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Original article

Beneficial effect of oocyte activation prior to and during nuclear transfer in cattle using *in vitro* matured oocytes 24 h of age

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Summary — This research was designed to study *in vitro* development of bovine nuclear transferred embryos using enucleated young *in vitro* matured oocytes 24 h of age as recipient cytoplasts activated prior to nuclear transfer. The oocytes were enucleated and then activated with electric pulse at 24 h of age followed by incubation with cycloheximide for 6–7 h before nuclear transfer and membrane fusion with a blastomere of the 16–32 cell stage. This new protocol effectively improved cleavage (68 *vs* 17%, $p < 0.05$) and morula/blastocyst development of reconstructed embryos (29 *vs* 6%, $p < 0.05$), compared to similar nuclear transfer procedure without prior activation of recipient oocytes. Corresponding rates of cleavage and morula/blastocyst development for oocytes activated similarly without nuclear transfer were 49 and 19%. Factors affecting nuclear transfer were also compared. Two electric pulses for fusion increased rates of fusion (76 *vs* 60%, $p < 0.05$) and subsequent development of cloned embryos (32 *vs* 11%, $p < 0.05$). Cytochalasin B treatment following nuclear transfer manipulation seemed not to be beneficial in improving development of cloned embryos ($p > 0.05$). Both co-culture systems with buffalo rat liver (BRL) cells and cumulus cells promoted development of cloned embryos compared to the controls (28, 21 *vs* 0%, $p < 0.05$). The BRL cell system seemed to be better for manipulated embryos by reducing embryolysis.

nuclear transfer / parthenogenetic activation / embryo culture / cattle

Résumé — Effet bénéfique de l'activation des ovocytes avant et pendant le transfert nucléaire chez les bovins dans le cas d'utilisation d'ovocytes maturés *in vitro* âgés de 24 h. *Ce travail a pour but l'étude du développement* in vitro *d'embryons bovins issus de transfert nucléaire dans des ovocytes jeunes maturés* in vitro *pendant 24 h. Les ovocytes sont ensuite énucléés puis activés par un champ électrique, puis incubés en présence de cycloheximide pendant 6–7 h avant fusion avec un blastomère de stade 16–32 cellules. Ce nouveau protocole améliore la segmentation (68 au lieu de 17%, p < 0,05) et le développement jusqu'au stade morula/blastocyte (29 au lieu de 6%, p < 0,05) si l'on compare avec un transfert nucléaire similaire sans préactivation. Leurs pourcentages de segmentation et de développement au stade morula/blastocyte pour des ovocytes activés sans transfert nucléaire sont 49 et 19%. Les facteurs qui influencent le transfert nucléaire sont aussi analysés. Deux impulsions élec-*

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triques sont plus efficaces pour obtenir une fusion (76 au lieu de 60%, p < 0,05) et un développement subséquent (32 au lieu de 11%, p < 0,05). Un traitement par la cytochalasine B après transfert nucléaire ne semble pas améliorer le développement (p > 0,05). La coculture avec des cellules de foie de rat buffle (cellules BRL) ou des cellules de cumulus favorise le développement des embryons clonés par rapport aux témoins (28 et 21 au lieu de 0%, p < 0,05). Le système avec les cellules BRL semble empêcher la lyse des embryons manipulés.

transfert nucléaire / activation parthénogénétique / culture d'embryons / bovins

INTRODUCTION

Activation of recipient oocytes is an important step in nuclear transfer for cloning embryos (Robl and Stice, 1989; Smith and Wilmut, 1990). Previous nuclear transfer studies involved introducing a donor nucleus into an enucleated mature (metaphase II or MII) oocyte. Activation was usually conducted at the time of membrane fusion applied by electric stimulation (Willadsen, 1986; Prather *et al,* 1987, 1989; Stice and Robl, 1988; Smith and Wilmut, 1989; Yang *et al,* 1992a, 1993a; Stice and Keefer, 1993). Because of the high level of maturation promoting factor (MPF) in the MII oocyte, the transferred nucleus would be quickly forced into metaphase, known as premature chromosome condensation (PCC) (Collas and Robl, 1991; Collas *et al,* 1992a; Barnes *et al,* 1993; Campbell *et al,* 1993). PCC was believed to be beneficial for remodeling the donor nucleus following nuclear transfer (Collas and Robl, 1991).

