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Preimplantation development and viability of *in vitro* cultured rabbit embryos derived from *in vivo* fertilized gene-microinjected eggs: apoptosis and ultrastructure analyses

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Summary

Microinjection (Mi) of gene constructs into pronuclei of fertilized eggs is a widely used method to generate transgenic animals. However, the efficiency of gene integration and expression is very low because of the low viability of reconstructed embryos resulting from cell fragmentation and cleavage arrest. As a consequence, only a few viable embryos integrate and express transgene. Since cellular fragmentation and cleavage stage arrest in embryos may be associated with apoptosis, we aimed to test the hypothesis that the low viability of Mi-derived eggs is caused by a high rate of apoptosis in embryos, as a result of the detrimental effect of Mi. Pronuclear stage eggs (19–20 hours *post-coitum*, hpc) were microinjected with several picolitres of DNA construct into the male pronucleus (gene-Mi); the intact eggs (non-Mi) or eggs microinjected with phosphate-buffered saline (PBS-Mi) served as controls. Epidermal growth factor (EGF; 0, 20 and 200 ng/ml) was added to the culture medium and the embryos were cultured up to 94–96 hpc. Apoptosis was detected using the TUNEL assay, and the ultrastructure was analysed using electron microscopy of Durcupan ACM thin sections of the embryo. Gene-Mi embryos had significantly lower ($p < 0.05$) blastocyst yields and a higher percentage of cleavage-arrested embryos than those in the non-Mi group. In gene-Mi groups, approximately 40% of all cleavage-stage-arrested embryos had fragmented blastomeres. Both gene-Mi- and PBS-Mi-derived blastocysts had a significantly higher TUNEL index ($p < 0.001$) and lower total cell number ($p < 0.05$) than the non-Mi embryos. Comparison of the quality of gene-Mi embryos with that of PBS-Mi embryos indicated that the deleterious effect of Mi on the embryo was caused by the Mi procedure itself, rather than DNA. EGF (at 20 ng/ml) had beneficial effects on the quality of gene-Mi-derived embryos, eliminating the influence of the Mi procedure on apoptosis and embryo cell number. Ultrastructural analysis confirmed a higher occurrence of apoptotic signs (nuclear membrane blebbing, areas with electron-dense material, numerous apoptotic bodies) in Mi-derived cleavage-arrested embryos compared with untreated or Mi-derived normal-looking embryos. These findings suggest an association between embryo cleavage arrest and apoptosis in Mi-derived embryos. Inclusion of EGF in the embryo culture medium can eliminate the detrimental effect of Mi on embryo quality.

Keywords: Apoptosis, Epidermal growth factor (EGF), Microinjection, Rabbit embryos, Ultrastructure

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Introduction

In vitro manipulation of embryos has been widely used to produce genetically modified animals (transgenic, chimeric, cloned). Microinjection of foreign DNA into fertilized mammalian eggs is a convenient mean of introducing genes into the germ line. The main obstacle to these technologies is that each additional *in vitro* manipulation step diminishes the viability of the

resulting embryo. In particular, microinjection of DNA into the cytoplasm or pronucleus of rabbit ova resulted in the arrest of 36% of zygotes at the 1-cell stage (Delouis *et al.*, 1992), decreased the cleavage rate and the proportion of morulae and blastocysts (Voss *et al.*, 1996; Chrenek *et al.*, 1998) and increased the proportion of degenerated eggs from 21% (intact control) to 69% (Chrenek *et al.*, 1998). Several factors influence the success of gene microinjection including the skill of the researcher, the type of gene construct, the culture medium and the duration of the procedure. In mouse zygotes, pronuclear microinjection had a deleterious effect on subsequent embryonic development, probably because of excessive damage to ova and their lysis (Brinster *et al.*, 1985; Mann & McMahon, 1993). Most fragmented or cleavage-stage-arrested human embryos showed signs of apoptosis (Jurisicova *et al.*, 1996; Levy *et al.*, 1998; Hardy, 1999). The incidence of apoptosis in bovine (Neuber *et al.*, 2002), mouse (Liu *et al.*, 2002) and human (Levy *et al.*, 2001) blastocysts correlated with cell number and embryo quality. Blastocysts with fewer cells showed a range of TUNEL-positive cells from 0 to 30%, whereas blastocysts with more cells had less than 10% TUNEL-positive cells (Levy *et al.*, 2001). We hypothesize that the occurrence of apoptosis, as a response to the detrimental effect of microinjection or microinjection-related conditions, is a consequence of the low quality and developmental potential of microinjection-derived rabbit embryos. One of the aims of this study was to test this hypothesis. The measurement of apoptosis in developmentally arrested rabbit embryos after *in vitro* manipulations (gene microinjection) has not been reported previously.

The occurrence of apoptosis in the embryo can be decreased by adding growth factors to the culture media. Although the anti-apoptotic effect of insulin-like growth factor I (IGF-I; Herrler *et al.*, 1998; Makarevich & Markkula, 2002; Sirisathien & Brackett, 2003) or insulin (Byrne *et al.*, 2002; Augustin *et al.*, 2003) on blastocyst-stage embryos is clearly reported, the effect of epidermal growth factor (EGF) on embryo development and apoptosis is controversial and species-dependent. The effect of EGF on microinjection-derived embryos has not been reported for any species. Therefore in the present study we tested the effect of two concentrations of EGF added to the culture medium in the presence of fetal calf serum, on the preimplantation development of microinjection-derived or intact rabbit embryos.

The aims of our study were: (1) to determine whether the decreased developmental rate of microinjected eggs is due to apoptosis in subsequent embryos; (2) to determine whether EGF is able to improve the quality (cleavage, level of apotosis, cell number) of microinjection-derived embryos; and (3) to determine ultrastructural differences in the morphology of normally developed and cleavage-arrested microinjection-derived embryos.

Materials and methods

Unless otherwise specified, chemicals were obtained from Sigma (St Louis, MO).

Egg collection

Female New Zealand White rabbits, kept on a local farm, were treated with PMSG (Werfaser, Alvetra und WERFFT, Vienna, Austria) intramuscularly at 20 IU/kg live weight, 72 h before mating. Immediately prior to mating, the females were injected intramuscularly with hCG (Werfachor, Alvetra und WERFFT) at 40 IU/kg live weight. The females were then mated with a male of proven fertility from the same breed. At 19–20 h *post coitum* (hpc) the pronuclear-stage eggs were flushed from the oviducts of slaughtered animals with phosphate-buffered saline (PBS; Gibco, Auckland, New Zealand). The flushed eggs were evaluated morphologically and eggs with both pronuclei, two polar bodies and compact cytoplasm were selected and randomly divided into two groups: (1) eggs intended for gene microinjection (gene-Mi), and (2) non-microinjected (non-Mi, untreated) eggs.

