

Cellular evaluation of bovine nuclear transfer embryos developed *in vitro*

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Summary — Cloned blastocysts developed *in vitro* for 7 d had a mean number of cells (82.86 ± 5.35) as evaluated by nuclei counting in serial optical sections using confocal microscopy, after staining with propidium iodide. This number was not significantly different from that of control IVF embryos cultured under the same conditions during the same period ($\bar{X} = 88.89 \pm 7.53$). Semi-thin sections revealed that most of the blastocysts had an inner cell mass (10/12) and a blastocoele. Under transmission electron microscopy, the trophoctoderm appeared well differentiated as a polarized epithelium with apical microvilli and lateral junctions including desmosomes with bound intermediate filaments. The cytoplasm sometimes contained immature mitochondria or a large number of residual bodies. About half of the blastocysts examined had a large amount of cellular debris in the perivitelline space or inside the blastocoele cavity. The cloned blastocysts were also able to hatch *in vitro* by day 8 and SEM indicated a normal morphology of the trophoctoderm cells with numerous apical microvilli. The high number of excluded or degenerating cells found in some embryos may partially explain early embryonic mortality that follows transfer. However, these observations do not give a clear explanation for the high incidence of fetal losses.

embryon cloning / bovine / blastocyst / ultrastructure / cell number

Résumé — Morphologie des blastocystes bovins issus de transfert nucléaire et développés *in vitro*. Des blastocystes issus de transfert nucléaire et développés *in vitro* pendant 7 j sont constitués de $82,86 \pm 5,35$ cellules en moyenne d'après le comptage de noyaux colorés à l'iodure de propidium, effectué sur des coupes optiques à l'aide d'un microscope confocal ; ce nombre n'est pas significativement différent de celui observé pour des blastocytes témoins obtenus par fécondation *in vitro* et cultivés dans les mêmes conditions ($\bar{X} = 88,89 \pm 7,53$). Les coupes semi-fines ont révélé que la plupart des blastocystes clonés avaient un bouton embryonnaire (10/12) et une cavité blastocœlique. En microscopie électronique de transmission, le trophoctoderme apparaît bien différencié avec un épithélium polarisé muni de microvillosités apicales et de jonctions latérales. Le cytoplasme contient parfois des mitochondries immatures ou un grand nombre de corps résiduels. Environ la moitié des blastocystes étudiés présentent un excès de débris cellulaires dans l'espace périvitellin ou la cavité blastocœlique.

Les blastocystes clonés sont capables d'éclorre in vitro à J8 et la microscopie électronique à balayage révèle une morphologie normale de la surface des cellules trophoctodermiques. Dans quelques embryons, la présence d'un nombre élevé de cellules ségréguées ou en dégénérescence pourrait expliquer en partie la mortalité embryonnaire précoce après transplantation mais pas les pertes fœtales.

clonage embryonnaire / bovin / blastocyste / ultrastructure / nombre de cellules

INTRODUCTION

Bovine embryo cloning has been successfully performed during the past years by several groups and resulted in the birth of calf clones (Bondioli *et al*, 1990; Willadsen *et al*, 1991; Chesné *et al*, 1993). However, in many cases the nuclear transfer eggs were allowed to develop *in vivo* in the oviduct of the intermediate recipient animals before definitive transfer to the cow uterus (Westhusin *et al*, 1991). Recently, bovine embryos obtained by nuclear transfer developed completely *in vitro* on a variety of culture systems used for IVF embryos. This permitted the early development of reconstituted eggs to be followed closely, especially during the critical steps of the first cleavages (Yang *et al*, 1993; Heyman *et al*, 1994). Despite the fact that the possibility of *in vitro* development of such embryos up to the blastocyst stage is clearly established, there is limited information available on the quality of such blastocysts compared to that of IVF or *in vivo* produced blastocysts in cattle.