In early studies, nuclear transfer with *in vitro* matured bovine oocytes often resulted in poorer development compared to that with *in vivo* matured oocytes (Prather *et al,* 1987). Activation of oocytes was observed as oocyte-age dependent (Ware *et al,* 1989; Yang *et al,* 1991, 1993a; Leibfried-Rutledge *et al,* 1992). Oocyte ageing, however, may cause an abnormal distribution of cytoskeletal components (Webb *et al,* 1986). The alterations in the cytoskeleton can lead to inward migration of the metaphase plate, which may be the cause for the previously reported reduced rate of enucleation of the aged oocytes and thus affects nuclear transfer efficiency (Yang, 1991; Yang *et al,* 1991). Thus it would be ideal to use young oocytes for nuclear transfer if they could be activated adequately. This possibility has become feasible with the finding that young oocytes have been effectively activated by combined activation treatments (Yang *et al,* 1992b; Presicce and Yang, 1994).

Several recent studies demonstrated that activating oocytes prior to membrane fusion prevented the PCC process (Barnes *et al,* 1993; Campbell *et al,* 1993) but increased the development of cloned embryos (Barnes *et al,* 1993; Aoyagi *et al,* 1994; Campbell *et al,* 1994; Heyman *et al,* 1994; Kono *et al,* 1994; Stice *et al,* 1994). In this study experiments were designed to test this finding with the combined activation treatment of electric pulse and cycloheximide used routinely in our laboratory, and to modify nuclear transfer procedures for improving development of cloned embryos.

MATERIALS AND METHODS

Oocyte maturation in vitro

Bovine oocytes were matured *in vitro* as described previously (Yang *et al,* 1993b). Briefly, oocytes were aspirated from small antral follicles (2–7 mm in diameter) in abattoir ovaries. Oocytes with intact or at least 4 layers of cumulus cells were used in this study. Prior to maturation culture, they were washed 3 times in Dulbecco's phosphate-buffered saline (DPBS) (Gibco, NY) containing 0.1% polyvinyl alcohol (PVA, Sigma, MO) (DPBS + PVA) and one time in maturation

medium. The maturation medium was Medium 199 (M199) with Earle's salts, 25 mM Hepes (Gibco, NY) containing 7.5% fetal calf serum (FCS) (M199 + FCS) and hormones. The hormone doses adopted in this study were 0.5 μg/ml ovine FSH, 5.0 μg/ml ovine LH (NIDDK) and 1.0 μg/ml estradiol (Sigma, MO). Oocytes were cultured for 20–22 h under 5% CO_2 and 95% humidified air at 39°C. Cumulus cells were then removed by incubation of the oocytes for 3 min in calcium-free DPBS-PVA containing 0.2% hyaluronidase (Sigma, MO) followed by vortexing for 1.5 min.

Oocyte activation

Denuded oocytes with polar body and evenly distributed cytoplasm were selected and allocated randomly to various treatments. Enucleated or intact oocytes were activated by combined treatments of electric pulse and cycloheximide (First *et al,* 1992; Yang *et al,* 1992b). Newly matured intact or enucleated young oocytes at 24 h of maturation were placed in 0.3 M mannitol with 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.5 mg/ml BSA (Fraction V, Sigma, MO) and equilibrated in this solution for 3 min. Activation (or membrane fusion) was conducted in a 3.2 mm BTX electrofusion chamber (BTX Inc, CA) by applying 1.0 MHz, 0.1 kV/cm alternating current (AC) for 5–10 s followed by a pulse of 1.2 kV/cm direct current (DC) for 30 μs as described previously (Yang *et al,* 1993a). Pulsed oocytes were washed with M199 + FCS, and then incubated in M199 + FCS containing 10 μg/ml cycloheximidine (Sigma, MO) for 6 h in a 5% CO_2 incubator at 39°C.

Donor embryos and cell disaggregation

Donor cells for nuclear transfer were from embryos derived from *in vitro* fertilization (IVF). IVF was conducted as described previously (Yang *et al,* 1993b). Briefly, oocytes were inseminated with capacitated spermatozoa at 22–24 h of maturation. Frozen-thawed spermatozoa were treated with 0.1 μM calcium ionophore A23187 (Sigma, MO) for 60 s, and then introduced into fertilization drops containing 10–15 oocytes for 6 h. IVF oocytes were washed and cultured in M199 + FCS on cumulus cell monolayers for 5 d. Embryos at the 16–32 cell stage were used for nuclear transfer. Zonae pellucidae of the embryos were removed by acidic DPBS (pH 2.3) treatment for 30–45 s followed by incubation with 0.5% pronase for 1 min (Sigma, MO) in protein-free M199. Zona-free embryos were incubated for 2 min in 0.25% trypsin Hank's solution containing 0.2% EDTA and then gently pipetted with a fine and fire-polished capillary pipette in M199 + FCS until individual blastomeres were disaggregated.