DNA microinjection

Gene microinjection was carried out using an Olympus microscope equipped with Alcatel micromanipulation units (France) and an Eppendorf microinjector (Germany) in CIM (CO_2-independent) medium supplemented with 10% (v/v) fetal calf serum (FCS; both from Gibco). The eggs were immobilized with a holding pipette by air suction. The gene construct used in this study consisted of a 2.5 kb murine whey acidic protein promoter (mWAP), 7.2 kb cDNA of the human clotting factor VIII (hFVIII) and 4.6 kb of 3′ flanking sequences of mWAP gene.

A few picolitres of the gene construct (at 5.5 μg/μl, about 300 copies) were microinjected into male pronuclei using a micropipette with a filament inside. Swelling of the pronucleus indicated successful microinjection. Microinjection-intended (gene-Mi) and untreated (non-Mi) embryos were kept in separate wells of the same culture plate, so that the two groups were almost equally exposed to microscope light during the procedure. As a control for microinjection, the eggs were microinjected with the same volume of PBS, pH 7.2 (PBS-Mi) instead of gene solution. The microinjection procedure usually took 15–20 min (for about 25–30 eggs), i.e. 30–45 s was spent per microinjected egg. After microinjection, the eggs were cultured in 4-well dishes (Nunc, Roskilde, Denmark),

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containing 600 µl of k-DMEM medium (Gibco) supplemented with 10% FCS and EGF (0, 20, 200 ng/ml) in 5% CO_2 and 39 °C, up to 96 hpc (blastocyst stage).

Experimental design

In experiment 1, the effect of EGF, isolated from mouse submaxillary gland (Sigma) added to the culture medium at 0, 20 or 200 ng/ml (EGF-0, EGF-20 and EGF-200 respectively) was tested on the intact (non-Mi) rabbit eggs.

In experiment 2, eggs were gene-microinjected and subsequently cultured in the presence of 0, 20 or 200 ng/ml EGF; non-Mi (untreated) or PBS-Mi eggs were used as a control. After 96 hpc, embryos were evaluated for cleavage stages; all blastocysts in each group were separated from cleavage-arrested (at the 1-cell or at the 2- to 16-cell stage) embryos and were processed for apoptosis.

In experiment 3, all recovered eggs were gene-microinjected and cultured in standard culture medium (without EGF) for 96 hpc, sufficient time for them to reach at least the expanded blastocyst stage under standard culture conditions. The resulting embryos were visually evaluated, and morphologically normal blastocysts (light colour, compact inner cell mass, even trophectodermal region, clear blastocoele cavity), expanded or hatched, were separated from the embryos that were considered as developmentally delayed (morulae and early blastocysts with a small blastocoele) or arrested (earlier stages than morula). The embryos in these two groups were processed for TUNEL and total cell number using confocal microscopy, and for ultrastructure using transmission electron microscopy.

Of the 882 eggs used in this study, 453 were gene-microinjected, 50 were PBS-microinjected (control group) and 379 were untreated. Of these, 188 resulting embryos were processed for TUNEL and total cell number analysis, and 31 embryos were used for the ultrastructural study.

TUNEL assay

Embryos were removed from culture medium, washed (3 × 5 min) in PBS supplemented with polyvinylpyrrolidone (PBS-PVP, 4 mg/ml), and then fixed for 5 min in neutral buffered formalin (3.7% w/v, Sigma) and for 10 min in 70% ethanol. For membrane permeabilization, embryos were incubated for 15 min in 0.5% v/v Triton X-100 in PBS. Non-specific binding was suppressed by incubation in blocking solution (1% w/v bovine serum albumin (BSA) in PBS). The embryos were processed for TUNEL using a MEBSTAIN Apoptosis kit Direct (Immunotech, Marseilles, France) according to the kit instructions. Briefly, fixed and permeabilized embryos were incubated in TdT end-labelling cocktail (TdT buffer, FITC-dUTP and TdT) at 37 °C for 1 h. The TUNEL reaction was stopped after washing three times in PBS-PVP. Afterwards, the embryos were counterstained with propidium iodide (PI; 1 µg/ml in PBS) for 20 min.

After the washing, embryos were placed on a coverslip and covered immediately with 5 µl of Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The coverslip was mounted on a slide sealed with nail polish. The slides were stored at −20 °C until scanning, which was usually performed within 2 weeks.

As a positive control for TUNEL, a group of fixed embryos was incubated in the presence of bovine DNAse I (1 µg/ml in PBS) for 1 h at 37 °C before the TdT end-labelling reaction. Thereafter the protocol was continued as above. The specificity of the TUNEL reaction was verified by omitting the TdT end-labelling reagent from the protocol (negative control).

Laser confocal microscopy and counting of TUNEL-positive cells and total cell number

TUNEL (dUTP-FITC) and PI fluorescent signals were imaged from whole-mount embryos using a confocal laser scanning microscope (Olympus IX 70) equipped with a Fluoview 300 scanning unit and an argon/krypton mixed gas laser. A UplanApo ×40 objective with aperture 2, was used. The embryonal images were acquired and processed using Fluoview version 1.2 software.

The TUNEL index was determined as the ratio of TUNEL-positive nuclei to all nuclei counted from Z-optical section images, each representing a 1.0 µm optical section of the embryo. The total number of nuclei per embryo could not be counted from the section, since many nuclei were obviously present in several sections. Therefore, the results of counting the TUNEL- and PI-positive nuclei from overlay stereoimages were included.

Electron microscopy

Rabbit embryos were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde (v/v) in 0.15 M cacodylate buffer (pH 7.1–7.3) for 1 h and washed in cacodylate buffer. One hour post-fixation in 2% w/v osmium tetroxide in cacodylate buffer the embryos were rinsed in distilled water, dehydrated in an acetone series and embedded in Durcupan ACM (Fluka). The blocks with embryos were cut into semithin sections using an LKB-Nova ultramicrotome and subsequently stained with toluidine blue. Thin (silver-stained) sections were contrasted with uranyl acetate and lead citrate and viewed in a JEM 100 CXII electron microscope (Jeol, Japan) operating at 80 kV.