The evolution of pregnancy rates obtained after transfer of cloned blastocysts to recipients reveals a high early embryonic and fetal mortality. In a large scale study reported by Bondioli (1993), the calving rate was limited to about 20%. We also observed a large decrease between initial pregnancy rate (50% at day 35) and calving rate (30%) after transfer of such blastocysts developed *in vitro* (Heyman *et al*, 1994). This may indicate that the quality of the transferred blastocysts is suboptimal. There are no reports on the cellular evaluation of nuclear transfer

bovine embryos at the time of their transfer into the recipient uterus. Most ultrastructural studies focused on nucleolar morphology after transfer of the nuclei (King *et al*, 1992) or on the first cleavages after fusion (Kanka *et al*, 1991). To our knowledge no ultrastructural observation has been undertaken at the blastocyst stage to evaluate the normality of the inner cell mass and trophoctoderm of bovine blastocysts after nuclear transfer and development when it occurs exclusively *in vitro*. The aim of this study was to fill this gap in knowledge.

MATERIALS AND METHODS

Production of nuclear transfer embryos

Nuclear transfer embryos were reconstituted from *in vitro* matured oocytes, which were enucleated, aged *in vitro* and cold-treated before fusion with blastomeres from *in vivo* produced morula according to the technique previously described (Chesné *et al*, 1993).

Briefly, the recipient cytoplasm was prepared after a 24 h *in vitro* maturation of cumulus oocyte complexes from 2–6 mm follicles collected from slaughterhouse bovine ovaries. After dechorionization, metaphase II oocytes were stained with Hoechst dye (HO 33342, 0.5 µg/ml) and enucleated by micromanipulation under low level fluorescence microscopy as described for rabbit nuclear transfer (Heyman *et al*, 1990). The cytoplasm was then aged *in vitro* for another 12 h and stored at low temperature (10°C) until fusion with blastomeres isolated from freshly collected day 6 morulae (Chesné *et al*, 1993).

Fusion between the recipient cytoplasm and the embryonic cell was achieved by electrostim-

ulation (Grass Stimulator 1.3 kV/cm for 50 μ s twice in 0.3 M mannitol solution). One hour after stimulation, the fusion rate was evaluated and the eggs were placed in coculture for *in vitro* development. Cultures were performed on bovine oviducal epithelial cell monolayers in B2 medium supplemented with 10% fetal calf serum in 4-well Nunc Plates (0.75 ml/well). The incubator was equilibrated at 39°C under 5% CO₂ and humidified atmosphere. On day 7, embryos which had developed into normal-looking blastocysts were either transferred to the uterus of recipient heifers to generate offspring, or fixed for cytological and ultrastructural observations.

Cell number evaluation

The total number of cells that were contained in the blastocysts developed *in vitro* after nuclear transfer was evaluated by counting nuclei. Individual embryos were removed from coculture on day 7 and fixed in a solution of 2.5% paraformaldehyde for 1 h at room temperature, treated with 0.5 M NaOH for 30 min to permeabilize them, and stained with propidium iodide for 15 min at 37°C. Each cloned embryo was then mounted on a slide for observation using a laser scan confocal microscope (LSM Zeiss). Optical sections of 4 to 5 μ m were made and the number of nuclei for each blastocyst was counted through all the optical sections of the embryo. A total of 15 cloned blastocysts were evaluated and their cell number was compared to that of 19 randomly selected control blastocysts (day 7), derived from *in vitro* maturation and fertilization and developed under the same conditions of coculture as the cloned embryos. A small number of blastocysts produced *in vivo* from superovulated heifers and flushed on day 7 were also fixed and used as *in vivo* controls.

Transmission electron microscopy

After 7 d of coculture, the nuclear transfer embryos that had developed into blastocysts according to stereomicroscope evaluation of the culture plate, were rinsed twice in simple buffered medium (PBS), fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3 for 1 h and post-fixed in a mixture of 1% OsO₄ in the same buffer

for another hour. Each sample was dehydrated in a series of ethanol baths and embedded in Epon. Serial semi-thin sections (0.5 μ m) were stained with toluidin blue and specific areas were selected for ultra-thin sectioning. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined under a Philips CM 12 transmission electron microscope (TEM).

