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Review

Handmade cloning of mammals

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Handmade embryo reconstitution (HMER) has been used to study the parameters of nuclear transfer experiments such as fusion process, nuclear remodeling, nuclear reprogramming, biochemical processes, and biological processes during embryogenesis. These parameters have been widely investigated using the micromanipulator-based cloning technique (MBCT). This technique is a tedious, multi-step, time consuming and complicated procedure that utilizes expensive equipment. The HMER has emerged as an alternative for the MBCT. If the HMER is used to produce cloned animals it is known as handmade cloning (HMC). The HMC will allow the scientists to produce cloned animals with simple non-expensive equipment. Consequently, enormous data concerning all the facets of the nuclear transplantation experiments could be retrieved from various laboratories. This will allow a better future application of the cloning technique for the welfare of human, through production of animals with high genetic traits, rescue of endangered animal species and production of transgenic animals that can produce medicine for certain human diseases.

Key words: Cloning, chemical enucleation, oocyte bisection, mouse.

INTRODUCTION

Since the success of Briggs and King (1952) who were able to enucleate the oocytes of the leopard frog (*Rana pipiens*) manually and the success of Gurdon and Uehlinger (1966) to clone fertile frogs by transferring nuclei from the cells of intestinal endoderm of the frog (*Xenopus laevis*) into enucleated oocytes, other scientists thought to conduct such experiment in mammals. Before doing this, they should have to perfect three important techniques: A method for enucleating mammalian oocytes without destroying them, a method for isolating intact donor nuclei, and a method for transferring these nuclei into the enucleated oocytes (cytoplasts) without destroying either the nuclei or the cytoplasts (Gilbert 1997).

However, the mammalian oocyte is very small and difficult to manipulate manually. In 1981 a micromanipulator-based cloning technique (MBCT) has been described by Illimense and Hoppe (1981) who reported the birth of the first cloned mammalian (mouse) by transferring nuclei from inner cell mass (ICM) of mouse embryo into an enucleated mouse zygote. The most popular and widely used MBCT is the one described by McGrath and Solter (1983). By this technique the birth of cloned offsprings from various animal species has been reported, cattle (Prather et al., 1987; Bondioli et al., 1990; Prather and First, 1990), rabbit (Stice and Robl, 1988; Collas and Robl, 1990) sheep (Willadson, 1986; Smith and Wilmut, 1989; Campbell et al., 1996; Wells et al., 1997). The most famous an important breakthrough in cloning of mammals is the production of the sheep Dolly after the transfer of differentiated mammary gland cells into an enucleated sheep oocyte (Wilmut et al., 1997). Since that time scientists envisage to clone animals with superior genetic traits and endangered animal species. Also they envisage to spread the cloning technique to produce genetically identical animals to hasten genetic gain in animal production, reduce the number of animals needed for experiment, investigate genetic and environmental interactions, study nucleocytoplasmic interactions, study the cytoplasmic inheritance through production of animals with same genetics but with different cytoplasm, study mechanisms regulating cell cycle and cell division as well as production of transgenic animals. Despite improvement and numerous advances in all the facets of the MBCT, this technique needs expensive equipment, certain laboratory arrangements and a considerable technical skill which limits its wide spread.

The first to overcome these difficulties were Taniguchi et al. (1992) and Taniguchi and Kanagawa (1992). They utilized the manual oocyte dissection described by Tarkowski and Rossant (1976) to dissect mouse oocytes into karyoplasts and cytoplasts. They electrofused the cytoplasts obtained onto mouse late 2-cell stage blastomeres to produce reconstituted embryos. The same technique has been employed with a modification that makes the egg (oocyte and zygote) manual dissection simple, easy and rapid (Elsheikh et al., 1997b; Elsheikh and Kanagawa, 2003; Elsheikh et al., 2006). The modification is to deform the zona-free eggs into cylindrical rods by sucking them into a glass pipette with a narrow mouth. Then the rods are dissected by a fine glass needle (5 µm) on a surface of agar under stereomicroscope. Another group of researchers performed HMC after dissecting oocytes into karyoplasts and cytoplasts under stereomicroscope with Ultrasharp Splitting Blades (Vajita et al., 2001; Booth et al., 2001a; Booth et al., 2001b). In 1993, a non invasive chemical enucleation procedure for mouse oocytes was described by Fulka and Moor 1993. The basic idea of this procedure is to block DNA topoisomerase II enzyme (top II) during metaphase I (M I). This treatment will inhibit the oocyte chromosomes separation and the whole chromatin is expelled into the first polar body leaving a chromatin-free cytoplast, the so-called chemically enucleated oocyte (CEO). This procedure has been employed by Elsheikh et al. (1997 b) for HMC of mouse embryos, where they electrofused CEO to late 2-cell stage mouse blastomeres. The reconstituted embryos produced were not able to develop beyond the 4 cell stage. Blastocyst development was reported when the CEO have been exposed to 0.75 M sucrose and used for HMC (Elsheikh et al., 2006). All these attempts were carried to overcome the difficulties of MBCT.