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Table 1 Influence of EGF on the development of intact cultured rabbit eggs (data from four replications)

EGF concentration	No. of eggs cultured	Cleavage-arrested	Morulae	Blastocysts[a]
0 ng/ml (control)	58	2 (3.45)	4 (6.9)	52 (89.65)
20 ng/ml	49	1 (2.04)	3 (6.12)	45 (91.84)
200 ng/ml	58	1 (1.72)	1 (1.72)	56 (96.55)

Data are *n* (%).
[a] Early, expanded and hatching blastocysts were included in each group.

Statistics

Microinjection of eggs and embryo culture were performed in 14 replicates, of which the TUNEL assay was performed in seven. The influence of the treatment on developmental stages of embryos was tested using a chi-square test or Fisher's exact test extended for $r \times c$ contingency tables according to Freeman and Halton (1951) for data with frequency numbers less than 5. Small p values supported the alternative hypothesis of association between the row and column variables. When the evidence of difference between treatment groups was found, more specific comparisons were tested using chi-square tests without adjustment for the degree of freedom of multiple tests. A two-sample *t*-test (Student's test) was used to analyse differences between normal blastocysts and cleavage-arrested embryos. The variable "TUNEL index" was normally distributed. The hypothesis of normality was rejected for "TUNEL cells per embryo" and for "Embryo cell number". For these two variables log-transformation of original values was used to improve the statistical properties before applying statistical tests. All calculations were performed using the SAS software package (SAS Institute, 2002).

Results

Embryo development

All eggs cultured in k-DMEM medium, in either the presence or the absence of EGF, were cleaved beyond the 1-cell stage (Table 1). Only 1 or 2 embryos in each group were arrested in cleavage at the 2- to 16-cell stage. Approximately 90% of embryos cultured in the control medium (without EGF) were blastocysts (evaluated as early, expanded or hatching blastocysts). Although the addition of EGF resulted in a slight increase in the blastocyst yield (96%), Fisher's exact test did not reveal a significant difference compared with the EGF-free control (two-sided p value is 0.3628).

Embryos derived from the gene-Mi eggs showed a significantly lower yield of blastocyst-stage embryos ($p < 0.05$) and a higher percentage of cleavage-arrested embryos than was observed in the untreated group (Table 2). The addition of EGF to culture medium at any concentration had no significant effect on the proportion of embryos that reached the blastocyst stage. Approximately 40% (37.9–40.6%) of the total number of arrested embryos in the microinjected groups had extensive cytoplasmic fragmentation, whereas no fragmented embryos were observed in the untreated group. EGF did not affect the percentage of fragmented embryos in the gene-Mi groups.

Effect of microinjection and addition of EGF on apoptosis and total cell number in rabbit blastocysts cultured *in vitro*

In about 83–100% of all embryos analysed at least one cell was TUNEL-positive with no significant differences between the groups. A higher TUNEL index was found in blastocysts from the gene-Mi or PBS-Mi (control for gene construct) groups.

Table 2 Influence of gene microinjection (Mi), alone (EGF-0) or combined with EGF (20, 200ng/ml), on rabbit embryo development

Experimental groups	No. of eggs cultured	Cleavage-arrested/ of these fragmented	Morulae	Blastocysts
Untreated	126	17 (13.5)/0	7 (5.6)	102 (81.0)[a]
Mi + EGF-0	180	58 (32.2)/22 (37.9)	14 (7.6)	108 (58.4)[b]
Mi + EGF-20	125	32 (25.6)/13 (40.6)	11 (8.8)	82 (65.6)[a,b]
Mi + EGF-200	143	52 (36.4)/20 (38.5)	18 (14.4)	73 (51.0)[b,c]

Data are *n* (%).
Values with different superscripts differ significantly ($p < 0.05$, chi-square test).

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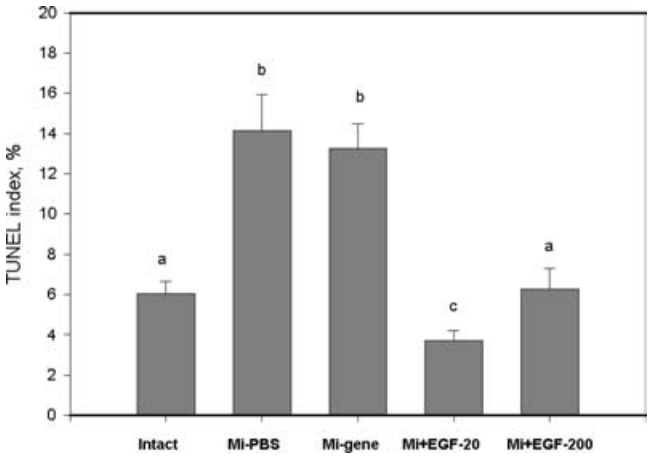

Figure 1 Influence of microinjection (Mi) and EGF additions (20 or 200 ng/ml) on the TUNEL index in rabbit blastocysts. Values are expressed as the mean ± SEM. [a,b,c]Significant differences between groups of embryos at $p < 0.05$ (two-sample *t*-test).

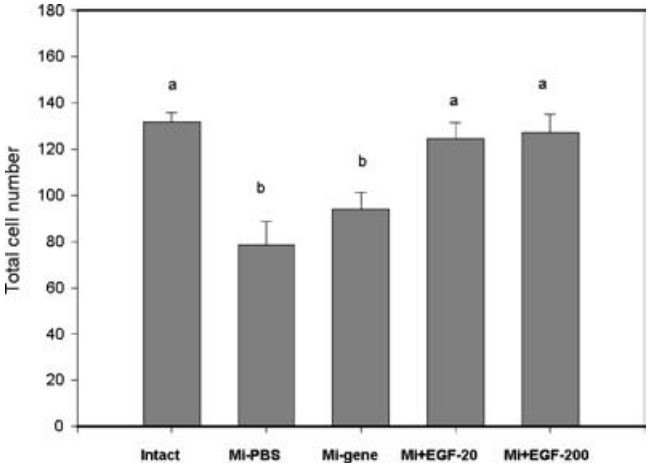

Figure 3 Influence of microinjection (Mi) and EGF (20 or 200 ng/ml) additions on the total cell number per blastocyst. Values are expressed as the mean ± SEM. [a,b]Significant differences between groups of embryos at $p < 0.05$ (two-sample *t*-test).

EGF at both doses significantly decreased ($p < 0.001$) the occurrence of apoptosis in microinjection-derived embryos compared with those cultured without EGF (Fig. 1). The effect of EGF at 200 ng/ml on the TUNEL index of microinjected embryos was comparable to that in the untreated control, whilst at 20 ng/ml the percentage of TUNEL-positive cells in the treated group was lower than in the intact control group ($p < 0.05$). No differences in TUNEL index between the gene-Mi and PBS-Mi groups were observed.