Scanning electron microscopy

A group of nuclear transfer embryos was cultured for 8 d to allow hatching. Five hatching or already hatched blastocysts were fixed in 1% glutaraldehyde in cacodylate buffer, and dehydrated in ethanol. Each sample underwent critical point drying (EMSCOPE CPD 750), then glued on a stub and coated with palladium gold (Sputter coater POLARON E 5150) and observed with a scanning electron microscope (SEM, HITACHI S 450).

RESULTS

Cell number in nuclear transfer blastocysts versus control embryos

From a total of 15 blastocysts that developed after nuclear transfer and were fixed on day 7, the mean number of cells (\pm SE) evaluated by counting nuclei using a confocal microscope was 82.86 ± 5.35 . This number of cells forming the cloned blastocysts was lower than that of normal bovine blastocysts developed *in vivo* after superovulation and recovered on day 7 ($\bar{X} = 161.57 \pm 8.38$), but no different from that of blastocysts derived from IVF and cultured for the same period of time (7 d) under the same conditions ($\bar{X} = 88.89 \pm 7.53$). However, the number of nuclei for individual blastocysts was quite variable ranging from 43 to 124 nuclei and could not be related to the gross morphological evaluation of the blastocyst under the binocular microscope at the time of fixation.

Ultrastructural observations on day 7 cloned embryos

Twelve blastocysts that were apparently normal, as evaluated from the culture plate on day 7, were fixed and processed for electron microscopy. Semi-thin sections of these blastocysts revealed that most of them had a clearly visible inner cell mass (10/12). However, in 2 cases, no ICM could be detected and these embryos, which looked apparently normal, were in fact 'trophectoderm vesicles'.

Ultrathin sections were made on each of the blastocysts at the ICM and trophectoderm level. In all the blastocysts analysed, the differentiation of the trophectoderm was clear; it appeared as a polarized epithelium with numerous apical microvilli. Lateral junctions between trophectoderm cells showed apical tight junctions and adherent junctions with bound microfilaments and several desmosomes with bound intermediate filaments. The cytoplasm of these cells contained mitochondria with transverse cristae

and also a variable number of lysosome-like bodies (fig 1a). Some nucleated cells were excluded from the blastocysts and presented very immature mitochondria (fig 1b). In about half of the blastocysts analysed (table I), the trophectoderm cells contained numerous cytoplasmic inclusions indicating a degenerative process (fig 2a). They also contained excessive cellular debris in the perivitelline space or inside the blastocoele cavity (fig 2a, b, c).

The ultrastructure of the inner cell mass appeared normal in 5 out of 10 embryos (fig 3a, b). It was characterized by the presence of rounded cells containing active nucleoli and well-formed mitochondria. However, in the other cases, despite the presence of an apparent ICM at the time of fixation, the cells constituting the ICM were in poor condition (fig 2a, b) or sometimes appeared to be lacking organelles and nuclei (fig 2c). The morphological observations on the 12 blastocysts studied by TEM are summarized in table I.

Table I. Morphological evaluation of bovine nuclear transfer blastocyst developed *in vitro*.

Embryo identification	Inner cell mass		Trophectoderm	Presence of cellular debris ^a	
	Semi-thin sections	Ultrastructure	Ultrastructure	Perivitelline space	Blastocoele cavity
V127	Yes	Good	Good	+	-
V128	Yes	Fair	Fair	++	-
V129	Yes	Poor	Poor	+++	-
V130	Yes	Poor	Poor	-	-
V132	Yes	Good	Good	-	-
V135	Yes	Good	Good	++	+
V136	Yes	Poor	Poor	+++	+
V137	Yes	Fair	Fair	+++	+
V139	No	No	Good	+	+
V140	Yes	Good	Good	+	+
V141	Yes	Poor	Good	+	+
V142	No	No	Poor	+	++

^a Cellular debris: - no ; + small amount of debris; ++ moderate; +++ much cellular debris.

