บทความปริทัศน์

Nuclear Transfer: Use of Biotechnology in Animal Reproduction

การถ่ายฝากนิวเคลียส : การใช้เทคโนโลยีชีวภาพในวิทยาการสืบพันธุ์สัตว์

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การถ่ายฝากนิวเคลียสเป็นเทคโนโลยีชีวภาพในทางวิทยาการสืบพันธุ์สัตว์ที่กำลังศึกษาในปัจจุบัน เพื่อจุดมุ่งหมายในการผลิตสัตว์ที่มีพันธุกรรมที่เหมือนกัน เทคนิคนี้ประกอบด้วย 5 ขั้นตอน คือ การเตรียมโอโอไซต์ การเตรียมนิวเคลียส การดูดเอาสารพันธุกรรมออกจากโอโอไซต์ การฝากบลาสโตเมียส์ซึ่งเป็นตัวนิวเคลียสใหม่ และการกระตุ้นให้เกิดการรวมตัวระหว่างนิวเคลียสที่ย้ายฝากและตัวโอโอไซต์ การทดสอบผลทำได้โดยดูจากการพัฒนาของตัวอ่อนในน้ำยาเพาะเลี้ยง หรือหลังนำฝากในสัตว์ตัวรับ การใช้นิวเคลียสจากตัวอ่อนที่เจริญหลังจากถ่ายฝากนิวเคลียสครั้งแรกไปฝากในโอโอไซต์ใหม่เรียกว่า "การถ่ายฝากนิวเคลียสซ้ำ" สามารถทำได้ ปัจจุบันปัจจัยที่เกี่ยวกับการปรับปรุงประสิทธิภาพของการถ่ายฝากนิวเคลียสกำลังศึกษากันอยู่

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Abstract : **Mongkol Techakumphu. 1992. Nuclear transfer : Use of biotechnology in animal reproduction. Thai J Hlth Rsch 6(2): 117-123**

Nuclear transfer is a recent biotechnological technique in animal reproduction for the production of identical individuals or "clones". The technique involves five essential steps: oocyte recipient preparation, nuclear donor preparation, enucleation of oocyte recipient, blastomere transfer and fusion/activation. The development of the cloned embryo can be performed by in vitro culture or by transfer in animal recipient. Subsequent recloning is possible. Studies on modification of the parameters involved in the technique are in progress and are aimed at improving cloning efficiency.

Key word : *Nuclear Transfer; Cloning; Oocyte; Nucleus.*

Introduction

Nuclear transfer or nuclear transplantation is a new method of animal reproduction for producing identical individuals with a highly desirable genotype; meat or milk production or fertility or disease resistance. The method was pioneered by Spemann in 1938 in amphibians, and the work was further developped by Briggs and King in 1952 in *Rana pipiens.* This work showed that a normal adult could develop from one nucleus selected from many in an embryo. At the present time, it has been performed in different species of animals such as sheep (Willadsen, 1986; Smith and Wilmut, 1989), cattle (Prather *et al.,* 1987; Bondioli *et al.,* 1990), pigs (Prather *et al.,* 1989) and rabbits (Stice and Robl, 1988) but not successfully in mice (McGrath and Solter, 1984, Robl *et al.,* 1986).

Methods of nuclear transfer

The nuclear transfer technique involves five essential steps :

1) Oocyte recipient preparation
2) Nuclear donor preparation
3) Enucleation of oocyte recipient
4) Blastomere transfer
5) Fusion and Activation

1) Oocyte recipient preparation

The matured oocytes can be obtained either by *in vitro* maturation or by *in vivo* maturation. The *in vitro* maturation presents an advantage of lower cost of production and a high number of oocytes. The technique is performed by puncturing antral follicles with a size of 2-8 mm from the ovaries obtained from a slaughter house. The immature oocytes completely enclosed by cumulus cells are cultivated for 24 hr in conditioned medium supplemented with gonadotropin (FSH/LH) and steroid hormones (essentially estradiol 17-β). The technique of *in vitro* maturation was described elsewhere (Marquant-Le Guienne *et al.,* 1989; Xu *et al.,* 1987). Usually, about 80-90% of cultured immature oocytes reach the stage of metaphase II. *In vivo* matured oocytes can be obtained by collecting ovulated oocytes following superovulation. In this case, the cost of operation is high, especially in cattle, but it is usually practical in laboratory animals such as rabbits. The matured oocytes must be decoronized and kept in a suitable medium before nuclear transfer.

