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ARTICLE REVIEW

Influence of Cell-Cycle Phase and Embryonic Stage of Donor Nucleus on Nuclear Transfer Success

อิทธิพลของวงจรเซลล์ของตัวนิวเคลียสและระยะของตัวอ่อนของตัวให้ต่อความสำเร็จในการถ่ายฝากนิวเคลียส

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บทคัดย่อ: **มงคล เตชะกำพุ และ อีวัง เฮมาน. 2536. อิทธิพลของวงจรเซลล์ของตัวนิวเคลียสและระยะของตัวอ่อนของตัวให้ต่อความสำเร็จในการถ่ายฝากนิวเคลียส. วารสารวิจัยวิทยาศาสตร์การแพทย์ 7(1): 27-33**

การถ่ายฝากนิวเคลียสเป็นเทคโนโลยีชีวภาพแบบใหม่ของวิทยาการสืบพันธุ์ เทคนิคนี้ทำโดยการนำนิวเคลียสของตัวอ่อนของตัวให้ที่ได้คัดเลือกแล้ว มาฝากในไซโตปลาสซึ่มของโอโอไซต์ตัวรับ อย่างไรก็ตาม แม้เทคนิคนี้จะมีการพัฒนามาอย่างดี แต่พบว่าอัตราความสำเร็จจากการเจริญของตัวอ่อนหลังทำการถ่ายฝากนิวเคลียสก็ยังต่ำอยู่ คือได้เพียง 20% ของตัวอ่อนที่เจริญในน้ำยาเพาะเลี้ยงจนถึงขั้นบลาสโตซีส และมีเพียง 3% เท่านั้นที่เจริญครบกำหนดหลังฝากในสัตว์ตัวรับ โดยมีปัจจัยต่าง ๆ ที่เกี่ยวข้องกับผลสำเร็จทั้งในหลอดแก้วและตัวรับฝากในการถ่ายฝากนิวเคลียส ซึ่งในปัจจัยต่าง ๆ นั้น วงจรเซลล์ของตัวนิวเคลียสและระยะของตัวอ่อนนับเป็นปัจจัยที่สำคัญ ซึ่งได้รวบรวมมารายงานไว้ในบทความนี้แล้ว

คำสำคัญ: *วงจรเซลล์; ระยะของตัวอ่อน; นิวเคลียสของตัวให้; การถ่ายฝากนิวเคลียส*

Abstract: **Mongkol Techakumphu, and Yvan Heyman 1993. Influence of cell-cycle phase and embryonic stage of donor nucleus on nuclear transfer success. Thai J Hlth Resch 7(1): 27-33**

*Nuclear transfer is a new biotechnology in reproduction. The technique is well developed by transfer the donor nuclei of selected embryos to cytoplasm of recipient oocyte. Unfortunately up to now, the success of nuclear transfer is quite low, only 20% can develop **in vitro** to blastocyst stage and not more than 3% of nuclear transfered embryos can survive to term after transfer to recipient. Various factors can affect **in vitro** and **in vivo** development. This paper discusses the two essential factors which are the cell-cycle phase of donor nuclei and the stage of embryos to the nuclear transfer success.*

Key words: *Cell-cycle phase; Embryonic stage; Donor nucleus; Nuclear transfer*

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Introduction:

In mammalian cells, the cell cycle consists of four phases, M (mitotic) phase, G1 (first gap) phase, S (synthetic) phase and G2 (second gap) phase. The M phase entails nuclear division (karyokinesis) and cytoplasmic division (cytokinesis) resulting two identical daugther cells. The daugther cells will subsequently enter in the new cycle directed to S phase where the DNA synthesis is taken place. The G1 phase is the gap between M and S phase and the G2 phase is between S and M phase. The G1, S and G2 phases are usually called "interphase" which present about 90% of length of cell cycle.

