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1 **The cell agglutination agent, phytohemagglutinin-L, improves the**
2 **efficiency of somatic nuclear transfer cloning in cattle (*Bos taurus*)**

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15 development

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22

1 **Abstract**

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

- 1 has no detrimental effect on embryo survival post vitrification, nor on pregnancy or
- 2 calving rates following embryo transfer.
- 3

1 **1. Introduction**

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

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

1 surface-specific carbohydrates by phytohemagglutinin (PHA) could have important
2 implications for critical biological processes, as well as practical applications in cellular
3 bioengineering. Phytohemagglutinins have been widely used in cell biology for
4 enhancing cell agglutination and fusion in plant protoplasts [33], and in various types of
5 mammalian cells including: erythrocytes [34], hybrid cell colonies [27,35], and bone
6 marrow mesenchymal stem cells [36]. Moreover, PHA was used for nuclear transfer
7 with human [37], goat [38] and bovine [39] oocytes, and for zona free NT in cattle [40-
8 43]. However, the toxicity of PHA to oocytes, donor somatic cells, and its consequence
9 to the developmental potential of NT embryos, has not been well established. It was
10 reported that high doses of PHA were cytotoxic to Chinese Hamster ovary (CHO) and
11 other mammalian cells [44].

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

18 **2. Materials and methods**

19 All the chemicals were manufactured in the USA. Most chemicals were obtained
20 from Sigma Chemicals Co (St. Louis, MO, USA) unless otherwise indicated. All embryo
21 cultures were maintained at 39 °C in 5% CO₂ and humidified air unless otherwise
22 specified.

23 *2.1. Bovine oocyte recovery, culture, and maturation in vitro*

1 Bovine cumulus oocyte complexes (COCs) were aspirated from antral follicles of
2 slaughterhouse ovaries as described previously [45]. COCs with at least 4 intact layers of
3 tight cumulus cells were selected, washed three times in Dulbecco's phosphate buffered
4 saline (D-PBS; Gibco, Grand Island, NY, 15240-013) containing 0.1% polyvinyl alcohol
5 (PVA; P-8136) (D-PBS+PVA). COCs, in groups of 20 to 25, were matured in 75 μ L
6 drops of maturation medium for 18 to 20 h. Maturation medium was a basal Medium 199
7 (M199) with Earle's salts, L-glutamine, 2.2 g/L sodium bicarbonate, and 25 mM HEPES
8 (Gibco, Grand Island, NY, 12340-014) containing 7.5% (v/v) fetal bovine serum (FBS)
9 (Hyclone, Logan, UT, SH0070.03), and supplemented with 0.5 μ g/mL ovine FSH
10 (NIDDK), 5.0 μ g/mL ovine LH (NIDDK), 1.0 μ g/mL estradiol (E-8875) and antibiotics
11 (Gibco, Grand Island, NY, 15240-013). The matured oocytes with well-expanded
12 cumulus layers were selected for proceeding of cumulus cell denuding for either
13 parthenogenetic activation or enucleation and NT.

14 *2.2. Culture of bovine skin fibroblast and cumulus donor cells*

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

1 35x10 mm culture dishes (Becton Dickinson, Franklin Lakes, NJ, 3001). Ear skin
2 explants were cultured in 10% FBS DMEM. Both COCs and skin tissues were cultured
3 at 37°C in 5% CO₂ humidified air until confluency. Cumulus cells formed a monolayer
4 around ova, and fibroblast monolayers formed from the tissue explants, in about two
5 weeks. For passaging, cells were washed with 1 mL of Dulbecco's PBS, then gently
6 digested by a three-minute incubation in 100 µl 0.05% trypsin (ICN, Aurora, OH,
7 103140) and 0.5 mM EDTA (Baker, Phillipsburg, NJ, 8991) at 37°C. The reaction was
8 terminated by adding 5% FBS in DMEM. Subsequently, the cell suspension was
9 centrifuged at 800 x g for 5 min, and cells were resuspended and divided into three new
10 dishes and maintained for 6 to 7 d. Cells cultured to different numbers of passages were
11 collected and frozen in 7% dimethylsulfoxide (DMSO, D-5879) and 7% glycerol (G-
12 2025) at -80°C for 1 d, then stored in liquid nitrogen.

