CHAPTER III

EFFECT OF RECIPIENT CYTOPLAST ON THE KINETICS OF DNA REPLICATION DURING THE 1-CELL STAGE IN BOVINE NUCLEAR TRANSFER EMBRYOS

Introduction

The use of pre-activated cytoplasts with somatic donor cells result in poorer development than that of non-activated cytoplasts (Tani et al., 2001; Du et al., 2002). It has demonstrated that the timing of initiation of DNA synthesis in NT embryos produced from different stages of cytoplast also affects the *in vitro* development (Kurosaka et al., 2002). Furthermore, the completion of DNA synthesis during the first cell cycle may contribute the high development of NT embryos when the S-phase blastomeres were used as donor nuclei (Barnes et al., 1993). The replication phase of the 1-cell NT embryos derived from somatic cells may be of importance in the process that donor nucleus has to become like those of the normal zygotic nucleus, since it corresponds to as phase where the chromatin structure becomes opened, allowing the access to replication.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

In this study, the influence of the recipient oocyte cell cycle stage on the kinetics of the first DNA synthesis in somatic nuclear transfer embryos using bovine enucleated oocytes, either non-activated or activated was investigated. The onset and the length of DNA synthesis in somatic nuclei after transfer in these cytoplasts were also determined, as well as the development potential of somatic nuclear transfer embryos was also evaluated. The DNA synthesis in parthenogenetic oocytes derived

หอสมุดกลาง สถาบันวิทยบริการ
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from both groups was also conducted to ensure the effective activation protocols used for the nuclear transfer experiment.

Materials and methods

Chemicals and media

Unless otherwise indicated, all chemicals and media were obtained from Sigma Chemical Co. (St. Louis, MO). LH from porcine was provided by Prof. J.F. Beckers, Faculty of Veterinary Medicine, University of Liège, Belgium. Culture media for preparation of donor cells were from Life Technologies (Grand Island, NY).

Preparation of donor cells

Donor cells were prepared from frozen cells derived from a skin fibroblast cell line of a 19 month-old Holstein Friesian (Vignon et al., 1998). The skin biopsies were washed three times in Dulbecco's phosphate-buffered saline (DPBS) and cut into small pieces before being attached to the bottom of 60-mm culture dishes. Two to three ml of culture medium was slowly added to the culture dishes and the cell explants were then cultured at 38.5°C in a humidified air atmosphere with 5% CO_2 for 4-5 d until a primary fibroblast monolayer was established. The culture medium was Dulbecco's modified Eagle medium with GLUTAMAX-1 (DMEM-G) supplemented with 10% fetal calf serum (FCS) and 50 IU/ml penicillin-streptomycin. Primary cells were trypsinized with 0.05% trypsin-EDTA, frozen in 10% dimethyl sulfoxide (DMSO) in FCS and stored in liquid nitrogen. For the experiments, the cells were thawed and cultured for 3-4 d. When the cells had grown to 80-90% confluence, they

were starved, by culturing in a medium supplemented with 0.5% FCS for 4-10 d. Before NT, the cells were harvested by trypsinization, resuspended in DMEM-G, supplemented with 10% FCS and immediately transferred into the recipient oocytes (Figure 2). Cells cultured between passages 5-15 were used for NT.

Preparation of recipient oocytes

Cumulus-oocyte complexes (COCs) were collected by aspiration of follicles with 2-8 mm diameter from slaughter ovaries, using a 19-guage needle. Selected COCs were washed two times in maturation medium, Medium199 (M199), supplemented with 10% FCS, 10 μg/ml of FSH (Rhône Mérieux, Lyon, France), 10 μg/ml LH and 1 μg/ml of estradiol 17β. Groups of 30-40 COCs were cultured in 500 μl of maturation medium for 20 h at 39°C, in a humidified air atmosphere with 5 % CO_2. After maturation, the cumulus cells were removed by culturing for 3 min in phosphate buffer saline (PBS) containing 0.5 % hyaluronidase, then stripped by gentle pipetting. Enucleation was performed as previously described (Vignon et al., 1998). Briefly, cumulus cell-free oocytes were cultured in M199, supplemented with 10 % FCS and 0.5 μg/ml Hoechst 33342, for 20-30 min at 39 °C in a humidified air atmosphere with 5 % CO_2 before enucleation. Oocyte enucleation was performed in M199-Hepes supplemented 10 % FCS, 5 μg/ml cytochalasin B (CB) and 0.5 μg/ml Hoechst 33342 overlaid with mineral oil on the stage of an inverted microscope equipped with Eppendorf micromanipulators and epifluorescent illumination. The metaphase plate and the first polar body were removed with a glass enucleation pipette (20 μm outside diameter). Confirmation of successful enucleation was achieved by visualizing the metaphase plate under ultraviolet light. The enucleated oocytes were assigned to different experimental groups as described below.