2) Nuclear donor preparation

A nuclear donor can be prepared from an embryo at the preimplantation stage. Blastomeres isolated from stages of development ranging from 8 to 64-cell stage can be used (Willadsen, 1989). In cattle, a 16 to 32-cell stage embryo is chosen because it allows non-surgical collection, provides a high number of clones, and the blastomeres are quite easily separated. In case of rabbits, pigs, sheep or goat, blastomeres are prepared from embryos collected by surgical techniques or after slaughtering. The blastomere nuclei are disaggregated

from embryos either chemically and/or mechanically. Usually embryos are incubated in a low concentration (0.25 to 0.5%) of a proteolytic enzyme such as pronase and are later aspirated with a fine micropipet with a diameter smaller than embryos. The incubation of embryos in a calcium-magnesium free medium at 37°C is necessary in the case of unseparated blastomeres. The separated blastomeres of each embryo will be kept in a small drop of medium at room temperature. Blastomeres obtained from frozen-thawed embryos at the 32 -cell stage can be used also in nuclear transfer in rabbits (Heyman *et al.*, 1990).

3) Enucleation of oocyte recipient

The enucleation can be performed by two methods:

a) a disruptive method which is done by bisection of oocytes as described by Willadsen (1986). The demi-oocytes are observed under ultraviolet microscopy after incubation for 20-30 min in the vital dye Hoechst. This allows identification of those demi-oocytes containing chromosomes (Wolfe and Kramer, 1992). The non containing chromosomes oocytes will be used as oocyte recipients.

b) a non-disruptive method which was borrowed from that developped by McGrath and Solter (1983) in amphibians. The enucleation is done by aspiration of the first polar body and the adjacent cytoplasm ($\simeq$20-30%) with a special micropipet (Fig 1). This part presumably contains metaphase chromosomes. The metaphase chromosomes are generally located near the first polar body although this is not always the case in rabbits or the case of a degenerated polar body (Modlinski and Smorag, 1991). Metaphase chromatin can be visualized by staining with a DNA-specific stain, Hoechst No.33342 before enucleation. With this technique, the enucleation rate is 100% either the *in vitro* or *in vivo* matured oocytes (Chesné *et al.*, 1991).

The oocyte recipient (cytoplast) should be incubated in Cytochalasin B in order to depolymerize the cortical actin of the cytoplasmic membrane. This facilitates the enucleation of oocytes without disruption of the cytoplasmic membrane during micromanipulation.

