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Development and viability of bovine preimplantation embryos after the *in vitro* infection with bovine herpesvirus-1 (BHV-1): immunocytochemical and ultrastructural studies

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Summary

The aim of our study was to examine whether: (1) the exposure of bovine embryos to the BHV-1 virus *in vitro* can compromise their further development and alter the ultrastructural morphology of cellular organelles; (2) whether the *zona pellucida* (ZP) can be a barrier protecting embryos against infection; and (3) whether washing with trypsin after viral exposure can prevent virus penetration inside the embryo and subsequent virus-induced damages. The embryos were recovered from superovulated Holstein-Friesian donor cows on day 6 of the estrous cycle. Only compact morulas or early blastocysts were selected for experiments with virus incubation. We used the embryos either with intact ZP (either with or without trypsin washing) or embryos in which the ZP barrier was avoided by using the microinjection of a BHV-1 suspension under the ZP. ZP-intact embryos ($n = 153$) were exposed to BHV-1 at $10^{6.16}$ $TCID_{50}$/ml for 60 min, then washed in trypsin according to IETS guidelines and postincubated in synthetic oviduct fluid (SOF) medium for 48 h. Some of the embryos ($n = 36$) were microinjected with 20 pl of BHV-1 suspension under the ZP, the embryos were washed in SOF medium and cultured for 48 h. Embryo development was evaluated by morphological inspection, the presence of viral particles was determined both immunocytochemically, using fluorescent anti-IBR–FITC conjugate and by transmission electron microscopy (TEM) on the basis of the ultrastructure of the cellular organelles.

It was found that BHV-1 exposure impairs embryo development to higher preimplantation stages independent of the presence of the ZP or the trypsin treatment step, as most of the embryos were arrested at the morula stage when compared with the control. Immunofluorescence analysis confirmed the presence of BHV-1 particles in about 75% of embryos that were passed through the trypsin treatment and in all the BHV-1-microinjected embryos. Ultrastructural analysis, using TEM, revealed the presence of virus-like particles inside the BHV-1-exposed embryos, where the trypsin washing step was omitted. Conversely, in trypsin-treated BHV-1-exposed embryos, TEM detected only the envelope-free virus-like particles adhered to pores of the ZP. The embryos that were microinjected with BHV-1 suspension showed the presence of BHV-1 particles, as well as ultrastructural alterations in cell organelles. Taken together these findings may suggest that BHV-1 infection compromises preimplantation development of bovine embryos *in vitro* and therefore the ZP may not be enough on its own to prevent virus-induced damage, unless it is not accompanied with trypsin washing.

Keywords: Bovine herpesvirus-1, Embryo infection, Trypsin washing, Ultrastructure

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Introduction

Bovine herpesvirus-1 (BHV-1) is from the Herpesviridae group of enveloped DNA viruses and belongs to the oldest known viruses that often occur among mammalian species. In cows, infections with BHV-1 can result in respiratory and reproductive disorders,

including rhinotracheitis, vulvovaginitis, endometritis and abortions. In bulls, BHV-1 is usually replicated initially in preputial mucosa, penis and urethra and subsequently the semen is probably contaminated from infected mucosa during ejaculation. If the infection in cows after insemination with infected semen will occur, depends mostly on the virus strain and the amount of virus in the insemination dose. It was found that the cows that were inseminated with semen containing more than $10^{6.3}$ $TCID_{50}$ of BHV-1 were irreversibly infected, however only six out of 25 cows that were infected with semen containing less than 200 $TCID_{50}$, were seroconversed (Van Engelenberg *et al*., 1994). Except for those studies on the possibility of pathogen invasion via sperm, many works to date have been done on embryos.

Guerin *et al*. (1990), using BHV-1-infected sperm and BHV-1-exposed oocytes in an *in vitro* embryo production (IVP) procedure, have found that BHV-1 did not influence oocyte maturation, but did significantly reduce the *in vitro* fertilization (IVF) outcome and elevated the frequency of sperm abnormalities such as sperm head decondensation. Similarly, Bielanski & Dubuc (1994) reported that oocytes that had recovered from BHV-1-infected cows could be matured and fertilized *in vitro* and resulted in transferable embryos, but the ratio of morphologically normal transferable blastocysts was decreased.

According to Vanroose *et al*. (2000), the *zona pellucida* (ZP) can protect preimplantation embryos against viral infection. However, the risk exists that viral particles can be trapped in the outer layers of the ZP. One way to remove BHV-1 virus from the surface of embryos is a procedure of washing with trypsin, which was firstly reported by Thibier & Nibart (1987) and subsequently was recommended by the IETS (Stringfellow, 1998).

Recent data suggest that the efficacy of BHV-1 removal depends on the origin of the embryos. Thus, in *in vivo* isolated bovine embryos with intact ZP, followed by co-incubation with the BHV-1 and subsequent washing in trypsin solution, neither presence of the virus in embryos nor their transmission to recipients and offspring, was proved (Edens *et al*., 2003). In contrast, several studies on IVP embryos reported that the trypsin washing procedure is not sufficient either to remove the BHV-1 virus from the embryo or to improve the rate of transferable embryos (Bielanski & Dubuc, 1993, 1994; Belanski *et al*., 1997; D'Angelo *et al*., 2002; Edens *et al*. 2003). These data suggest that there is no unified opinion on the efficacy of using trypsin washing for removal of virus and to prevent infectivity of embryos following transfer.

