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ULTRASTRUCTURAL ALTERATIONS IN SOME ORGANELLES OF BOVINE EMBRYOS EXPOSED TO BOVINE HERPESVIRUS-1 (BHV-1) *IN VITRO*

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ABSTRACT

The aim of this study was to evaluate the ultrastructure of early bovine embryos after the microinjection of bovine herpesvirus (BHV-1) viral suspension into the subzonal space and the possibility of virus penetration into the cellular structures of embryos. The embryos (morula, early blastocyst) were recovered from superovulated Holstein-Friesian donor cows on Day 6 of the estrous cycle. Part of embryos were microinjected with the suspension of BHV-1 under the ZP, 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 (fluorescent anti-IBR-FITC conjugate) and by transmission electron microscopy (TEM) on the basis of ultrastructure of cellular organelles. It was found that BHV-1 microinjection impairs embryo development to higher preimplantation stages compared with the control. Immunofluorescent analysis confirmed the presence of BHV-1 particles in all BHV-1-microinjected embryos. TEM detected the presence of BHV-1 particles and showed ultrastructural alterations in cell organelles. Our study describes for the first time the presence of virus-like particles and changes occurred as a consequence of viral infection of embryos. Virus-like particles were revealed in vacuoles of disintegrated cytoplasm, around nuclei and inside them. We suppose that consequences of viral infection on the embryo viability may depend also on the titre of virus. Based on these observations we conclude that BHV-1 infection compromises preimplantation development of bovine embryos *in vitro*.

Key words: bovine herpesvirus-1; embryo microinjection; ultrastructure

INTRODUCTION

Bovine herpesvirus-1 (BHV-1) from *Herpesviridae* group of enveloped DNA viruses belongs to the oldest known viruses, which often occurred among mammalian species. In cows, infections with BHV-1 can result in respiratory and reproductive disorders. Bielanski and Dubuc (1994) and Guerin et al. (1990) reported that oocytes recovered from BHV-1-infected cows can be matured and fertilized *in vitro* and resulted in transferrable embryos, but the ratio of morphologically normal transferrable blastocysts is decreased. According to Vanroose et. al. (2000) 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 of ways to remove BHV-1 virus from the surface of embryos is a procedure of washing with trypsin, which was firstly reported by Thibier and Nibart (1987) and several years after was recommended by International Embryo Transfer Society (IETS; Stringfellow, 1998).

For detection of viruses in embryos immunofluorescent technique and electron microscopy (EM) were applied. Vanroose et al. (1997), using immunofluorescent

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technique, localized virus replication in blastomeres of ZP-free morulas. 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 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. On the other hand, Bowen et al. (1985) using EM demonstrated replication of viral agents.

The aim of this work was to evaluate the ultrastructure of early bovine embryos after the microinjection of viral suspension of BHV-1 into the subzonal space and the possibility of virus penetration into the cellular structures of embryos.

MATERIAL AND METHODS

For production of bovine pre-implantation embryos *in vivo*, Holstein-Friesian (HF) donor cows were superovulated between 10th and 12th days of the previous oestrous cycle by intramuscular administration of 24mg 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 first standing of oestrous with the semen from a sire of proven fertility. Embryos were recovered by uterine flushing with Dulbecco phosphate-buffered saline (PBS) supplemented with 1% bovine serum after slaughter of donor cattle on Day 6 of the estrous cycle (Day 0= first standing oestrous). The embryos were classified according to general criteria of developmental competence and quality evaluation at 100x magnification using stereomicroscope (Pivko et al., 2000; Wright, 1998) and only compact morulas and early blastocysts were selected for further experiments.

After the evaluation a part of embryos (n = 36) were microinjected with approx. 20 picolitres of BHV-1 viral suspension at 106.16 TCID50 /ml under the *zona pellucida* using manual micromanipulation units (Alcatel, France) connected to automathic microinjector (Eppendorf) under the Olympus (IMT-2) microscope. After the microinjection the embryos were washed three times in SOF medium and cultured for 48 hours at 39°C in an atmosphere of 5% CO_2, 5% 0_2, 90% N_2. After the microinjection of bovine herpesvirus-1 (BHV-1) and 48 hours of *in vitro* post-culture the embryos were processed and analyzed for the presence of bovineherpesvirus-1 (BHV-1) using immunocytochemical technique and electron microscopy (EM).

For immunofluorescent analysis embryos were washed four times in 100 µl drops of PBS-PVP by transferring the embryos drop by 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% (v/v) Triton X-100 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 antibody conjugated with FITC (30 min). Following three-times washing 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 the Leica fluorescent microscope equipped with digital camera DFC-480.