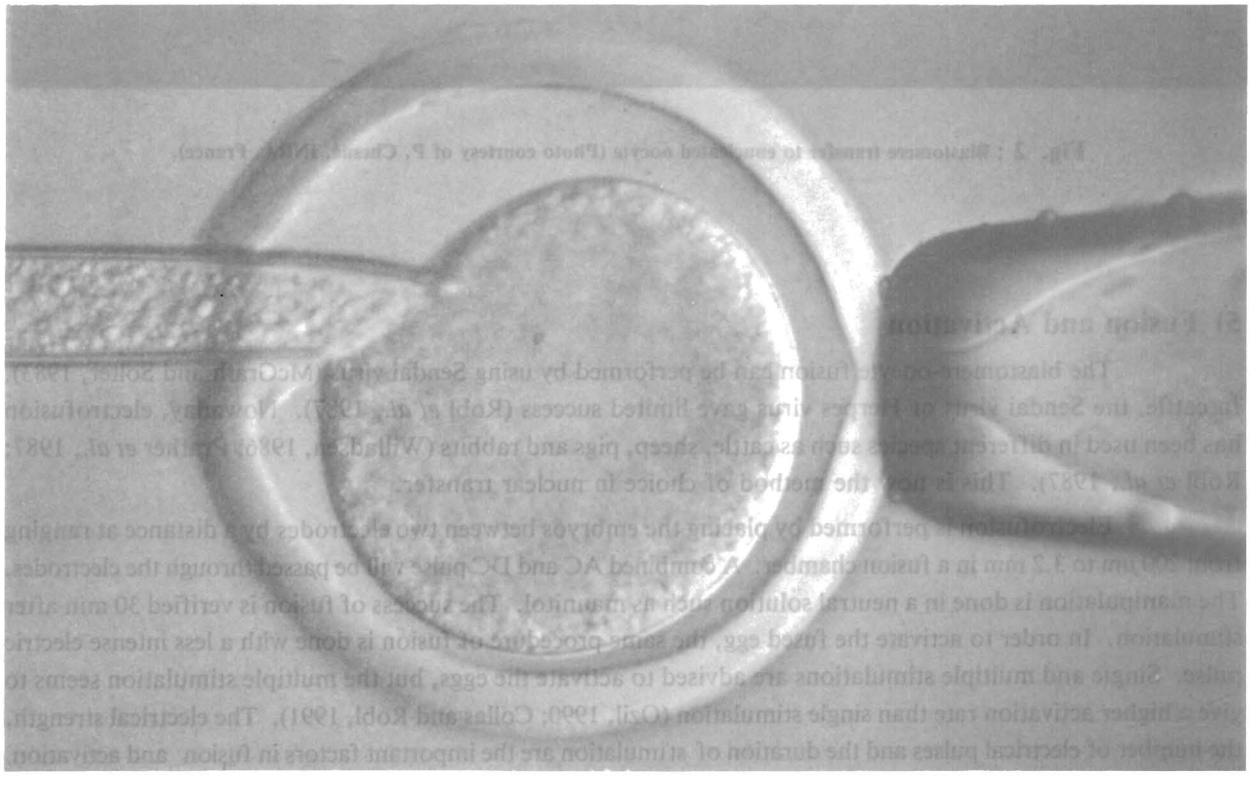

Fig 1 : Enucleation of oocyte recipient by aspirating the metaphase chromatin and the first polar body (Photo courtesy of P. Chesné, INRA, France)

4) Blastomere transfer

The donor blastomere (karyoplast) will be transferred into the enucleated oocyte via a penetration of a bevelled pipet (Fig 2). The oocyte recipient will be held by holding pipet with a diameter of approximately 120 μm. The nucleus is aspirated in a bevelled pipet and transferred into the perivitelline space. It is obligatory to attach the transferred nucleus to the cytoplasm of oocyte recipient in order to have good fusion rate. All steps from the enucleation of oocytes to blastomere transfer are performed by micromanipulation under an inverse microscope.