13 Donor cells at passage 5 to 10, derived either from cattle cumulus or fibroblast
14 cells were used for nuclear transfer. After reaching confluency, bovine fibroblast cells
15 were serum starved in 0.5% FBS DMEM for 4 to 5 d. The bovine cumulus cells were not
16 starved, but were continuously cultured for an additional 5 to 6 d after confluency, in
17 20% FBS DMEM. Nuclear donor cells were then disassociated by 2 to 3 min of
18 trypsinization at 37°C, and resuspended in 1 mL 5% FBS in DMEM. Prior to NT, cell
19 suspensions were allowed to recover for about 20 to 30 min at 37°C.

20 *2.3. Nuclear transfer, phytohemagglutinin treatment, and cell fusion*

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

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

5 Bovine NT treatment groups were incubated for 20 min in M199-FCS
6 supplemented with 0 or 150 µg /mL phytohemagglutinin-L (PHA-L, L-4144). PHA-L
7 was classified as a lecuoagglutinin, derived from *Phaseolus vulgaris* (red kidney bean).
8 Donor-cytoplasm complexes were incubated for 5 min in Zimmerman cell fusion medium
9 [47], then manually oriented by fine electrical rods (Fig. 2D,E) under light microscopy.
10 Electric fusion of the donor cells to the recipient oocytes was accomplished by a double
11 electrical pulse of 2.3 kV/cm for 10 µsec. After the electric stimulation, oocyte-donor
12 cell complexes were incubated for 15 to 30 min in 20% FBS PBS at room temperature
13 before being subjected to further activation procedures. Fusion rates were determined 90
14 min after electrical pulse.

15 *2.4 Activation and culture of cloned bovine embryos in vitro*

16 After electrical stimulation, cloned bovine embryos were cultured in M199+FCS
17 supplemented with 2.5 µg/mL cytochalasin D (CD) (C-8273) and 10 µg/mL
18 cycloheximide (CHX) (C-6255) for 1 h, and then for an additional 4 h in M199+FCS
19 with 10 µg/mL CHX. The culture medium for bovine NT embryos was a defined CR1
20 medium (referred to as CR1aa [48]) consisting of: 114.7 mM NaCl, 3.1 mM KCl, 26.2
21 mM NaHCO₃, 1 mM L-Glutamine, 0.4 mM sodium pyruvate (P-2256) and 5.5 mM
22 hemicalcium lactate (L-4388), and was supplemented with 1X MEM (M-7145) and 1X
23 BME amino acid (B-6766). Activated bovine NT embryos were cultured in CR1aa

1 containing 6 mg/mL BSA for 2 d (initiation of activation = day 0), under 5% CO₂, 5% O₂
2 and 90% N₂ with high humidity. Embryos were then switched to 10% FBS CR1aa co-
3 culture with a bovine cumulus monolayer for an additional 5 d. For somatic cell co-
4 culture of NT embryos, the medium in the maturation droplets that contained a layer of
5 80 to 90% confluent cumulus cells was changed to the appropriate CR1aa culture
6 medium.

7 *2.5. Vitrification of cloned embryos*

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

1 To test the viability of vitrified NT embryos *in vitro*, frozen embryos were
2 sequentially warmed, rehydrated in 20% FBS M199 with different concentrations of
3 sucrose, and washed in 20% FBS M199 (Vitrification and Warming Kit, provided by
4 Evergen Biotechnologies Inc, Storrs, CT) for 5 min. Cell counts and nuclear evaluations
5 of embryos were performed under fluorescent microscopy following staining with
6 10µg/mL Hoechst 33342.

7 *2.6. Evaluation of fresh and vitrified NT embryos in vitro*

8 The different stages of bovine embryo development were recorded as cleavage (2
9 to 8 cell), morula, and blastocyst, on day 2, day 4, and day 7, respectively, according to
10 the standard of the International Embryo Transfer Society (IETS manual, 1998) [51].
11 Vitrified bovine NT embryos were cultured for three days in M199 supplemented with
12 7.5%FBS. Survival and development to hatching were recorded at 0h, 24h, 48h, and 72h
13 of culture (warming = 0 h).