For EM the embryos were fixed in aldehyde mixture (2.5% glutaraldehyde and 2% paraformaldehyde in 0.15M 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 aceton series (30, 50, 70, 95 and 100%) and embedded in Durcupan ACM (Fluka). Blocks with embryos were cut on a LKB-Nova ultramicrotome into semithin 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, Japan) electron microscope operating at 80 kV.

RESULTS AND DISCUSSION

The evaluation of developmental potential of intact embryos (control), cultured up to Day 8, revealed that about 73% of embryos reached the early blastocyst, blastocyst and expanded blastocyst stage and about 27% of embryos had been arrested at morula stage and could not develop further. When the barrier of ZP was omitted using the microinjection of BHV-1 suspension under the ZP, all the embryos were infected (based on the presence of IBR-FITC conjugate under fluorescent microscope) and the infection exhibited embryocidal character. After 48h following microinjection none of the all 36 embryos advanced to blastocyst stage, they were cleavage-arrested and subsequently degenerated.

In those embryos, where ZP was omitted using the BHV-1 microinjection, fluorescent signal of BHV-FITC conjugate was localized inside the embryo in blastomere nuclei. Control embryos (non-microinjected with BHV-1) showed only slight non-specific background.

Electron microscopy analysis showed that the embryos from control group with intact ZP showed undamaged blastomeres and transparent perivitelline space. ZP was about 10-15 µm in width and consisted of two layers – external (8-10 µm) and internal (3-5 µm).

External layer of ZP surface had a rough, sponge-like appearance with numerous pores. The pore size in external layer reached about 200 nm. Internal layer is formed by dense fibrillar reticulum without larger or smaller pores (data not shown). Cytoplasm of trophoblastic and embryoblastic cells contained numerous mitochondria, granular endoplasmic reticulum and Golgi complex without changes. Embryoblastic nuclei were of round shape (Fig.1), whilst trophoblastic nuclei were oval, euchromatic with several reticular nucleoli. Microvilli on trophoblast cell surface were intact.

In embryos with intact ZP, microinjected with BHV-1 suspension, EM revealed an occurrence of microfilaments, disintegration of cytoplasm and expressed vacuolization in embryoblastic (Fig. 2) and trophoblastic (Fig. 3) cells. Significant accumulation of viral nucleocapsules within the perivitelline space (Fig. 3) and in the nuclei, as well as the appearance of complete virus-like particles around nuclei and cytoplasm caused changes mainly in endoplasmic reticulum and mitochondria. Rough endoplasmic reticulum was substantially dilated and virus-like particles (nucleocapsids) appeared as granules in cytoplasm with the formation of vacuoles (Fig. 4). Several particles were observed adhering to trophoblast cof the cytoplasmic membrane. The viral envelope, after absorption to trophoblast membrane receptors, fused with the membrane and the capsid was released into the cytoplasm (Fig. 3). Viruses were transported via vacuoles and dilated channels of endoplasmic reticulum towards the nuclear membrane, through which viral DNA-protein