In embryonic cells, the length of cell cycle is dependent on stage of embryo and animal species. In amphibians, for example, before the midblastula stage the length of cell cycle is short about 35 min without a pause between M and S phases. The cell cycle begins to lengthen progressively with the addition of both G1 and G2 phases while the length of M and S phases remain unchanged. In domestic animals, the length of cell cycle is longer than that of amphibians. In mouse and bovine embryos, it is generally about 12 to 15 hr except in the first cell cycle and the cell cycle in which the maternal/zygotic transition is occurred.

It is suggested that the compatibility of cell-cycle phase between donor nuclei and recipient cytoplasm and the stage of embryo influence the developmental ability of nuclear transfer embryos. Experiments in amphibians, mice, rats, rabbits and domestic animals showed the importance of these two factors which are reviewed in this paper.

Cell cycle of embryo:

The cell cycle is the period covering the cell division which allows the cell multiplying and maintaining its population. Few observations have been done for the determination of the length of cell cycle in embryo. Only in mouse and bovine embryos that the cell cycle is analysed.

After fertilization, embryo divides synchronously until 4-cell stage and after that the rate of division of each blastomere is different. The first cell cycle is usually taken about 24-30 hr because it covers the time of sperm capacitation, of activation and the time for the completion of oocyte maturation. Furthermore, the length of cell cycle is usually corresponded to the time of maternal/zygotic transition (MZT) which is the time of zygotic genome activation and major change in protein patterns. This occurs during the second cell cycle of mouse, the third cell cycle of human, rabbit and pig and the fourth cell cycle of sheep and cattle. The cleavage of embryos at this stage is sensitive to the environment and development arrest is usually occurred in *in vitro* cultivation. Only co-culture and incubation in intermediate recipient such as rabbit or sheep oviduct allow to overcome this arrestment.

In mouse as in cattle, the S phase occupies about 80% of cell cycle. The duration of G1 except in the first cycle is variable and usually short, not more than 2 hr in mouse (Smith and Johnson, 1986; Chisholm, 1988) and 30-45 min for the fifth cycle in rabbit (Collas *et al.*, 1992a). The duration of G1 phase in cattle is not reported except in the first cell cycle which is 6 hr (?) (Barnes and Eyestone, 1990). The summary of different phases of each cell cycle in mouse and in bovine embryos is presented in Table 1 and 2.

Table 1 Summary of each phase duration in hr of the first 4-cell cycles of mouse embryos

Cell cycle	G1	S	G2+M
First[a]	4.5-1.2	4-7	1-8
Second[a]	0-1.3	4-7	12-18
Thrid[a]	1-1.5	7	0.5-5
Fourth[a]	2	7	1-3
Fifth[b]	<2	8-9	2

from a) R.K.W. Smith and M.H. Johnson, J. Reprod. Fert, 76, 393-399, 1986
b) J.C. Chisholm, J. Reprod. Fert, 84, 29-36, 1988

Table 2 Values for the duration in hr of the first 4-cell cycles in bovine embryos

Cell cycle	G1	S	G2	M	Ranges
First	6	8	4	2	28-32
Second	-	8	0-2	2	12-13
Third*	-	8	4-6	2	13-14
Fourth**	?	?	?	?	21-30

*First observed zygotic transcription

**First observed rRNA synthesis: *from F.L Barns and W.H. Eyestones. Theriogenology, 33(1), 1990*

Method of cell cycle synchronization:

a) control at M phase

The cell cycle synchronization is useful for the study of the influence of cell-cycle phase on the development of nuclear transfer embryos. In **amphibians**, a short cell cycle and a high mitotic index allow to control the cell cycle by manual removal of cell in metaphase stage. This pick off can be done regularly for the interval of 30 to 40 min depended on the stage of nuclei used. The cold storage is suggested to use to synchronize the cell cycle. Tsunoda *et al.* (1987) revealed that using donor nuclei stored at 4° C for 4-6 hr prior to use provided a better chance for synchronization of the cell cycle of donor nuclei with recipient cytoplasm. The other technique for cell cycle synchronization is by using the drug which can depolymerize the microtubules of cells. For example, the drug as nocodazole or colcemid has been used to synchronize the cell cycle of **mouse** 2-cell embryos (Kato and Tsunoda, 1992). The embryos were incubated in a medium contained of the drug for 12.5 to 14.5 hr which covers the period of cell cycle of this stage. The results indicated that 87 to 100% of mouse embryos stopped at metaphase stage and after drug removal the embryos can continue to develop until blastocyst with a high percentage, more than 80%. Nocodazole seems to be a preferable drug to synchronize the cell cycle of mouse embryos than colcemid because of a higher development to young was obtained.