This article emphasizes the HMC that utilizes cytoplasts obtained by manual dissection and chemical enucleation as an alternative for MBCT to allow the wide spread of cloning of mammals and reduce the cloning expenses.

PROCEDURES OF EGG ENUCLEATION

Two procedures for egg enucleation (oocyte and zygote) have been described to produce recipient cytoplasts for the HMC.

Manual dissection

This procedure has been described by Tarkowski and Rossant (1976). They dissected the zona-free mouse zygote, manually under a dissecting microscope, into cytoplast and karyoplasts. A modification for the manual egg dissection has been described (Elsheikh et al., 1997; Elsheikh and Kanagawa, 2003; Elsheikh et al., 2006). This modification suggests the culture of the zona-free mouse eggs, in phosphate buffered saline (PBS) supplemented with 3 mg/ml bovine serum albumin (BSA) and 5 µg Cytocalsin B (CB), for 30 min. They are then transferred to a dissection medium (a surface of 1% agar in physiological saline covered with PBS + BSA). After this they are sucked into a glass pipette of 20 µm internal diameter under a stereomicroscope. This treatment will deform the zona-free eggs into cylindrical rods (Figure 1a, b). The rods are then dissected manually with a fine glass needle (5 µm) into two fragments. The oocytes will produce demioocytes (Figure 1c) and the zygote will produce karyoplat or nucleated fragment and cytoplast or enucleated fragment (Figure 1d). By this procedure at least 70 oocytes can be dissected within 1 h. 90% of the oocytes deformed into rods and manually dissected survived and the demioocytes obtained become spherical immediately (Elsheikh and Kanagawa, 2003). The enucleated and nucleated fragments of the oocytes will be classified after Hochest staining.

Chemical enucleation

This procedure has been described by Fulka and Moor (1993) and Elsheikh et al. (1997b). In this procedure the mouse oocytes have been enucleated with etoposide (ETO) a specific top II blocker. Top II is essential for chromosomes segregation during mitosis (Wright and Schatten, 1990; Downes et al., 1991). The basic idea of chemical enucleation is to block top II during M I. This treatment will inhibit chromosome segregation and the total chromatin of the oocyte will be expelled into the first polar body leaving a chromosome-free cytoplast the so called CEO (Figure 2). In this procedure, groups of cumulus-free or cumulus-intact germinal vesicle (GV) stage mouse oocytes are cultured, for 3 - 6 h in tissue culture medium 199 (TCM 199) supplemented with 10% fetal calf serum (FCS), at 37°C in 5% CO_2 in air. The oocytes that undergo GV breakdown (M I) are further cultured for 3 h in TCM 199 + FCS supplemented with 50 µg/ml ETO (Sigma, Chemical Company, St. Louis, Mo. USA). Thereafter, they are transferred to TCM 199 + FCS supplemented with 50 µg/ml ETO and 50 µg/ml cycloheximide (CHXM, Sigma) and the culture is allowed to continue for 12 h. The oocytes that will extrude the first polar bodies will be stained with Hoechst stain to confirm their enucleation. To confirm enucleation the demioocyte obtained by manual bisection and the oocytes that extruded the first polar bodies after chemical enucleation treatment are transferred to PBS containing 2 µg/ml Hochest 33342 stain and incubated for 5 min at 37°C in 5% CO_2 in air. After staining they are washed in TCM 199 + FCS and are mounted on a glass micro-chamber (Multitest slide 12 well, Flow Laboratories, Rockville, MD, USA). The glass micro-chamber is examined under a