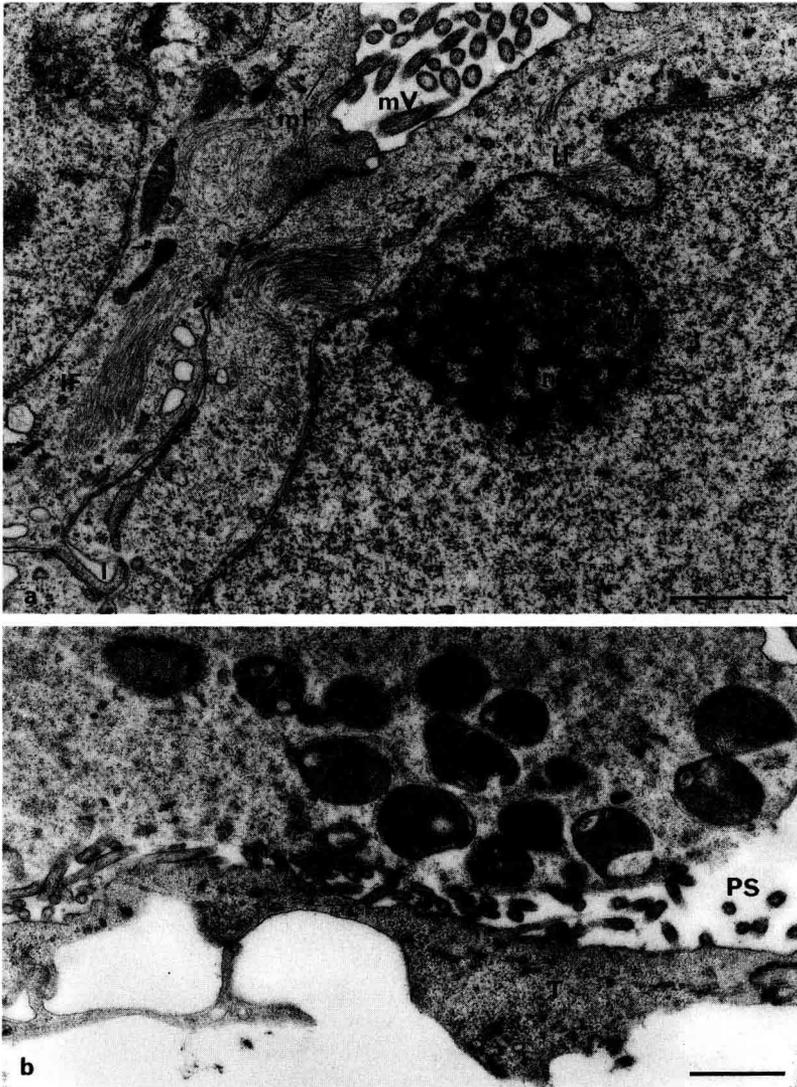


Fig 1. TEM observations on trophectoderm (bar = 1 μm). **a)** Normal-looking trophectoderm cells appear as polarized epithelial cells: the apical plasma membranes forms microvilli (**mV**) containing microfilaments (**mF**). Small coated vesicles and microfilaments are visible in the apical cortex. The lateral plasma membranes are bound by apical tight junctions, adherent junctions binding microfilaments (**mF**), desmosomes (>) binding intermediate filaments (**IF**) which are also surrounding the nucleus, and show few interdigitations (**I**). Lysosome-like bodies are visible in the cytoplasm of one of the cells (\rightarrow). Note the active nucleolus (**n**). **b)** Part of a cell containing mitochondria (**M**) of the type found in oocytes or early cleavage stages (hood morphology, few cristae distributed in peripheral areas), observed in the perivitelline space (**PS**), on the surface of a thin trophectoderm cell (**T**).