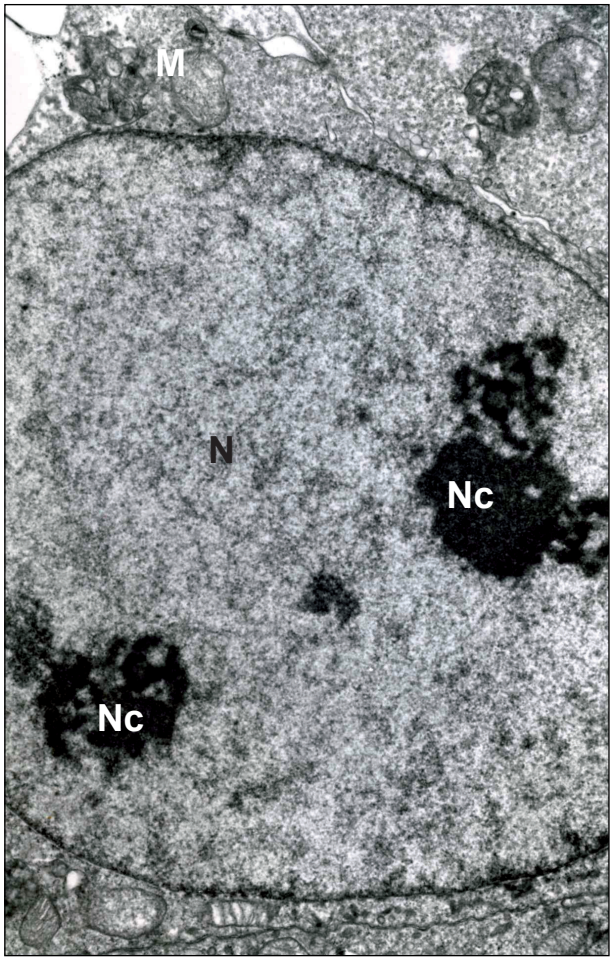

Fig. 1. Ultrastructural morphology of bovine intact blastocysts (control). Normal reticulated architecture of typical nucleolus (Nc) with fibrillar centres and morphologically normal mitochondria (M) in cytoplasm. Round shaped euchromatic nucleus (N) of embryoblastic cell. (x 15120).

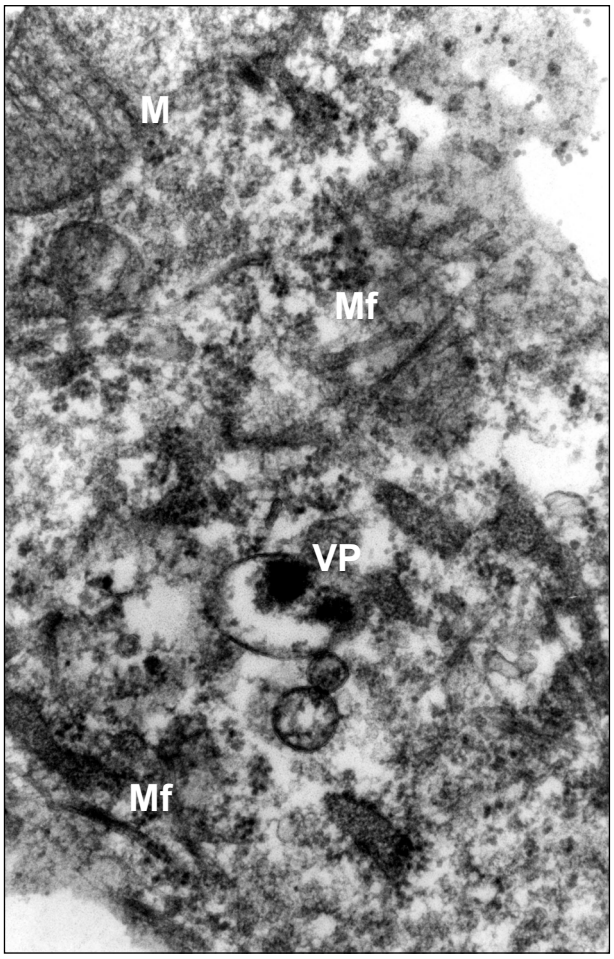

Fig. 2 Ultrastructural morphology of bovine embryoblastic cells after microinjection of BHV-1 suspension. Virus – like particles (VP) within vacuole, swollen mitochondria (M), disintegrated cytoplasm with visible microfilaments (Mf) (x 60800).

