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Ultrastructural characterization of *in vivo*-produced ovine morulae and blastocysts

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Summary

The ultrastructure of *in vivo*-produced ovine embryos, at the morula, early blastocyst and late blastocyst stages, was evaluated using transmission electron microscopy. Embryonic cells were characterized by the presence of intact intercellular junctions, numerous mitochondria, smooth endoplasmic reticulum cisternae and light vesicles. Polyribosomes, rough endoplasmic reticulum cisternae, secondary lysosomes, Golgi complexes and lipid droplets were also observed in the cytoplasm. The nucleus was well defined and organized, with an intact envelope rich in nuclear pore complexes, and one or more reticular nucleoli. Microvilli were present in external blastomeres of morulae and became more abundant in trophectoderm cells of early and late blastocysts. Light vesicles seemed to be associated with small cisternae of Golgi and endoplasmic reticulum origin. These cisternae fused and created light vesicles with engulfed heterogeneous cytosolic structures, small cisternae and vesicles. Their labile membrane enabled them to rapidly coalesce into medium-sized vesicles that began to engulf mitochondria and lipid droplets, forming giant vacuoles mostly filled with fat. Incomplete matured secretory vesicles were observed to exocytose into the perivitelline space of morulae, whereas fully matured secretory vesicles appeared only in trophectoderm cells, being exocytosed into the blastocoelic cavity. These observations suggested that these endoplasmic-/Golgi-derived vesicles behave as active autophagic organelles presenting probably a maturation process from compact morulae to blastocyst.

Keywords: Blastocyst, Morula, Ovine, Ultrastructure, Vesicles

Introduction

Several invasive and non-invasive methods for embryo evaluation have been reported (Van Soom *et al.*, 2001; Abe *et al.*, 2002). Despite the fact that more accurate data can be acquired from invasive techniques, embryo quality must be estimated by non-invasive methods if the selected embryo has to be transferred (Van Soom *et al.*, 2001). Embryo morphology evaluation using stereomicroscopy is the method used most widely to evaluate embryo quality, but it remains one of the most subjective and qualitative aspects of all the multiple ovulation and embryo transfer (MOET) processes (Abe *et al.*, 2002).

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The application of the morphological classification, developed for bovine embryos (Linder & Wright, 1983), to other species may result in some misclassifications. The shortcomings of the morphological evaluation are even more serious after cryopreservation, as ultrastructural studies carried out with cryopreserved embryos (Cocero *et al.*, 2002; Bettencourt *et al.*, 2009) have revealed that certain abnormalities and alterations cannot be detected using stereomicroscopy alone.

Mammalian embryos are routinely cultured until the morula or blastocyst stages before being either cryopreserved or transferred to recipients and therefore the accurate evaluation of embryo quality at these developmental stages is crucial for both the researcher and the clinician (Van Soom *et al.*, 2001; Abe *et al.*, 2002). In order to correctly interpret the level of cellular damage after cryopreservation a precise knowledge of the ultrastructural characteristics of fresh embryos is needed. There are several studies that describe the ultrastructure of fresh and cryopreserved embryos of various species (Ferrer *et al.*, 1995; Vajta *et al.*, 1997; Fair *et al.*, 2001; Rizos *et al.*, 2002; Cuello *et al.*, 2007) but detailed data on the ultrastructural characterization of ovine *in vivo*-produced embryos are scarce (Calarco & McLaren 1976; Ferrer *et al.*, 1995; Rizos *et al.*, 2002). In the present ultrastructural study we describe the ultrastructure of *in vivo*-produced morula and blastocyst embryos, with a focus on the association of light vesicles with other cellular organelles.

Materials and methods

Embryo production

In vivo-produced embryos ($n = 14$) were obtained from Portuguese Black Merino ewes, superovulated during the spring. Donors were synchronized with intravaginal sponges that contained 40 mg flurogestone acetate (Chronogest®, Intervet Laboratories, Boxmeer, The Netherlands) for 12 days. Starting on day 9 of sponge treatment and for 4 consecutive days, ewes were treated (intramuscular) with 1.25 ml ovine follicle stimulating hormone (oFSH; Ovagen®, Immunological Products Ldt. Aukland, New Zealand), twice a day (at 9:00 and 21:00 h). Sponges were removed at the time of the seventh FSH administration. Laparoscopic intrauterine insemination was performed with fresh diluted semen in all ewes, 48 h after sponge removal, as previously described (McKelvey *et al.*, 1985). A minimum of 50×10^6 motile spermatozoa was placed in each uterine horn. Additionally, ewes were hand mated at 36 and 48 h after sponge withdrawal and left with rams for 4–6 h at a ewe:ram ratio of 3:1. Embryos were recovered by abdominal laparotomy, under general anesthesia, on days 6 or 7 after sponge removal. Each uterine horn was flushed using a Foley catheter as described in detail elsewhere (Smith & Murphy, 1987). The collection medium (40 ml for each uterine horn) was phosphate-buffered saline (PBS) solution that contained 2% bovine serum albumin (BSA).

Morphological scoring of embryos was performed based on morphological criteria and developmental stage in accordance with the guidelines of the International Embryo Transfer Society (Stringfellow & Seidel, 1998). Embryos were scored as grade 1 (excellent or good) when presenting a symmetrical and spherical cell mass with ≥75% of intact cells (<25% of degenerated cells/cell fragments), with individual cells showing a uniform size, colour and density and as grade 2 embryos (fair) when showing a cell mass with ≥50% of intact cells (25–50% of degenerated cells/cell fragments), with individual cells presenting moderate irregularities in size, colour and density. Fresh embryos, at the compact morula ($n = 4$) early blastocyst ($n = 6$) and blastocyst ($n = 4$) stages, classified as grade 1 and 2, were processed for transmission electron microscopy.