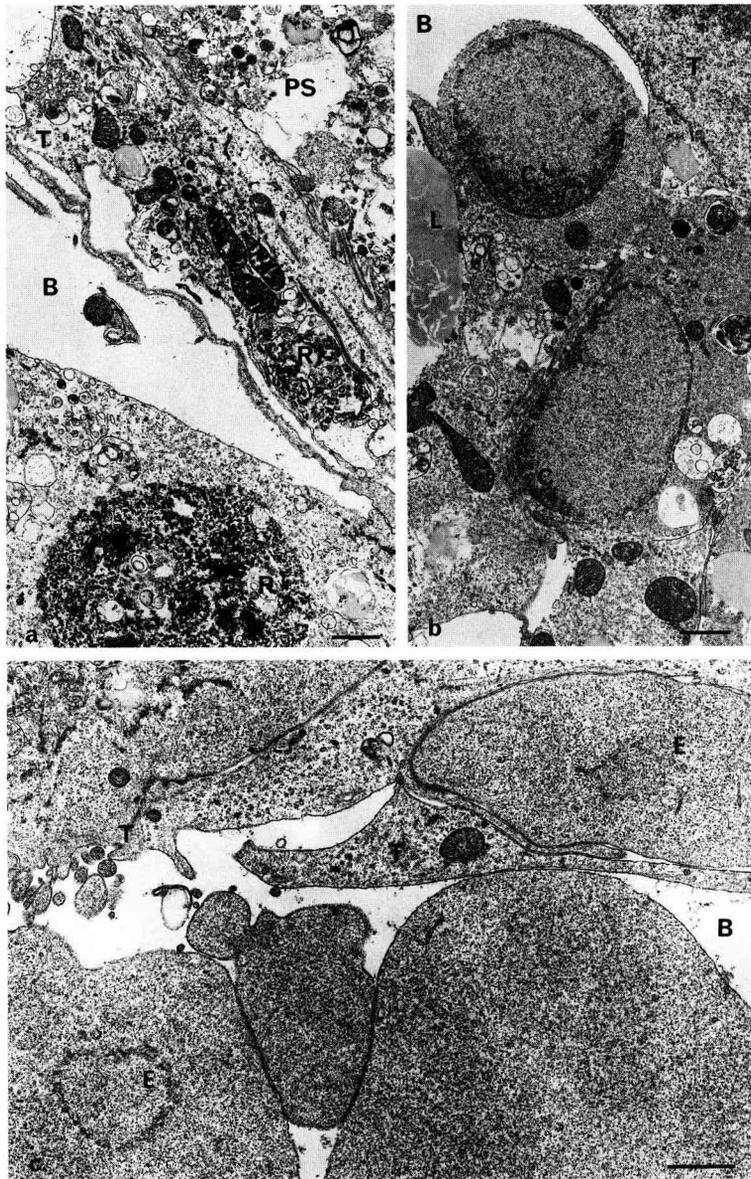


Fig 2. TEM aspects of low quality embryos (bar = 1 μ m). **a**) In this case, degenerate cytoplasmic material is visible in the perivitelline space (PS) outside a flat trophoblast cell (T) containing a residual body (R). In the blastocoele (B), a cell of the ICM contains a large residual body (R). **b**) Under the trophoblast (T), in the blastocoele (B), 2 cells of the ICM show a reduced cytoplasm and condensed chromatin (C) on one side of the nucleus (apoptosis?); L = lipid inclusion. **c**) The ICM appears to be made of cytoplasmic fragments devoid of organelles. In 2 cases, endoplasmic reticulum forming a kind of empty nuclear envelope is visible (E); B: blastocoele; T: trophoblast cells.