14 *2.7. Transfer of vitrified embryos and pregnancy monitoring*

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

1 5% CO₂ gassing of 20% FBS M199 culture medium, and transferred to a farm for direct
2 embryo transfer. Frozen NT embryos were thawed by transferring the vitrified spheres
3 containing embryos through a series of steps as described above. Two embryos were
4 transferred into each synchronous recipients by non-surgical means. Pregnancy was
5 determined by palpation per rectum on day 70 after transfer.

6 2.8. Specific experiments

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

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

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

1 to an electrical pulse, and the fusion rates were examined 90 min later. After determining
2 the best fusion parameters (150 $\mu\text{g}/\text{mL}$ for 20 min) (Expt 3a), somatic donor cell-oocyte
3 cytoplasm pairs were randomly allocated to a 2 X 2 factorial design, with
4 phytohemagglutinin treatment for 20 min at a concentration of either 0 or 150 $\mu\text{g}/\text{mL}$ in
5 7.5% FBS M199, and with either cumulus or fibroblast cells as nuclear donors;
6 subsequently, the NT embryos were allocated to *in vitro* tests to determine their
7 developmental potentials (Expt 3b).

8 *2.9. Statistical analyses*

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

17 **3. Results**

18 *3.1. Experiment 1*

19 This experiment was designed to test the effect of phytohemagglutinin (PHA)
20 dosage on oocyte survival and subsequent development. As shown in Table 1, the
21 survival rates of oocytes were not different when treated with PHA at a dose from 0 to
22 300 $\mu\text{g}/\text{mL}$. In contrast, a PHA concentration of 600 $\mu\text{g}/\text{mL}$ proved to be highly toxic to
23 oocyte development. Likewise, the total efficiency of blastocyst development was

1 similar among groups treated with 0, 150, or 300 $\mu\text{g}/\text{mL}$ (28, 21, and 20% respectively,
2 $P>0.05$), whereas, the blastocyst development rate for oocytes treated with 600 $\mu\text{g}/\text{mL}$
3 PHA went as low as 8% ($P<0.05$).

4 *3.2. Experiment 2*

5 To further elucidate the effects of the duration of phytohemagglutinin (PHA)
6 treatment on parthenogenetic development, oocytes were treated with PHA at a dose of
7 150 $\mu\text{g}/\text{mL}$ (determined in Expt 1) for 0, 10, 20 or 60 min, prior to activation . Data
8 (Table 2) showed, following PHA treatment of oocytes for 0 to 20 min, the survival (91
9 to 94%), cleavage (67 to 85%), and blastocyst development rates (23 to 38%) were not
10 affected; neither was there an adverse effect on total efficiency of blastocyst
11 development, calculated as a percentage of the total number of oocytes treated (21 to
12 35%). However, incubation with 150 $\mu\text{g}/\text{mL}$ PHA for 60 min not only reduced the
13 cleavage rates (56%), as well as 8-cell (30%), morula (29%), and blastocyst development
14 (21%), but also reduced the total efficiency (19%), compared to those observed with
15 shorter treatments ($P<0.05$). Therefore, we conclude that a prolonged incubation with
16 phytohemagglutinin is detrimental to oocyte development, similar to the adverse effect
17 observed with a high dosage.