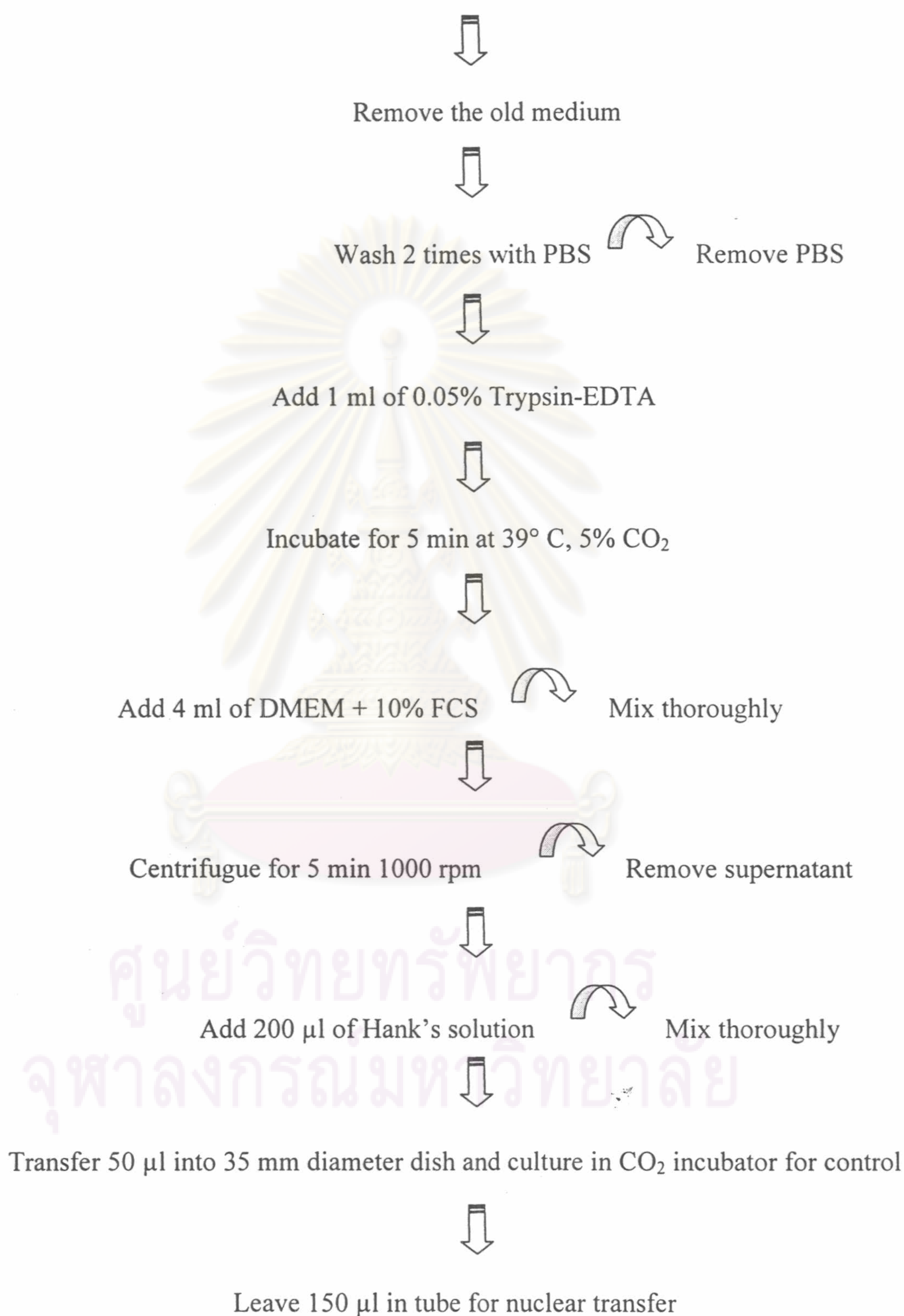

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Figure 2. Diagram of donor cells preparation prior to transfer into recipient oocytes

Somatic cell nuclear transfer

In the non-activated group (NT-MII), the enucleated oocytes were fused with donor cells at 25-26 h post *in vitro* maturation (hpm) by applying 2 DC pulses of 2.2 kV/cm for 30 μs in the cell fusion medium containing 0.3 M mannitol, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$. The cell-cytoplasts were immediately activated by incubation in 10 μg/ml cycloheximide (CHX) and 5 μg/ml CB for 5 h. For the activated group (NT-ACT), the enucleated oocytes were activated at 23 hpm by incubation in 7% ethanol for 5 min and cultured in CHX for 2 h (LeBourhis et al., 2002). Fusion was done at 25-26 h post fusion (hpf), corresponding time of the NT-MII group, with the same parameter as was in the NT-MII group. The reconstructed oocytes were then incubated in CHX for an additional 3 h (Figure 3). Parthenogenetic controls for both groups, parthenogenetic activation of non-activated (P-MII) and activated oocytes (P-ACT), were also run in parallel with the same manipulation but without removal of metaphase plate. The nuclear transfer and parthenogenetic embryos were then co-cultured with monolayer of Vero cells in B_2 medium (CCD Laboratory, Paris, France) supplemented with 2.5% FCS at 39°C in a humidified air with 5% CO_2 (Menck et al., 1997) until the time of DNA synthesis detection or 168 hpf. The cleavage and development rates of NT embryos were determined at 24 and 168 hpf. At the end of the culture period, the NT blastocysts were stained with 0.5 μg/ml Hoechst 33342 for 20 min. The stained nuclei were visualized and recorded using a fluorescent microscope equipped with Viewfinder Life program and then counted by ImageTool software.