Electron microscopy

Fourteen embryos were fixed, within 1 h of collection, in Karnowsky's fixative (2 h, 4ºC), washed with 0.15 M sodium cacodylate buffer, pH 7.3 (overnight, 4ºC), post-fixed (1 h, 4ºC) in 1% OsO_4 in buffer that contained 0.8% hexanocyanoferrate potassium [$K_3Fe_3^+(CN)_6$], and washed for 15 min in buffer. They were then dehydrated through a graded series of ethanol (50, 70, 90, 2 ×100%; 30 min each), at room temperature followed by propylene oxide (15 min), impregnated with propylene oxide:epon (3:1, 1 h; 1:1, overnight, 4ºC; 1:3, 4 h) and embedded in Epon (7 h at room temperature, 3 days at 60ºC). Sections were cut with a Leica ultramicrotome with a Diatome knife. Semi-thin sections (0.5 μm) were stained with aqueous azur II:methylene blue (1:1). Ultrathin sections (700 Å) were collected onto 200 mesh copper grids (Taab) and stained with 3% aqueous uranyl acetate (20 min) and Reynolds lead citrate (10 min). Sections were observed in a transmission electron microscope JEOL 100CXII operated at 60 kV.

Except when otherwise indicated, all chemicals were obtained from Sigma Chemicals Company (Barcelona, Spain).

Results

Semi-thin sections showed that at the compact morula stage, there were almost no intercellular spaces, being

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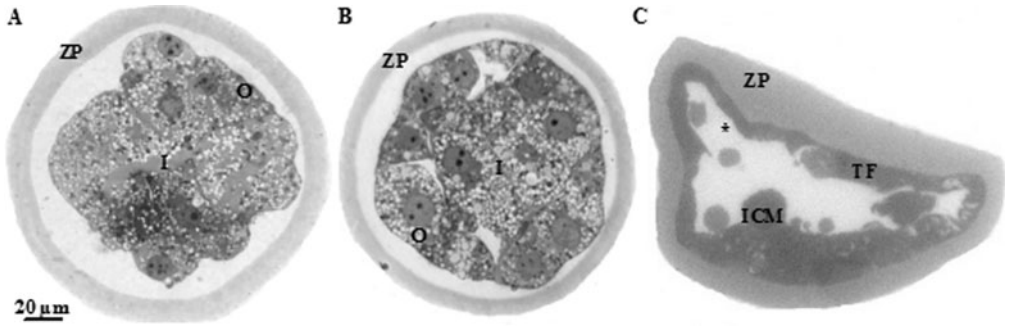

Figure 1 Fresh *in vivo* ovine embryos fixed 1 h after collection. Semi-thin sections of compact morulae (*A*), early blastocyst (*B*), and blastocyst (*C*). Zona pellucida (ZP), inner cells (I), outer cells (O); trophectoderm (TF); inner cell mass (ICM); blastocoelic cavity (*).

difficult to distinguish the individual cells, whereas the perivitelline space was enlarged due to cell compaction (Fig. 1*A*). Early blastocysts were characterized by the appearance of small cavities below peripheral cells (Fig. 1*B*), which grew progressively as the embryos developed to the late blastocyst stage (Fig. 1*C*). Two cell types were already distinguishable at the early blastocyst stage: (1) those on the periphery that will give rise to trophectoderm; and (2) those located centrally that will give rise to the inner cell mass (Fig. 1*B*). The blastocoelic cavity accounted for more than half of the embryo size at the blastocyst stage and the cells of the inner cell mass were grouped together and surrounded by trophectoderm cells. At this stage the perivitelline space was no longer visible and trophectoderm tightly cells lined the inner zona pellucida (Fig. 1*C*).

At the ultrastructural level there were several characteristics that were common to all studied stages of development (Fig. 2). The main differences found between outer and inner cells of morulae (Fig. 2*A*, *B*), or between trophectoderm (Fig. 2*C*, *E*) and inner cell mass cells (Fig. 2*D*, *F*) of blastocysts were their shape, the presence of external microvilli and very large secretory vesicles in outer cells of morulae (Fig. 2*A*) and trophectoderm cells of late blastocysts (Fig. 2*C*, *E*) and an increase in the number of perinuclear Golgi complexes in early blastocysts.

Microvilli were sparse and shorter in compact morulae (Fig. 2*A*), then becoming longer and more abundant in early (Fig. 2*C*) and late blastocyst (Fig. 2*E*) stages. At the later stage, due to the absent perivitelline space, a dense continuous cover of bended microvilli lay between trophectoderm and the zona pellucida (Fig. 2*E*).

Polyribosomes, rough endoplasmic reticulum, secondary lysosomes, perinuclear Golgi complexes and lipid droplets were also observed (Fig. 2). Mitochondria, presenting peripheral and transverse cristae, were frequently surrounded by cisternae of the smooth endoplasmic reticulum (Fig. 2).

The nucleus was well defined and organized, with an intact envelope rich in nuclear pore complexes and one or more reticulated nucleolus with evident fibrillar centres surrounded by the dense fibrillar and granular components (Fig. 2).

Outer and inner embryonic cells were characterized by the presence of intact intercellular junctions, numerous mitochondria, smooth endoplasmic reticulum and light vesicles (Fig. 2). Embryonic cells of morulae and inner cell mass showed adherent and gap junctions without prominent associated filament bundles. Inner cell mass cells also presented long cytoplasmic extensions that overlapped and interdigitated with neighbouring cells forming additional intercellular links (Fig. 3*A*); while trophectoderm cells of blastocysts showed prominent junctional complexes (Fig. 3*B*).