For the detection of viruses in embryos, immunofluorescence techniques and electron microscopy (EM) were applied. Vanroose *et al*. (1997), using an immunofluorescence technique, localized virus replication in blastomeres from ZP-free morulas. The embryos with intact ZP were protected from infection, however hatching blastocysts, after exposure to virus, exhibited fluorescein-conjugated viral antigen in about 13% of cells and subsequently degenerated (Vanroose *et al*., 1996). The detection of the presence of BHV-1 (Schlafer *et al*., 1990) or BVDV (Gillespie *et al*., 1990) viruses in embryos by electron microscopy did not bring the expected results. Although the infection with BHV-1 resulted in embryonic degeneration and death, morphological identification of viruses was complicated, making assessment by EM unreliable. Conversely, Bowen *et al*. (1985), using EM, demonstrated replication of viral agents.

The aim of this work was to evaluate the developmental capacity and ultrastructure of early bovine embryos incubated *in vitro* with bovine herpesvirus-1 (BHV-1) and the possibility of virus penetration across the ZP into the subzonal space and cell structures of embryos. For exposure to BHV-1 we used both ZP-enclosed embryos and embryos in which the ZP barrier was avoided by microinjection of a viral suspension into the subzonal space. The presence of viral particles in embryos was evaluated using an immunofluorescence technique of whole embryo preparations and by transmission electron microscopy (TEM) of embryo sections.

Materials and methods

Collection of bovine embryos *in vivo*

For the production of bovine preimplantation embryos *in vivo*, Holstein-Friesian (HF) donor cows were superovulated between the tenth and twelfth days of the previous estrous cycle by intramuscular administration of 24 mg FSH (folicotropin inj. ad us. vet., Spofa, Prague, Czech Republic), given in a series of decreasing doses over a 4-day period. Oestrus was induced by i.m. administration of 0.75 mg prostaglandin $F_{2\alpha}$, i.e. 750 µg cloprostenolum (Oestrophan inj. ad us. vet., Léčiva, Prague, Czech Republic) in the morning and evening of the third day of FSH treatment. Oestrous detection was performed twice per day beginning 24 h after the first prostaglandin $F_{2\alpha}$ injection. Donor cows were artificially inseminated 12 and 24 h after the first standing of oestrous with the semen from a sire of proven fertility. Embryos were recovered by uterine flushing with Dulbecco's phosphate-buffered saline (PBS) supplemented with 1% bovine serum after the slaughter of donor cattle on day 6 of the estrous cycle (day 0 = first standing estrous). The embryos were classified according to a general criteria of developmental competence and quality evaluation at ×100 magnification using a stereomicroscope (Wright,

1998) and only compact morulas and early blastocysts were selected for further experiments.

Exposure of embryos to bovine herpesvirus-1 (BHV-1)

After evaluation, the embryos were exposed to bovine herpesvirus-1 (BHV-1) at $10^{6.16}$ $TCID_{50}$/ml for 60 min in an atmosphere containing 5% CO_2. Following viral exposure, embryos were washed in trypsin according to the IETS guidelines (Stringfellow, 1998). The first five and the last five washings in PBS supplemented with penicillin, streptomycin and BSA were interspersed by the sixth and seventh washes, which were in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} plus sterile trypsin (1:250). Following washing, the embryos were postincubated for 48 h in synthetic oviduct fluid (SOF) medium with BSA.

Microinjection of bovine herpesvirus-1 (BHV-1) under the zona pellucida of bovine embryos

BHV-1 were directly inoculated by microinjection of approx. 20 pl of viral suspension under the ZP of morulas using manual micromanipulation units (Alcatel, France) connected to an automatic microinjector (Eppendorf) under the Olympus (IMT-2) microscope. Briefly, embryos were placed to TCM-199 medium with GlutaMAX 1 (Gibco BRL) into the micromanipulation chamber and fixed by a glass holding pipette. The ZP was penetrated, the BHV-1 was injected subzonally and the injection pipette was quickly withdrawn. The embryos were washed three times in the SOF medium and cultured for 48 h at 39 °C in an atmosphere made up of 5% CO_2, 5% O_2 and 90% N_2.

Determination of virus particles in the embryos using an immunofluorescent assay

After infection with bovine herpesvirus-1 (BHV-1) and 48 h of *in vitro* postculture, the embryos were processed and analyzed for the presence of BHV-1. Embryos were washed four times in 100 µl drops of PBS–PVP by transferring the embryos from drop to drop. Then the embryos were fixed in 4% (w/v) paraformaldehyde in PBS pH 7.4 for 30 min at room temperature, washed three times (2 min/wash) in PBS–PVP and permeabilized in 0.5% Triton X-100 (v/v) for 15 min. After washing in PBS–PVP (2 min) and blocking in PBS with 20% of bovine fetal calf serum (20 min), the embryos were incubated with anti-IBR/IPV FITC conjugated antibody (30 min). Following washing three times in PBS–PVP (2 min/wash) the embryos were mounted on slides with 5.5 µl of mounting medium Vectashield (Vector Laboratories, Burlingame, CA, USA) and evaluated under a Leica fluorescence microscope equipped with a digital camera DFC-480.

