Ultrastructural Morphometry of Precompacted Bovine Embryos Produced *in vivo* and *in vitro* After Activation by Electric Pulse AC/DC

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Abstract. Early bovine precompacted embryos (at 1- to 8-blastomere stage) were analyzed by electron microscopy. The volume density of cellular components was determined by morphometric analysis to quantify the ultrastructure of early bovine embryos produced either in vivo or parthenogenetically after stimulation of oocytes by electric pulse AC/DC. In embryos obtained in vivo, most of cellular volume was occupied by cytoplasm (82.93 %). The relative volume of lipids, vacuoles, mitochondria was relatively low (5.46; 5.07; 3.78 %, respectively), and the relative volume of Golgi apparatus and cell inclusions was the lowest (1.51 %). AC/DC-derived parthenogenotes had a relative high area occupied by vacuoles and lipids (18.68 *vs.* 14.33 %) and a significantly lower relative volume was occupied by cytoplasm (60.63 %) when compared with the control in vivo embryos. These observations demonstrated that parthenogenetic embryos had significantly altered ultrastructure, indicating extensive subcellular damages. These findings are discussed from the physiological and functional point of view.

Key words: Bovine — Early embryos — Electron microscopy — Parthenogenetic — Electric pulse AC/DC

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Introduction

The interaction of the spermatozoon with the oocyte causes a series of physiological changes in the oocyte, which trigger the process of activation. However, mature oocytes can be activated also *in vitro* by a variety of chemical and physical stimuli in mammals (Kaufmann 1983). The details of this parthenogenetic activation and subsequent development are important for studies of nuclear transfer and cloning of embryos (Prather et al. 1989). Parthenogenesis is also one of the useful means of analysis of epigenetic changes, e.g. genomic imprinting of maternal genes, which leads to expression or repression of certain genes of either the maternal or paternal loci during embryonic development (Kono et al. 1996; Surani et al. 1987). To date, a large number of studies on oocyte activation were made. However, most of the information on parthenogenetic development has been derived from the mouse (Surani et al. 1987), and a little is known about ultrastructural features of *in vitro* produced parthenogenotes. Despite the fact that morphometric analysis represents a more objective method of assessing differences in cellular ultrastructure (Weibel and Bolender 1973; Farin et al. 1986), which may occur in embryos as a result of *in vitro* culture, no systematic morphological comparison of *in vivo* and *in vitro* produced bovine embryos has been reported. Morphological studies have usually been restricted to particular stages of development or cellular structures (Brackett et al. 1980; Shamsuddin et al. 1992). It is known, that bovine embryos produced *in vitro* are characterized by altered morphology compared with those produced *in vivo*. In particular, embryos produced *in vitro* have fewer total number of blastomeres, demonstrate incomplete compaction and cell-to-cell coupling and have a greater variance in morphological quality and development rate (Iwasaki et al. 1990; Van Soom et al. 1997). Crosier et al. (2000), on the basis of morphometrical study of bovine embryos produced *in vitro*, showed that these embryos were not comparable at an ultrastructural level to those produced *in vivo*. *In vitro* culture resulted in compact morula with an increased amount of lipids and vacuoles and decreased density of mature mitochondria.

The objective of this study was to quantify the changes in the ultrastructure of bovine precompacted 1- to 8-cell embryos produced either *in vivo* or after parthenogenetic activation of bovine oocytes by electric pulse AC/DC (alternative current/direct current).

Materials and Methods

Bovine embryos produced in vivo

For production of bovine preimplantation embryos *in vivo* (early blastocysts and blastocysts), Holstein-Friesian (HF) donor cows were superovulated between 10 and 12 days of the previous estrus cycle by intramuscular administration of 24 mg follicle stimulating hormone (FSH) (Folicotropin, inj. ad us. vet., Spofa, Prague, Czech Republic) given in a series of decreasing doses over 4 day period. Estrus

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. Estrus detection was performed twice a 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 estrus with semen from a proven sire. Embryos used in this study were recovered by uterine flushing with Dulbecco phosphate-buffered saline (PBS) supplemented with 1 % bovine serum after slaughter of donor cattle either on Day 1 or Day 4 of the cycle (Day 0 = first standing oestrus). The embryos (1- to 8-blastomere stages) were then classified according to the general criteria of developmental competence and evaluated as morphologically normal at magnification ×100 by a stereomicroscope (Wright 1998; Pivko et al. 2000).

