EFFECTS OF STORAGE AT 4°C AND CHEMICAL-ENZYMATIC DENUDATION ON IN VITRO DEVELOPMENT OF 8-CELL-STAGE RABBIT EMBRYOS FOR APPLYING TO NUCLEAR TRANSPLANTATION

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Abstract - The effects of chemical-enzymatic denudation and refrigeration of eight-cell-stage rabbit embryos were studied. Treatment with acidic PBS (pH=2.5) and 0.5% pronase in PBS plus 0.5% PVP determined that 96% (72/75) of denuded embryos were intact. When denuded embryos were cultured in Ham's F-10 plus 20% homologous serum at 39°C in a mixture of 7% CO_2 in air for three days, the percentage of blastocyst stage obtained was 95.8% (69/72). In control (untreated) group this percentage was 100% (84/84).

After preservation by refrigeration at 4°C, for 0 hrs., 3 hrs., 24 hrs., 48 hrs. and 72 hrs., all cultured eight-cell rabbit embryos reached the morula stage. Embryos were fully developed to the blastocyst stage (control group -0 hours-: 100%, 21/21; 3 hour group: 100%, 20/20) when their storage period was for three hours. The storage at 4°C for 24 hours reduced non-significantly the embryo viability (87.5%, 21/24). For longer storage, significant losses in the development to blastocyst stage was observed (48 hour group: 66.6%, 16/24 and 72 hour group: 66.6%, 16/24).

INTRODUCTION

The isolation of blastomeres from nuclear donor embryos is a previous step of the nuclear transplantation procedures.

Blastomere separation is carried out either by mechanical procedures or, more efficiently, by chemical-enzymatic procedures (YANG *et al.* 1990). However, little is known about effects of the chemical-enzymatic treatment on embryo viability.

On the other hand, because of the relatively high number of recovered embryos by doe in rabbits and of time-consuming nuclear transfer procedure, to have a longer period for manipulating may be advantageous. For this, two ways are possible: the use of frozen-thawed embryos (HEYMAN *et al.* 1990) followed by storage of isolated blastomeres at 4°C for around 2-6 hours, or the storage of embryos at low temperatures above freezing for a longer period (ANDERSON and FOOTE 1975b).

In order to apply the nuclear transplantation techniques in our Laboratory, we consider appropriated to test separately the effects of embryo refrigeration and the chemical-enzymatic denudation on *in vitro* embryo development.

MATERIAL AND METHODS

Embryo collection

Animals used were virgin sexually adult New Zealand does. The does were kept in individual cages for 20 days before use. Does were mated with a fertile buck, then each animal received an intramuscular GnRH (20 mg., Fertagyl) injection. Almost 38-40 hours post-GnRH treatment, does were killed and their oviducts were perfused. The 8-cell-stage embryos were collected and placed in phosphate-buffered solution (PBS) supplemented with 20% homologous serum.

Two independent experiments were realized.

Experiment 1 : Effects of chemical-enzymatic denudation - A total of 159 eight-cell-stage embryos were obtained from 18 does (2-3 does by session). The recovered embryos were pooled and assigned randomly to untreated (n=84) and treated (n=75) groups.

On treated group, the zona pellucida and mucin coat of the 8-cell embryos were removed following the report by YANG *et al.* (1990). It involves combining chemical (acidic PBS) and enzymatic (pronase) treatments. Embryos were incubated in prewarmed acidic PBS (pH=2.5) for approximately 3 minutes at room temperature (20-22°C) followed by incubation in 0.5% pronase in PBS, containing 0.5% PVP, for 5 minutes at 39°C. After 0.5% pronase incubation, the embryos were washed again in PBS supplemented with 20% homologous serum and partially digested zona was removed by pipetting with a fire-polished micropipette.

Embryos in all groups were finally cultured in Ham's F-10 plus 20% homologous serum at 39°C, in a mixture of 7% CO2 in air for three days.
Embryo development was recorded daily.
The results were analyzed by Chi-square test.

Experiment 2 : Effects of refrigeration - This experience was designed to test viability *in vitro* of embryos, following storage at 4°C in PBS supplemented with 20% homologous serum for 0, 3, 24, 48 and 72 hours.
From 14 does (2-3 does by session), a total of 113 eight-cell-stage embryos were obtained.
In each session, embryos were pooled, washed and stored in a conventional refrigerator at 4°C as described above, excepting the 0 hour group which, instead of storing it, it was cultured directly.
After each post-storage period, a group of embryos was randomly picked out from the pool, prewarmed at room temperature (20-22°C), washed twice in PBS supplemented with 20% homologous serum and cultured in the conditions described in Experience 1.
Embryo development was recorded daily during four days.
All data on embryo development were analyzed by Chi-square test.