Small dense cortical vesicles were observed in inner and outer cells of early blastocysts (Figs. 2*C* and 3*B*) whereas receptor-mediated endocytosis was observed only in inner cells of early blastocysts (Fig. 2*D*).

Abundant light vesicles were observed in the vicinity of the perinuclear region, at the *trans*-Golgi network, as well as at the cortical region of peripheral cells in morula stage (Fig. 4). Golgi-derived small cisternae were observed to bend and fuse at their extremities, trapping inside cytosolic structures, small cisternae and vesicles. At the cell periphery, small smooth endoplasmic reticulum cisternae began to encircle light cytosolic areas (Fig. 4*B*), and then also fused to form light vesicles. Protrusion and coalescence between vesicles was also seen. Expansions of the outer nuclear membrane were also observed to be closely associated with the membrane of the vesicles (Fig. 5*A*). These vesicles incorporated other organelles through protrusion and engulfment of other light vesicles, mitochondria and lipid droplets (Figs. 5*B* and 6*A*, *B*). Large light vesicles were observed to open into the perivitelline space of morulae (Fig. 2*A*), whereas very large and dense vesicles were restricted

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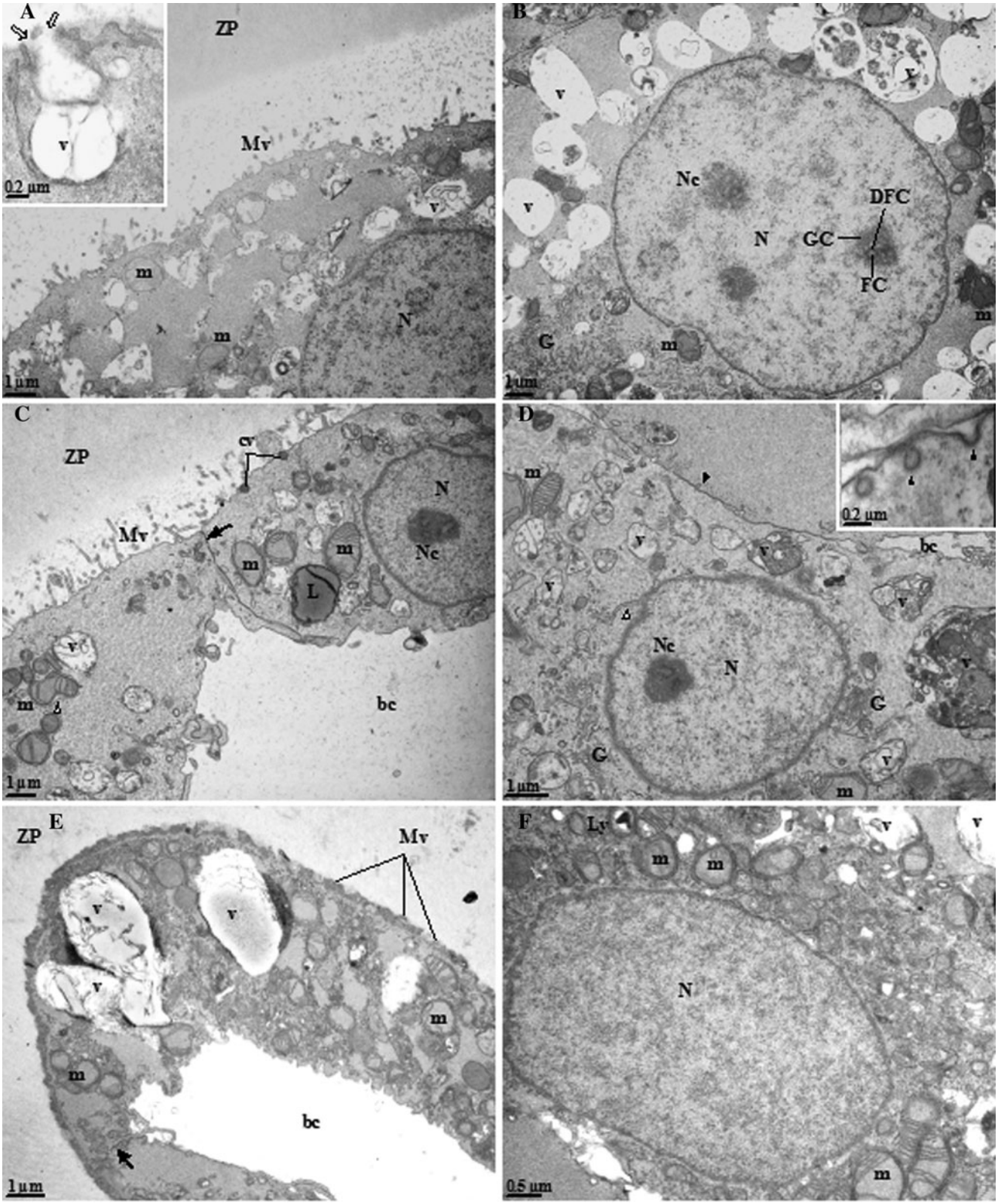

Figure 2 Ultrastructure of fresh *in vivo* ovine embryos at compact morulae (*A*, *B*), early blastocyst (*C*, *D*) and blastocyst (*E*, *F*) stages. Outer (*A*, *C*) and inner cells (*B*, *D*), trophectoderm (*E*) and inner cell mass (*F*) cells. Zona pellucida (ZP), microvilli (Mv), blastocoelic cavity (bc), exocytosis (thick white arrows), intercellular junctions (black arrows), mitochondria (m), smooth endoplasmic reticulum (white arrowheads), light vesicles (v), lipid droplets (L), Golgi complexes (G), secondary lysosomes (LY), cortical vesicles (cv), nucleus (N), nucleolus (Nc), nucleolar fibrillar centers (FC), nucleolar dense fibrillar component (DFC), nucleolar granular component (GC), receptor-mediated endocytosis (black arrowheads).