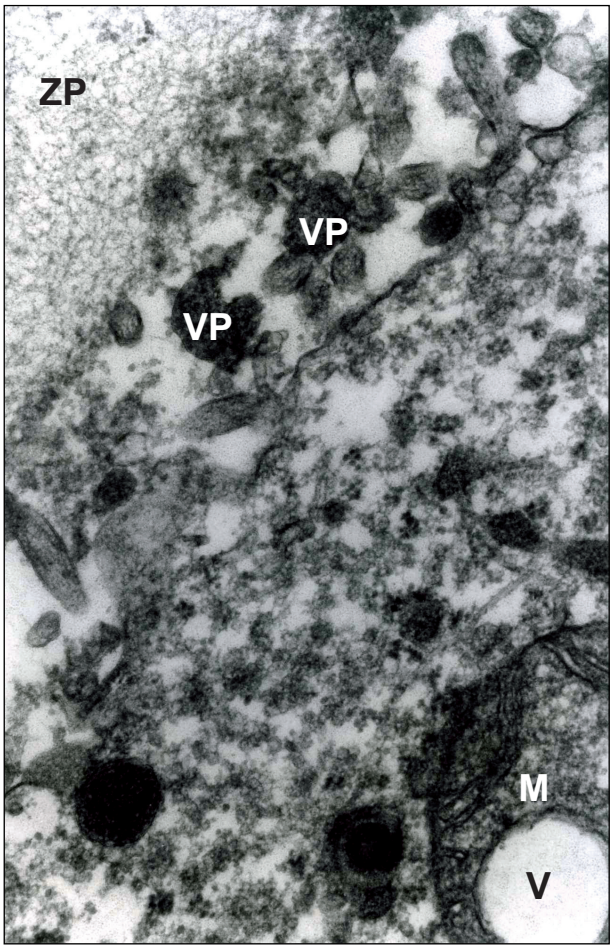

Fig. 3. Ultrastructural morphology of bovine ZP-surrounded blastocysts after microinjection of BHV-1 suspension subzonally. Accumulation of virus-like particles (VP) within the perivitelline space. Viral envelope is fused with the cell membrane (*asterisk). Virus-like particle is released into the cytoplasm. Mitochondria (M) are swollen, devoid of cristae, with incorporated vacuoles (V, vacuolation of mitochondria; x 60900).

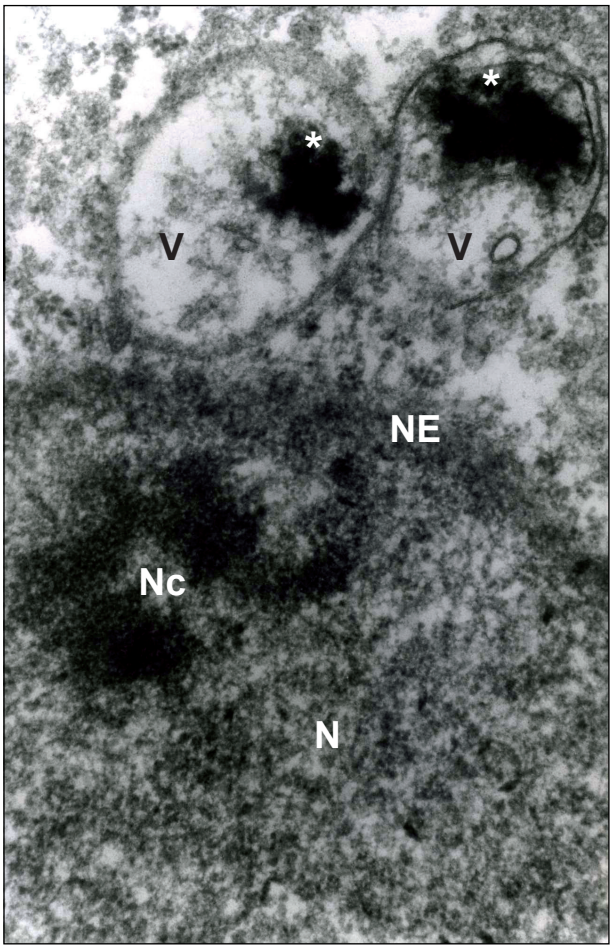

Fig. 4. Ultrastructural pattern of bovine ZP – surrounded blastocyst after microinjection of suspension of herpesvirus-1. Virus-like particles (asterisk) localized inside the vacuoles (V). Nuclear envelope (NE) is disintegrated and nucleolus is destroyed (x 60900).

complex migrated into the nucleus. In the nucleus, newly formed nucleocapsides, passing through the nuclear membrane or other cellular membranes, acquired the envelope (data not shown).

It was found that BHV-1 microinjection impairs embryo development to higher preimplantation stages compared with the control. Immunofluorescent analysis confirmed the presence of BHV-1 particles in all the BHV-1-microinjected embryos. TEM detected the presence of BHV-1 particles and showed ultrastructural alterations in cell organelles.

It is known that the *zona pellucida* 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, risk exists in that the viral particles can 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 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, virus may stick to that structure.

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 were not proved (Edens et al., 2003). The efficiency of trypsin washing of BHV-1-infected embryos was confirmed by Thibier and 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.

Using EM 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 earlier study using electron microscopy Bowen et al. (1985) demonstrated replication of viral agents in bovine *in vivo*-derived hatched embryos, i.e. released from the ZP. Viral nucleocapsides were observed in 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 1 of the 5 embryos. However, 48 h after exposure virus was demonstrated from a majority of trophoblastic cells in each embryo. Our study describes for the first time the presence of virus-like particles and changes occurred as a consequence of viral infection of embryos. Most of morphological alterations were presented by the vacuolization of embryonic cells, disintegrated cytoplasm, swollen mitochondria, and dilatation in rough endoplasmic reticulum. Appearance of viral particles was revealed in numerous vacuoles of disintegrated cytoplasm, around nuclei and inside them. We suppose that consequences of viral infection on the embryo viability may depend also on the titre of virus. Based on these observations we conclude that BHV-1 infection compromises preimplantation development of bovine embryos *in vitro*.

