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SOMATIC CLONE AND METHODOLOGY OF NUCLEAR TRANSFER-EGG RECONSTRUCTION

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SUMMARY

The somatic cloning technology, As a response to the rapid loss of animal genetic resource salute at present very important solutions for the conservation of the biodiversity, the foundation of new productive strains and the transgenic animals production.

In the case of the two first applications is indispensable requisite to arrange of cryopreservation techniques of somatic cells, sufficiently efficient to permitting the establishment of cryobank of nuclear donor cells.

The present work of thesis was under taken in the animal and it put about the techniques required to obtain, cryopreserve, to cultivate and to prepare the two cellular types better candidates to apply the somatic cloning to the conservation of the biodiversity and the genetic improvement: cumulus cells and very especially epithelial fibroblasts.

Two procedures of non-equilibrium cryopreservation (rapid-freezing and vitrification) had been tested to conserve the two cell types, reaching very high levels of survival and cellular proliferation after thawing, sufficient to cover the applied objective that is pursued.

Besides, they have been able to establish the temporary postmortem limits inside which, and in function of the environmental temperature, they can be achieved to recuperate life cells from dead specimens.

Finally, the different steps of the somatic cloning process have been studied, so that has already reached a direct and precise knowledge of their more critical phases, coming to propose possible solutions. In every case should be indicated that the technical efficiency, evaluated in terms of embryo cleavage rates after nuclear transplant, is similar to it reached by other authors in this same species.

INTRODUCTION

With the conservation on Biological Diversity (United Nations, 1992) conservation of genetic variability has become a generally accepted responsibility of United Nations member states. While developed countries have taken on the responsibility- and the means- to preserve their endangered breeds in the area of animal agriculture thereby reducing the rate of extinction, breeds are still disappearing on a global level at a worrying rate (Scherf, 2000).

Cloning and conservation of genetic animal is the solute of the reducing and disappearing of animal agriculture. Cloning is the production of multiple, identical offspring. A clone is an animal who is genetically identical to its donor "parent". We now know that this can be achieved using cells derived from a microscopic embryo, a

fetus, or from an adult animal. Cloning from adult animals was introduced to the public in 1997 when scientists announced the birth of Dolly, the first animal cloned in this way. There have now been hundreds of clones produced from skin cells taken from adult sheep, cattle, goats, pigs and mice. The real key to cloning an adult animal is the ability to reprogram the skin cell nucleus and cause it to begin developing as if it was a newly fertilized egg.

Criopreservation Techniques:

Freezing of donor cells does not impede the in vitro development and the development to term of nuclear transplants either in embryo cloning (Heyman,*et al*., 1990) and in somatic cloning (Kato *et al*., 1998). However, as cells are often damaged during freezing and thawing, this process should be carefully examined.

Following the same genetic selection strategy used in hyperprolific squemes, adult somatic cloning will provide obtention of live individuals with the same genotype that the selected genetically exceptional does and so the possibility of application of the highest rate of selection intensity because in such case the genetic material is only of females of high genetic merit origin. Although this also needed the complementarily of cryopreservation of nuclear donor cells (Kato *et al*., 1998).

Immediately after recovery, cumulus cells, as the majority of somatic cells, can be successfully cryopreserved by the classical and more extensively used equilibrium protocol of slow freezing (freezing rate about -1ºC/min) in a low final cryoprotectant concentration, usually DMSO or glycerol at 5 to 10% (v/v) (Hay, 1992; Morgan and Darling, 1993). However this procedure is time consuming requires specific equipment for temperature regulation during cryopreservation and the number of samples (in ampoules or straws) that can be cryorized at the same time is limited. Moreover, taking into account the probable geographical dispersion of the genetically exceptional animals from which the donors cells must be recovered in situ, this conventional procedure is not the more recommended. Other recently developed non-equilibrium cryopreservation procedures, such as quick-freezing or vitrification, have the advantages of their equipment and protocol simplicity and their rapidity without reduction of final efficiency. Actually such procedures are extensively used to cryopreserve animal gametes and embryos and, more recently and in a more limited degree, somatic cells (Kurata *et al*., 1994). However in the revised bibliography there are not any references of their use for cryopreservation of cumulus cells.