The number of apoptotic cells per blastocyst was higher in the gene-Mi ($p < 0.05$) and PBS-Mi embryos compared with the untreated embryos (Fig. 2). EGF at 20 ng/ml (but not at 200 ng/ml) significantly lowered the average number of apoptotic cells per blastocyst compared with both microinjected ($p < 0.001$) and untreated ($p < 0.05$) groups of embryos. Significant differences in the average number of apoptotic cells per blastocyst ($p < 0.05$) between the groups treated with 20 ng/ml EGF and 200 ng/ml EGF groups were observed.

Microinjection of rabbit blastocysts with either gene (gene-Mi) or PBS (PBS-Mi) significantly ($p < 0.05$) reduced the total cell number versus non-Mi embryos, with no differences between the microinjected groups (Fig. 3). EGF added at either concentration reversed the influence of microinjection on cell number. The average blastocyst cell number at the two EGF doses was similar.

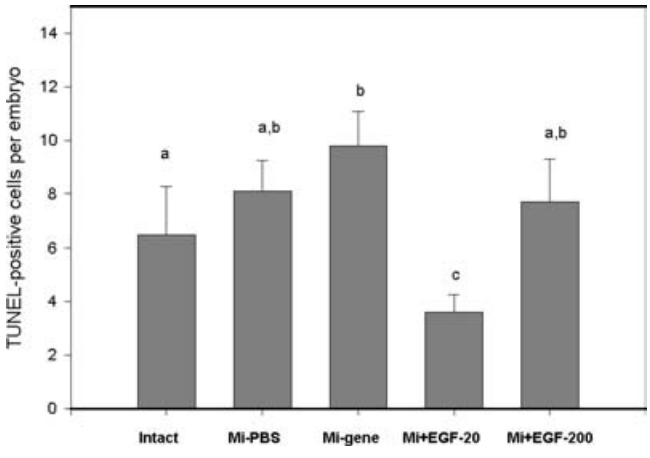

Figure 2 Influence of microinjection (Mi) and EGF (20 or 200 ng/ml) additions on TUNEL-positive cells per blastocyst. Values are expressed as the mean ± SEM. [a,b,c]Significant differences between groups of embryos at $p < 0.05$ (two-sample *t*-test).

Apoptosis in gene microinjection-derived embryos

In order to determine differences between normally developing and arrested embryos derived from microinjected eggs, we carried out a separate experiment with gene microinjection. All embryos, following culture up to 96 hpc, were divided into morphologically normal-looking blastocysts (Fig. 4A, B) and developmentally arrested embryos (Fig. 4C–F), which were processed and analysed separately for TUNEL and for ultrastructural analysis by electron microscopy.

Of 39 microinjection-derived embryos, 18 (46%) were morphologically normal blastocysts and 21 (54%) were cleavage-arrested, of which 10 embryos (48%) had cytoplasmic fragments. No fragmentation was observed in the normal-looking blastocyst group. Arrested embryos had a threefold higher TUNEL index

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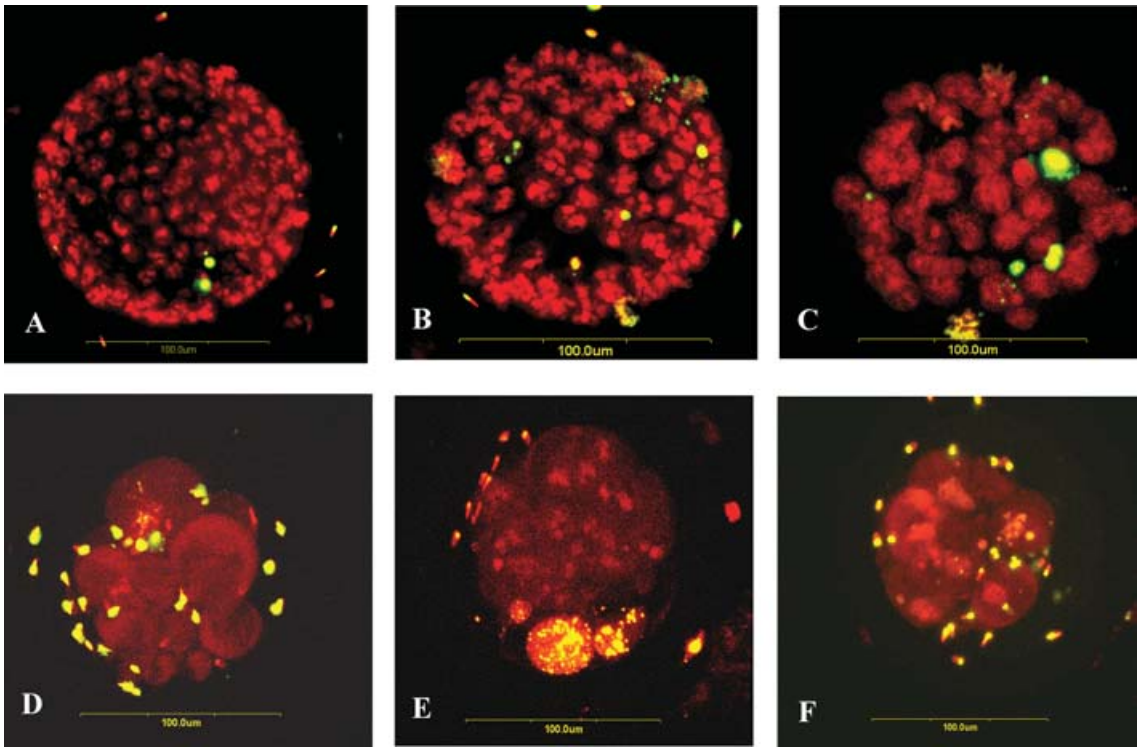

Figure 4 Representative images of confocal laser scanning microscopy of rabbit embryos. TUNEL staining (green) combined with propidium iodide (red) staining of embryonal nuclei. *Upper panel*: Blastocyst-stage embryos. (*A*) Blastocyst from the untreated (non-Mi) group; (*B*) normal-looking blastocyst from the Mi group; (C) cleavage-retarded early blastocyst from the Mi group. Yellow-stained nuclei are TUNEL-positive. Scale bar represents 100 µm. *Lower panel*: Cleavage-arrested embryos with blastomere fragmentation. (*D*) Several nucleus-free blastomeres and one TUNEL-positive nucleus; (*E*) two TUNEL-positive nuclei; (*F*) morphologically apoptotic nuclei with no TUNEL staining. Note numerous TUNEL-positive spermatozoa, which can complicate identification of apoptotic nuclei. Scale bar represents 100 µm.

and a more than threefold lower embryo cell number than morphologically normal blastocysts (Table 3).