RESULTS AND DISCUSSION

Table 1 : Number of treated and untreated embryos which had reached the blastocyst stage after 72 hours of culture.

	Number of embryos	
	Cultured (initial stage: 8-cells)	Blastocyst number at 72 hours of culture (%)
Treated	72	69 (95.8)
Control	84	84 (100.0)

Table 2 : *In vitro* embryo development after 96 hours of culture.

	Number of embryos	
Duration of storage at 4°C(hours)	Cultured	Blastocysts (%)*
0	21	21 (100.0)a
3	20	20 (100.0) a
24	24	21 (87.5)ab
48	24	16 (66.6)b
72	24	16 (66.6)b

(*) Different superscripts indicate significant differences (P<.05).

Effect of chemical-enzymatic denudation

Removal of the zona pellucida and mucin coat was effective (72 intact denuded embryos, 96%). The damaged embryos (n=3) were because the mechanic removal of the mucin coat rests. Results of in vitro development are presented in table 1.
Observed differences between treated and control groups were not significant. In our culture conditions, 98% of embryos attained the blastocyst stage.

Effects of refrigeration

Post-treatment embryo survival was firstly checked at 24 hours of culture. At this time, all of embryos (100%) were able to resume development, reaching the morula stage.
In relation to the later *in vitro* development capacity, it was evaluated by the number of embryos which reached the blastocyst stage after 96 hours of culture (Table 2).

Storage at 4°C for three hours, did not affect the *in vitro* embryo development (100% blastocysts). However, if the storage period at 4°C was expanded to 24 hours, a non-significant decrease in embryo development rate to the expanded or hatched blastocyst stage was observed (87.5% blastocysts).
A significant decrease in embryo viability was observed when the embryos were stored at 4°C for 48 or 72 hours, although between these two groups, differences were not significant (66.6% blastocysts).
The results of the present experience show that the embryos to be used for nuclear transfer can be efficiently stored at 4°C during 24 hours. Slightly lower results have been obtained by ANDERSON and FOOTE (1975b) using 2-cell-stage embryos.
However, the losses in viability observed when the embryos were stored at 4°C for 48 or 72 hours might be not relevant for nuclear transfer. NIWA *et al.* (1991) suggested that nucleus is more resistant to the effects of preservation at low temperatures than the cytoplasm. Moreover, ANDERSON and FOOTE (1975a) found that 2-cell-stage rabbit embryos stored at 10°C for 24 hours had no effect upon the DNA, RNA or protein synthesis of embryos which survived and developed to the blastocyst stage.

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Efecto de la conservación a 4°C y de la denudación químico-enzimática sobre el desarrollo in vitro de embriones de conejo en 8 células para su applicación en transplante nuclear - En este trabajo, se estudiaron los efectos que la denudación química-enzimática y la refrigeración ejercen sobre embriones de conejo en estadío de 8 células.

El tratamiento con PBS acídico (pH=2.5) y con 0.5% de pronasa en PBS más 0.5% de PVP, determinó una eficacia de denudación del 96% (72/75). Tras un periodo de cultivo de tres días en Ham's F-10 más 20% de suero homólogo a 39°C en una atmósfera de 7% de CO_2 en aire, el porcentaje de blastocistos obtenidos fué del 95.8% (69/72).

En los ensayos de conservación a 4°C, durante 0 hr., 3 hr., 24 hr., 48 hr. y 72 hr., todos los embriones de 8 células puestos en cultivo alcanzaron el estadío de mórula. Los embriones refrigerados durante un periodo de 3 horas alcanzaron plenamente, en su cultivo posterior, el estadio de blastocisto (grupo control, 0 h.: 100%, 21/21; 3 h.: 100%, 20/20). La refrigeración durante 24 h. produjo una pérdida no significativa en la capacidad de desarrollo de los embriones hasta blastocisto (87.5%, 21/24). Con periodos de conservación más prolongados, se produjeron pérdidas significativas en la capacidad de desarrollo hasta blastocisto (grupo 48 h.: 66.6%, 16/24 y grupo 72 h.: 66.6%, 16/24).