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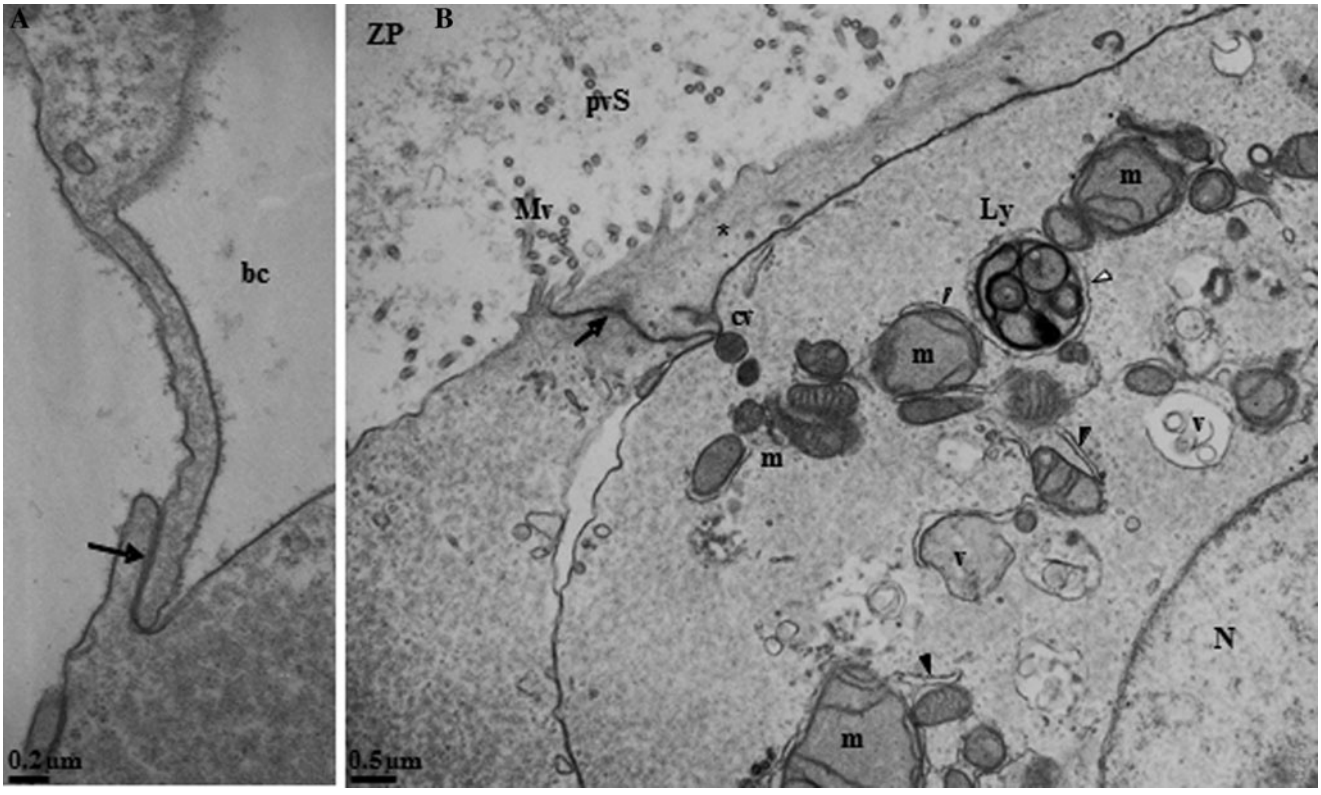

Figure 3 Ultrastructure of fresh *in vivo*-produced ovine early blastocysts. (*A*) Inner cell mass cells. (*B*) Trophectoderm cells. Blastocoelic cavity (bc), zona pellucida (ZP), perivitelline space (pvS), microvilli (Mv), mitochondria (m), light vesicles (v), smooth endoplasmic reticulum (white arrowheads), rough endoplasmic reticulum (black arrowheads), cortical vesicles (cv), intercellular junctions (black arrows), filament bundles (*), nucleus (N).

to trophectoderm cells of late blastocysts (Fig. 7). Some of these large vesicles exhibited signs of swelling, with a decrease in the total area occupied by dense lipid droplets and by appearance of an amorphous substance and numerous membrane profiles that remained attached to the surface of lipids. The exocytosis of the three components into the blastocoelic cavity was seen (Fig. 7*C*).

Discussion

Ovine *in vivo*-produced embryos are usually collected at the morula or blastocyst stage (Cognié *et al.* 2003). The ultrastructural characterization of the cell organization at these stages of development provides essential information, which enables the establishment of objective criteria for embryo evaluation. This is particularly useful in order to understand cell damage observed after cryopreservation and allows the correct characterization of the different lesions induced along the process (Dobrinsky, 1996; Cocero *et al.*, 2002; Smith & Silva, 2004; Bettencourt *et al.*, 2009). Some ultrastructural characteristics of ovine *in vivo*-produced embryos have been previously described (Calarco & McLaren, 1976; Ferrer *et al.*, 1995; Cocero *et al.*, 2002; Rizos *et al.*, 2002; Bettencourt *et al.*, 2009). This study provides additional information on cellular structures that can be used to better characterize embryos at this stage of development.