18 *3.3. Experiment 3*

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

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

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

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

3 *3.4. Developmental potentials of phytohemagglutinin treated NT embryos in vitro and in*
4 *vivo after vitrification*

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

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

1 **4. Discussion**

2 In this study, we have demonstrated that the efficiency of somatic cloning can be
3 markedly increased by the use of phytohemagglutinin (PHA), presumably due to its
4 agglutinating factors that assist in the adhesion between a nuclear donor cell and the
5 recipient cytoplasm. Obtaining adequate contact between a donor cell and a recipient
6 oocyte, and thereby, achieving a higher rate of fusion has been one of the challenges of
7 somatic nuclear transfer (NT). This modification of the NT procedure will help to
8 increase its overall efficiency, particularly for the cloning novices. Our study provides a
9 systematic investigation of effects of PHA dosage and duration on the efficiency of
10 bovine somatic cloning. Following PHA treatment, the fusion rates of NT donor-oocyte
11 complexes were increased from 33% to 61% for skin fibroblasts, and from 59% to 88%
12 for cumulus cells. Embryonic development of the fused clone embryos to the blastocyst
13 stage (from 29 to 38%) was not significantly affected by PHA treatment. However, the
14 overall blastocyst developmental efficiency was significantly improved following pre-
15 incubation of donor-cytoplasm complexes in PHA. In our study, using cumulus cells as
16 donor nuclei, nuclear transfer into metaphase II oocytes resulted in the highest percentage
17 (34%) of cloned blastocysts. Incubation with PHA has been shown to increase the
18 efficiency of cell fusion, as well as the number of viable hybrid cells, depending on the
19 cell types [53]. It is believed that PHA may have great potential in tissue engineering and
20 cell therapy [36]. Furthermore, phytohemagglutinin has been successfully used for
21 membrane induced fusion in human oocytes [37], nuclear transfer with inner cell mass as
22 nuclear donors [35], and hand-made somatic cell cloning- a zona free somatic cloning
23 method in cattle [39,40,43,54]. Since human oocytes are easily activated by

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

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

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

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

1 little effect on their migration, proliferation, or differentiation [36]. However, a high
2 dosage of PHA was cytotoxic to Chinese hamster ovary (CHO) cell mutants [44]. In the
3 present study, we found high dosages (up to 600 $\mu\text{g}/\text{mL}$ for 20 min) and/or prolonged
4 treatment (150 $\mu\text{g}/\text{mL}$ for 60 min) of phytohemagglutinin-L (PHA-L) was detrimental to
5 the survival and subsequent development of parthenogenetically activated bovine oocytes
6 *in vitro*. In human oocyte nuclear transfer, 300 $\mu\text{g}/\text{mL}$ PHA has been supplemented in
7 the medium, and an incubation of up to 60 min used to induce chemical fusion [37],
8 however, the subsequent developmental potential of those fused oocytes was not
9 examined. We expect that the developmental potential of those NT embryos would be
10 adversely affected by such a prolonged incubation with PHA. A very short exposure, 3
11 sec, of the cytoplasm to PHA, at concentrations of 200 $\mu\text{g}/\text{mL}$ [42] or 500 $\mu\text{g}/\text{mL}$ [54],
12 was carried out during nuclear transfer/fusion to zona-free oocytes. This indicated that
13 PHA attachment to the cell membrane could be completed within an extremely short
14 period of time when zona-free oocytes directly contact the PHA, thus, reducing the
15 possibility of PHA toxicity. However, we believe that this hand-made cloning protocol
16 requires intensified technical skills for rapid hand-manipulations, in order to handle the
17 small donor and cytoplasm pairs and to ensure their correct alignment. Our experiment
18 demonstrated that PHA could penetrate the zona pellucida, however, it required at least
19 20 min, probably to allow for absorption into the sub-zonal space, and to facilitate the
20 agglutinating process. The results of Expt 1 showed that treatments with two doses of
21 PHA (150 and 300 $\mu\text{g}/\text{mL}$) for 20 min were equally harmless to the oocytes (Table 1). It
22 is possible that, similarly, the fusion rate could be increased by a dose of 300 $\mu\text{g}/\text{mL}$
23 PHA (for 20 min) without affecting oocyte viability. As a precaution, however, we

1 deemed it more reasonable and safer to select a regime utilizing a lower PHA dosage
2 (150 µg/mL). The development of fused NT embryos generated from PHA mediated
3 fusion was not different from those without PHA treatment *in vitro*, suggesting the toxic
4 effect of PHA was minimized in our system. Through a series of experiments, a
5 concentration of 150 µg/mL was found safe and effective, among all PHA treatments
6 tested, and the acceptable duration for a non-toxic, optimal fusion efficiency was
7 determined to be 20 min.