The genetics of litter size and its components in rabbits and pigs are similar (Blasco *et al*. 1993). In both species hyperprolific selection, in which the selection intensity is very high, is an effective way to increase prolificacy (Herment *et al*., 1994; Cifre *et al*., 1998). Some years ago, the availability of efficient embryo criopreservation techniques in rabbits has allowed the application of highly restricted selection criteria and consequently an increase enhanced on the applied selection intensity in rabbit breeding programmes (Garcia-Ximenez *et al*. 1996). This is not the case in pigs, because the embryo cryopreservation in such species have been more recently reached (Kuwayama *et al*., 1997) and at this time remain less efficient than in rabbits.

Vitrification solution (VS) consisted of 20% (v/v) ethylene glycol (EG) and 20% (v/v) DMSO in F-PBS (33, 34). Intact or disaggregated cumuli were distributed into as many cell groups as possible in F-PBS; then, every group was collected with a thinned pipette and transferred into 0.5ml VS. Cell groups were immediately loaded

into 0.25ml plastic straws, sealed with modeling clay and plunged vertically into liquid nitrogen (-196ºC). The straw loading took less than 30s and was performed as follows: F- PBS, air bubble, VS containing cells, air bubble and F-PBS (Saeed *et al*., 2000).

Quick-Freezing solution (QF) was prepared in F-PBS and its final composition was 0.25M sucrose, 2.25M EG and 2.25M DMSO (32). The cells were handled as indicated above and then placed into 0.5ml QF solution for 30s and loaded into 0.25ml straw as follows: F-PBS, air bubble, QF-solution with cells, air bubble and F-PBS. Straw was sealed with modeling clay and plunged vertically into liquid nitrogen immediately after loading (Saeed *et al*., 2000).

Somatic clone and methodology of nuclear transfer:

Live individuals obtained by somatic cloning using differentiated cells from adult animals has been reached in sheep (Wilmut *et al*.,1997), cattle (Kato *et al*., 1998, Vignon *et al*., 1998), mouse (Wakayama *et al*.,1998) and goat (Baguisi *et al*., 1999) but not, to date, in rabbit (Dinnyes *et al*.,1999) and pigs (Du *et al*., 1999). Between the assayed types of donor cells derived from adult animals, to date only the mammary epithelial cells from pregnant ewes (Wilmut *et al*.,1997), the non-cultured cumulus cells collected from metaphase II mouse oocytes (Wakayama *et al*., 1998) and the cultured quiescent (by starvation) cumulus cells obtained from ovarian oocytes at the germinal vesicle stage from a cow in an unknown stage of the oestrus cycle (Kato *et al*., 1998) or mural granulosa cells (Wells *et al*., 1999 , Biol. Repr.), primary fetal somatic cell from 40 days transgenic female fetus (Baguisi *et al*., 1999) have been shown capable to support development to term. Cumulus cells submitted to the LH and FSH ovulatory surge reached its final degree of cellular differentiation that, perhaps, alter its reprogramming capability.

At present, tow main approaches have been advocated to induce reprogramming of somatic cell nuclei. The first focuses on exposing the transplanted nucleus for prolonged periods to the oocyte cytoplasmic environment during M phase when levels of maturation-promoting factor (MPF) are high. The second approach is to induce nuclear quiescence in culture cells before their use as nuclear donors. Both approaches aim to modify the structure and function of donor chromatin to render it more accessible to reprogramming factors in the oocyte cytoplasm. For example, the displacement of transcription factors is thought to be a common feature resulting both from the entry into G0 after serum deprivation and from chromosome condensation induced by MPF.

Nuclear transfer requires activation of enucleated recipient oocyte, being necessary to define the need or not of an additional support (electrical or chemical) to fusion pulse. Previous reports on nuclear transfer have indicated the beneficial effect of CCB-treatment after fusion upon subsequent reconstructed egg development (sheep: Smith and Wilmut, 1989; rabbit: Collas and Robl, 1990; Yang *et al*., 1992). However, to our knowledge, no paper about post-fusion CCB effect after nuclear transfer in porcine has been published.

Source of cells:

Cumulus cells from in vitro maturated oocytes and fibroblast cells from new born and adult animal were used as nuclear donors. After partial disaggregation with hyaluronidase, some cell groups were treated by trypsine (Sigma) for 30 s. Then,

cells were washed in PBS plus 20% fetal calf serum to block the enzyme action. Individual cells were separated by repeated pipetting with a fire-polished micropipette.