Some of the embryos, after exposure to BHV-1 and subsequent washing, were disaggregated using freezing–thawing in TCM-199 medium and subsequent vortexing, in order to determine virus titre. Virus infectious titre was evaluated by counting virus plaques after co-incubation of disaggregated embryos on a monolayer of Madin–Darby bovine kidney (MDBK) cells. This titration test showed that 36 h following postculture, the viral titre was approximately 10–50 fold higher than immediately after exposure to virus.

Electron microscopy

Bovine embryos after infection with bovine herpesvirus-1 (BHV-1) were fixed in aldehyde mixture (2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M cacodylate buffer, pH 7.1–7.3) for 1 h and washed in cacodylate buffer. One hour following fixation in 1% osmium tetroxide in cacodylate buffer the embryos were rinsed in distilled water, dehydrated in acetone series (30, 50, 70, 95 and 100%) and embedded in Durcupan ACM (Fluka). Blocks with embryos were cut on a LKB-Nova ultramicrotome into semi-thin sections (1–2 µm) and stained with toluidine blue. Thin sections were contrasted with uranyl acetate and lead citrate and viewed in a JEM 100 CX II (Jeol) electron microscope operating at 80 kV.

Negative staining of BHV-1 virus

Virus suspension was mixed with an equal volume of negative dye, 2% phosphotungstic acid (PTA, pH 6.0–7.0) and put onto an electron microscope grid covered with formvar (Agar Scientific Ltd). After 30 s, excessive fluid was sucked off and the preparation was viewed by a JEM 100 CX II (Jeol, Japan) microscope at accelerating voltage 60 kV.

Results

Preimplantation development of BHV-1-exposed embryos

The evaluation of the developmental potential of intact embryos (control), cultured up to the eighth day, revealed that 36% of embryos reached the expanded blastocyst stage and 18% of embryos reached early blastocyst or blastocyst stage. About 27% of embryos had been arrested at the morula stage and could not develop further. When the embryos with intact ZP were incubated for 60 min with BHV-1, following washings in trypsin and 48 h postculture in virus-free medium, only 7.8% of embryos had developed to expanded blastocysts, 9.8% to blastocyst and about 2% to early blastocyst stage. Most embryos (80%) were developmentally arrested and subsequently degenerated (Table 1).

Table 1 Preimplantation development of intact or BHV-1-exposed bovine embryos, *n* (%)

Experimental groups	Total no. embryos	Developmental stages			
		Arrested at morula stage	EB1	B1	ExB1
Intact (control)	66	18 (27.2)[a]	12 (18.2)[a]	12 (18.2)[a]	24 (36.4)[a]
BHV-1 incubated (ZP+)	153	123 (80.4)[b]	3 (2.0)[b]	15 (9.8)[b]	12 (7.8)[b]
BHV-1 microinject (ZP–)	36	36 (100)[b]			

Bl, blastocyst; EBl, early blastocyst; ExBl, expanded blastocyst.
[a,b]Values with different superscripts differ significantly ($p < 0.05$, chi-squared test).

When the barrier formed by the ZP was omitted, by using microinjection of BHV-1 suspension under the ZP, all of the embryos were infected (based on the presence of IBR–FITC conjugate, Fig. 1*G*, *H*) and the infection exhibited embryocidal character. After 48 h following microinjection none of the total of 36 embryos developed to blastocyst stage, they were cleavage-arrested and subsequently degenerated.

The presence of BHV-1 in embryos using immunofluorescent staining

The embryos with intact ZP, following virus incubation and subsequent washing according to IETS procedure, were subjected to direct immunofluorescent analysis using polyspecific anti-IBR/IPV–FITC conjugate. Out of the 82 embryos analyzed, the presence of BHV-1 was revealed in 61 embryos (74.4%) inside the embryo, either in the inner cell mass (ICM) area (Fig. 1*A*) or in the trophectoderm (TE) area (Fig. *1B*) or both compartments (Fig. 1*C*, *D*), whilst in only 21 embryos (25.6%) BHV-1 virus was not detected within the embryo, but IBR–FITC fluorescence was noted on the surface of ZP (Fig. 1*E*, *F*).

In the embryos in which the ZP was omitted using BHV-1 microinjection, a BHV–FITC conjugate fluorescent signal was localized inside the embryo in the blastomere nuclei (Fig. 1*G*). Control embryos (non-microinjected with BHV-1) showed only slight non-specific background (Fig. 1*H*).

Ultrastructure of intact or BHV-1 exposed embryos using TEM

Negative staining revealed that bovine herpesvirus-1 (BHV-1) is 120–150 nm in size. The virion (complete virus particle) was spherical in shape and was formed by four structural components: matrix, capsid, tegument and envelope. Central matrix (core) was formed by DNA and protein, surrounded by a capsid that was 100 nm in diameter (data not shown).