Arrested embryos in the microinjected groups had cytoplasmic fragmentation and were characterized by a low blastomere number and irregularly sized blastomeres, most of which were nucleus-free (Fig. 4D, E). Although several embryos showed morphologically apoptotic nuclei (Fig. 4F), no TUNEL-positivity was detected. Fragmented embryos were characterized by a low cell number whereas most blastomeres did

Table 3 Apoptosis and total cell number in morphologically normal (blastocysts) and arrested rabbit embryos derived from gene-microinjected eggs

Groups of embryos (*n*)	TUNEL index,* %	TUNEL cells per embryo†	Embryo cell number†	Fragmented embryos *n* (%)
Mi-derived embryos, total (39)	10.36 ± 2.25	3.94 ± 0.72	70.9 ± 11.5	10 (25.6)
Of these:				
Blastocysts (18)	3.65 ± 1.2[a]	3.34 ± 0.25[a]	111.9 ± 5.6[a]	0
Arrested embryos (21)	17.8 ± 1.49[b]	5.7 ± 0.75[a]	33.8 ± 4.3[b]	10 (47.6)
p value [a] versus [b] across columns	$p < 0.0001$	$p < 0.3035$	$p < 0.0001$	

Data are the mean ± SEM.
Two-sample *t*-test (Student's test) was used either without transformation (∗) or after log transformation (†) of the data.

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not contain nuclei. Of 10 fragmented embryos, 8 were TUNEL-positive and 2 were TUNEL-negative. Microinjection-derived embryos, arrested at the early blastocyst stage, showed fewer nuclei. Inner cell mass (ICM) cells and trophectoderm (TE) cells were not clearly distinguishable and the blastocoele was not expressed (Fig. 4C), in contrast to morphologically normal microinjection-derived (Fig. 4B) or non-microinjection (Fig. 4A) blastocysts. Several nuclei of these blastocysts were TUNEL-positive (Fig. 4C). Although the number of apoptotic cells per embryo in the arrested group (5.7 ± 0.75) was not different from that in normal blastocysts (3.34 ± 0.25), the TUNEL index in arrested embryos was significantly higher ($p < 0.0001$) because of the lower cell number in these embryos (33.8 ± 4.3) versus normal blastocysts (111.9 ± 5.6; Table 3).

Ultrastructure of intact and gene microinjection-derived embryos

Rabbit blastocysts flushed from oviducts (*in vivo* control; Fig. 5A), as well as blastocysts derived from cultured pronuclear-stage eggs (*in vitro* control; Fig. 5B), were selected for electron microscopy. The zona pellucida of normal embryos was intact, blastomeres were evenly developed and the perivitelline space was transparent without fragmented cells. The cellular structure of rabbit embryos (either *in vivo* or *in vitro*) was characterized by cytoplasmic cell inclusions, lipids, vacuoles, elongated mitochondria, Golgi apparatus, rough endoplasmic reticulum and normal nuclei and nucleolus.

In the group of microinjection-derived normal-looking blastocysts the cytoplasm of normal blastomeres contained a few lipid droplets. Vesicles containing flocculent material and phagosomes/lysosomes were abundant in morulae and blastocysts. Elongated mitochondria were observed in morulae and most blastocysts. The Golgi apparatus (observed in the nuclear region), rough endoplasmic reticulum and normal nuclei were present in all embryos. All morulae and blastocysts contained well-developed mature nucleoli, in which fibrillar centres as well as fibrillar and granular components were observed (Fig. 6).

In the group of microinjection-derived abnormal (cleavage-arrested) embryos most were fragmented and some blastomeres were localized in the subzonal space. An alteration was detected in the nuclear and nucleolar morphology; blebbing of the nuclear envelope was observed in most blastomere nuclei (Fig. 7A). In contrast to normal-looking developing embryos, an extensive area with dispersed electron-dense material was formed as a result of cytoplasmic breakdown; electron-dense bodies (apoptotic bodies) were numerous (Fig. 7B). Apoptotic bodies were characterized by membrane-bound, condensed, homogeneous material in juxtaposition to the nucleus. Many recognizable mitochondria were vacuolated with a very dense matrix.

Discussion

It has been shown that microinjected DNA can result in DNA rearrangement during integration, and cause developmental arrest (Covarrubias *et al.*, 1987) and decreased cleavage rates in microinjected zygotes (Voss *et al.*, 1996). Based on our previous results, the efficiency of gene integration in newborn rabbits derived from microinjected eggs in our laboratory was approximately 3% (Chrenek *et al.*, 2002). Our present results, obtained with embryos microinjected with PBS solution, demonstrate that the apoptotic index and total cell number of these embryos were similar to those in gene-microinjected embryos. These data and earlier observations of Canseco *et al.* (1994) suggest that factors associated with microinjection itself (mechanical damage, pronuclear lysis, etc.), rather than DNA integration, may be responsible for the decreased quality of the microinjected embryos and the high percentage of embryonic losses. Among such factors, exposure of zygotes to a microscope light during microinjection and the type of microinjection pipette may be of particular importance (Voss *et al.*, 1996). Thus, exposing rabbit zygotes to a microscope light of a higher intensity significantly reduced their cleavage rate. Also, using pipettes with a filament resulted in a higher cleavage rate of zygotes (30.3% vs 20.6%) than did using pipettes without a filament (Voss *et al.*, 1996). Since in our microinjection protocol we used pipettes with a filament, we can assume that mechanical damage might be reduced. In our procedure both microinjected and intact eggs were held during microinjection in the same culture plate, but the microinjected group was exposed to direct microscope light whereas intact eggs were a little off the direct light beam. Although the whole microinjection procedure lasted only 15–20 min, we cannot exclude a possible deleterious influence of light exposure. Therefore, differences in viability between microinjected and intact zygotes may be explained partially by consequences of microinjection-induced mechanical damage and partially by the exposure to direct microscope light.