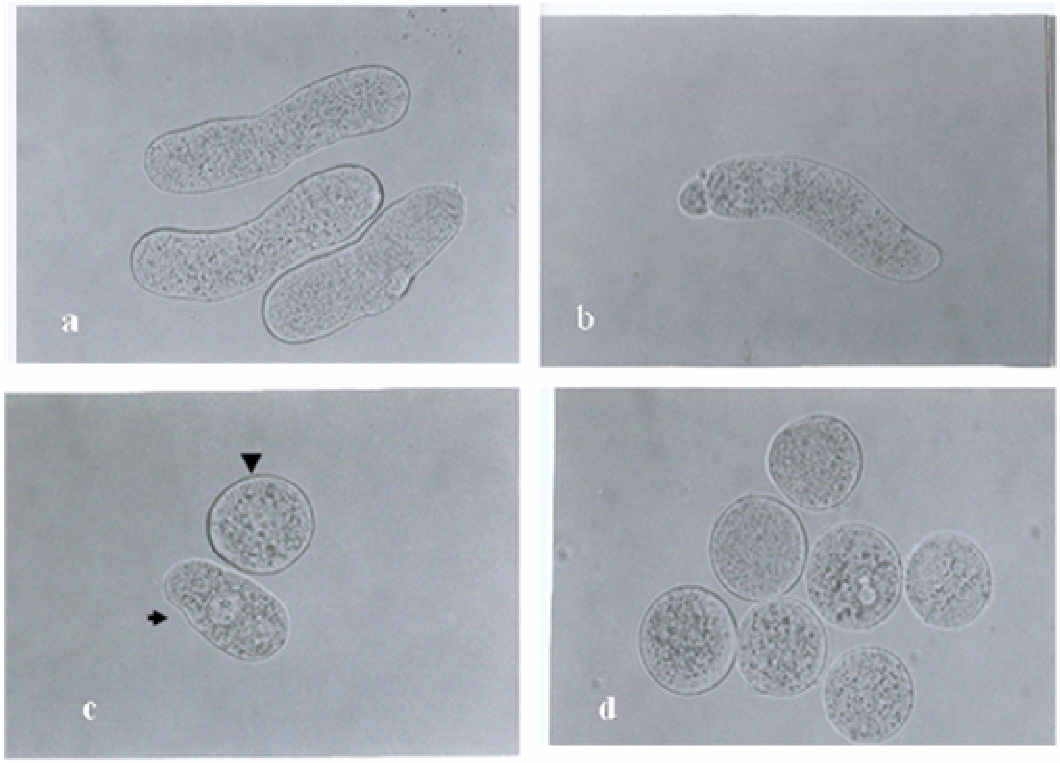

Figure 1. a) Oocytes deformed into rods, b) pronuclear stage embryos deformed into rods, c) cytoplast (large arrow) and kayoplast (small arrow) obtained after manual dissection of pronuclear stage embryo and d) demioocytes obtained after manual bisection of oocytes.

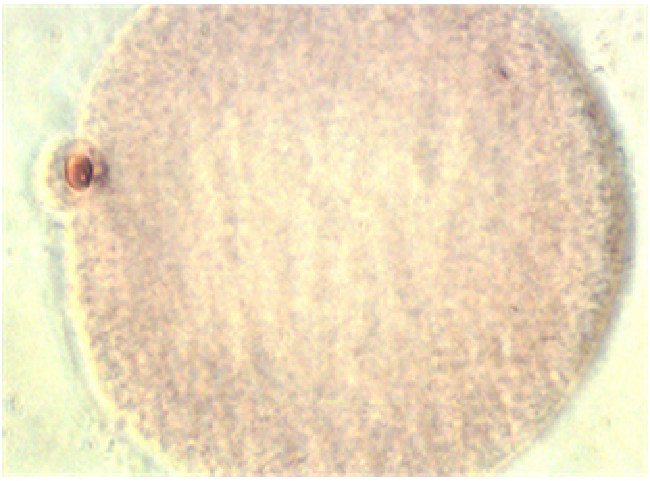

Figure 2. Chemically enucleated oocyte (CEO) stained with aceto-orcien. The total chromatin is extruded into the first polar body.

microscope with florescence condenser and super pressure mercury lamp. An ultraviolet excitation filter UG1 is used in combination with barrier filter Y 435 (Elsheikh et al., 2006). The oocytes or oocyte fragments are considered enucleated when their cytoplasm is free from any chromosome. The rate of chemical enucleation success has been reported to be more than 90% (Fulka and Moor, 1993; Elsheikh et al., 1997 b).