Using electron microscopy we have confirmed that the embryos from the control group with intact ZP showed undamaged blastomeres and transparent perivitelline spaces. The ZP was about 10–15 μm in width and consisted of two layers – external (8–10 μm) and internal (3–5 μm). The surface of the external ZP layer had a rough, sponge-like appearance with numerous pores. The pore size in the external layer reached about 200 nm. The internal layer is formed by a dense fibrillar reticulum without larger or smaller pores (data not shown). The cytoplasm of trophoblastic and embryoblastic cells contained numerous mitochondria, granular endoplasmic reticula and Golgi complexes without changes. Embryoblastic nuclei had a round shape (Fig. 2*A*), whilst trophoblastic nuclei were oval, euchromatic (Fig. 2*B*) with several reticular nucleoli. Microvilli on trophoblast cell surface were intact.

The embryos, surrounded by ZP (ZP+), after incubation with BHV-1 virus and subsequent trypsin treatment according to IETS protocol, had viral particles that adhered to pores of the ZP. Most of these had destroyed envelopes and disintegrated teguments. Several of the envelope-free nucleocapsids were adhered to the surface or had entered the pores of the outer ZP layer (Fig. 3*A*). In ZP+ embryos, which were not treated with trypsin ($n = 9$), the presence of BHV-1 virus was detected inside the embryo. The accumulation of a large amount of envelope-enclosed virus-like particles was observed in numerous vacuoles of the disintegrated cytoplasm and in multivesicular bodies (Fig. 3*B*).

The embryos with intact ZP, microinjected with BHV-1 suspension, exhibited an expressed vacuolization in trophoblastic and embryoblastic cells. Significant accumulation of viral nucleocapsules within the perivitelline space (Fig. 4*A*) and in the nuclei, as well as the appearance of complete virus-like particles around the nuclei and cytoplasm caused changes mainly in endoplasmic reticula and mitochondria. Rough endoplasmic reticula were substantially dilated and virus-like particles (nucleocapsids) appeared as granules in the cytoplasm with the formation of vacuoles (Fig. 4*B*). Several particles had adhered to the trophoblast cytoplasm membrane. The viral envelope, after absorption to trophoblast membrane receptors, fused with the membrane (Fig. 4*A*) and

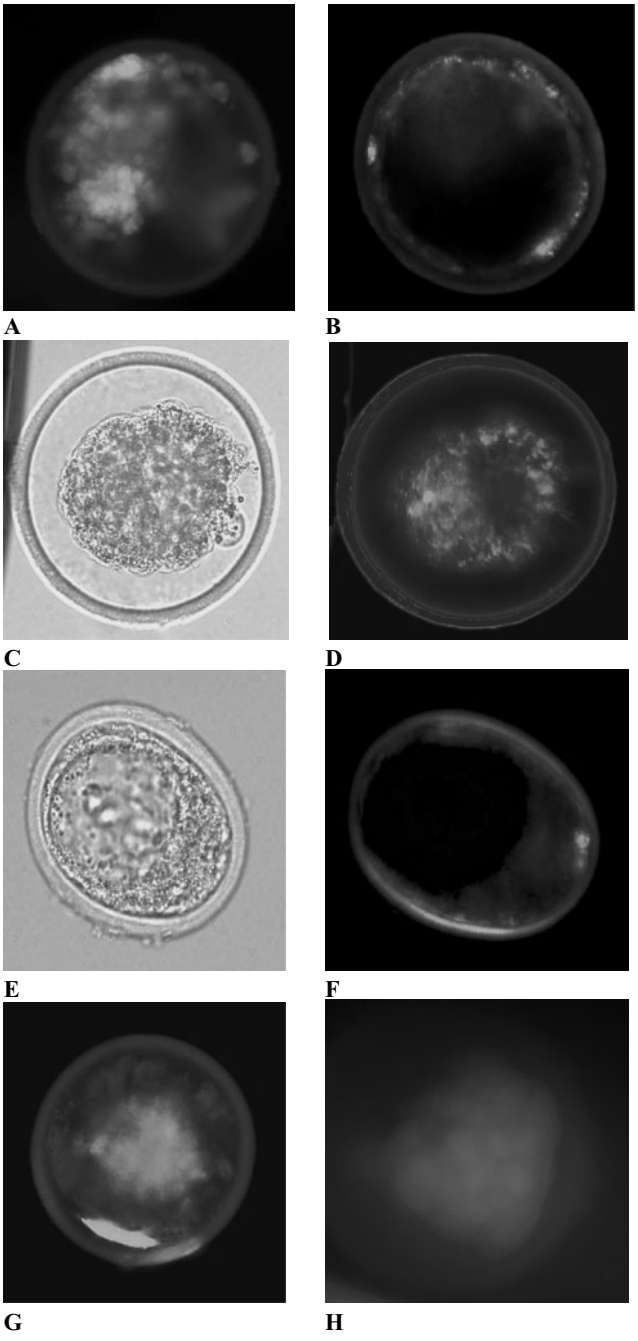

Figure 1 The presence of BHV-1 in bovine embryos shown by of anti-IBR–FITC-positive staining. Localization of BHV-1 in the inner cell mass (*A*) or in the trophectoderm (*B*) area of bovine blastocysts. Light microscope (*C*) and fluorescent (*D*) images of BHV-1-infected blastocysts with IBR–FITC-positive blastomeres. Light microscope (*E*) and fluorescent (*F*) images of BHV-1-exposed blastocysts, negative for IBR–FITC. (*G*) IBR–FITC-positive embryo 48 h following microinjection with a BHV-1 suspension. (*H*) IBR–FITC-negative embryo not microinjected with BHV-1 (control).