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

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

1 68] and Liquid Nitrogen Surface Vitrification (LNSV), recently developed by our group
2 [50]. In the present study, we used the same LNSV vitrification protocol for the
3 cryopreservation of PHA treated or un-treated NT embryos. Because most of the
4 embryos were half-hatched, through the slit in the zona made during the nuclear transfer
5 process, the developmental potential of cloned embryos *in vitro* was determined by
6 completed hatching from the zona. We observed an extremely high, up to 96%, survival
7 rate of vitrified embryos from both PHA treated and non-PHA control cloning groups,
8 and subsequent complete hatching rates of up to 86 to 92%. These results demonstrated
9 that bovine vitrified cloned embryos derived from PHA mediated fusion have equal
10 ability to survive and subsequently develop as do non-PHA treated embryos *in vitro*. The
11 full developmental potential and health status of newborns derived from PHA cloned
12 embryos were also shown to be similar to those from non-PHA treated embryos,
13 indicating that PHA, at the optimal dosage and incubation duration, did not have a toxic
14 effect on *in vivo* development of cloned embryos. In conclusion, an optimized procedure
15 of somatic cloning using phytohemagglutinin (PHA) was shown to be effective by
16 significantly increasing the fusion rate, and thus, the overall efficiency of somatic cloning
17 in cattle. *In vitro* and *in vivo* development of NT embryos was not harmed by PHA
18 assisted membrane fusion treatment. This efficient cloning technology should be
19 applicable to improve nuclear transfer efficiency in other domestic animals, such as pigs,
20 sheep, rabbits, goats and horses, where electrical fusion is used. The combination of NT
21 and PHA mediated cell fusion could also be applied to the efforts to preserve endangered
22 species.

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

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

3 ^{a,b}Values with different superscripts within columns differ, P<0.05. BL, blastocyst, D7, day 7. Oocytes were
 4 treated with different concentrations of PHA for 20 min. Oocyte survival was evaluated morphologically under
 5 stereomicroscope. Oocyte development to cleaved (2 to 8 cell), 8-cell stage and morula was evaluated on day
 6 2 and day 4, respectively, according to the standard of the International Embryo Transfer Society [51].

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

9

1 **Table 2. Effect of phytohemagglutinin (PHA, 150 µg/mL) treatment duration on parthenogenetic**
 2 **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