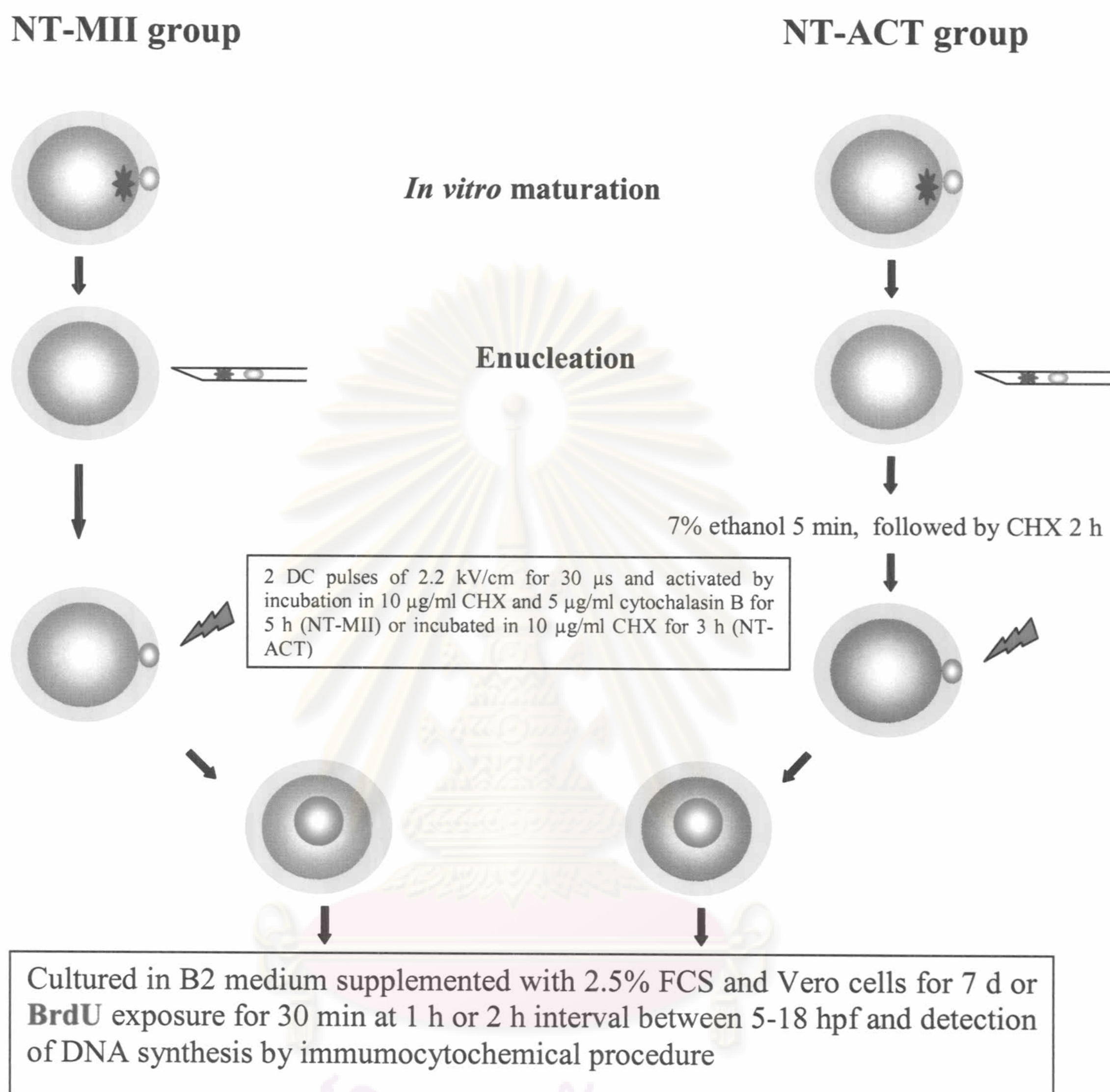

Figure 3. Nuclear transfer procedure used to produce bovine embryos with non-activated and activated cytoplasts

CHX: cycloheximide, BrdU: 5'-bromo-2'-deoxyuridine, min: minute
h: hour, hpf: hour post fusion, FCS: fetal calf serum

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Characterization of DNA synthesis in nuclear transfer embryos and parthenogenetic embryos

At 1 h interval between 5 –18 hpf for the NT-MII group or at 2 h intervals between 4-22 hpf for the NT-ACT group, embryos were incubated for 30 min in a culture medium containing 1 mM 5'-bromo-2'-deoxyuridine (BrdU) and then fixed in 2.5% paraformaldehyde (PAF). At least two replicates at each time point were carried out for all experiments. The continuous exposure to BrdU from 5 hpf and fixed at 18 hpf were performed in both groups. DNA synthesis was also examined in the parthenogenetic embryos produced from each group. The optimal DNA replication was characterized by immunocytochemical detection of BrdU incorporation (APPENDIX B) by the method of Adenot et al. (1997). Briefly, the fixed BrdU incorporated embryos/oocytes were permeabilized in 0.5%Triton X100 then treated with a first monoclonal antibody (mouse IgG anti-BrdU) and a second polyclonal antibody (goat anti-IgG of mouse conjugated with fluorescein isothiocyanate, FITC). The nuclei were also stained with 10 μg/ml of propidium iodide (PI), mounted on a grass slide then observed under a fluorescent microscopy (LEICA DMRB, Piece 543, Germany).

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Statistical analysis

Numbers of embryos labeled with FITC at the onset and the ending times of DNA synthesis were compared by means of Chi-square test or Fisher's exact test. The cleavage and development rates between two groups were also compared by Chi-square test. The cells number of embryos were analyzed by Student's *t*-test. Differences were considered significant when $P < 0.05$.