Micromanipulation and membrane fusion

Detailed procedures for nuclear transfer have been described previously (Prather *et al,* 1987; Stice and Robl, 1988; Yang *et al,* 1992a; 1993a). In this study oocyte enucleation and blastomere insertion were conducted separately 6–7 h apart (Yang *et al,* 1991). Enucleation of oocytes was conducted at 22–23 h, activation at 24 h and nuclear transfer manipulation at 30–31 h. Briefly following *in vitro* maturation of the oocytes for 22–23 h, they were denuded and then placed in M199 + FCS with 7.5 μg/ml cytochalasin B for 20 min before enucleation micromanipulation. The polar body and about 20% adjacent cytoplasm of the oocyte presumably containing the MII chromosomes were removed as described previously (Yang *et al,* 1992a). The presumptive karyoplasts removed from the oocytes and some manipulated oocytes from each trial were stained with 10 μg/ml Hoechst 33342 (Sigma, MO) for 10 min and observed by fluorescence to evaluate the enucleation efficiency. However, all manipulated oocytes were assigned for activation treatments for 6 h. This allowed the oocytes to develop to early 'pronuclear stage' (Presicce and Yang, 1994) presumably at G1 or early S phase as reported earlier (Barnes and Eyestone, 1990; Campbell *et al,* 1993; Stice *et al,* 1994). Embryonic cells were then inserted into perivitelline space of the activated cytoplasts. Care was taken to ensure close contact between the donor cell and the oocyte. Membrane fusion was induced at 31 h with 1 or 2 times of electric pulses (30 min apart) with the same stimulation protocol as described earlier for activation. After the first fusion pulse donor cell–oocyte complexes were cultured in M199 + FCS alone or containing 7.5 μg/ml cytochalasin B for 1 h depending on experiments. Fusion rate was examined 90 min after the first pulse. Fused and unfused embryos were recorded and cultured for 7 d to evaluate development to cleavage, morula and blastocyst stages.

Embryo culture and evaluation

Reconstructed embryos were cultured in M199 + FCS on buffalo rat liver (BRL) cell monolayers except for the co-culture comparison experiment. For preparation of BRL cell monolayer, frozen BRL cells were thawed, suspended in M199 + FCS and centrifugated at 500 *g* for 15 min to remove cryoprotectant, and then resuspended to a concentration of 1.0 x 10^6 cells/ml. Six droplets (each 100 μl) were made in each small Petri dish (Falcon, 1008, NJ) and overlaid with medical fluid (Dow Corning). Cells were cultured for 2–3 d to allow formation of a confluent monolayer. Cloned embryos and activated oocytes were cultured on confluent BRL cell monolayers for 7 d (initiation of activation = day 0). Cleavage to 2- to 8-cell stage was determined at day 3 and development to morula and blastocyst stages was evaluated at day 7. All blastocysts at day 7 were fixed in 2.3% sodium citrate/95% ethanol (3v/1v), and then stained with 10 μg/ml Hoechst for nuclear evaluation. Cell number for each blastocyst was recorded for analysis.

Specific experiments

Experiment 1 was designed to test nuclear transfer efficiency with preactivated cytoplasts. *In vitro* matured (IVM) oocytes were enucleated at 22–23 h of maturation culture. Presumptive enucleated oocytes were then activated by electric pulse and cycloheximide as described earlier. Nuclear transfer and membrane fusion were conducted 6–7 h later (at approximately 30–31 h IVM). In this experiment, 2 pulses were used to induce membrane fusion. Specific treatments include **A**) nuclear transfer with pre-activated cytoplasts; **B**) nuclear transfer/membrane fusion as in treatment **A** but no prior activation treatment; and **C**) activation of intact oocytes with same procedure as treatment **A** but no nuclear transfer manipulation. Treatments **B** and **C** served as nuclear transfer and activation controls.

Experiment 2 was designed to test whether the improved embryo development in *Experiment 1* was due to the double fusion pulses. Nuclear transfer with pre-activated cytoplasts was conducted as for treatment **A** in *Experiment 1*. The rates of fusion and subsequent embryo development were compared following application of 1 *vs* 2 fusion pulses.

Experiment 3 was designed to test whether exposing nuclear transferred embryos to cytochalasin B for 1 h improves rates of membrane fusion and subsequent embryo development. Treatments with and without cytochalasin B exposure were compared directly in this experiment.