In **rabbit**, culture of blastomeres in 0.1-5.0 µg/ml colcemid for 10 hr can mostly block 16-cell stage embryos at M phase and this effect is reversible. More than 80% of treated embryos develop to blastocyst after removal of the drug (Collas *et al.*, 1992a). In **cattle**, Techakumphu *et al.*, (1993) revealed that nocodazole can be used to synchronize the cell cycle of bovine embryos at 16-cell stage with the mitotic index of 50%. The embryos produced from *in vitro*maturation and *in vitro* fertilization (*IV*M-*IV*F) responded to the drug as well as the embryos produced *in vivo*, the mitotic index was 53% and 60% respectively. This value is significantly higher than non-treated embryos which is not over than 6% (Table 3). The metaphase arrested nuclei by nocodazole treatment is shown in figure 1. The effect of nocodazole is reversible due to the reduction of mitotic index and the increases of number of nuclei/embryo after 24 hr of culture (Figure 2). However, it exists the variation of mitotic index between embryos after treatment, this means that not all blastomeres are sensitive to nocodazole to stop at metaphase. Furthermore, the side effect of nocodazole such as dispersed chromosomes, multiple micronuclei formation or highly condensed nuclei after treatment (Techakumphu, 1992, personal observation) was presented. A sligth defect can affect on the development of treated embryos especially at the time of differentiation. A lower of blastocyst formation rate (24%) from *IV*M-*IV*F embryos was observed after treatment compared to 50% in non-treated embryos. This side effect seems less in *IN VIVO* embryos because the treated embryos can develop as the same percentage as control. Brinkley *et al.* (1967) suggested that higher doses of depolymerize drugs may have negative effect on inhibition of nucleoside transport, in some cell types.

Table 3 Mitotic index and viability of embryos after nocodazole treatment

Embryos	Mitotic index (No. of cells in metaphase/ No. of observed cells	% of Blastocysts (No. of embryos)
IVM-IVF	53% [a] (143/270)	24% [c](55)
Control	6% [b](22/370)	50% [d](42)
IN VIVO	60% [a](122/204)	50% [d](16)
Control	1% [b](1/132)	59% [e](22)

(a,b,p < 0.01; c, d, P < 0.05; d, e, ns; X2)

from M. Techakumphu *et al.* (1993), IETS, Baton rouge, Louisiana, USA

b) control at G1/S phase

The synchronization of blastomeres at the G1/S phase needs a two -step control. Firstly by using an inhibitor of microtubule polymerization and secondly by incubation in an DNA synthesis inhibitor, aphidicolin or mitomycin C for example following the time of release from the first drug. The results of Collas *et al.* (1992a) showed that inhibition of DNA synthesis was reversible if the concentration of aphidicolin does not exceed than 1.0 µg/ml and the optimal dose is 0.1 µg/ml. The initiation of ^{3}H thymidine uptake after mitosis will correspond to S phase. The determination of G1 can be calculated from the releasing time of M phase to the initiation time of thymidine uptake. Use colcemid and aphidicolin for blastomeres synchronization did not affect chromosome configuration in G1 transplants (Collas *et al.*, 1992b).



Figure 1. Metaphase arrested nuclei from nocodazole treatment in bovine embryos at 16-cell stage (arrow).