All embryos evaluated in this study were at the compact morula, early blastocyst or late blastocyst stage, based on chronological development of ovine embryos (Winterberger-Torres & Sevellec, 1987). In ovine embryos, compaction has been described to occur between days 5 and 6 (day 0: estrous) and, as embryos progress from the morula to the blastocyst stage (between days 6 and 7), it was noticed that the relative volume of the intercellular space decreased as the blastocoele became more organized and occupied an increased proportion of the embryo (Calarco & McLaren, 1976; Winterberger-Torres & Sevellec, 1987; Ferrer *et al.*, 1995).

The general ultrastructural characteristics here described are in agreement with previous findings (Calarco & McLaren, 1976; Ferrer *et al.*, 1995; Cocero *et al.*, 2002; Rizos *et al.*, 2002).

The well defined nucleus with two or more reticulated nucleoli observed here at the morula

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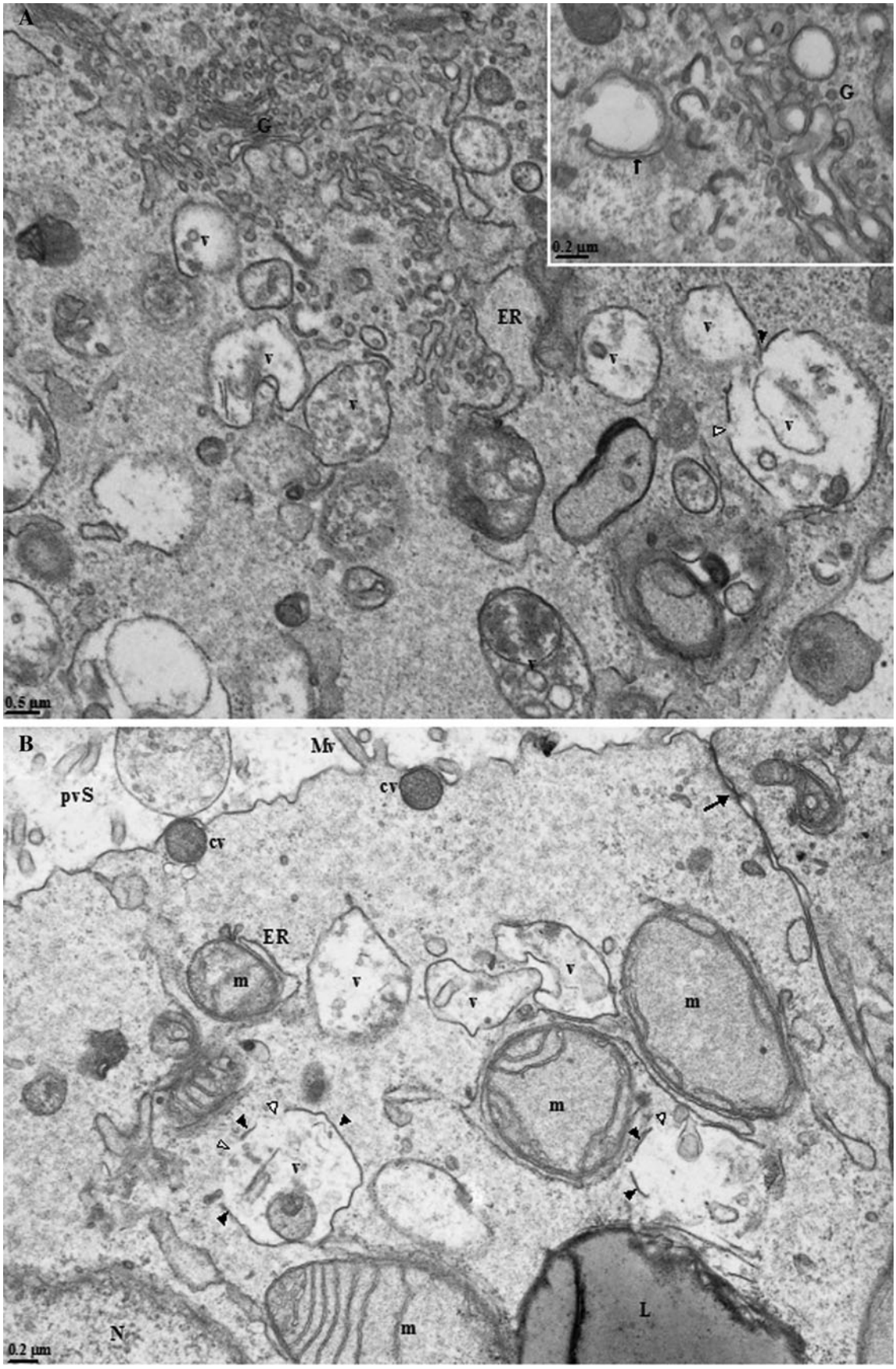

Figure 4 Origin of light vesicles at the perinuclear region (*A*) and cortical region of peripheral cells (*B*) in ovine morula. Golgi complexes (G), Golgi cisternae (thick black arrow), smooth endoplasmic reticulum (ER), vesicles (v), mitochondria (m), labile membranes (white arrowheads), membrane fusion (black arrowheads), perivitelline space (pvS), microvilli (Mv), nucleus (N), cortical vesicles (cv), tight junction (black arrow), lipid droplet (L).