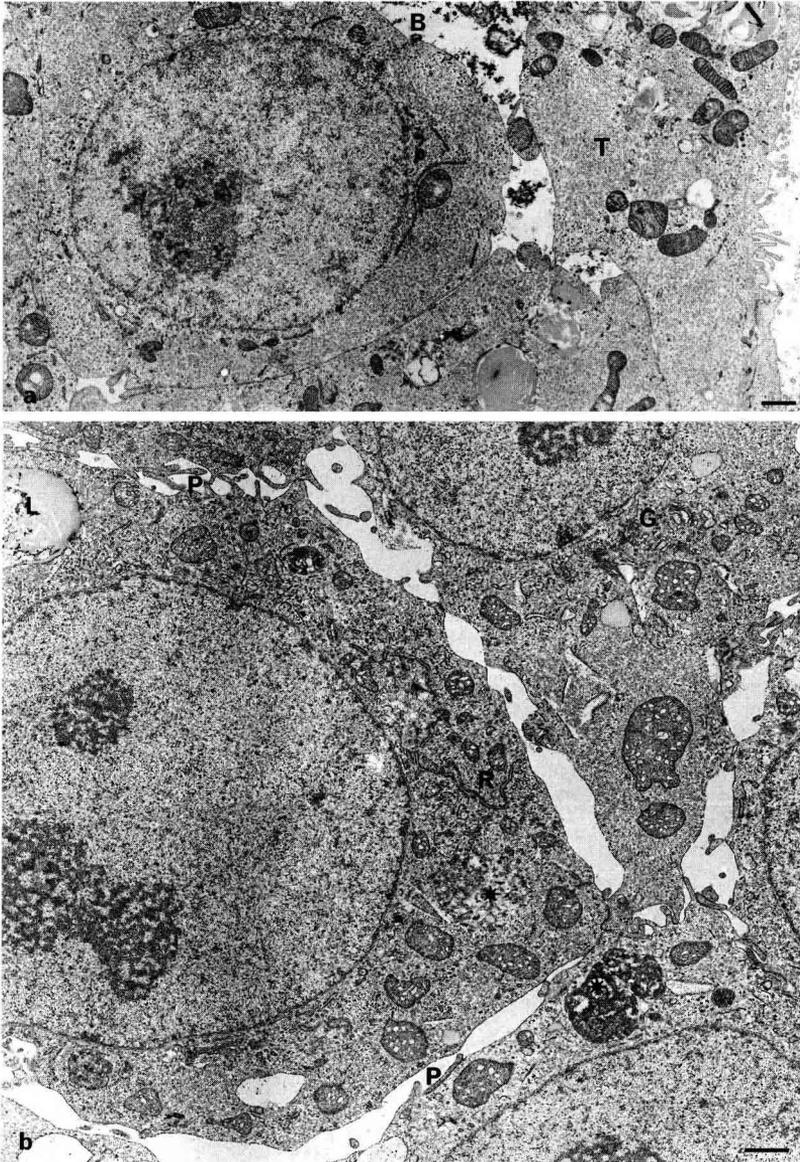


Fig 3. TEM observations on the ICM (bar = 1 μ m). **a**) Three closely apposed cells of the ICM located under the trophectoderm (T). The space between 1 cell of the ICM and the adjacent T cell may correspond to an extension of the blastocoel (B). **b**) Details of the cells of the ICM. Although spaces between the cells may be exaggerated by fixation in this case, adhesion foci are present between the plasma membranes, which also show a few cytoplasmic projections (P). The cytoplasm contains rough endoplasmic reticulum (R) and golgi cisternae (G), large lipid inclusions (L) and a few secondary lysosomes (*). The mitochondria are polymorphic, sometimes very large. The nucleoli are of the active type.

Scanning microscopy on hatching blastocysts

Out of 23 blastocysts developed *in vitro* until day 7 after nuclear transfer and cultured for another 24 h up to day 8, 15 were hatching or totally hatched (65.2%). This proportion of *in vitro* hatching for cloned embryos was similar to that we observed (74%) for IVF-derived blastocysts cultured

under the same conditions in our laboratory.

Five of these hatching blastocysts were fixed and processed for SEM. A typical aspect of hatching is shown in figure 4a, b where extrusion of trophoctoderm occurred at 2 different places in the zona. Hatched blastocysts showed an apical surface regularly and completely covered with microvilli (fig 4c).