Experiment 4 was to compare BRL and cumulus cell co-culture systems for the cloned embryos. Embryos were either cultured on monolayers of BRL cells or cumulus cells. Embryo culture in medium alone served as controls.

Data analysis

The development of embryos to cleavage, morula and blastocyst stages were compared by chi-square. Data on nuclear count were analysed by 1-way ANOVA or Student's *t* test.

RESULTS

Improved development of cloned embryos with pre-activated recipient oocytes

In a preliminary trial, we examined enucleation of oocytes at 22–23 h after maturation and found that 122 out of 156 (78%) manipulated oocytes were successfully enucleated. In *Experiment 1*, treatments included i) nuclear transfer with prior activation; ii) nuclear transfer control; and iii) activation control. The results in table I showed that 49% ($n = 104$) *in vitro* matured bovine oocytes were activated (cleavage) and 39% of the cleaved embryos developed to morula and blastocyst stage at day 7 following combined treatments of electric pulse and cycloheximide. The same activation procedure used for nuclear transfer effectively improved the development of reconstructed embryos. Significantly higher rate of cleavage ($p < 0.05$) was obtained for the nuclear transfer treatment group (68%, $n = 112$) compared to those for the control

Table I. Embryo development after nuclear transfer and activation.

Nuclear transfer treatment	*No of oocytes*	*No (%) fused*	*% development* [a]			*Cells/blastocysts (mean ± SEM)*
			Cleaved	*Morula*	*Blastocyst*	
Prior activation	112	85 (76)^{A}	68^{A}	42^{A}	44^{A}	58 ± 11^{A}
Transfer control	106	86 (81)^{A}	17^{B}	33^{A}	83^{A}	31 ± 8^{A}
Activation control	104	N/A	49^{C}	39^{C}	35^{A}	58 ± 9^{A}

[a] % cleaved was based on total manipulated oocytes; % morula and blastocyst were progressive development from previous stages. ^{A,B,C} Values within columns with different superscripts differ, $p < 0.05$.

nuclear transfers (17%, $n = 106$) and the activation control groups (49%, $n = 104$). However, subsequent developments of the cleaved embryos were similar (33–39%) regardless of the treatments. The fusion rate of the nuclear donor cell and the recipient oocyte was similar with or without prior activation of the recipient oocytes (76 *vs* 81%, $p > 0.05$). Overall prior activation resulted in higher rates of embryonic development to cleavage (68 *vs* 17%, $p < 0.05$) and morula plus blastocyst stages (29%, $n = 112$ *vs* 6%, $n = 106$, $p < 0.05$). Cell number analysis for blastocysts produced by nuclear transfer with prior activation ($n = 11$, 26–140), transfer control ($n = 5$, 18–60) and activation control ($n = 7$, 32–98) showed no difference between treatments at the P value of 0.05. However, embryos from the transfer control group appeared to have poorer morphology and the cell number was smaller at P value of 0.10 when compared to that of the treated groups.

Effects of repetitive fusion pulses and cytochalasin B

Based on the results of the first experiment, we continued to investigate factors affecting nuclear transfer. In *Experiment 2*, we tested whether 2 fusion pulses with intervals of 30 min apart *vs* a single pulse would improve membrane fusion and embryo development (table II). Fusion rate increased from 60% ($n = 186$) to 76% ($n = 132$, $p < 0.05$) when a sigle DC pulse was replaced by 2 pulses. Meanwhile the overall rates of cleavage and morula plus blastocyst development were also improved (44 *vs* 65% and 11 *vs* 32%, $p < 0.05$) for 1 *vs* 2 pulses, respectively. When expressed as development of the cleaved embryos to morula and blastocyst stages a higher rate of development was also observed for the 2 *vs* 1 pulse group (49 *vs* 26%, $p < 0.05$).

The effect of cytochalasin B was tested in *Experiment 3* (table II). The results show that incubation of cloned embryos for 1 h with cytochalasin B after nuclear transfer manipulation seemed not to be beneficial ($p > 0.05$) either for membrane fusion (72%, $n = 81$ *vs* 76%, $n = 82$), cleavage (68 *vs* 74%) or subsequent embryo development of the cleaved embryos (31 *vs* 26%). This was also the case when the results were expressed as overall embryo development of all manipulated oocytes to morula and blastocyst stages (29 *vs* 26%, $p > 0.05$). Development from morula to blastocyst stage, however, was reduced for the cytochalasin B treatment group (75 *vs* 35%, $p < 0.05$).