the capsid was released into the cytoplasm (Fig. 4*C*). Viruses were transported via vacuoles and dilated channels of endoplasmic reticula towards the nuclear membranes, through which the viral DNA–protein complex migrated into the nucleus. In the nucleus, newly formed nucleocapsid, passing through the nuclear membrane or other cellular membranes, acquired the envelope (data not shown).

Discussion

In our study we examined whether: (1) the exposure of bovine embryos to the BHV-1 virus *in vitro* can compromise their further development and alter the ultrastructural morphology of cellular organelles; (2) whether the ZP can be a barrier protecting embryos against infection; and (3) whether trypsin washing after viral exposure can prevent virus penetration inside the embryo and subsequent virus-induced damages. We used in our experiments for virus incubation, embryos that had either intact ZP (either with or without trypsin washing) or embryos in which the ZP barrier was avoided by microinjecting a BHV-1 suspension under the ZP.

It was found that BHV-1 exposure impairs embryo development to higher preimplantation stages independent of the presence of ZP or the trypsin treatment step, since most of embryos were arrested at the morula stage compared with the control. Immunofluorescence analysis confirmed the presence of BHV-1 particles in about 75% of the embryos that were passed through trypsin treatment and in all the BHV-1-microinjected embryos. Ultrastructural analysis revealed the presence of virus particles only inside those BHV-1-exposed embryos in which the trypsin step was omitted, whereas in trypsin-treated BHV-1-exposed embryos TEM analysis detected virus particles adhered to the pores of the ZP. Moreover, all BHV-1-microinjected embryos exhibited the presence of BHV-1 particles and showed ultrastructural alterations in cell organelles.

It is generally known that the ZP plays an important role in the protection of preimplantation embryos against viral infection. Intact ZP of bovine *in vitro*-matured (IVM) oocytes and *in vitro*-produced (IVP) embryos is constructed in such a way that BHV-1 should not be able to traverse the ZP and reach the embryonic cells (Vanroose *et al*., 2000). However, the risk exists that virus particles could be trapped in the outer layers of the ZP. An earlier study reported that bovine embryos (from 16-cell to blastocyst stage) with intact ZP that had been exposed to BHV-1 for 24 h did not show embryonic infection, however virus was recovered from most of these embryos even after extensive washing (Singh *et al*., 1982). These observations mean that although the ZP is an effective barrier to BHV-1, the virus may stick to this structure.

Guerin *et al*. (1990), using oocytes exposed to BHV-1 during maturation or fertilization, have found