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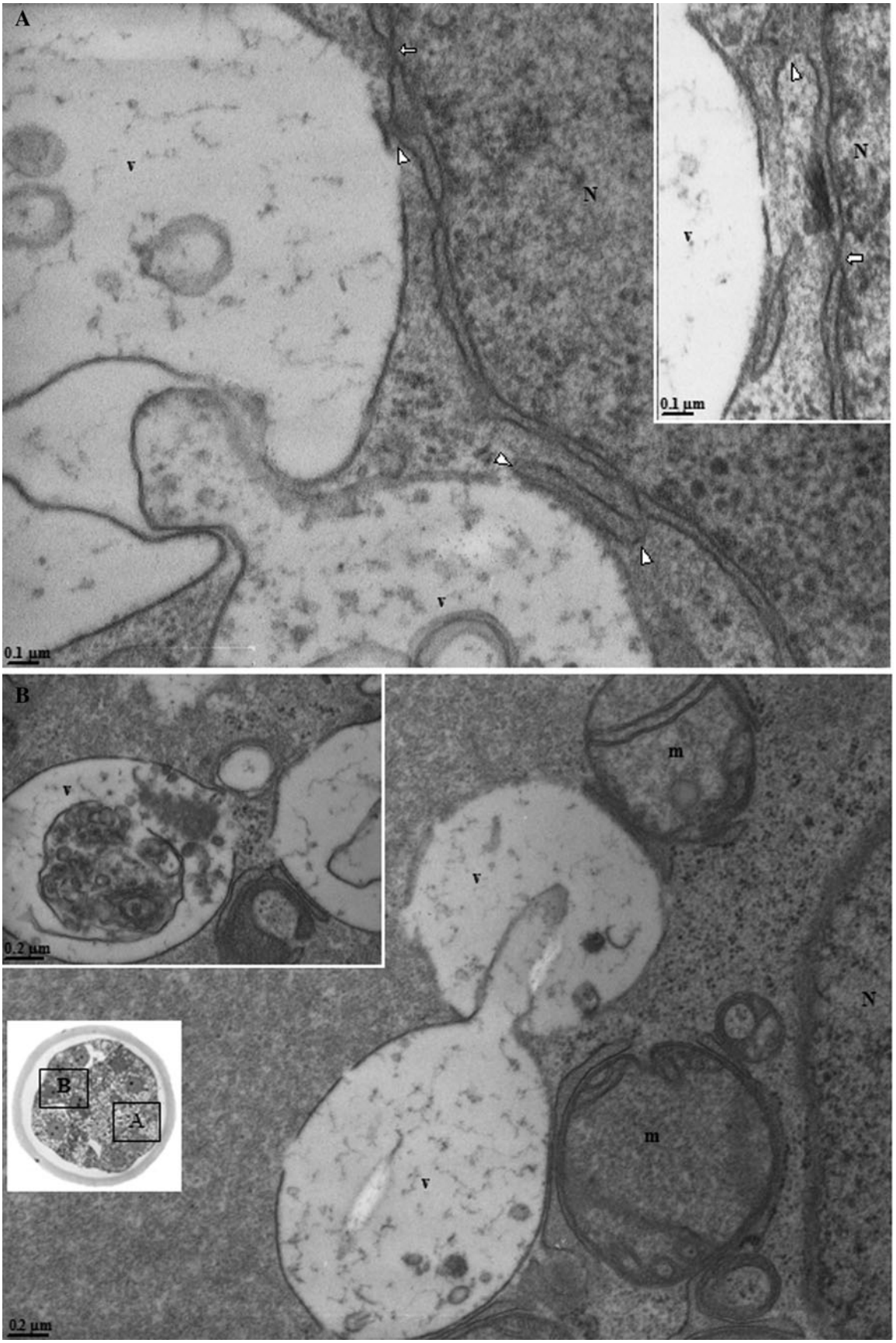

Figure 5 (*A*) cortical region of peripheral cells. (*B*) Cell periphery of peripheral cells. Vesicle protusion and coalescence in ovine early blastocysts Semi-thin section (inset left down), incorporation of organelles by light vesicles (inset left up). Nucleus (N), nuclear pore complexes (thick white arrow; inset right), light vesicles (v), expansions of the outer nuclear membrane (white arrowhead, inset right), mitochondria (m).

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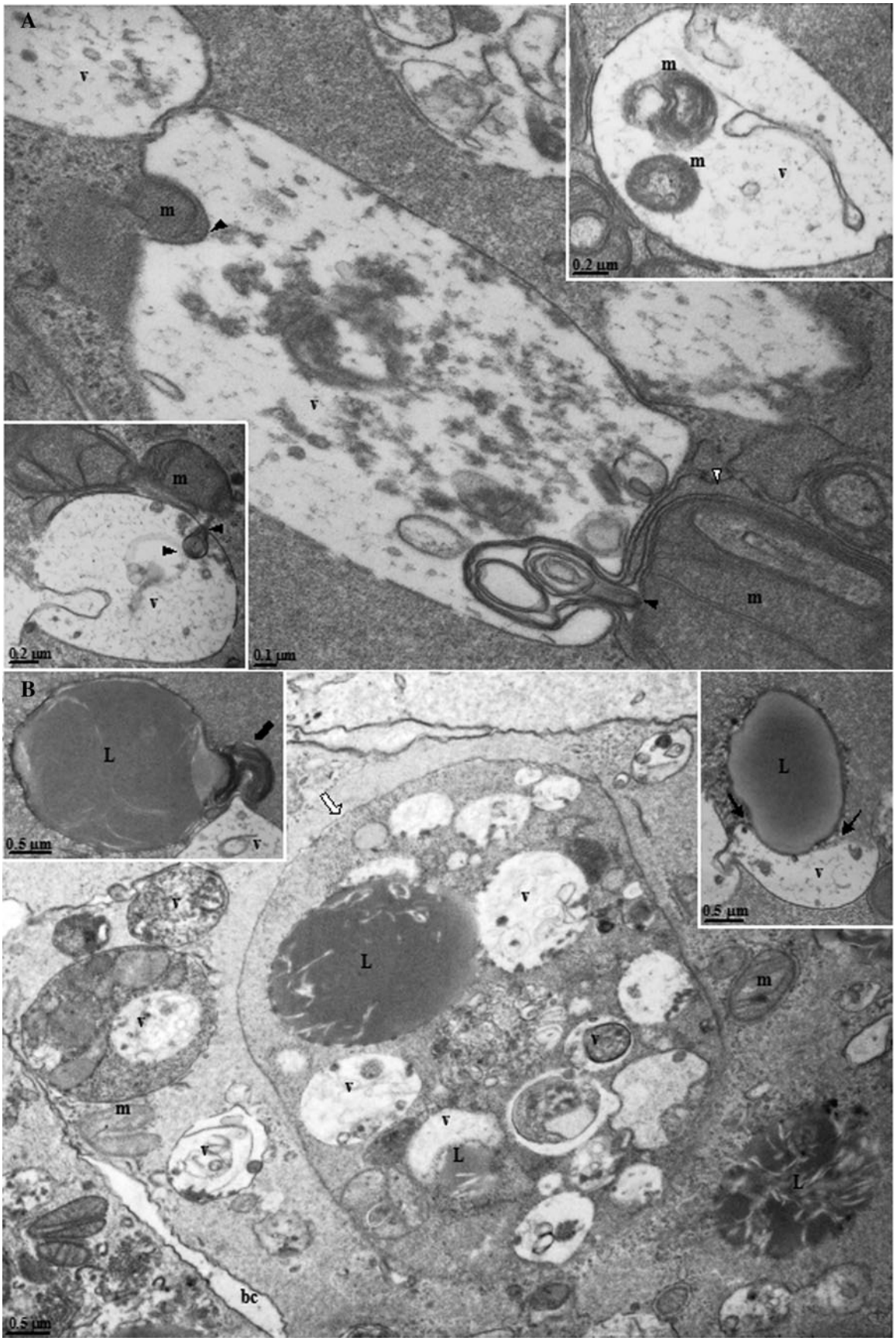