Table II. Effect of repetitive fusion pulses and cytochalasin B (CB) on nuclear transfer.

Experiment	*Fusion treatment*	*No of oocytes*	*No (%) fused*	*% development* [a]			*Cells/blactocysts (mean ± SEM)*
				Cleaved	*Morula*	*Blastocyst*	
2	1x pulse	186	111 (60)^{A}	44^{A}	26^{A}	38^{A}	64 ± 17^{A}
	2x pulse	132	101 (76)^{B}	65^{B}	49^{B}	43^{A}	58 ± 9^{A}
3	with CB	81	58 (72)^{A}	68^{A}	31^{A}	35^{A}	32 ± 5^{A}
	without CB	82	62 (76)^{A}	74^{A}	26^{A}	75^{B}	61 ± 14^{A}

[a] % cleaved was based on total manipulated oocytes; % morula and blastocyst were progressive development from previous stages. ^{A,B} Values within columns within experiments with different superscripts differ, $p < 0.05$.

In vitro *culture systems*

BRL cells and cumulus cells were compared as culture systems for cloned embryos (table III). Both BRL and cumulus cells promoted overall development of the cloned embryos to morula and blastocyst stages (28%, $n = 70$ and 21%, $n = 71$ *vs* 0%, $n = 58$ for controls, $P < 0.05$). Rates of progressive development from each previous stage were also similar between the 2 co-culture treatments ($p > 0.05$, table III). Cumulus cells showed higher tendency of overgrowth and forming multilayer cell clusters. They often squashed embryos and sometimes destroyed manipulated embryos by pushing cytoplasm or blastomeres out of the zonae pellucidae through the micromanipulation punctures. This effect of the cumulus cell co-culture system reduced embryo cleavage development as compared to that of the BRL cells system (53 *vs* 66%, $p < 0.05$). However, the subsequent embryo development to morula and blastocyst stages was not affected ($P > 0.05$).

Table III. Comparison of co-culture system for cloned embryos.

Culture system	*No of oocytes*	*% development* [a]			*Cells/blastocysts (mean ± SEM)*
		Cleaved	*Morula*	*Blastocyst*	
BRL cells	70	66^{A}	43^{A}	40^{A}	55 ± 15^{A}
Cumulus cells	71	53^{B}	39^{A}	40^{A}	38 ± 9^{A}
M199 alone	58	53^{B}	0^{B}	–	N/A

[a] Fusion rate was not checked and cleavage values were based on total numbers of manipulated oocytes; % for morula and blastocyst development were calculated based on previous stages. ^{A,B} Values within columns with different superscripts differ, $p < 0.05$.

DISCUSSION

In earlier nuclear transfer studies, *in vivo* matured oviduct oocytes were used as cytoplasm recipients because these were believed to support better development of the cloned embryos compared to *in vitro* matured oocytes (Willadsen, 1986; Prather *et al,* 1987; Bondioli *et al,* 1990). Indeed, electrical activation of the *in vitro* matured bovine oocytes was found to be oocyte-age-dependent and poor activation results were obtained with oocytes matured *in vitro* less than 30 h (Ware *et al,* 1989; Yang *et al,* 1991). Thus, at one time, most nuclear transfer programs involved using aged oocytes as recipients (Willadsen *et al,* 1991; Westhusin *et al,* 1992; Yang *et al,* 1993a). More recently, improvements on activation procedures have resulted in successful activation and led to the use of young oocytes for nuclear transfer (First *et al,* 1992; Barnes *et al,* 1993; Yang *et al,* 1993b; Aoyagi *et al,* 1994; Heyman *et al,* 1994; Stice *et al,* 1994). This study confirmed this finding and demonstrated that oocytes as young as 22–23 h supported up to 30% development of viable embryos (day 7) following a modified nuclear transfer procedure. With a modified activation protocol, a pregnancy has been established following transfer of several cloned embryos using young 24 h IVM oocytes as recipients for nuclear transfer (Du and Yang, unpublished results).

