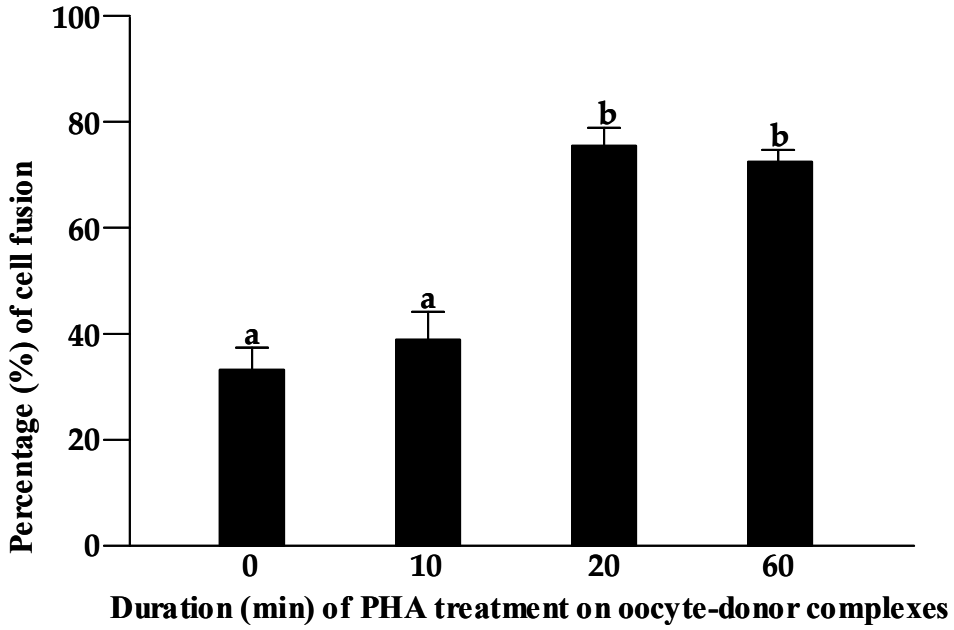

Figure 1 Fusion rate of oocyte-donor complexes after phytohemagglutinin (PHA) treatment (Expt 3a). Cumulus donor cell-cytoplast complexes were incubated in 150 μg/mL PHA and 7.5% FBS M199 for 0, 10, 20 and 60 min before electrical current was applied. The fusion rate was 35% (n=67), and 38% (n=72) with a phytohemagglutinin treatment duration of 0 and 10 min, respectively($P>0.5$). In contrast, a significantly high rate of donor cell-cytoplast pairs were fused when the duration of phytohemagglutinin treatment was increased to 20 min (78%, n=61), and 60 min (75%, n=71)($P<0.05$).

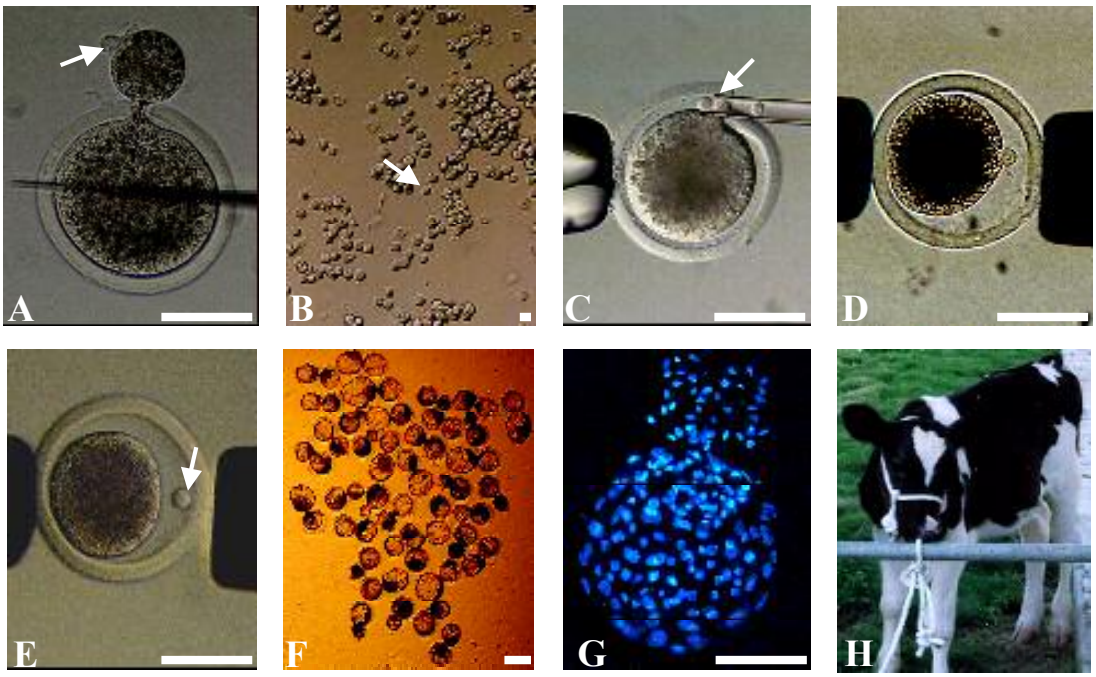

Figure 2 Somatic nuclear transfer using phytohemagglutinin as an agglutination agent in cattle. (**A**) Enucleation of matured bovine oocyte. A slit in the zona pellucida was made by a micro-needle, then the first polar body (arrow) and its surrounding cytoplasm, approximately 1/8 the total volume, presumably containing metaphase II chromosomes, were pressed out of the oocyte. The extruded cytoplasm was then stained and examined under fluorescent microscopy to ensure successful enucleation. Only enucleated oocytes, whose MII chromosomes were confirmed to be contained in the excluded cytoplasm, were used for nuclear transfer. Cumulus or fibroblast cells were disassociated by 0.25% trypsin, small and round cells (arrow in **B**) that contained a compact nucleus were selected, then transferred into the perivitelline space of an enucleated oocyte (arrow in **C**). (**D-E**)Oocyte-nuclear donor complex was subjected to cell fusion induced by direct electrical pulses using micro-electro rods. The donor cell and recipient oocyte adhered together after PHA treatment (**D**), while the donor cell often stuck to the oocyte's zona

pellucida (arrow) without PHA treatment (**E**). After optimal electrical activation, 39 hatching/hatched blastocysts (**F**) developed from fused NT embryos on day 7 of culture; these were shown, under fluorescent microscopy, to possess substantially propagated nuclei from a donor cell nucleus (**G**). A calf born following embryo transfer of vitrified, PHA-treated embryos (**H**). Bar represents 80 μm, in **A**, **C, D**, **E**, **F, G**, and 20 μm in **B**.

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