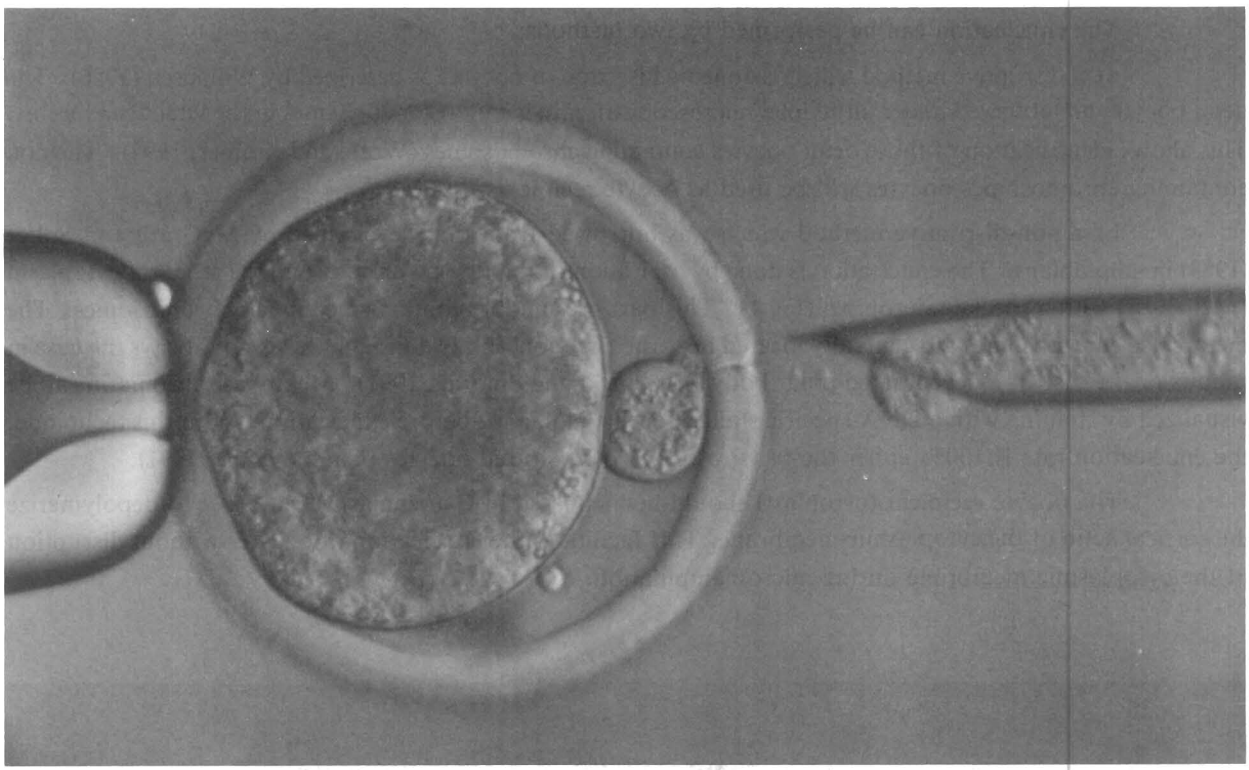

Fig. 2 : Blastomere transfer to enucleated oocyte (Photo courtesy of P. Chesné, INRA, France).

5) Fusion and Activation

The blastomere-oocyte fusion can be performed by using Sendai virus (McGrath and Solter, 1983). In cattle, the Sendai virus or Herpes virus gave limited success (Robl *et al.*, 1987). Nowaday, electrofusion has been used in different species such as cattle, sheep, pigs and rabbits (Willadsen, 1986; Prather *et al.*, 1987; Robl *et al.*, 1987). This is now the method of choice in nuclear transfer.

Electrofusion is performed by placing the embryos between two electrodes by a distance at ranging from 200 μm to 3.2 mm in a fusion chamber. A combined AC and DC pulse will be passed through the electrodes. The manipulation is done in a neutral solution such as mannitol. The success of fusion is verified 30 min after stimulation. In order to activate the fused egg, the same procedure of fusion is done with a less intense electric pulse. Single and multiple stimulations are advised to activate the eggs, but the multiple stimulation seems to give a higher activation rate than single stimulation (Ozil, 1990; Collas and Robl, 1991). The electrical strength, the number of electrical pulses and the duration of stimulation are the important factors in fusion and activation. After fusion and activation, the eggs are cultivated in conditioned medium in order to observe cleavage and development to the blastocyst. A schematic of all process of nuclear transfer is presented in Fig. 3.