We hypothesize that a higher frequency of apoptosis may be a response to the adverse influence of microinjection. Increased incidence of apoptosis is an indicator of suboptimal culture conditions (Byrne *et al.*, 1999). The present observations demonstrate that blastocysts developed from microinjected eggs had a significantly higher occurrence of apoptosis and lower

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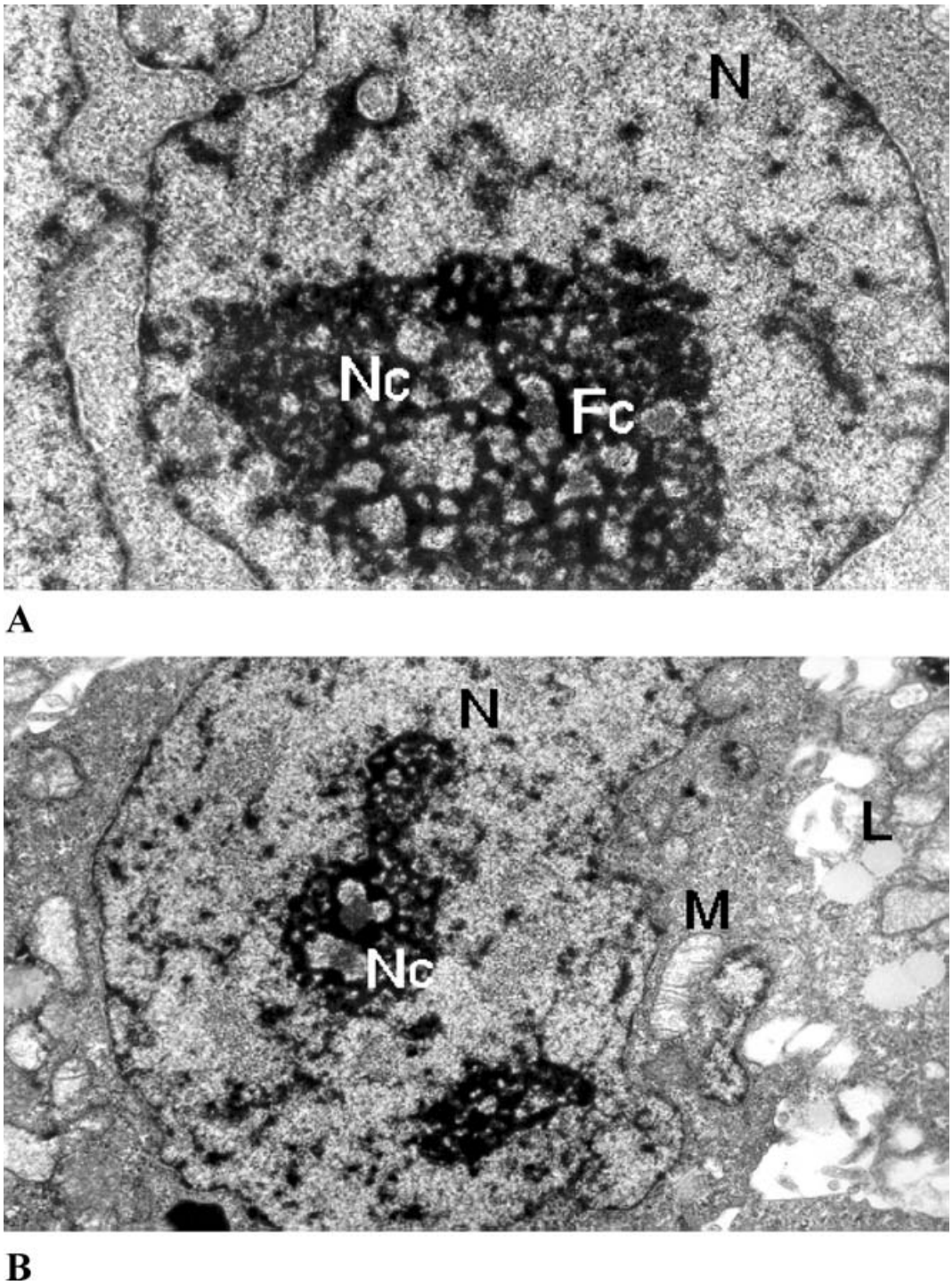

Figure 5 Fine structure of rabbit embryos. The blastocysts were prepared for electronic microscopy as Durcupan ACM thin sections. (*A*) Ultrastructure of nucleus (N) and nucleolus (Nc) with fibrillar centres (Fc) of *in vivo* control blastocyst (× 21 500). (*B*) Ultrastructure of nucleus (N), nucleolus (Nc), mitochondria (M) and lipids (L) of inner cell mass (ICM) cell of *in vitro* cultured intact blastocyst (× 15 500).

cell number than those with intact blastocysts. From our data it is evident that the higher apoptotic index in gene-Mi embryos (13.3%) versus untreated (non-Mi) embryos (6.0%; Fig. 1) was not due to a high average number of apoptotic cells per embryo (7 in the non-Mi group vs 9.8 in the Mi group; Fig. 2) but to a low cell number in cleavage-arrested (degenerated) embryos (33.8 ± 4.3; Table 3). We observed that not all cleavage-arrested embryos in our study were TUNEL-positive. It may be suggested that either (1) TUNEL was unable