Results

DNA synthesis in nuclear transfer embryos and parthenogenetic embryos

As shown in Figure 4B and in Table 3, at 5 hpf, DNA synthesis started in the NT-ACT embryos (37%, 10 of 37 embryos) whereas it was not observed in the NT-MII group (0%, 0 of 25 embryos, P< 0.001). At 6 hpf, only 10 of 53 (18.9%) embryos in the NT-MII group synthesized DNA but 15 of 19 (78.9%) embryos in the NT-ACT group did so (P < 0.001, Figure 5A, 5B). The DNA replication ended at 18 hpf in NT-MII group (Table 3, Figure 6E, F), but it did not end in the NT-ACT group. It was found that 9 of 25 embryos (36%, Table 3) in the NT-ACT group still synthesized DNA (Figure 6C) at 18 hpf (P<0.001). Three of 28 (10.7%, data not shown) parthenogenetic embryos from P-MII group started synthesizing DNA at 7 h post activation (hpa). All oocytes stopped synthesizing DNA at 18 h after activation (Figure 4A). Two of 16 (12.5%, data not shown) parthenogenetic embryos from P-ACT group had synthesized DNA within 5 hpa and none of embryos synthesized DNA at 16 hpa (Figure 4B).

DNA synthesis increased rapidly, in the same manner, within 1-2 hpf/hpa in the NT embryos and in the parthenogenetic embryos after the onset, as seen in Figure 4A-B. In the NT-MII group, almost 100% of nuclei were in S-phase at 8-9 hpf, and thereafter the proportion of replicating nuclei rapidly decreased until 18h post-fusion (Figure 4A). In contrast, in the NT-ACT group, DNA replication gradually decreased after 10 h post fusion until 18 h post fusion and resumed thereafter (Figure 4B). The replication period was 13 h and 12 h in the NT-MII group and in the parthenogenetic embryos from P-NT group, respectively (Figure 4A). The duration of DNA synthesis in the parthenogenetic embryos from the P-ACT group was 11 h, however it could not

be examined in the NT-ACT embryos due to the curve of replicating nuclei did not touch at a zero point (Figure 4B).

To determine the optimal ability on synthesizing DNA of the NT embryos from both groups, they were continuously exposed to BrdU from 5 hpf and fixed at 18 hpf. As summarized in Table 4, almost all embryos in both groups can synthesize DNA (96.8% versus 89.7% in the NT-MII and NT-ACT groups, respectively, $P > 0.10$).

Developmental potential of nuclear transfer embryos

The development of NT embryos *in vitro* obtained from non-activated and activated oocytes using the quiescent somatic cell nuclei was evaluated (Table 5). The cleavage rates of NT embryos in both groups were not different ($P > 0.30$). Development rates to the blastocyst stage were significantly higher in the NT-MII group than in the NT-ACT group (51.1% versus 22.8%, respectively, $P < 0.001$). The mean cell numbers (± SEM) of the NT embryos were not significantly different (Table 5).

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Table 3. BrdU incorporation in bovine NT embryos after 30 min BrdU-exposure at 5, 6 and 18-hour post fusion

Group	No. of embryos incorporated to BrdU / total embryos fused (%)		
	at 5 hpf	at 6 hpf	at 18 hpf
NT-MII	0/25 (0) [a]	10/53 (18.9) [a]	2/55 (3.6) [a]
NT-ACT	10/37 (37.0) [b]	15/19 (78.9) [b]	9/25 (36.0) [b]

Data are from 2-6 replicates at each time point. min: minute, hpf: hour post fusion

[a, b] Values with different superscripts within the same column differ significantly ($P < 0.001$).

Table 4. BrdU incorporation in bovine NT embryos after 18 h BrdU-continuous exposure

Group	No. of embryos fused (No.replicates)	No. of embryos incorporated to BrdU (%)
NT-MII	31 (3)	30 (96.8)
NT-ACT	29 (2)	26 (89.7)

BrdU: 5'-bromo-2'-deoxyuridine, hpf: hour post fusion, h: hour

NT-MII: nuclear transfer embryos derived from non-activated cytoplasts reconstructed with donor nuclei at presumptive G0 stage

NT-ACT: nuclear transfer embryos derived from activated cytoplasts reconstructed with donor nuclei at presumptive G0 stage