Source of the donor nuclei

All kinds of nuclei from early embryonic stage such as pronuclei from zygote, embryonic blastomeres, ICM of the embryo, embryonic stem cells (ESC), cumulus cells or any other somatic cell can be used as nuclear donor in HMC if they can be isolated without destroying them. The karyoplasts of pronuclear stage embryos can be obtained by manual bisection of zygotes (Elsheikh and Kanagawa, 2003; Elsheikh et al., 2006). The blastomeres can be isolated by culturing zona-free embryos in calcium-free culture media and then they are isolated by pipetting the zona-free embryos with a narrow pore pipette of 60 - 70 µm internal diameters (Elsheikh et al., 1997 b). The cumulus cells can be isolated by vortexing and careful pipetting using 1 ml automatic pipette after culture in 0.5 mg/ml hyalurondase dissolved in Hepes-buffered TCM 199 (Vajita et al., 2001). The ICM cells, ESC and somatic cells in culture can be isolated after washing of cells in calcium and magnesium free PBS and incubated at 37 - 39^{o}C for 5 min (according to animal species) in 100 µl of 0.05% trypsin in PBS (Vajita et al., 2001).

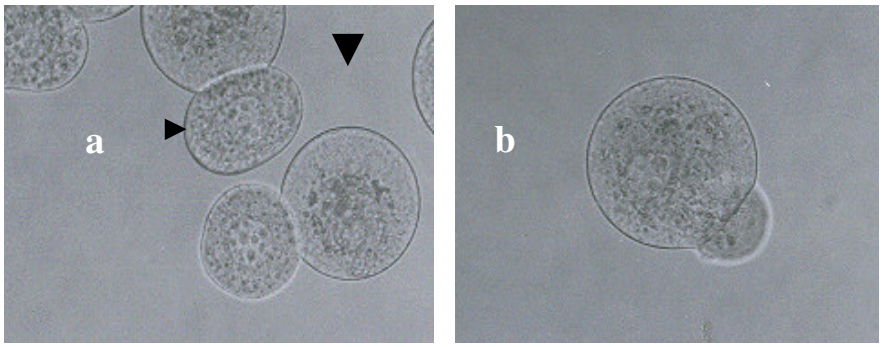

Figure 3. a) Aggregated cytoplast (large arrow head) and karyoplast (small Arrow head) and b) the couplets in the process of fusion.

PROCEDURE FOR HMC

Removal of the zona pellucidae from eggs

The eggs are exposed to acid Tyrode's solution (pH 2.7) for 20 – 40 s (Elsheikh et al., 1997a; Elsheikh and Kanagawa, 2003; Elsheikh et al., 2006). This treatment will remove the zona pelluceda. The zona-free eggs should be washed in several drops of TCM 199 + FCS. They are then pooled and kept in the incubator at 37°C in 5% CO_2 in air until used. Also zonae can be removed by exposing eggs to 0.5% pronase in PBS as described by Elsheikh et al. (1995).

Synchronization of the donor cell cycle

The cell cycle stage of the cytoplast to be used in HMC might be different from that of the donor nuclei. Thus, the development of the reconstituted embryos might be hampered. Many methods are used to synchronize the environment between the donor nuclei and the recipient cytoplast. The famous method is the serum starvation which has been described by Wilmut et al. (1997). In this method, the donor nuclei are cultured in a medium with low level of serum (0.5%). This treatment will keep the cell that divided at the gap phase (G_0) of the cell cycle. During the G_0 the DNA will never replicate and remains dormant as long as the cells are kept in this medium. When these nuclei are transferred into recipient cytoplasts and activated, they will start the processes of remodeling and reprogramming to form a new zygote like reconstituted embryo. Also the cell cycle of donor nuclei can be synchronized with chemicals such as nocodazole (Tanaka et al., 1995; Otaegui et al., 1994).