Figure 6 Different steps of the maturation process of the light vesicles in ovine blastocysts. Light vesicles (v), mitochondria (m), mitochondrial membranes (black arrowheads), smooth endoplasmic reticulum (white arrowheads), lipid droplets (L), myeline like droplets (thick black arrow), large vacuole (thick white arrow), lipid engulfment (arrows), blastocoelic cavity (bc).

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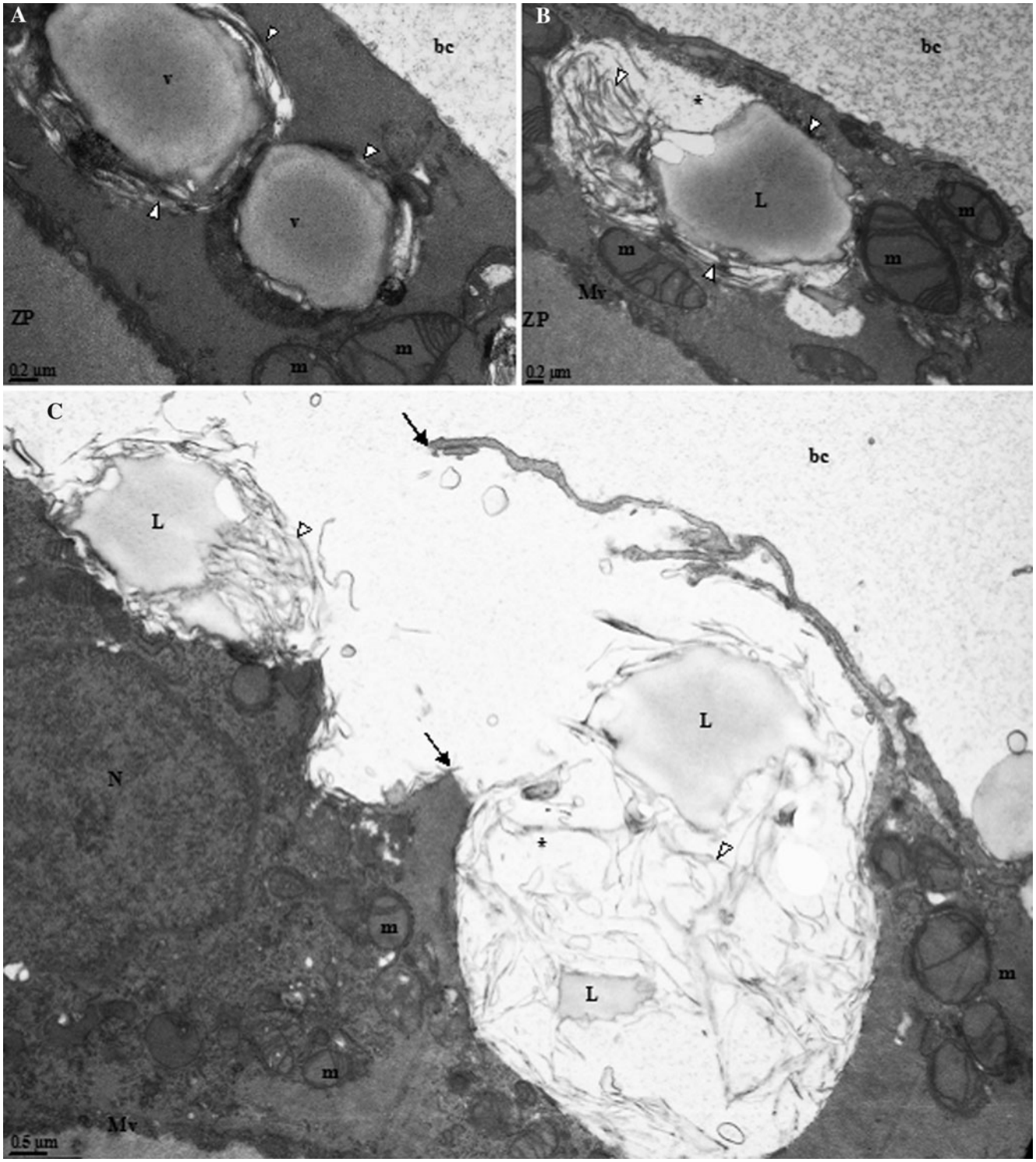