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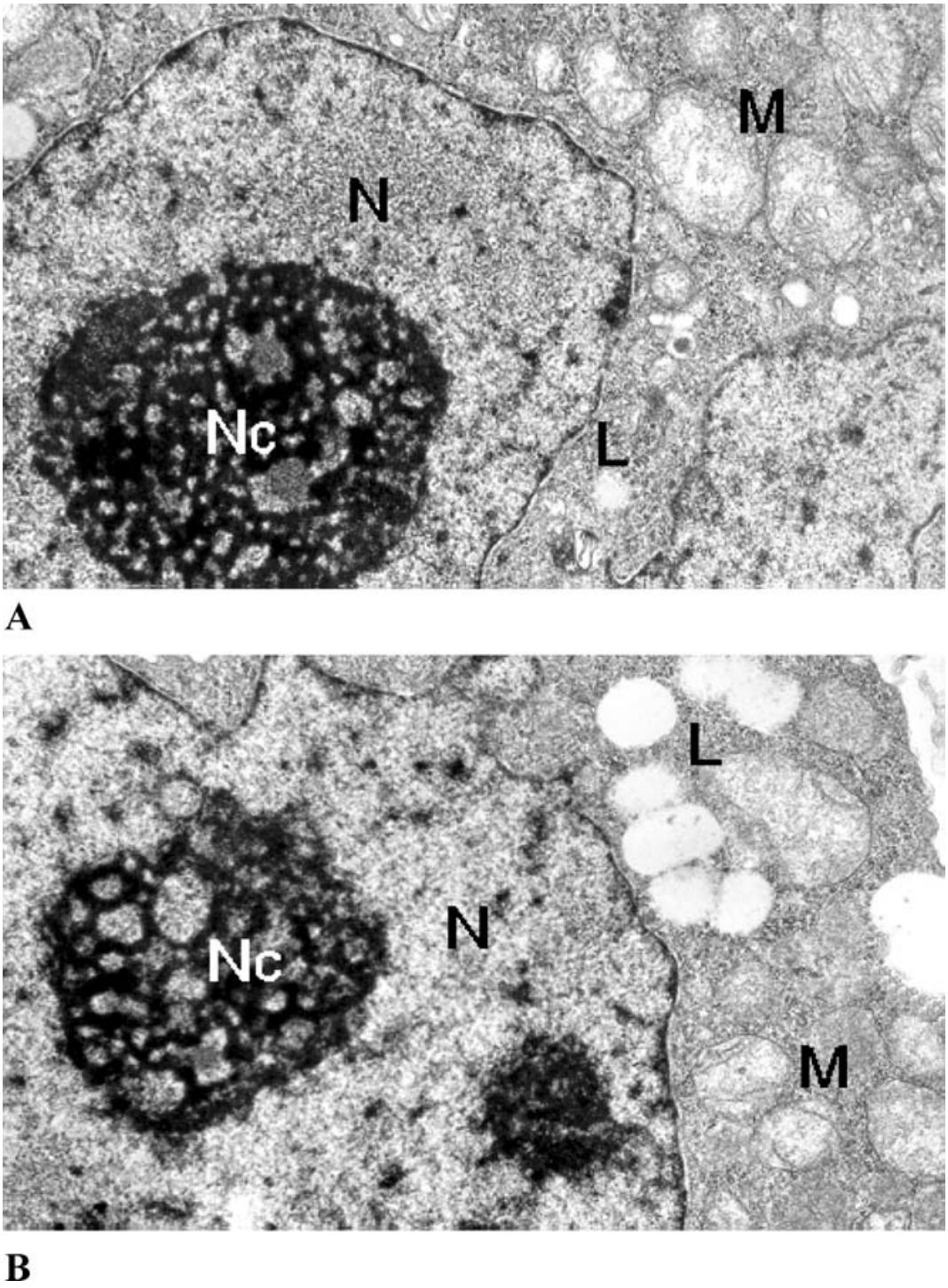

Figure 6 Fine structure of microinjection-derived normal-looking expanded blastocyst. (*A*) Ultrastructure of nucleus (N) and nucleolus (Nc) of ICM cell of expanded blastocyst. Lipids (L) and mitochondria (M) were localized in the cytoplasm (× 21 500). (*B*) Ultrastructure of nucleus (N) and nucleolus (Nc) of trophectoderm (TE) cell of normal-looking expanded blastocyst. Mitochondria (M) and lipids (L) were localized in the cytoplasm (× 21 500).

to detect earlier signs of forthcoming apoptosis in these early-stage embryos, or (2) that apoptosis is not always the primary cause of a decrease in embryo cell number and/or embryo degeneration. We cannot exclude the possibility that another mechanism, such as the organization and function of the cytoskeleton, which controls movement of mitochondria and polarized distribution of specific macromolecules (Van Blerkom *et al.*, 2000), may be partially involved in embryo cleavage arrest.

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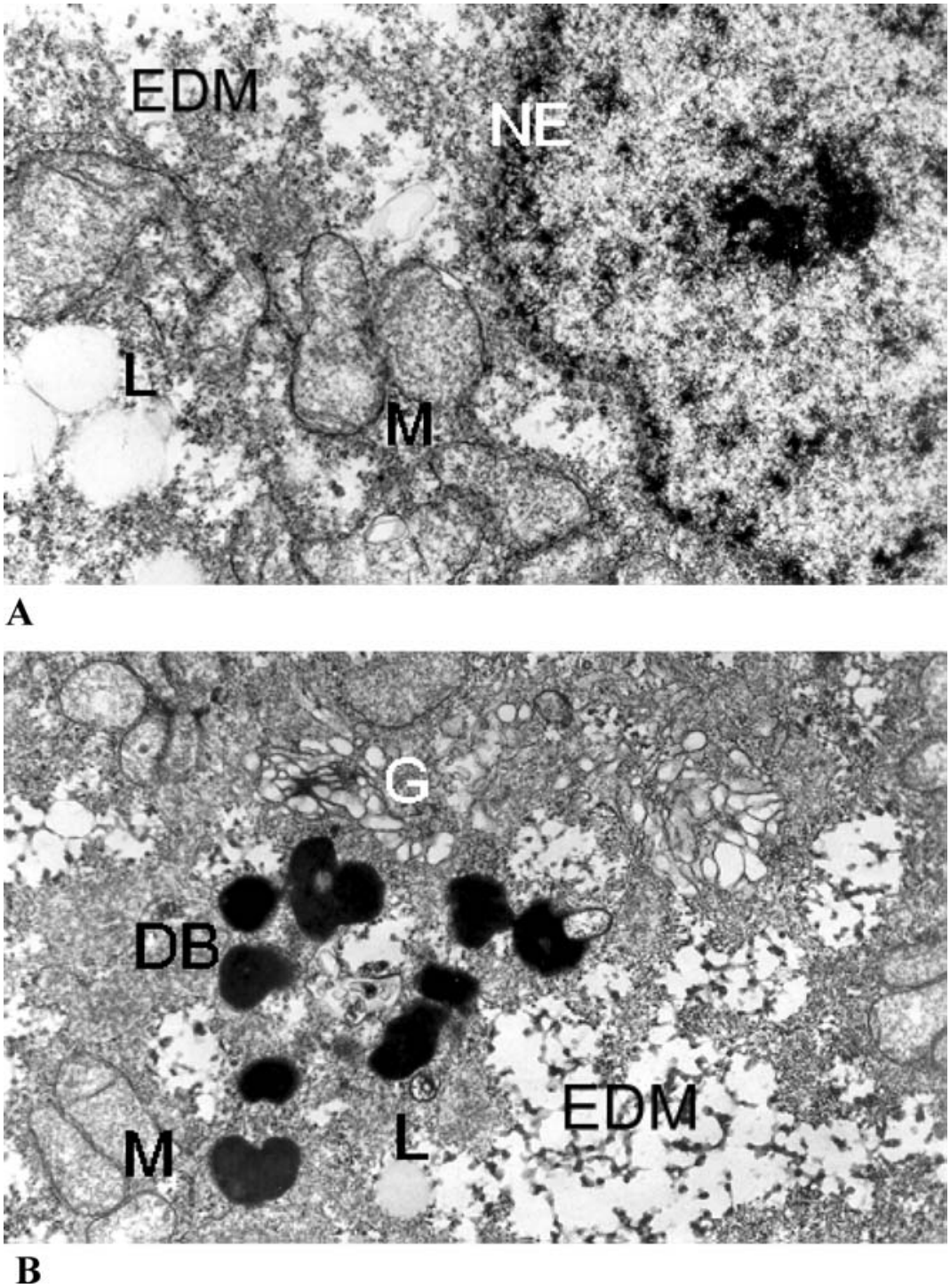