Several recent studies suggested a beneficial effect of oocyte activation prior to nuclear transfer (Campbell *et al,* 1993, 1994; Kono *et al,* 1994; Stice *et al,* 1994). The rationales for this are the following: when donor nucleus was introduced into the MII oocyte with high MPF level, the interphase nucleus was immediately induced to nuclear envelope breakdown and premature chromosomal condensation or PCC (Collas *et al,* 1992b; Barnes *et al,* 1993; Campbell *et al,* 1993). Nuclear membrane was subsequently reformed without cell division (*ie* without release of a second polar body) and DNA re-replication occurred (Barnes *et al,* 1993; Campbell *et al,* 1993). At the same time extensive nuclear swelling appeared (Czolowska *et al,* 1986; Szollosi *et al,* 1988; Prather *et al,* 1990; Collas and Robl, 1991). This nuclear remodeling was believed to be essential for rabbit cloned embryos (Collas and Robl, 1991) and it was thought particularly beneficial for regulation of G_1 or early S nuclei (Collas *et al,* 1992a, b). Recently, it was found that the effect of PCC was likely deleterious to mid to late S and G_2 nuclei (Collas *et al,* 1992a; Barnes *et al,* 1993; Campbell *et al,* 1993), resulting in abnormal chromosomal constitution and incorrect ploidy of reconstructed embryos due to DNA re-replication, as suggested by Campbell *et al* (1993). In blastomeres of early embryos, almost the whole cell cycle of these cells was occupied by the S phase (Barnes and Eyestone, 1990; Collas *et al,* 1992a). This explains the poor results of early nuclear transfer studies without cell cycle synchronization of donor cells (see review by Yang, 1991). Activation treatment which causes inactivation of MPF activity would induce parthenogenetic development of the oocytes. It was reported that fertilized oocytes had an extended G1 (6 h) phase (Barnes and Eyestone, 1990). This suggests that in our experiment, membrane fusion, induced at 6–7 h after activation, occurred at G1 or early S phase of the recipient oocyte. Cell fusion studies suggest that G1 or S phase recipient oocyte cytoplasm would not induce PCC or change the stage of the donor nucleus and thus this treatment would make better 'synchronization' between the donor nucleus and the recipient cytoplast (Campbell *et al,* 1993, 1994). Our study supports the observation that activation of recipient cytoplasts prior to nuclear transfer improves development of cloned embryos (Barnes *et al,* 1993; Campbell *et al,* 1994; Kono *et al,* 1994; Stice *et al,* 1994).

In this research, we introduced a double pulse fusion protocol following nuclear transfer regardless of the fusion status between the blastomere and the recipient oocyte. The 2-electrical pulse treatment not only improved the fusion rate but also the subsequent development of the reconstructed embryos (table II). This enhancement in embryo development was probably through improving adequate activation by further lowering the MPF activity after the second electric pulse.

The effect of cytochalasin B on nuclear transfer was controversial. Incubation of the nuclear transferred embryos with cytochalasin B for 1 h after fusion pulse was conducted in sheep (Smith and Wilmut, 1990), rabbits (Collas and Robl, 1991) and cattle (Levanduski and Westhusin, 1990). It was suggested that cytochalasin B prevented the activated oocytes to extrude a 'false' polar body containing donor nuclear materials and thus sustained normal ploidy of reconstructed embryos (Smith and Wilmut, 1989). In bovine nuclear transfer, however, a short period of cytochalasin B treatment was found unnecessary and not beneficial (Levanduski and Westhusin, 1990; Van Stekelenburg-Hamers *et al,* 1993). This variation was probably due to differences of activation procedures and different species. In cattle, our experiment confirmed the latter observations that cytochalasin B treatment after nuclear transfer had no beneficial effect. In fact, even though development of the cloned embryos was similar up to the morula stage with and without cytochalasin B treatment, the cytochalasin B treatment reduced the development from morula to blastocyst stage (table II).

It is known that the co-culture system was effective in promoting development of bovine embryos *in vitro* (Eyestone and First, 1989; Hernandez-Ledezma *et al,* 1993) including cloned embryos (Barnes *et al,* 1993; Van Stekelenburg-Hamers *et al,* 1993; Yang *et al,* 1993a; Kono *et al,* 1994). Results, however, were also controversial about selecting cell systems (Kono *et al,* 1994; Myers *et al,* 1994; Rehman *et al,* 1994). Our study showed that cloned embryos also experienced an 8- to 16-cell culture block as we reported earlier (Yang *et al,* 1993a). Both BRL cells and cumulus cells systems improved development of cloned embryos *in vitro* up to morula and blastocyst stages. BRL cells showed the characteristics of contact inhibition and stayed as monolayer throughout culture. In contrast, cumulus cells formed multilayer clusters which often squashed the cloned embryos resulting in embryolysis particularly at the early culture period. Furthermore, BRL cells form a commercially available cell line and the same batch of cells can be used throughout the research.