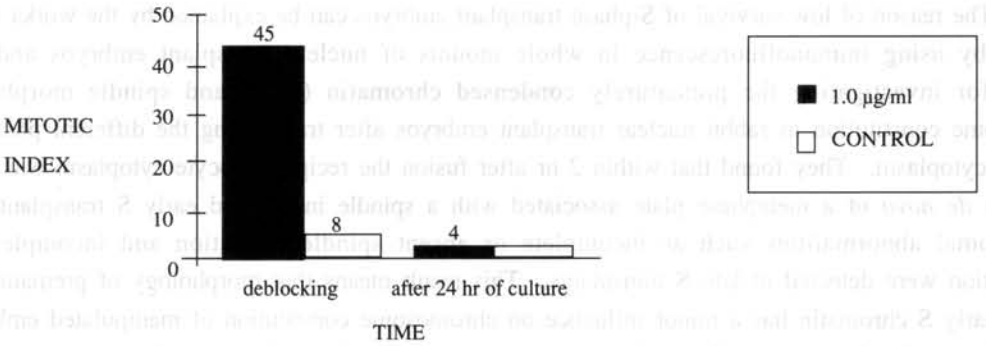


Figure 2. Mitotic index of nocodazole-treated embryos after 24 hr of culture *(from Techakumphu* ***et al.****, 1992, AETE)*

Effect of cell-cycle phase:

Nuclear transfer studies in ***Rana pipiens*** has shown that donor cell-cycle phase does have an effect on nuclear transfer success. Donor nuclei at G1 phase have a more limited developmental capacity beyond the blastula stage than nuclei from mid-S, late-S and G2 cells (von Beroldingen, 1981). The enlargement of transfered nuclei which is related to the developmental ability of the reconstituted embryos was less in G1 and early-S than in G2 and late S.

In **mouse**, this effect of cell-cycle phase was tested by using donor nuclei at different stages transfered to enucleated at telophase II oocytes. These nuclei were obtained from 2-cell stage embryos at 30-32 hr (early), 34-36 hr (middle), 40-42 hr (late 1), and 48-50 hr (late 2) after hCG injection. These times corresponded to G1, S middle G2 and late G2 phase. No effect of stage of the cell cycle of donor karyoplast on the success rate of fusion and activation was found. Most of the reconstitued oocytes emitted a polar body with 2 hr and formed one pronucleus, especially when nuclei from 2-cell embryos were transfered to the early and middle period between the first and second mitotic divisions of embryos. Contrary to the fusion and activation rate, the developmental capacity of the reconstituted embryos was significantly affected by the mitotic stage. The reconstituted embryos that received a nucleus from late 2-cell embryos had a greater capacity to develop in blastocyst stage (Kono *et al.*, 1991).

The influence of cell-cycle phase was also studied in **rabbit** (Collas *et al.*, 1992a). They found that *in vitro* development to blastocyst stage of cloned embryos was reduced as donor nuclei progressed in the cell cycle. Nuclei in the early stages of the cell cycle have a greater potential development than those in later stages of the cycle. Use of early stage (G1) nuclei gave 59% of blastocyst while they were only 32 and 3% with mid and late stage (early S or late S phase). At the present, no studies on the relationship of the stage of cell cycle of donor cells and the development of nuclear transfer embryos in other domestic animals were reported. We studied in cattle by using the drug, nocodazole, for blocking the cell cycle at 16-cell stage of bovine embryos at metaphase. The donor nuclei at metaphase just after the deblockage and the nuclei from the approximate time presumably correspond to G1 (1.5 hr after deblocking) and S phase (5-8 hr after deblocking) was transferred into cytoplasm of *in vitro* maturated-enucleated metaphase II oocytes. The result showed that a lower development to morulae was found in the reconstituted embryos that received nuclei from S phase while no difference had been found between the group of M or G1 phase (Table 4, Techakumphu *et al.*, 1993).