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REFERENCES

BIELANSKI, A. - DUBUC, C. 1994. In vitro fertilization and culture of ova from heifers infected with bovine herpesvirus-1 (BHV-1). In: *Theriogenoogy,* vol. 41, 1994, p. 1211-17.

BOWEN, R. A. - ELSDEN, R. P. - SEIDEL, Jr., G. E. 1985. Infection of early bovine embryos with bovine herpesvirus-1. In: *Am. J. Vet. Res*. vol. 46, 1985, p. 1095-97.

EDENS, M. S. D. - GALIK, P. K. - RIDDELL, K. P. - GIVENS, M. D. - STRINGFELLOW, D. A. - LOSKUTOFF, N. L. 2003. Bovine herpesvirus-1 associated with single, trypsin-treated embryos was not infective for uterine tubal cells. In: *Theriogenol*. vol. 60, 2003, p. 1495-504.

GUERIN, B. - Le GUIENNE, B. - ALLIETTA, M. - HARLAY, T. - THIBIER, M. 1990. Effects de la contamination par le BHV-1 sur la maturation et fecundation *in vitro* des ovocytes des bovines. In: *Rec.Med. Vet.,* vol. 66, 1990, p. 911-17.

GILLESPIE, J. H. - SCHLAFER, D. H. - FOOTE, R. H. - QUIC, S. - DOUGHERTY, E. - SCHIFF, E. - ALLEN, S. 1990. Comparison of persistence of seven bovine viruses on bovine embryos folloving in vitro exposure. In: *Dtsch. Tierarztl. Wochenschr*. vol. 97, 1990, p. 65-68.

STRINGFELLOW, D. 1998. Recommendations for the sanitary handling of in-vivo-derived embryos. International Embryo Transfer Society (IETS). In: STRIGFELLOW, D. A. - SEIDEL, S. M. editors. *Manual of the international embryo transfer society: a procedural guide and general information for the use of embryo transfer technology, emphasising sanitary procedures*. 3rd ed. Savoy, IL : International Embryo Society Inc., 1998, ISBN 0-9662386-05

PIVKO, J. - GRAFENAU, P. - SOKOL, J. 2000. *Prenos raných embryí zvierat.* 1. vyd. Nitra : Garmond, 2000, s. 212. ISBN 80-7146-038-X

SCHLAFER, D. H. - GILLESPIE, J. H. - FOOTE, R. H. - QUICK, S. - PENNOW, N. N. - DOUGHERBY, E. P. - SCHIFF, E. I. - ALLEN, S. E. - POWERS, P. A. - HALL, C. E. et al. 1990. Experimental transmission of bovine viral diseases by insemination with contaminated semen or during embryo transfer. In: *Dtsch. Tierarzel. Wochenschr.* vol. 97, 1990, p. 68-72.

SINGH, E. L. - THOMAS, F. C. - PAPP-VID, G. - EAGLESOME, V. D. - HARE, W. C. 1982. Embryo transfer as a means of controlling the transmission of viral infections. [II. The in vitro exposure of preimplantation bovine embryos to infectious bovine rhinotracheitis virus.] In: *Theriogenol*. vol. 18, 1982, p. 133-140.

THIBIER, M. - NIBART, M. 1987. Disease control and embryo imports. In: *Theriogenology,* vol. 27, 1987, p. 37-47.

VANROOSE, G. - NAUVYCK, H. - VAN SOOM, A. - VANOPDENBOSCH, E. - de KRUIF, A. 1997. Susceptibility of zona-intact and zona-free *in vitro* produced bovine embryos at different stages of development to infection with bovine herpesvirus-1. In: *Theriogenology,* vol. 47, 1997, p. 1389-402.

VANROOSE, G. - NAUVYCK, H. - VAN SOOM A. - YSEBAERT, M.T. - CHARLIER, G. - VAN OOSTRELDT, P. et al. 2000. Structural aspects of the zona pellucida of *in vitro* - produced bovine embryos: a scanning electron and confocal laser scanning microscopic study. In: *Biol Reprod.* vol. 62, 2000, p. 463-469.

WRIGHT, J. 1998. *Photomicrographic illustration of embryo codes*. In: STRINGFELLOW, D. A - SEIDEL S.M. (eds.) IETS Manual. 3rd ed. Savory, IL : International Embryo Society. Inc, 1998, p. 167-170, ISBN 0-9662386-05

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