In conclusion, young 24 h IVM oocytes can be successfully activated and used for nuclear transfer when the young cytoplasts were activated for 6–7 h followed by nuclear transfer and membrane fusion. Two electric pulses to induce membrane fusion significantly improved the rates of fusion and subsequent embryo development, suggesting adequate activation at fusion may also be important for embryo development. Cytochalasin B treatment for 1 h after nuclear transfer did not show a beneficial effect in cattle in any criteria examined in this study. Both BRL cells and cumulus cells supported reasonable embryo development, but BRL cells were recommended due to reduced embryolysis.

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REFERENCES

Aoyagi Y, Konishi M, Wada T, Takedomi T (1994) Unaged bovine oocytes successfully develop to blastocysts after parthenogenic activation or nuclear transfer. *Theriogenology* 41, 157 (abstr)

Barnes FL, Eyestone WH (1990) Early cleavage and the maternal zygotic transition in bovine embryos. *Theriogenology* 33, 141-152

Barnes FL, Collas P, Powell R, King WA, Westhusin M, Shepherd D (1993) Influence of recipient oocyte cell cycle stage on DNA synthesis, nuclear envelope breakdown, chromosome constitution, and development in nuclear transplant bovine embryos. *Mol Reprod Dev* 36, 33-41

Bondioli KR, Westhusin ME, Looney CR (1990) Production of identical bovine offspring by nuclear transfer. *Theriogenology* 33, 165-174

Campbell KHS, Ritchie WA, Wilmut I (1993) Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: implications for deoxyribonucleic acid replication and development. *Biol Reprod* 49, 933-942

Campbell KHS, Loi P, Cappai P, Wilmut I (1994) Improved development to blastocyst of ovine nuclear transfer embryos reconstructed during the presumptive S-phase of enucleated activated oocytes. *Biol Reprod* 50, 1385-1393

Collas P, Robl JM (1991) Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos. *Biol Reprod* 45, 455-465

Collas P, Balise JJ, Robl JM (1992a) Influence of cell cycle stage of the donor nucleus on development of nuclear transplant rabbit embryos. *Biol Reprod* 46, 492-500

Collas P, Pinto-Correia C, Ponce De Leon FA, Robl JM (1992b) Effect of donor cell cycle stage on chromatin and spindle morphology in nuclear transplant rabbit embryos. *Biol Reprod* 46, 501-511

Czolowska R, Waksmundzka M, Kubiak JZ, Tarkowski AK (1986) Chromosome condensation activity in ovulated metaphase II mouse oocytes assayed by fusion with interphase blastomeres. *J Cell Sci* 84, 129-138

Eyestone WH, First NL (1989) Co-culture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium. *J Reprod Fertil* 85, 715-720

First NL, Leibfried-Rutledge ML, Northey DL, Nuttleman PR (1992) Use of *in vitro* matured oocytes 24 h of age in bovine nuclear transfer. *Theriogenology* 37, 211 (abstr)

Hernandez-Ledezma JJ, Villanueva C, Sikes JD, Roberts RM (1993) Effects of CZB *versus* medium 199 and of conditioning culture media with either bovine oviductal epithelial cells or buffalo rat liver cells on the development of bovine zygotes derived by *in vitro* maturation-*in vitro* fertilization procedures. *Theriogenology* 39, 1267-1277

Heyman Y, Chesne P, Lebourhis D, Peynot N, Renard JP (1994) Developmental ability of bovine embryos after nuclear transfer based on the nuclear source: *In vivo versus in vitro*. *Theriogenology* 42, 695-702

Kono T, Sotomaru Y, Aono F *et al* (1994) Effect of ooplast activation on the development of oocytes following nucleus transfer in cattle. *Theriogenology* 41, 1463-1471

Leibfried-Rutledge ML, Northey DL, Nuttleman PR, First NL (1992) Processing of donated nucleus and timing of post-activation events differ between recipient oocytes 24 or 42 h of age. *Theriogenology* 37, 244 (abstr)

Levanduski MJ, Westhusin ME (1990) Effect of cytoskeletal inhibitor on fusion and development of bovine nuclear transfer embryos. *Theriogenology* 33, 273 (abstr)

Myers MW, Broussard JR, Menezo Y, Prough SG, Blackwell J, Godke RA, Thibodeaux JK (1994) Established cell lines and their conditioned media support bovine embryo development during *in vitro* culture. *Human Reprod* 9, 1927-1931

Prather RS, Barnes FL, Sims MM, Robl JM, Eyestone WH, First NL (1987) Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. *Biol Reprod* 37, 859-866