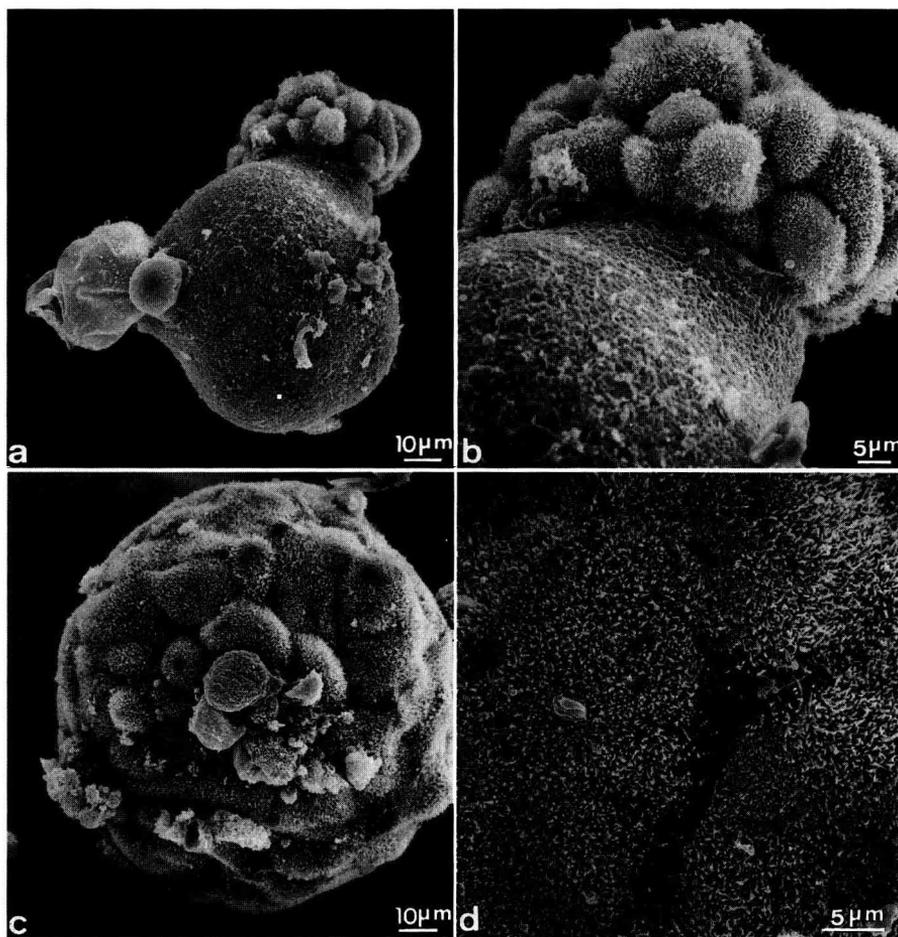


Fig 4. SEM observations on hatching. **a)** General view of zona pellucida perforated in 2 sites. **b)** Detail of extruding blastomeres (same blastocyst). **c)** Hatched blastocyst. Note compacted aspect of the trophoctoderm cells and a few cells extruded from the surface epithelium. **d)** Surface microvilli of trophoctoderm cells (same blastocyst).

DISCUSSION

The present results show that the trophoctoderm is effectively differentiated in almost all cases of bovine blastocysts (defined by the presence of a blastocoele), developing *in vitro* after nuclear transfer.

In the blastocysts showing the best morphology, the trophoctoderm appears normal with polarized epithelial morphology. The main characteristics of the cells are the apical distribution of microvilli (polarization) and the presence of desmosomes located in the lateral plasma membranes and binding cytoplasmic filaments the size of intermediate filaments. These observations are similar to that of normal bovine blastocysts developed *in vivo* (Betteridge and Fléchon, 1988). The presence of apical junctional complexes (tight junctions, adherent junctions and desmosomes) between adjacent trophoblast cells is also characteristic of the blastocysts of a large number of mammalian species (Enders, 1971) and is a necessary condition for the establishment of the blastocoele cavity (review by Fléchon and Guillomot, 1992).

In poor quality embryos, signs of trophoctoderm anomalies in progressive order are: presence of cytoplasmic inclusions resembling small secondary lysosomes or large residual bodies containing degenerate cell parts; and the occurrence of excluded cell debris in the perivitelline space. The latter cells probably escaped the phenomenon of eventual compaction through a lack of cell adhesion molecules such as E cadherin or uvomorulin (Takeichi, 1988) and still contain mitochondria of the oocyte type (Brackett *et al*, 1980).

In good quality embryos, the ICM cells appeared as non-polarized adherent cells similar to that described for normal, *in vivo*, day 7 blastocysts (Linares and Plöen, 1981; Massip *et al*, 1981). In fair quality embryos, the ICM cells contain varying indications of

degeneration which may be the consequence of suboptimal culture conditions compared to the *in vivo* microenvironment. In the pig species, some major ultrastructural deviations of the embryo such as nucleolus-like structures found outside of the nuclei in the cytoplasm, are induced by *in vitro* culture (Hyttel and Niemann, 1990).