Manual aggregation of cytoplasts and donor nuclei into couplets

The donor karyoplasts, blastomeres or cells can be aggregated with cytoplasts obtained by manual bisection or chemical enucleation as described by Elsheikh et al. (1997). The cytoplasts and donor cells or karyoplasts are transferred to PBS supplemented with 10 µg/ml phytohemagglutinin-p (PHA-P, Difco Laboratories Detroit Michigan, USA). The cytoplasts and donors are brought in contact with the end of a narrow glass pipette of 30 – 40 µm internal diameter. To augment the contact between the donor and the recipient, they are pippetted with the same pipette (Figure 3a).

Fusion of the couplets

Electrofusion has been widely used to transfer donor nuclei into recipient cytoplasts in nuclear transfer experiments (Robl et al., 1987; Smith and Wilmut, 1989; Rechards and White, 1992; Wilmut et al., 1997; Elsheikh et al., 1997b). This technique can be applied in electrolyte solution (Zimmermann and Vienken, 1982; Kubiak and Tarkowski, 1985; Rechards and White, 1992; Elsheikh et al., 1995; Elsheikh et al., 1997b; Elsheikh and Kanagawa, 2003; Elsheikh et al., 2006) and in non-electrolyte solution (Berg et al., 1983; Willadsen, 1986; Kato and Tsunoda, 1987; Iwasaki et al., 1989; Smith and Wilmut , 1990; Kanka et al., 1991; Taniguchi et al., 1992; Heyman et al., 1994; Vajita et al., 2001, Booth et al., 2001a, b). The electrolyte fusion media (PBS) has been used for the first time to produce HMC embryos when CEO are fused to late 2-cell stage blastomeres of mouse (Elsheikh et al., 1997b). The fusion rates of HMC embryos in PBS were proved superior to those in manitol solution which is non-electrolyte (Elsheikh and Kanagawa, 2003). The fusion rates of HMC embryos recorded were 72.5% for PBS and 57.5% for manitol. This has been followed by successful development of HMC embryos to blastocysts when fusion has been carried in PBS (Elsheikh et al., 2006).

The fusion parameters have been a field pulse of 0.6 MHZ and alternating current pulse (AC) of 5 Vmm^{-1} applied for 5 s to induce alignment and to bring the membranes of the donor and recipient in direct contact. The electrofusion pulses have been three direct current (DC) pulses of 70 mm^{-1} applied for 70 µs at an interval of

1 s. A fusion chamber consisted of two stainless steel plate electrodes (25.0 × 15.0 × 0.5 mm) glued onto a glass slide 1 mm apart, was used. The fusion chamber has been connected to the fusion machine (LF.100Life Tec. Co. Tokyo, Japan). A fusion medium (PBS) supplemented with 3 mg/ml BSA was employed. The couplets are oriented manually between the electrodes to keep the fusion plains parallel to electrodes. This fusion technique utilizes a machine which renders the cloning technique a semi-HMC. To make this a complete HMC, fusion should be done by Sendai virus (Hallet et al., 1982) which aids the fusion without machines. Therefore, the use of viruses and chemicals that can induce cell fusion is suggested as an alternative for fusion machines

Activation of the reconstituted embryos

When the sperm enters the oocyte during the fertilization process it induces certain chemical changes known as activation. After oocyte activation the process of embryogenesis starts. The HMC embryo needs an action that mimics the sperm action on the oocyte to start embryogenesis. The activation of HMC embryos is carried by electric pulses (Vajita et al., 2001; Booth et al., 2001a, b). However, some researchers prefer the use of ethanol for activation of HMC embryos (Elsheikh et al., 1997b; Elsheikh and Kanagawa, 2003; Elsheikh et al., 2006). To activate HMC embryos they are exposed to 7% ethanol in culture medium 60 – 90 min after fusion. If the cytoplasts used in HMC are obtained from enucleated parthenogenetic oocytes or zygotes the activation is not essential.