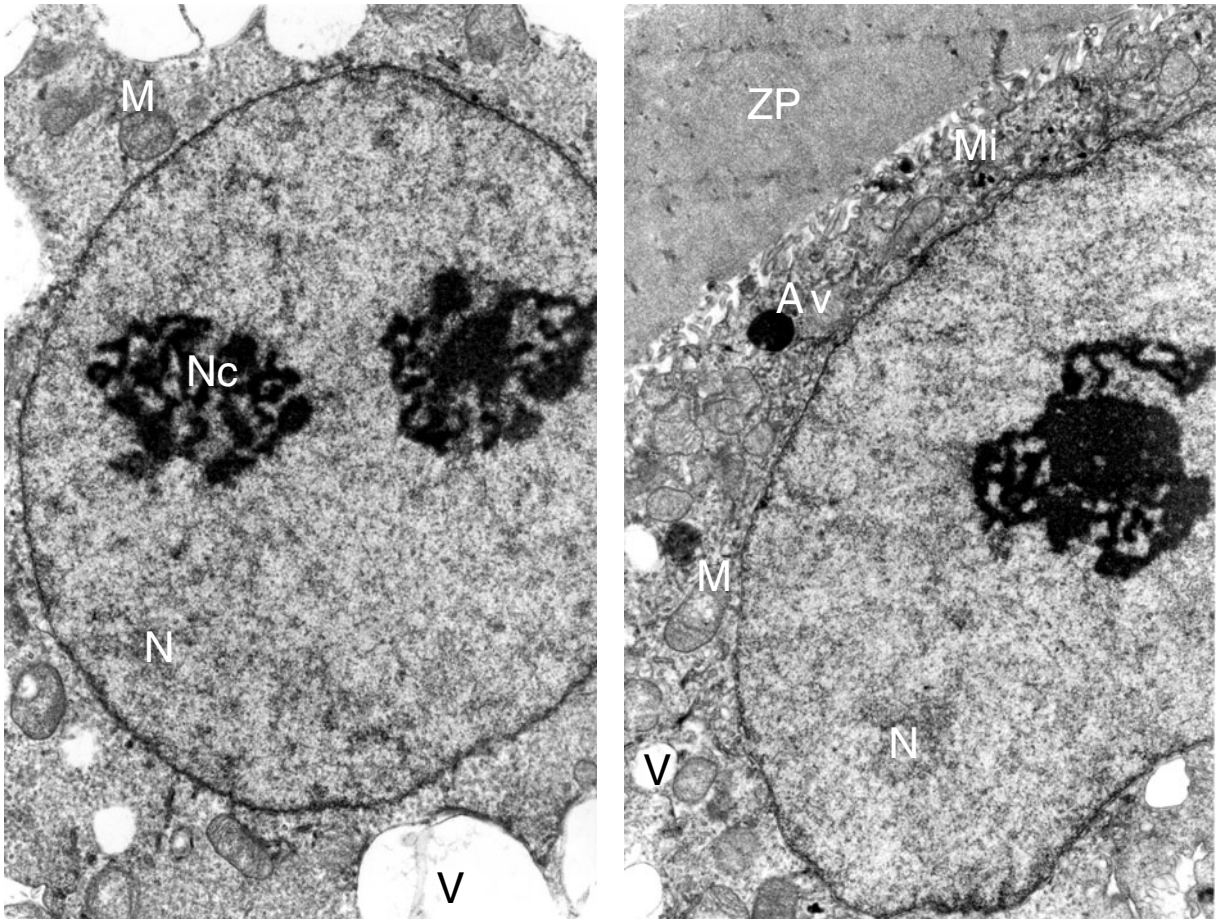

Figure 2 Ultrastructural morphology of bovine intact blastocysts (control). Normal reticulated architecture of typical nucleolus (Nc) with fibrillar centres and morphologically normal mitochondria (M) in the cytoplasm. (*A*) Round-shaped nucleus (N) of embryoblastic cell. (*B*) Oval euchromatic nucleus of trophoblast cells with reticular nucleoli. (Av, autophagic vacuole; Mi, microvilli; V, vacuole; ZP, zona pellucida; ×11 520).

that BHV-1 did not influence oocyte maturation, but significantly reduced IVF outcome (65%) compared with control (85%). Moreover, the frequency of sperm abnormalities, such as sperm head decondensation, was elevated to 49% versus 4% in control. The authors concluded that BHV-1 not only absorbed to gametes, but also diminished their fertilizing capacity. Bielanski & Dubuc (1993) after adding 10^6 $TCID_{50}$/ml of BHV-1 to fertilizing medium observed only a slight retardation of embryonic development, but they succeed to isolate virus from all embryos, although the embryos were washed in trypsin more times than the IETS protocol recommended (Stringfellow, 1998). In another experiment, Bielanski & Dubuc (1994) found that embryos derived from oocytes that were recovered from experimentally and naturally infected cows were positive for BHV-1, and the ratio of morphologically normal transferable blastocysts was decreased despite trypsin washing.

In vivo isolated bovine embryos with intact ZP were co-incubated with the BHV-1 virus and subsequently washed in trypsin solution. Presence of the virus in embryos, or their transmission to recipients and offspring, was not proven (Edens *et al*., 2003). The efficiency of trypsin washing of BHV-1-infected embryos was confirmed by Thibier & Nibart (1987), where, after transfer of fresh and frozen-thawed embryos, all recipients were seronegative. In our study, we also used *in vivo* derived embryos. In our experiments, 80% of ZP-intact embryos, after BHV-1 incubation, were arrested at the morula stage, however TEM did not reveal viral particles inside the embryos, excepting those in which trypsin washing was omitted. Similarly, Vanroose *et al*. (1999) failed to detect virus replication in the embryonic cells of IVP embryos exposed to BHV-1, although these embryos had lower rates of cleavage and blastocyst formation.