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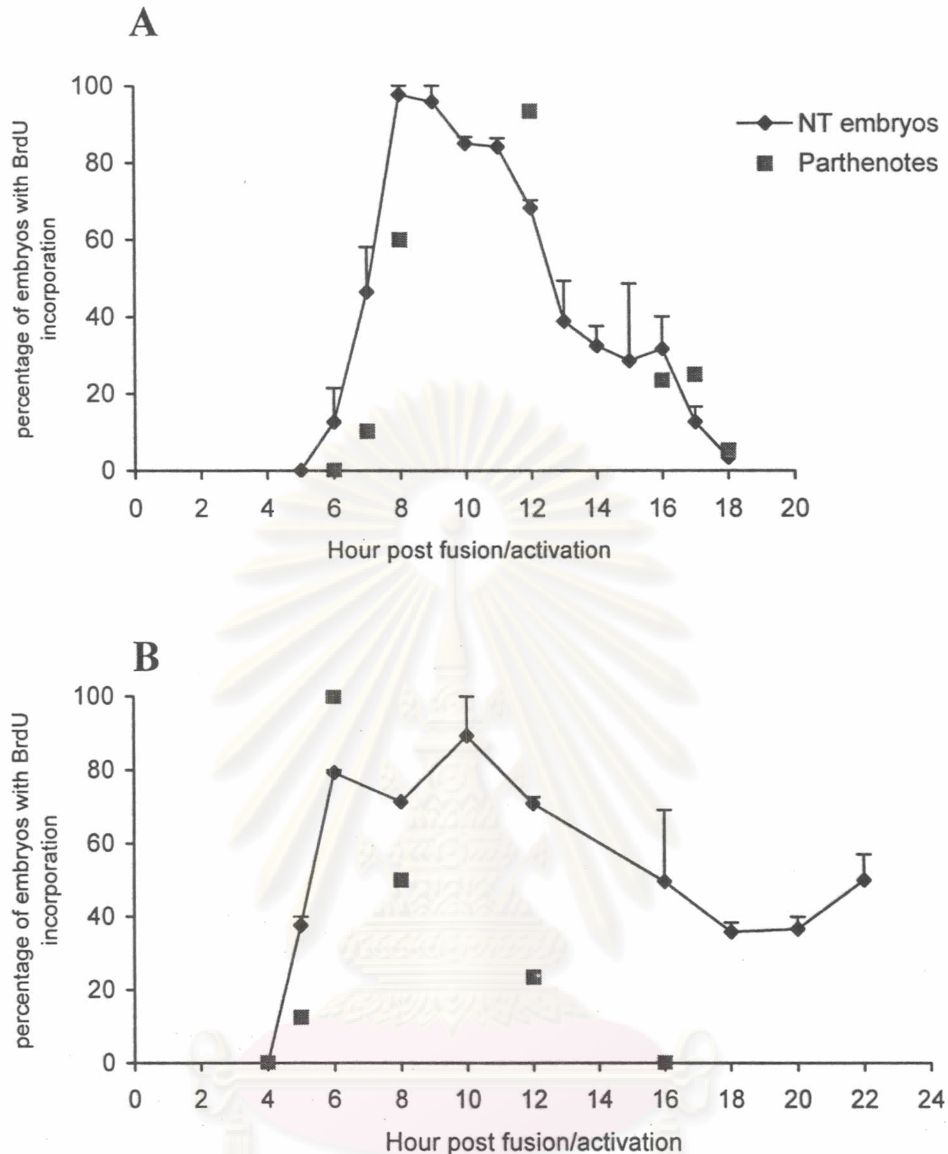

Figure 4. DNA synthesis of the first cell cycle in bovine NT embryos and parthenogenetic oocytes derived from non-activated and activated cytoplasts

A. DNA synthesis of NT embryos at the 1-cell stage derived from non-activated oocytes reconstructed with serum starved ear skin fibroblasts (NT-MII group). Data are from 2-6 replicates, 24-61 embryos, at each time point for NT embryos and 10-20 embryos at each time point for parthenogenetic embryos (P-MII group). Each point represents the mean ± standard error of the mean (± SEM).

B. DNA synthesis of NT embryos at the 1-cell stage derived from activated oocytes reconstructed with serum starved ear skin fibroblasts (NT-ACT group). Data are from 2 replicates (except at 8 hpf), 14-25 embryos, at each time point for NT embryos and 10-33 embryos for parthenogenetic embryos (P-ACT group). Each point represents the mean ± standard error of the mean (± SEM).