Figure 7 Trophectoderm cells of late blastocysts (*A*–*C*). Vesicles (v), lipid droplets (L), amorphous substance (*), membrane profiles (white arrowheads), exocytosis (arrows), zona pellucida (ZP), microvilli (Mv), blastocoelic cavity (bc), mitochondria (m), nucleus (N).

and blastocyst stages, has been associated with the high protein synthesis described for these stages of development (Hyttel *et al.*, 2001), and that is needed for both housekeeping functions and cell specialization of the developing preimplantation embryo (Hyttel *et al.*, 2000, 2001). At earlier stages, only a dense and spherical nucleolar precursor was observed at the eight-cell stage (Ferrer *et al.*, 1995).

Abundant mitochondria, surrounded by smooth endoplasmic reticulum cisternae, as reported here,

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have also been described in both in *in vivo*-produced (Calarco & McLaren, 1976; Ferrer *et al.*, 1995; Cocero *et al.*, 2002) and in *in vitro*-produced ovine embryos (Rizos *et al.*, 2002). It has been suggested that the mitochondrial structure is altered throughout bovine and ovine preimplantation embryo development, with mitochondria in morulae presenting an increased amount of transverse cristae compared with those found at earlier stages (Ferrer *et al.*, 1995; Crosier *et al.*, 2000, 2001). Mitochondrial function may be used as a predictor of the developmental potential of embryos after cryopreservation, as lipid accumulation depends on the metabolism of mitochondria (Dorland *et al.* 1994; Crosier *et al.*, 2000, 2001; Abe *et al.*, 2002) and resistance to cryopreservation depends on lipid contents (Dobrinsky 1996; Smith & Silva, 2004). Thus evaluation of the degree of ultrastructural damage of mitochondria in frozen–thawed embryos may be a useful indicator of embryo developmental capabilities.

Microvilli were more abundant in blastocysts compared with morulae, confirming previous observations (Ferrer *et al.*, 1995). Junctional complexes only connected to neighbouring peripheral and trophectoderm cells of blastocysts, anchoring them in place. These intercellular junctions and the associated system of intracellular filaments are known to contribute to the retention of the blastocoelic fluid (Van Soom *et al.*, 2001), allowing trophectoderm cells to work as an epithelial barrier (Boni *et al.*, 1999) and have also been previously described in *in vitro*- and *in vivo*-produced ovine embryos (Ferrer *et al.*, 1995; Cocero *et al.*, 2002; Rizos *et al.*, 2002). We show here that inner cell mass cells also develop long cytoplasmic extensions that overlap and interdigitate with neighbouring cells, further contributing to intercellular communication that is essential for the coordination of the ensuing process and leading to the early embryogenic process (Boni *et al.*, 1999). Damage to this cytoplasmic extension network during the freezing/thawing cycles may compromise further embryo development.

The most noticeable aspect of the cytoplasm of ovine embryos was the presence of abundant cytoplasmic small light vesicles with heterogeneous contents. These vesicular structures have been previously described both in *in vivo*- and in *in vitro*-produced bovine, swine and ovine embryos (Calarco & McLaren, 1976; Ferrer *et al.*, 1995; Abe *et al.*, 2002; Cocero *et al.*, 2002; Fabian *et al.*, 2005). The origin and specific role of these vesicles remain uncertain (Calarco & McLaren, 1976; Ferrer *et al.*, 1995).

Due to the close association between structures it could be speculated that light vesicles originate from small cisternae of Golgi and endoplasmic reticulum. Vesicles with heterogeneous contents, including cytosolic structures, small cisternae and vesicles were also seen. It will be interested to verify if they originated from fusion of smaller light vesicles. Even larger vesicles were made through protrusion and engulfment of other light vesicles and lipid droplets, therein forming giant vacuoles mostly filled with fat. Lipid-poor medium-sized vesicles were observed to exocytose into the perivitelline space of compact morulae, while lipid-rich, very large vesicles were restricted to trophectoderm cells and observed to exocytose into the blastocoelic cavity. Light vesicles have been previously found to decrease in number from morulae to blastocyst stages (Calarco & McLaren, 1976; Ferrer *et al.*, 1995), while increasing in size (Ferrer *et al.*, 1995), and have been suggested to behave as lysosome-like organelles that somehow could contribute to the formation of the blastocoelic cavity (Linares & Plõen, 1981; Ferrer *et al.*, 1995; Abe *et al.*, 1999). A similar origin and maturation process of endoplasmic reticulum- and Golgi-derived vesicles was previously described in crustacean oogenesis, during which they were the origin of yolk vesicles, energy-storage organelles that are essential for embryo development, and cortical vesicles, which also secrete materials of the hyaline layer that covers and protects external surfaces of embryonic cells (Carvalho *et al.*, 1998a, 1998b, 1999).

Thus, it seems plausible that the described vesicular structures may play a role in the formation of the blastocoelic cavity, morula compaction and trophectoderm development.

In conclusion, the findings of this study have added detailed information on the cellular components of *in vivo*-produced ovine embryos, some of which were described here for the first time. Taken together, the information provided can be used as a basis to assess structural differences between fresh *in vivo*- and *in vitro*-produced embryos, as well as to contribute to better evaluation of the damage produced by the freezing and thawing processes.

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