Figure 7 Fine structure of microinjection-derived abnormal (cleavage-arrested) embryos. (*A*) Large region of blebbing of the nuclear envelope (NE), swollen mitochondria (M), lipids (L) and an area with dispersed election-dense material (EDM) were visible in the cytoplasm. The fibrillar center as well as the fibrillar and granular components of the nucleolus are poorly developed (× 31 200). (*B*) Ultrastructure of the cytoplasm with numerous dense bodies (DB), Golgi apparatus (G), mitochondria (M), lipids (L) and an extensive area with dispersed electron-dense material (EDM) (× 21 500).

In our study, blastomere fragmentation was detected in about 40% of microinjected embryos, whereas no fragmented embryos were observed in the intact embryos. This suggests that cytoplasmic fragmentation in our embryos is caused by an unfavourable effect of microinjection. Fragmentation was observed in embryos at the 2- to 32-cell stage and these embryos were not cleaved further. Since embryo fragmentation

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became visible at an early preimplantation stage it is possible that developmental arrest and increased apoptosis in microinjected embryos is a consequence of cytoplasmic fragmentation. Cytoplasmic fragments can be formed due to asynchrony between the nuclear and cytoplasmic cell cycles, as a result of a lack of karyokinesis, even though cytokinesis occurs normally. Several studies on human (Jurisicova *et al.*, 1996) and mouse (Jurisicova *et al.*, 1998) preimplantation embryos suggest that blastomere fragmentation may be an indicator of apoptosis and that cytoplasmic fragments are equivalent to apoptotic bodies. No association has been found between cytoplasmic fragmentation and apoptosis, measured using TUNEL, annexin V or caspase-3, in human (Antczak & Van Blerkom, 1999), bovine (Van Soom *et al.*, 2003) and mouse (Xu *et al.*, 2001) embryos. On the other hand, Antczak & Van Blerkom (1999) do not exclude the possibility that fragmentation itself may be an initiator of apoptosis if some alterations in the polarized distribution of important proteins occur. In porcine *in vitro* produced embryos, developmental arrest was associated with subsequent apoptosis, whilst cytoplasmic fragmentation did not occur (Hao *et al.*, 2003). These authors also suggest that cytoplasmic fragmentation, developmental arrest and nuclear condensation are typical morphological changes of embryos undergoing apoptotic cell death. Therefore, despite a growing body of data, the interrelationship between apoptosis, cytoplasmic fragmentation and embryo cleavage arrest remains unclear. It is possible that this relationship is species-specific or dependent on the origin of embryos (*in vivo*/*in vitro* derived, parthenogenetic, nuclear transferred, microinjected, etc.).

The frequency of apoptosis in the embryo can be decreased by adding growth factors to the embryo media (Herrler *et al.*, 1998; Makarevich & Markkula, 2002; Augustin *et al.*, 2003). We tested the ability of EGF to decrease apoptosis and therefore to reduce the adverse effect of microinjection on the quality of preimplantation rabbit embryos. There is still no consistency in data about the effects of EGF on embryo development and apoptosis in different animal species. In particular, EGF, when added to maturation medium, stimulated meiotic and cytoplasmic maturation, cleavage rate, blastocyst development and embryo viability in pig (Grupen *et al.*, 1997; Abeydeera *et al.*, 2000), cattle (Watson *et al.*, 2000; Goff *et al.*, 2001) and sheep (Grazul-Bilska *et al.*, 2003). However, it did not affect the total cell number of blastocysts or apoptosis in cattle (Watson *et al.*, 2000). The addition of EGF to the medium during the culture of previously *in vitro* matured oocytes improved the percentage of 4-cell bovine embryos reaching the blastocyst stage (Sirisathien *et al.*, 2003), although neither cell number nor the percentage of TUNEL-positive nuclei were affected by EGF treatment (Sirisathien & Brackett, 2003). On the contrary, in mouse embryos, blastocyst formation, cell proliferation and zona hatching were improved if EGF was added to the culture medium (Paria & Dey, 1990). EGF in our culture experiments neither increased blastocyst yields of either intact or microinjected embryos, nor significantly reduced the number of arrested 1-cell stage embryos. However, EGF significantly decreased the TUNEL index in microinjected embryos and increased the total cell number of blastocysts compared with microinjected embryos cultured without EGF. This suggests that EGF (at certain concentrations) is able to diminish or completely reverse the unfavourable effects of microinjection, therefore improving the quality of the resulting blastocysts.

To support the results from the TUNEL assay of rabbit embryos, we studied the ultrastructure of embryos undergoing gene microinjection, using electron microscopy. Our data showed that microinjection-derived normal-looking blastocysts did not differ from the untreated *in vitro* cultured or *in vivo* originated blastocysts in either the structure of nucleus and nucleolus, or the appearance of other cellular organelles. In contrast, microinjection-derived cleavage-arrested embryos exhibited a poorly developed nucleolus, swollen mitochondria, several lipid vesicles, an extensive area with dispersed electron-dense material as well as other typical apoptotic signs, such as nuclear membrane blebbing and numerous electron-dense bodies. These data in combination with the TUNEL results confirm the higher occurrence of apoptosis in cleavage-arrested embryos derived from microinjected eggs.

In conclusion, the present study demonstrates that microinjection-derived embryos are characterized by a higher apoptotic rate than intact embryos, suggesting an association between embryo cleavage arrest and apoptosis in embryonal blastomeres. Therefore, apoptosis is probably one of the major causes of the low quality and viability of microinjected embryos. Improvements to the developmental potential of gene-microinjected embryos may be possible by preventing apoptosis. EGF can diminish or completely reverse (depending on the concentration) the detrimental consequences of microinjection, therefore improving the quality of resulting blastocysts. However, further research is required in order to determine whether apoptosis is the primary or secondary cause of embryo arrest.

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