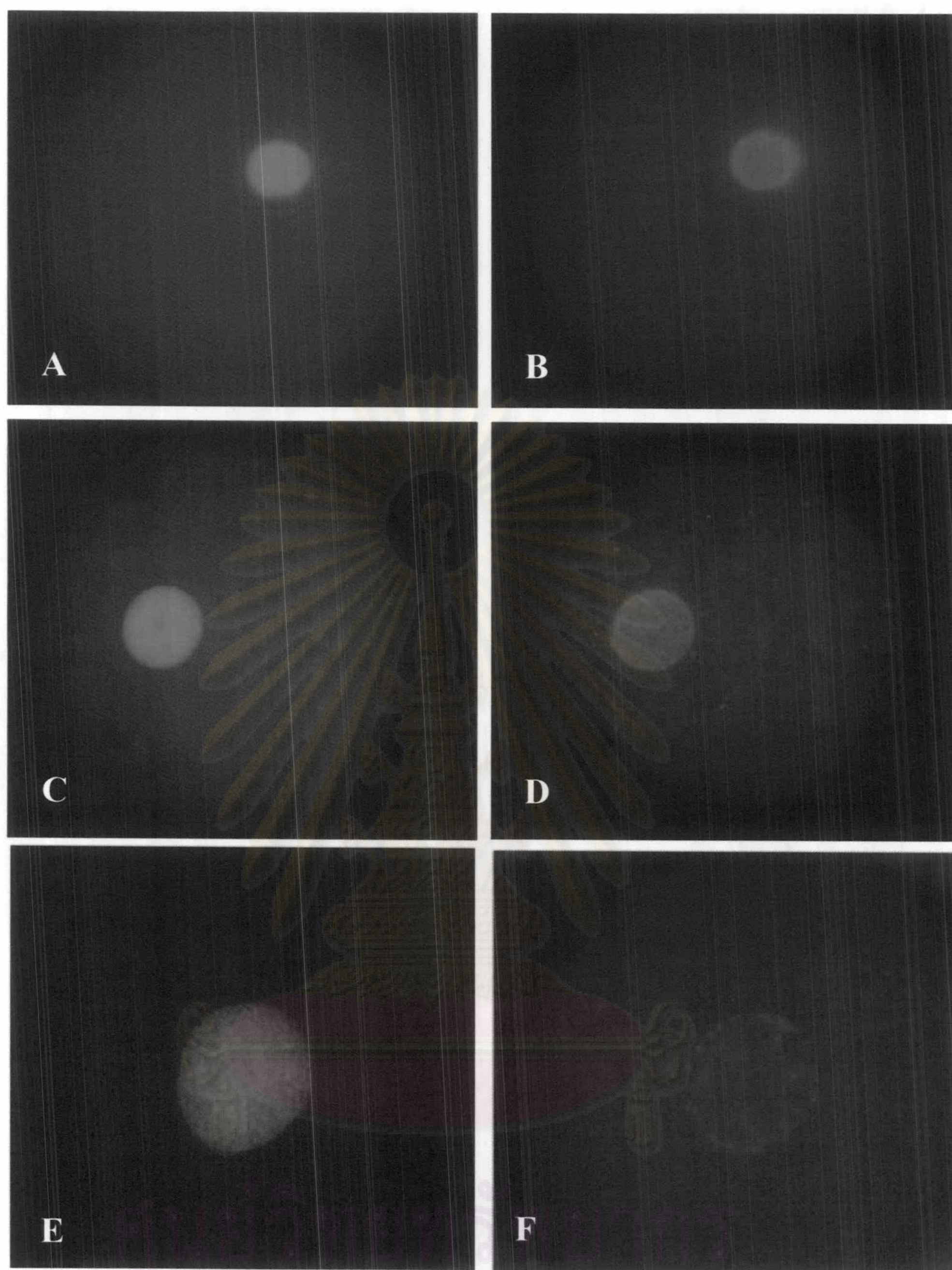

Figure 5. BrdU incorporation in bovine NT embryos at the 1-cell stage after 30-exposure at 6, 10 and 16 hour post fusion (X400)

A, B. One-cell NT embryo from NT-ACT group had incorporated to BrdU, after 30-min exposure,at 6 hour post fusion. green fluorescence: FITC, red fluorescence: propidium iodide.

C, D. One-cell NT embryo from NT-ACT group had incorporated to BrdU, after 30-min exposure, at 10 hour post fusion.

E, F. One-cell NT embryo from NT-MII had incorporated to BrdU, after 30- min exposure, at 16 hour post fusion. BrdU: 5'-bromo-2'-deoxyuridine, FITC: fluorescein isothiocyanate

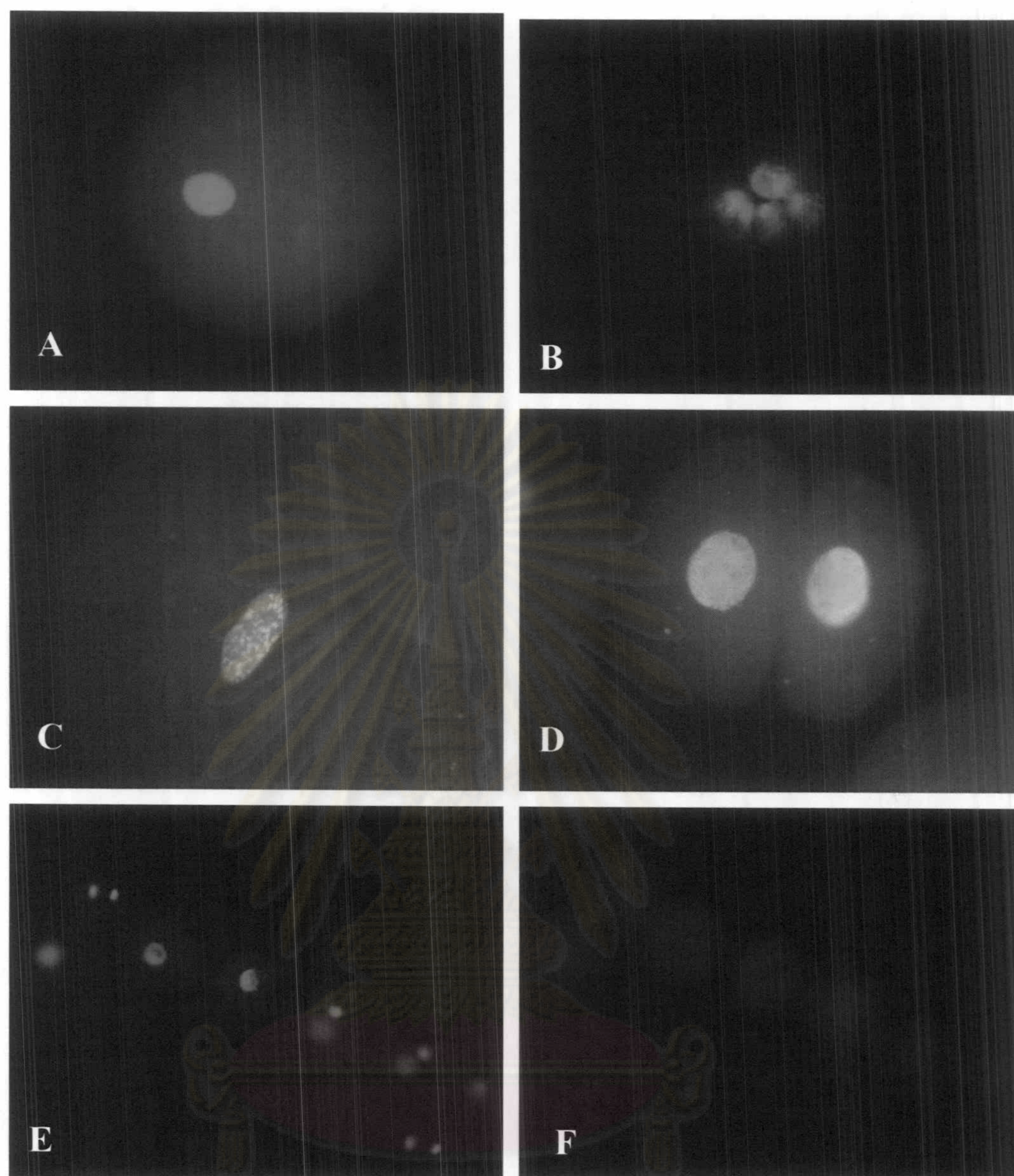

Figure 6. BrdU incorporation in the bovine NT embryos at the 1-cell stage at 4, 18 and 22-hour post fusion and at 6 hour post activation in the parthenogenetic embryo