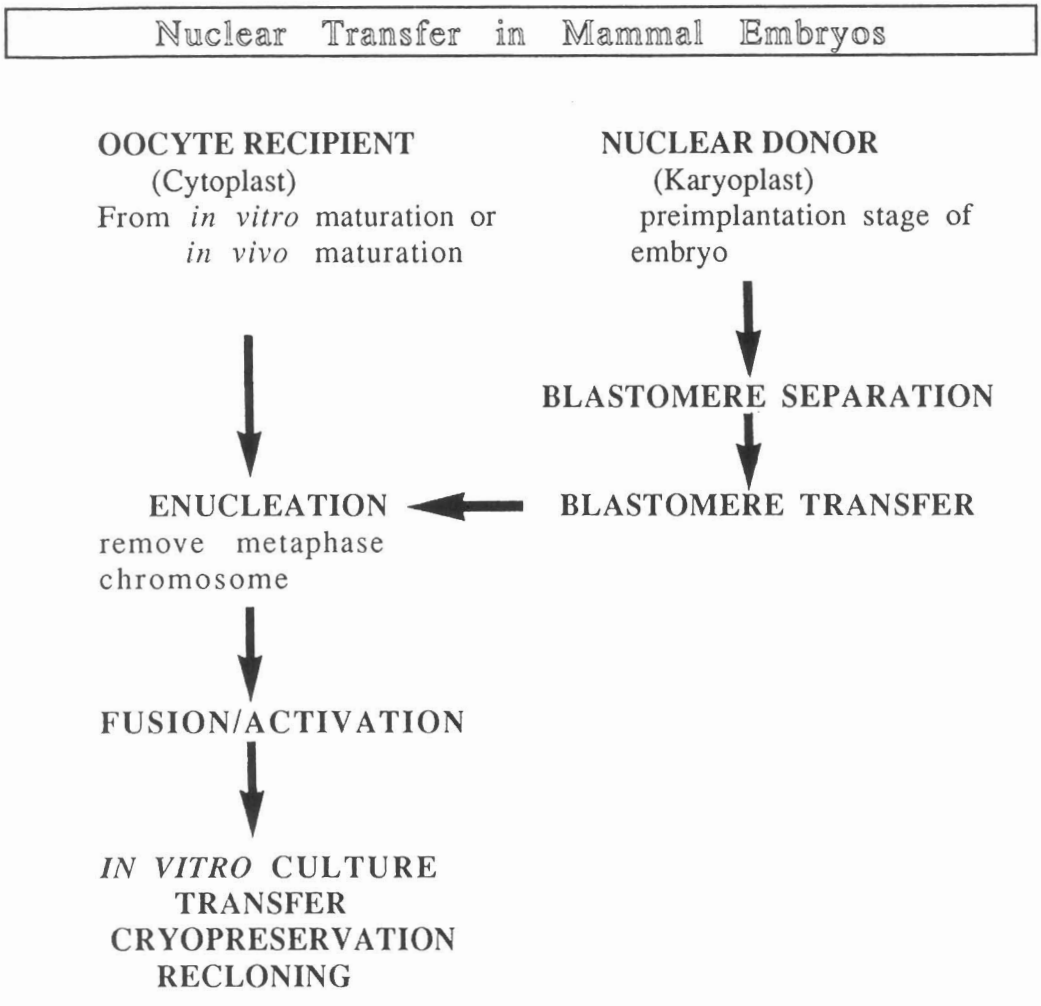

Fig 3 : Different steps of nuclear transfer

Efficiency of nuclear transfer technique

The efficiency of each step of nuclear transfer procedure depends on species as shown in Table 1. The enucleation rate obtained in different species is similar while the activation rate and fusions rate vary between 40 to 85% and 70 to 90% respectively. The reconstituted embryos rate obtained before cleavage is around 40 to 90%.

Table 1 : Efficiency of different steps in nuclear transfer

Species	Enucleation rate	Activation rate[a]	Fusion rate	Reconstitution rate
Cattle*	60-80%	57-61%	70-88%	44-70%
Sheep*	68-75%	43%	82-90%	56-68%
Pigs**	74%	81%	87%	–
Rabbits*	60-100%	46-85%	84-91%	51-91%

* Modified from Y Heyman *et al.* (1991)

** Modified from R S Prather and N L First (1990)

a) The criteria of activation rate is cleavage to the 2-cell stage

The results of *in vitro* development have shown that at least 50% of cloned eggs cleaved to the 4 to 8-cell stage but the development to the morula and to the blastocyst stage is about 30% and 10% respectively. The culture in ligated oviduct of an intermediary recipient such as in rabbits or in sheep is an alternative method which provides a good environment for development. This method gave a higher development than that of culture *in vitro* (Northey *et al.*, 1992).

At present, the result of transfer of cloned embryos in a recipient in different species is low at the overall efficiency rate of under 6% with few clones obtained after transfer (Yang, 1991; Seidel, 1992). Nevertheless, in cattle Willadsen *et al.* (1991) reported a 33% pregnancy rate after transfer of cloned embryos to 302 recipients. The majority of the calves produced by nuclear transfer were apparently normal and viable, although a small number of them presented some abnormalities such as hydrallantois, contracture malformation or cardiac septal defect. Recently, at symposium on cloning mammals by nuclear transplantation this year in Colorado USA, showed that in bovine cloning about 60-70% of calves are normal while about 20-30% are larger than normal, up to twice normal size. The abnormality is called **"large-calf syndrome"**. The cause of which is still unknown and it requires caesarian intervention. This high weight at birth is followed by a normal size within a few months (Seidel, 1992).

Embryo cloning in the bovine species is now commercialized in at least three companies, particularly in the USA. On the other hand, research on the different factors which affect the efficiency of nuclear transplantation such as age or types of oocyte recipient, stage of nucleus before transfer, fusion and activation scheme etc. are being studied in different laboratories in the world. Nuclear transfer is in an early phase of development since the first nuclear transplant lambs were born by Willadsen in 1986. The technique is still requires improvement. The nuclear transfer technique **(cloning and recloning)** combined with the cryopreservation of cloned embryos will allow stocking of large numbers of genetically selected animals for breeding purposes in the future.

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