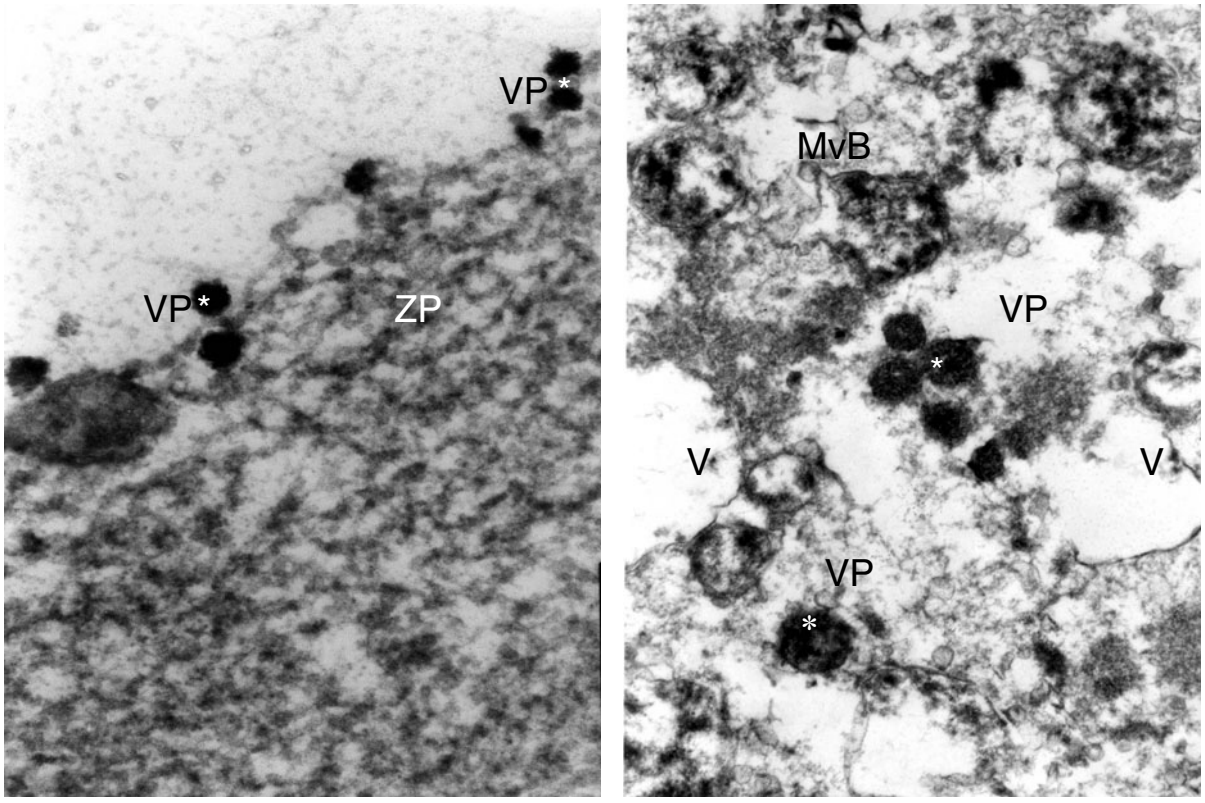

Figure 3 Ultrastructural morphology of bovine ZP-surrounded blastocysts after incubation with a BHV-1 suspension. (*A*) Following trypsin washing according to the IETS Manual. Envelope-free virus-like particles (VP) only adhered to pores of the ZP (marked by an asterisk), but are not detected inside the embryo (×96 000). (*B*) Trypsin washing was omitted. Virus-like particles were detected inside the embryo and accumulated in numerous vacuoles (V) of the disintegrated cytoplasm and in multivesicular bodies (MvB) (×21 000).

After several verifying studies Bielanski *et al*. (1997) concluded that IVP embryos, compared to *in vivo*-derived embryos, have a higher predisposition to transmit BHV-1 and it is much more difficult to eliminate virus even using IETS trypsin washing. Observations of D'Angelo *et al*. (2002) and Edens *et al*. (2003) that trypsin washing was not sufficient to remove BHV-1 from bovine IVP blastocysts support this statement.

Using electron microscopy analysis the presence of BHV-1 was not proved (Gillespie *et al*., 1990; Schlafer *et al*., 1990), although the infection resulted in embryonal degeneration and death. However, in an earlier study using electron microscopy, Bowen *et al*. (1985) demonstrated replication of viral agents in bovine *in vivo*-derived embryos, but these embryos were hatched, i.e. released from the ZP. Viral nucleocapsides were observed in the nuclei of a majority of trophoblast cells, while complete viral particles were present in the area of the nuclear envelope and in cytoplasmic vacuoles. At 24 h after exposure, complete virus was found only in one of five embryos. However, at 48 h after exposure, virus was demonstrated within a majority of trophoblastic cells in each embryo. Our study describes for a first time the presence of virus-like particles and the changes that occurred because of viral infection of embryos. Most morphological alterations were presented by the vacuolization of embryonic cells, disintegrated cytoplasm, swollen mitochondria and dilatation in rough endoplasmic reticula. Appearance of virus particles was revealed in numerous vacuoles of disintegrated cytoplasm, around nuclei and inside them. These changes appeared to be at a similar level either in ZP-intact or BHV-microinjected embryos.

The consequences of viral infection on the embryo's viability may depend on the titre of virus. Viral infectivity test based on the number of virus plaques in MDBK cells showed approximately a 10–50 fold increase in BHV-1 titre in our embryos infected in culture. Our observations of BHV-1 presence inside ZP-enclosed embryos may be explained by substantially higher concentrations of the BHV-1 virus in our culture conditions than in the natural milieu of the reproductive tract, as well as a consequence of physical impairment of ZP because of possible non-specific effects.