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

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

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

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

9

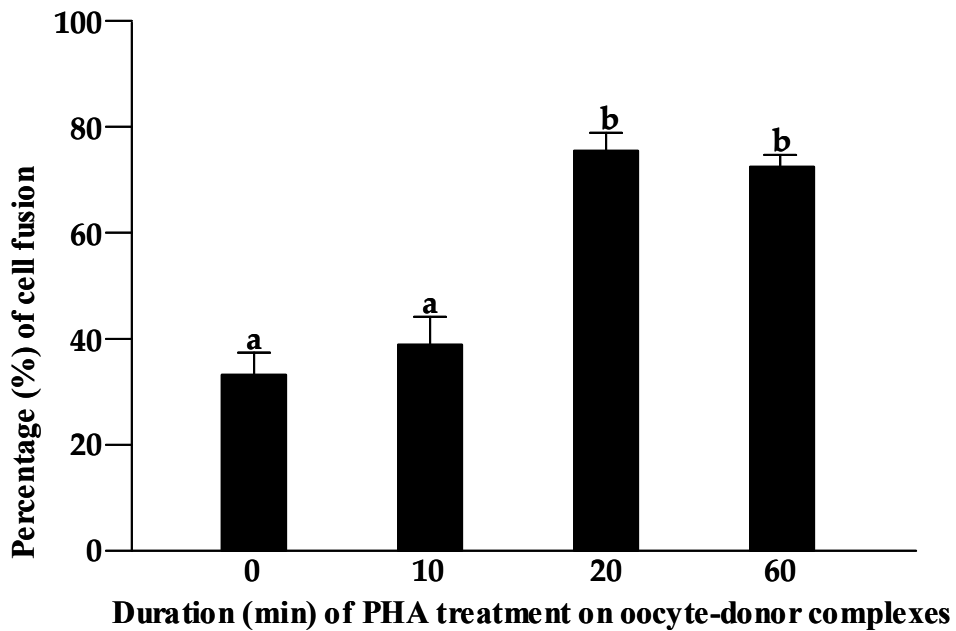


Figure 1 Fusion rate of oocyte-donor complexes after phytohemagglutinin (PHA) treatment (Expt 3a). Cumulus donor cell-cytoplasm complexes were incubated in 150 $\mu\text{g}/\text{mL}$ PHA and 7.5% FBS M199 for 0, 10, 20 and 60 min before electrical current was applied. The fusion rate was 35% (n=67), 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-cytoplasm pairs were fused when the duration of phytohemagglutinin treatment was increased to 20 min (78%, n=61), and 60 min (75%, n=71) ($P<0.05$).

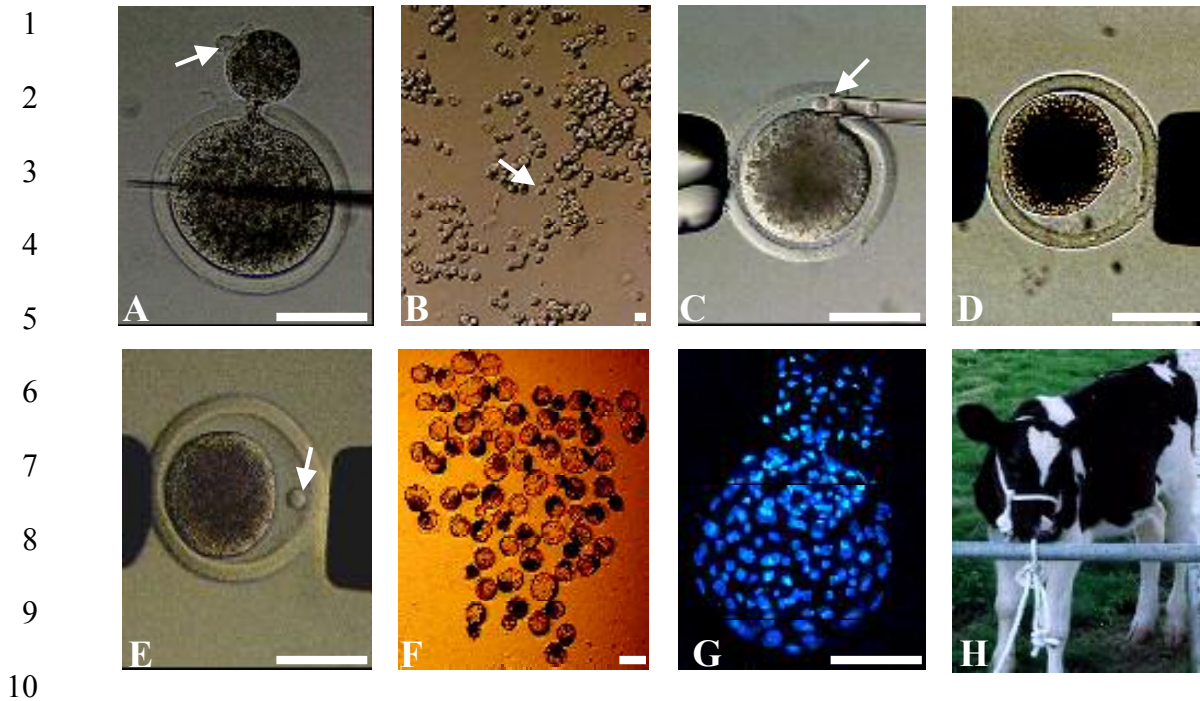


Figure 2 Somatic nuclear transfer using phytohemagglutinin as an agglutination agent in cattle. (A) Enucleation of matured bovine oocyte. A slit in the zona pellucida was made by a micro-needle, then the first polar body (arrow) and its surrounding cytoplasm, approximately 1/8 the total volume, presumably 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

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

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