A. One-cell NT embryo from NT-ACT group had incorporated to BrdU, after 30- min exposure, at 4-hour post fusion (X400). The nucleus exhibiting green fluorescence of FITC and red fluorescence of propidium iodide indicates DNA are replicating.

B. Parthenogenetic embryo from activated oocytes had incorporated to BrdU, after 30-min exposure, at 6-hour post fusion (X400). Four pronuclei exhibiting green fluorescence of FITC and red fluorescence of propidium iodide are observed.

C. One-cell NT embryo from NT-ACT group had incorporated to BrdU, after 18-hour continuous exposure, at 18-hour post fusion (X600). The nucleus exhibiting some spots of green fluorescence of FITC and red fluorescence of propidium iodide indicates DNA starts replicating.

D. Two-cell NT embryo from NT-ACT group had incorporated to BrdU, after 30- min exposure, at 22-hour post fusion (X400). Note the DNA replicating in both nuclei.

E, F. One-cell NT embryos derived from NT-MII group after 30- min exposure at 18-hour post fusion (X100). No replicating nucleus is observed.

Table 5. Developmental potential of bovine NT embryos derived from non-activated and activated cytoplasts reconstructed with donor nuclei at presumptive G0 stage

Group	Replicates	No. of embryos cultured	No. of embryos cleaved (%)	No. of embryos developed to blastocyst stage (%)	Cell number per blastocyst (*n*) (mean ± SEM)
NT-MII	6	88	68 (77.3)	45 (51.1) ^{a}	115.2 ± 26.1 (21)
NT-ACT	8	123	87 (70.7)	28 (22.8) ^{b}	108.0 ± 25.9 (22)

n: numbers of embryos examined ; SEM: standard error of the mean

NT-MII: nuclear transfer embryos derived from non-activated cytoplasts reconstructed with donor nuclei at presumptive G0 stage

NT-ACT: nuclear transfer embryos derived from activated cytoplasts reconstructed with donor nuclei at presumptive G0 stage

$^{a, b}$ Values with different superscripts within the same column differ significantly ($P < 0.001$)

Discussion

The present study clearly demonstrates that the kinetics of DNA synthesis during the first cell cycle in the 1-cell stage nuclear transfer embryos is affected by the cycle stage of recipient cytoplasts. The DNA synthesis starts earlier and is longer in somatic nuclei transferred into activated cytoplasts than in somatic nuclei transferred into non-activated cytoplasts. The DNA synthesis starts earlier and is shorter in parthenogenetic embryos produced from activated cytoplasts than in parthenogenetic embryos produced from non-activated cytoplasts. The rates of development to the blastocyst stage were significantly higher in nuclear transfer embryos produced from non-activated cytoplasts than in nuclear transfer embryos produced from activated cytoplasts. This clearly demonstrates that the cell cycle of recipient cytoplast affects on the development to blastocyst stage of NT embryos when serum-starved somatic cells were used as donor nuclei.

In the NT-ACT group, the initiation of DNA synthesis, at 5 hpf, in somatic nuclei after they transferred into activated cytoplasts, as well as in the P-ACT group (Figure 4B), was similar to that of the parthenogenetic embryos produced from aged oocytes as previously reported by Soloy et al. (1997). In this study, the DNA synthesis starts earlier in the somatic nuclei transferred into activated cytoplasts than that transferred into non-activated cytoplasts. The activated cytoplasts may accelerate nuclear progression, which corresponded to DNA synthesis (Soloy et al., 1997), faster than the non-activated cytoplasts. It has been demonstrated that the nuclear progression of old or aged oocytes occurs more rapidly than young oocytes (Presicce and Yang, 1994). The DNA synthesis starts with a high speed, the proportion of nuclei that synthesized DNA increased to 80% at 6 hpf (Figure 4B, Table 3), then

decreased rapidly to around 70% and increased again to 90% at 10 phf. This may result from the heterogeneous of donor nuclei transferred. The high-speed increase in synthesizing DNA of NT-ACT embryos, at these points, was similar to that of the parthenogenetic embryos derived from aged oocytes (Soloy et al., 1997). However, the data at 8 hpf are only from 1 replicate, due to limitation of samples, may not be a good representative of the proportion of replicating nuclei at this time point.