In poor quality embryos, the blastocoele contained various amounts of cellular debris. Such signs of degeneration as detached cells and cell remnants in the blastocoele cavity have been observed in rabbit embryos after asynchronous transfer (Fisher, 1989). In 2 cases out of 12 blastocysts, there was complete absence of a compact inner cell mass (ICM). In one case the embryo was in fact a trophoblastic vesicle which would never have developed into a fetus if transferred into a recipient uterus. In the second case, only a few non-aggregated cells were found in the place of the ICM.

In some of the cloned blastocysts studied by TEM, lysosomes are present in the trophoctoderm cells as a result either of the active endocytotic activity of these cells or autophagic process. However, a large accumulation of these late endosomes is only observed in elongating embryos (Fléchon and Guillomot, 1992). Most of the residual bodies observed in this study contain parts of cells or whole cells in the process of degenerating. It is possible that cell degeneration is a normal feature of blastocyst development, particularly during its expansion phase (Carnegie *et al*, 1985). The chances of survival then depend on the balance between proliferation and degeneration. It is difficult to say, in the present case, the reason for excessive degeneration rate in some poor quality embryos, because the dead cells are already at a late degenerative stage. In only a few cases (fig 2b) have we observed small cells with condensed chromatin, which could reflect apoptosis.

The hatching process for cloned blastocysts sometimes showed a typical occur-

rence of trophectoderm extrusion at 2 different locations on the zona. A possible explanation of such a phenomenon is that after nuclear transfer, the zona is made more fragile by micromanipulation during enucleation and blastomere insertion. Such a double extrusion was not seen for *in vivo* derived blastocysts (Fléchon and Renard, 1978). After hatching, the external morphology of the trophectoderm cells, as revealed by SEM, was normal, that is they were completely covered with microvilli. This confirms the TEM observations on the polarization of these cells. It can be concluded from the SEM observations that the surface aspects of the blastocysts is not a sufficient criteria of quality. Moreover, hatching *in vitro* may reflect the adequacy of the culture medium (Fléchon and Renard, 1978), but is not a criterion for the ability to develop further.

The cloned blastocysts developed *in vitro* for 7 d had a similar number of cells to the control IVF embryos cultured under the same conditions. The cell number was evaluated by counting nuclei in successive optical sections of the whole embryo using a confocal microscope. Such an evaluation is more precise than the *in toto* estimation of cell number in previously reported for IVF embryos. However the mean number of cells in day 7 *in vitro* produced blastocysts remained lower than for blastocysts of the same age developed *in vivo* as reported by Iwasaki and Nakahara (1990).

In conclusion, we do not know the origin of the various abnormalities observed at high rate in poor quality embryos (isolated cells, degenerated cells in trophectoderm or ICM or absence of ICM). These may be related to incomplete reprogramming of the transferred nucleus. This phenomenon consists of the repression of gene expression in the nucleus before a re-onset of gene expression as observed recently in our laboratory (Kanka *et al*, unpublished results). Bovine nuclear transfer embryos are able

to develop *in vitro* up to the blastocyst stage at the same rate as control IVF embryos (Heyman *et al*, 1994). *In vitro* culture conditions are known to induce a deviation in the fine structure of bovine embryos (Shamsuddin *et al*, 1992) and to reduce further viability of the blastocyst compared to that of *in vivo* developed ones (McLaughlin *et al*, 1990). The success of implantation is probably dependent upon the normal development of trophectoderm, whereas the fetal development relies on the integrity of the ICM. In the case of cloned embryos, the total loss is the cumulative result of defects induced by nuclear transfer and culture *in vitro*. After transfer of cloned blastocysts, the rate of loss of implanted embryos seems to overweight the percentage of blastocysts with an abnormal ICM. Therefore we cannot use our ultrastructural observations to provide a complete explanation for late embryo mortality occurring after nuclear transfer.

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