Table 4 Effect of stage of donor nuclei on *in vitro* development of bovine cloned embryos

Stage of donor nuclei	Reconstructed embryos	Fusion rate	Cleavage rate	Morulae (%)
M phase	34	29(85%)	26(90%)	6(21%)
G1 phase	100	74(74%)	61(82%)	15(20%)
S phase	46	33(72%)	25(76%)	2(6%)

from M. Techakumphu *et al.* (1993), IETS, Baton rouge, Louisiana, USA

The reason of low survival of S-phase transplant embryos can be explained by the works of Collas *et al.* (1992b) by using immunofluorescence in whole mounts of nuclear transplant embryos and chromosome spreads for investigating the prematurely condensed chromatin (PCC) and spindle morphology and on chromosome constitution in rabbit nuclear transplant embryos after transferring the different phase of nuclei to recipient cytoplasm. They found that within 2 hr after fusion the recipient oocyte cytoplasm was able to induce formation *de novo* of a metaphase plate associated with a spindle in G1 and early S transplants, whereas the chromosomal abnormalities such as incomplete or absent spindle formation and incompleted chromatin condensation were detected in late S transplants. This result means that morphology of prematurely condenses G1 and early S chromatin has a minor influence on chromosome constitution of manipulated embryos. That of late S chromatin, however, affects chromosome constitution in embryos and may account for reduced development of nuclear transplant embryos when late S phase donor nuclei are used (Collas *et al.*, 1992b).

Effect of embyonic stage:

In **amphibians,** the latest stage of nuclear donor used successfully for nuclear transfer was 3,000-4,000 cells of blastula and gastrula stage. In mammals, the success of development of reconstituted embryos is related to the stage of donor nuclei. In mouse, donor nuclei from embryos at the 4-and 8-cell stage have only a limited potential to support further development when fused to enucleated zygotes (McGrath and Solter, 1983). In rat as in mouse, the enucleated rat zygotes which received pronuclear karyoplasts can develop to term while the reconstituted embryos stopped to cleave at 2-cell stage when they received nuclei from 2, 4 or 8-cell stage (Kono *et al.*, 1991). Contrary to cattle, sheep or rabbit that the full term development is related to the timing of activation of the embryonic genome. Genomic activation would either renden nuclei incapable of reactivating developmentally important genes and/or that stage-specific genes continue to be expressed after transfer and that their products affect further development of the reconstituted embryos. Briggs and King (1957) suggested that the majority of abnormal nuclear transfered embryos obtained from advanced donor nuclei contained marked chromosomal abnormalities which subsequently cause development arrest.

The latest stage of embryos used for donor nuclei for domestic animals is yet unknown. In **rabbit** is usually used as a model of nuclear transfer in cattle. Because the genomic shift from maternal to zygotic control of development in rabbit is similar to that in cattle. Progeny by nuclear transfer of blastomeres from 8-cell (Stice and Robl, 1988) and 8-16-cell (Collas and Robl, 1990) embryos have been produced in rabbit. Recently, Yang *et al.* (1992) showed that blastomeres of morulae (32-64-cell stage) obtained from culture of 8-cell stage for 20-24 hr are still totipotent. The culture does not modify the totipotency of blastomeres donor as similar as the freezing. The young can also produced after using frozen blastomere donors from early morulae stage (Heyman *et al.*, 1990). In **cattle**, no significant difference of calving rate was found after using donor nuclei from 8-16 cell stage or 16-32 cell stage or 32-64 cell stage which were 45, 31 and 32% respectively (Willadsen, 1989). The rate of morulae/blastocysts was higher when nuclei from day 6 embryo is transferred in cytoplasm recipient than day 5.5 and day 5.0 embryos respectively 35% v/s 28% v/s 23% (Bondioli *et al.*, 1990). Blastomeres from late morula stage about 48 to 64 cells can be used for nuclear transfer and resulted the development to blastocysts (35%). At the present, early or late morula stage is normally used as donor nuclei for a nuclear transfer program because the embryos can be collected repeatedly by non-surgical method. In sheep, donor cells from inner cell mass resulted 56% of blastocysts after nuclear transfer (Smith and Wilmut, 1989). The studies in **sheep** indicated that donor nuclei until blastocyst stage were still totipotent and were able to be reprogrammed when they were transfered into enucleated oocytes recipient. This will be useful for clonal multiplication of genetically valuable embryos due to a higher number of blastomeres obtained per embryo.

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