Recipient oocyte preparation:

Oocytes with a well-defined PB1 and perivitelline space (PVS) were selected for manipulation. These oocytes were incubated in MM199 containing 7.5 µg/ml CCB. Enucleation and nuclear transfer were carried out by a single micropipette (25 µm, outer diameter) with a sharpened and 45º beveled tip. Enucleation was performed by aspiration of one-forth of cytoplasm adjacent to the portion of the PB1. A maximum of two batches per session were performed. After manipulation, the OCCs were cultured in CM199 for 1 h to remove CCB. All manipulations were performed at room temperature.

Electrical activation:

Oocyte activation was accomplished by electrical stimulation started at 46-48h post-initial maturation culture. MII oocytes were allowed to equilibrate in pulsing medium (EM): 0.3M mannitol containing 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.01% PVA (pH = 7.0; Grupen *et al*., 1999) for 5 min before being placed between two stainless-steel round wire electrodes 0.5 mm apart and overlaid with EM. Each sets consisted of two square wave electrical DC pulses of 1.5 kV/cm for 60 µs each at 1 sec. apart. Pulses were delivered with an electro-cell operator (BTX Electro-Cell Manipulator 2001) and monitored with and oscilloscope (TDS 320, Tektronix, Spain). Following Grupen *et al*., (1999) in this experiment, oocytes were submitted either to one or two sets of electrical pulses. When a second additional set of pulses was given, at 30 min delay. Oocytes were cultured in Medium 199 supplemented with 10% (v/v) foetal calf serum (FCS; Sigma, Spain) and antibiotics (CM199) between sets.

CCB treatment and embryo culture:

Immediately after last electrical stimulation, eggs were randomly incubated in CM199 with or without 7.5 µg/ml CCB for 1 hr. Then, eggs were washed twice and definitely cultured in CM199 in the above referring culture conditions.

At 12 hr of culture, lysed eggs were checked and removed from culture. Non-lysed eggs were assessed for the second polar body (PB2) extrusion, but not separately culture was performed. After the first 48 hr of culture cleaved eggs were checked as assessment of global activation (Kaufman, 1978; Ozil *et al*., 1990; Escribá and García-Ximénez, 1999). Embryos were not culture thereafter.

Electrofusion:

For electrical fusion, couplets were equilibrated in EM for 5 min before being placed in the electroporator chamber and aligned manually, with the aggregation plane of the donor cells parallel to the wire electrodes.

OCCs were submitted to two different electrical fusing treatments. In the first one, OCCs were submitted to one set of electrical pulses and only those non fused were pulsed again at 30 minutes after the first set. In the second treatment, all OCCs, fused

or not, were submitted to two electrical sets separated 30 min. After every electro fusing treatment, immediately lysis was assessed.

For what one recounts to the applications of the cloning somatic in animal production, these are of diverse types:

Animal Transgenic for productive characters: In Animal Production, till now only there have been identified a small number of genes which modification can have applied interest. Though it is of hoping that more genes should be identified with effects on productive characters. In any case it will be important to know all the genes they can be modified in the same individual at the same time and without they interfere his effects

lots Uniform of production. In animals of farm, the cloning for transplant nuclear theoretically would allow to obtain a great number of replies of individuals of the genetic elite who will present combinations of very favorable genes, though it will always have to be born in mind that the genetic expression is much determined by the environmental.

Foundation of new lines. Somatic cells obtained of individuals who present the such only characters as resistance to illnesses, can be in use for producing more animals of the same genotype, being able to join this way programs of selection, if several manage to be located of these animals

Conservation of biodiversity. During the last hundred years, the valuation of disappearance of species and races has been very high. Though the conservation of the environment is the most important simple factor to protect the species in danger, certain reproductive skills and especially the somatic cloning, they must be taken in account. Cells obtained of individuals of these species can be cryopreservated and be used in a moment given as cells donors in a program of somatic clone

Finally, I want to point out that the studied skills in this dissertation allow already finding in Egypt a bank cryogenic of fibroblasts and granulosa cells to obtain the copies of the different local Animals, creating this way a reservation cryogenic of his genomes, which will avoid his disappearance by a very low cost.

Besides, exceptional individuals will be located for characters of interest in the productive conditions of this zone.

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