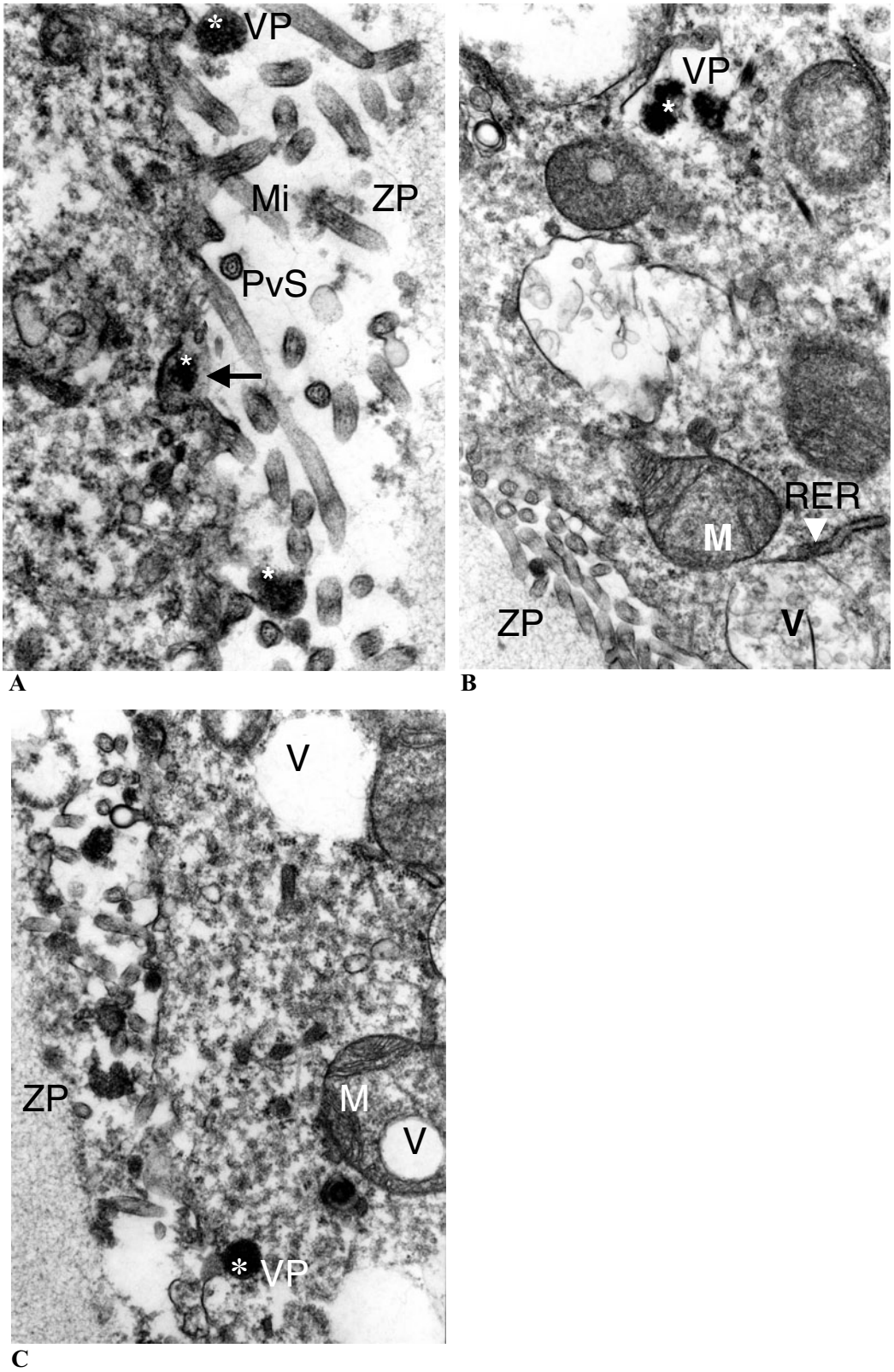

Figure 4 Ultrastructural morphology of bovine ZP-surrounded blastocysts after microinjection of a BHV-1 suspension subzonally. (*A*) Accumulation of virus-like particles within the perivitelline space (PvS). Viral envelope is fused with the cell membrane (arrow, ×43 000). (*B*) Virus particles appear as granules in the cytoplasm with the formation of vacuoles. Rough endoplasmic reticulum (RER) is substantially dilated (white arrow) forming a pathway for the moving of virus particles (×29 000). (*C*) Virus-like particles are released into the cytoplasm. Mitochondria (M) are swollen, loss of cristae, with incorporated vacuoles (V, vacuolation of mitochondria; ×29 000).

Based on these observations, we propose that either: (1) our embryos have somehow permeable or damaged ZP; or (2) the ZP is not reliable barrier against BHV-1 infection. Since ZP-intact embryos washed with trypsin did not show any virus particles inside the embryo, but ZP-embryos without trypsin treatment did, this may indicate that trypsin washing according to the IETS guidelines can be an effective way to eliminate BVH-1 virus from the surface of embryos. Our TEM findings showed that, in the trypsin-treated embryos, virus particles adhered to the ZP and were either presented by envelope-free nucleocapsid or had destroyed the envelope and disintegrated the tegument. This observation may, at least in part, explain the mechanism of the trypsin treatment effect in preventing BHV-1 infection in BHV-1 exposed embryos. Taken together these findings may suggest that BHV-1 infection compromises preimplantation development of bovine embryos *in vitro* and that the ZP itself may not be enough to prevent virus-induced damage, unless it is unaccompanied by trypsin washing.

Acknowledgments

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