The DNA synthesis, in NT-ACT embryos, gradually decreased after 10 hpf until 18 hpf when 36% of them still synthesized DNA and DNA synthesis continued at least until 22 hpf (Figure 4B). It means that DNA synthesis in NT-ACT embryos was not complete during the first cell cycle. In addition, a high proportion of chromatin in S-phase did not enter metaphase (M phase) at 18 hpf, that is the lowest point of replication. It was found that there was a low proportion of nuclei at M phase 18 hpf (data not shown). Furthermore, the replicating chromosomes damaged in nuclei at M phase and 2-cell stage embryos were also observed during 20-22 hpf, in this study (data not shown). This may result in a low developmental rate to blastocysts of NT-ACT embryos in the present study. A possible explanation of the low number of M phase nuclei associated with extended replicating DNA of nuclei is that may be due to lack of cell cycle regulatory factors stored in cytoplasts. Control of the cell cycle consists of two major control points, START and ENTRY, referring to the cell commits to start DNA synthesis and to enter M phase, which corresponds to the activation of MPF at both two points (Parrish et al, 1992). The activation of oocytes before and at the time of cell fusion may destruct these factors, leading to the depletion of essential cytoplasmic factor (s) for terminating the DNA synthesis and then driving of a large proportion of nuclei into M phase. Another possibility may explain chromosome damage in some of NT-ACT embryos is that limited nuclear

swelling and smaller than normal size (personal observation) whereas there are double of DNA content when replicating nuclei had completed synthesizing DNA. It has been demonstrated that fusion of donor nuclei to activated cytoplasts resulted in reduced nuclear swelling and smaller in nuclear size than normal (Collas and Robl, 1991).

In NT-MII group, after starting DNA replication at 6 hpf, almost 100% of nuclei were in S-phase at 8-9 hpf thereafter the proportion of replicating nuclei decreased until 18 hpf. The initiation of DNA synthesis was similar to that reported by Kurosaka et al. (2002). As considered from the pattern of DNA replication, seen in Figure 4A, it seems that the somatic nuclei in NT embryos were well synchronized or regulated in starting and ending of DNA synthesis. This may result in a higher rate of embryo development. In addition, a large proportion of M phase nuclei (> 70%) were observed at 18 hpf (data not shown), indicated that the replication was completely stopped before entry to mitosis. The results support the observations of Barnes et al. (1993), who revealed that the synchronous completion of DNA synthesis, before entry to mitosis, affects *in vitro* development potential of nuclear transplant embryos. Taken together, it is hypothesized that the synchrony between initiation and termination of DNA replication of quiescent transplanted nuclei yields a high frequency of developing embryos. Although it is likely that the somatic nuclei in NT-MII embryos were incomplete reprogramming during the first cycle, the period of S-phase was, about 6.5 h (Figure 4A), particularly short compared to that of *in vivo* embryos, 7-9 h (Laurincik et al., 1994) and in IVF embryos, 10.4 h (Comizzoli et al., 2000). The short period of S-phase in NT-MII embryos raises the question of whether DNA replication is complete during the first cell cycle. In the mouse embryo, it is clearly evident that the pattern of gene expression occurring during the 2-cell stage is

dependent on the first round of DNA replication (Davis and Schultz, 1997). A recent report in the cow showed the first and the second rounds of DNA replication are important regulators of early gene expression (Memili and First, 1999). In fact, DNA synthesis must occur only one time in each cell cycle associated with every segment of DNA that must be copied before it passed to the next phase (Parrish et al., 1992).

There were no significant differences in the mean cell numbers of the NT embryos produced by both protocols (Table 5). This indicated that the same quality of NT embryos, not the same numbers of embryos, could be obtained either from non-activated or activated cytoplasts. It was similar to the results of a previous study (Lavoir, 1996).

In this study, the combined ethanol and 2- h-CHX treatment, a little modified from Kurosaka et al. (2002) was used to activate *in vitro* matured oocytes. As seen in Figure 4B, DNA synthesis in the P-ACT group started and ended at the same times (4 and 16 hpa) as that reported previously (Soloy et al., 1997) when activated bovine *in vitro* matured oocytes, using Ca^{2+} ionophore followed 6-h CHX incubation, at 36 h post maturation. Recent report by LeBourhis et al. (2002) and a preliminary experiment also revealed that the oocytes either were electrically pulsed or treated with ethanol and incubated for 2 h in CHX had reached the post-telophase stage, indicating the activated condition of those oocytes. In addition, the profiles of DNA synthesis in parthenogenetic embryos derived from both groups (Figure 4A, 4B) were similar to that of parthenogenetic embryos derived from 24-h post maturation oocytes and 36-h post maturation oocytes (Soloy et al., 1997), confirm the effective activation protocols in this study. It should be noted that, the activation protocol for producing NT-ACT or P-ACT embryos, in this study, the additional 3 h incubation in CHX after cell fusion/activation, 3 h shorter than that of the normal protocol (6 h, Soloy et al.,

1994; Kurosaka et al., 2002; Tani et al., 2001; Du et al., 2002), is enough to support the development of embryos. This may be an alternative protocol for somatic nuclear transfer.

The nuclear transfer procedures that have been generally used for producing NT embryos may not be the optimal protocol for producing the normal NT embryos. Several recent studies showed the success of producing high proportion of blastocysts, but high incidence of fetal losses associated with postnatal mortality of cloned offspring has been frequently observed (reviewed by Renard et al., 2002). This occurrence may come from the transfer of the abnormal NT embryos produced, which could not be judged by morphology, either from somatic or embryonic cells as donor nuclei by general NT procedures. Therefore, a new NT procedure should be carefully designed to ensure fully reprogramming of nuclei associated with the effective examination of NT embryos before transfer to recipient animals.

In conclusion, the results of this study indicate that the stage of recipient cytoplasts affects the kinetics DNA replication during the first cell cycle and developmental potential of somatic nuclear transfer embryos in bovine. Non-activated oocytes can support the completion of DNA synthesis during the first cell cycle of somatic nuclei after transfer into these cytoplasts, resulting a higher developmental rate. The DNA replication did not complete when transfer of somatic nuclei into activated cytoplasts, resulting in a lower number of developing embryos.

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