Culture of HMC embryos

Many media are used to culture mammalian embryos. The mouse embryo can be cultured in Whitten mediums (Whitten, 1971), Whittingham medium (Whittingham, 1971) or in simplex optimization medium (KSOM) with low potassium (Erbach et al., 1994). The rabbit embryos can be cultured in BO medium (Brachett and Oliphant 1975). The synthetic oviduct fluid medium (mSOF) is widely used to culture bovine embryos (Takahashi and First, 1993). The culture is done in Co_2 incubators (5% CO_2 in air) or O_2 incubator (5 - 20%). The oxygen tension depends on the species.

Developmental potentials of HMC embryos

The desired result of HMC of mammalian embryos is to produce cloned embryos capable to develop blastocyst *in vitro* and to normal adult when transferred to a recipient animal (Vajita et al., 2005). The totipotency of nuclei of different cells used in nuclear transfer experiments is controversial. However, the success of different nuclei to develop to term include nuclei of 4-cell pig embryos (Prather et al., 1989), nuclei from 4-cell stage mouse embryo (Cheong et al., 1993), cell line culture of sheep (Campbell et al., 1996; Wells et al., 1997), and nuclei of mammary gland of sheep (Wilmut et al., 1997), made us to conclude that all body cells can be used as donor nuclei in HMC. The use of all body cells as donors in cloning cannot be achieved unless reliable techniques to isolate these nuclei without destroying them are developed.

PROBLEMS LIMITING THE SUCCESS OF HMC

Problems limiting the success of egg bisection

There are several problems that limit the success of egg manual dissection to obtain cytoplasts for HMC.

Fluidity of zona-free eggs

When the zona-free eggs are cultured in media supplemented with Cytocalsin B (CB), their cytoplasm will become more fluid and deforming them into cylindrical rods will become very easy. If the fluidity is not enough the egg will lyses. Therefore, before starting the rods making, be sure that the fluidity is enough by testing one or two eggs.

Egg deforming pipette diameter and end

The diameter of the micropipette used for egg deforming into rods should be 20 µm for mouse eggs and about 30 µm for bovine eggs. The end of this pipette should be well fire-polished to avoid injury of the zona-free eggs.

Suction of the zona-free eggs into the deforming pipette

Suction of eggs into the deforming pipette should be carefully, slow and very smooth to avoid egg lyses.

Site of bisection of rods

The site of bisection of oocyte rods should be near the end of the rod where the polar body is available. By doing this you will get a cytoplast of a considerable size and for sure free from any chromosomes since they are usually near the polar body. If the polar body is lost or the egg is a zygote it is better to dissect the egg into equal parts.

Aggregation of the couplets

During aggregation of cytoplasts and donor nuclei the area of contact between them should be wide to achieve

high fusion rates. To increase the area of contact between the cytoplast and donor nuclei a glass pipette with an internal diameter of 30 – 50 µm should be employed. The couplets are sucked into this pipette and the cytoplast and the karyoplast will be pressed and the contact between them will be increased.

Fusion parameters

The fusion parameters which have been described here should be followed carefully, unless superior fusion conditions are discovered for HMC.

Problems of HMC using CEO

In addition to the problems mentioned above, CEO might have residues of ETO and CHXM that will affect the transplanted nuclei. Thus, the step of washing in sucrose is very essential for the success of HMC when CEO is used. Until now chemical enucleation is limited to the mouse oocytes and it remains to be examined for other species. Furthermore, new materials that induce chemical enucleation remain to be discovered.

Problems of zona-free embryos

Normal embryos are equipped with zona-pellucida which protects them not only from predators and toxic substances in culture media (Elsheikh et al., 1997a) but it is essential for even undisturbed embryonic development (Suzuki et al., 1995; Elsheikh et al., 1997 a). The HMC embryos are zona-free and they might be affected by toxic substances in culture media. Furthermore, their development might be disturbed. These problems can be overcome by using an artificial zona – pellucida (Elsheikh et al., 1997a).

CONCLUSION

This procedure will allow many researchers around the world, who are interested in cloning, to do these experiments with low cost, at a simple laboratory and without purchasing expensive equipment. Consequently, enormous data concerning all the facets of cloning could be retrieved and many cloned animals could be produced. In the future better cloning programs could be developed to produce cloned animals with superior genetics, rescue endangered animal species and create transgenic animals for medical purposes.

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