Prather RS, Sims MM, First NL (1989) Nuclear transplantation in early pig embryos. *Biol Reprod* 41, 414-418

Prather RS, Sims MM, First NL (1990) Nuclear transplantation in the pig embryo: nuclear swelling. *J Exp Zool* 225, 355-358

Presicce GA, Yang X (1994) Nuclear dynamics of parthenogenesis of bovine oocytes matured *in vitro* for 20 and 40 h and activated with combined ethanol and cycloheximide treatment. *Mol Reprod Dev* 37, 61-68

Rehman N, Collins AR, Suh TK, Wright RW, Jr (1994) Development of *in vitro* matured and fertilized bovine oocytes co-cultured with buffalo rat liver cells. *Theriogenology* 41, 1453-1462

Robl JM, Stice SL (1989) Prospects for the commercial cloning of animals by nuclear transplantation. *Theriogenology* 31, 75-84

Smith LC, Wilmut I (1989) Influence of nuclear and cytoplasmic activity on the development *in vivo* of sheep

embryos after nuclear transplantation. *Biol Reprod* 40, 1027-1035

Smith LC, Wilmut I (1990) Factors affecting the viability of nuclear transplanted embryos. *Theriogenology* 33, 153-164

Stice SL, Robl JM (1988) Nuclear reprogramming in nuclear transplant rabbit embryos. *Biol Reprod* 39, 657-664

Stice SL, Keefer CL (1993) Multiple generational bovine embryo cloning. *Biol Reprod* 48, 715-719

Stice SL, Keefer CL, Matthews L (1994) Bovine nuclear transfer embryos: oocyte activation prior to blastomere fusion. *Mol Reprod Dev* 38, 61-68

Szollosi D, Czolowska R, Szollosi MS, Tarkowski AK (1988) Remodelling of mouse thymocyte nuclei depends on the time of their transfer into activated homologous oocytes. *J Cell Sci* 91, 603-613

Van Stekelenburg-Hamers AEP, Van Inzen WG, Van Achterberg TAE, Kruip TAM, De Laat SW, Weima SM (1993) Nuclear transfer and electrofusion in bovine *in vitro*-matured/*in vitro*-fertilized embryos: effect of media and electrical fusion parameters. *Mol Reprod Dev* 36, 307-312

Ware CB, Barnes FL, Maiki-Laurila M, First NL (1989) Age dependence of bovine oocyte activation. *Gamete Res* 22, 265-275

Webb M, Howlett SK, Maro B (1986) Parthenogenesis and cytoskeletal organization in ageing mouse eggs. *J Embryol Exp Morph* 95, 131-145

Westhusin ME, Levanduski MJ, Scarborough R, Looney CR, Bondioli KR (1992) Viable embryos and normal calves after nuclear transfer into Hoechst stained enucleated demi-oocytes of cows. *J Reprod Fertil* 95, 475-480

Willadsen SM (1986) Nuclear transplantation in sheep embryos. *Nature (Lond)* 320, 63-65

Willadsen SM, Janzen RE, McAlister RJ, Shea BF, Hamilton G, McDermand D (1991) The viability of late morulae and blastocysts produced by nuclear transplantation in cattle. *Theriogenology* 35, 161-172

Yang X (1991) Embryo cloning by nuclear transfer in cattle and rabbits. *Intl Embryo Transfer Newsletter* 9, 10-22

Yang X, Jiang S, Kovacs A, Foote RH (1991) Age dependent activation, enucleation and nuclear transfer of bovine oocytes matured *in vitro* and *in vivo*. *Biol Reprod* 44 (suppl), 141 (abstr)

Yang X, Jiang S, Kovacs A, Foote RH (1992a) Nuclear totipotency of cultured rabbit morulae to support full-term development following nuclear transfer. *Biol Reprod* 47, 636-643

Yang X, Jiang S, Shi Z (1992b) Improved activation by combined cycloheximide and electric pulse treatment of bovine follicular oocytes matured *in vitro* for 23–24 h. *Biol Reprod* 46 (suppl), 117 (abstr)

Yang X, Jiang S, Farrell P, Foote RH, McGrath AB (1993a) Nuclear transfer in cattle: effect of nuclear donor cells, cytoplast age, co-culture, and embryo transfer. *Mol Reprod Dev* 35, 29-36

Yang X, Jiang S, Foote RH (1993b) Bovine oocyte development following different oocyte maturation and sperm capacitation procedures. *Mol Reprod Dev* 39, 94-100