Collection and in vitro maturation of oocytes

Bovine ovaries were collected from abbatoir Zbrojníky near Nitra and transported within 2 h to the laboratory (at 39 ℃). Follicles of diameter 1–5 mm were aspirated, and only oocytes with compact layers of cumulus cells were selected for *in vitro* maturation. Washed oocytes were transferred into 35 mm Petri dishes (Sigma, Germany) with 3 ml of medium TCM 199 with GlutaMAX-I (Gibco^{TM}, UK) supplemented with 25 mmol/l sodium pyruvate, 10 % fetal cow serum (Sigma, Germany), 100 μg/ml gentamycin sulphate, 2 μg/ml FSH (Sigma, Germany), 10 μg/ml luteinizing hormone (LH) (Sigma, Germany) and matured at 39 ℃ in 5 % CO_2 in humidified air for 24–26 h. At the end of *in vitro* culture, oocytes with expanded cumulus mass were selected, and cumulus cells were removed by repeated pipetting. Only oocytes with distinct polar bodies were used for parthenogenetic activation.

Parthenogenetic activation and in vitro culture

Parthenogenetic activation of bovine oocytes was induced using the method previously described by Landa and Hájková (1989). Three to five oocytes were transferred into a drop (10 to 15 μl) of medium TCM 199 with GlutaMAX-I, placed between electrodes, and exposed to AC (1000 kHz, 6 V) for 20 s, followed by a single pulse of DC (20 μs, 60 V). After the DC pulse, AC voltage was extincted to 0 V in 5 s. Afterward the oocytes were washed and cultured *in vitro* for 24 to 72 h in 10 μl of medium TCM 199 with GlutaMAX-I at 39 ℃ and 5 % CO_2 in air.

Electron microscopy

Bovine embryos were fixed in 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.15 mol/l cacodylate buffer (pH 7.1–7.3) for 1 h, and washed in cacodylate buffer. One hour following fixation in 2 % osmium tetroxide in cacodylate buffer the embryos were rinsed in distilled water, dehydrated in aceton series and embedded in Durcupan ACM (Fluka). The blocks with embryos were cut on LKB-Nova ultramicrotome into semithin sections (1–2 μm) and stained with toluidine blue. Ultrathin 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.

Morphometry

The volume density of cellular components was determined using the point-count method. Briefly, a transparent grid consisting of 154 total test points was laid over each micrograph (Weibel and Bolender 1973). The square occupied by each component was expressed as percentage of the number of points covering the structure component to the total number of test points of the test grid. Within an individual embryo, the average volume density represents summary counts from micrographs for given embryo. We measured a relative volume of lipids, vacuoles, mitochondria, Golgi apparatus, cell inclusions and cytoplasm.

Statistical analysis

Relative volume of all cell components was measured from 10 micrographs *per* embryo. Obtained data were processed using statistic analytical system (SAS, version 8.1., procedure Univariate, Freq. Univariate, SAS Institute, Cary, NC, USA). Analysis of variability and Scheffe's test of multiple comparations were used for calculation of basic statistical characteristics (least square mean, SEM) and the Student's *t*-test was used for determination of differences between *in vivo* derived and parthenogenetic embryos.

Results

Precompacted bovine *in vivo*-derived embryos, intended to electron microscopy, were mostly at the 1- to 8-blastomere stage. The *zona pellucida* of the normal early embryos was intact, blastomeres were evenly developed and perivitelline space was transparent without fragmented cells. The most cellular volume of bovine embryos obtained *in vivo* was represented by cytoplasm (82.93 %) what is usual for normally developing early bovine embryos. On the other hand, relative volume of cell inclusions (1.25 %) and Golgi apparatus (1.51 %) was minimal (Table 1, Figs. 1–3).

In the group of embryos after parthenogenetic activation by AC/DC some of embryo blastomeres were fragmented, or smaller, nucleus-free. Cytoplasm of

Table 1. Volume density (%) of cellular components in bovine early embryos (1- to 8-blastomeres) produced *in vivo* or *in vitro* after parthenogenetic activation (AC/DC)

Experimental groups	(n)	Cellular components ($x \pm m$)					
		L	V	M	G	IB	C
in vivo	(20)	$5.46^a \pm 0.71$	$5.07^a \pm 0.73$	3.78 ± 0.24	1.51 ± 0.14	1.25 ± 0.14	$82.93^a \pm 0.89$
AC/DC	(20)	$14.33^b \pm 1.13$	$18.68^b \pm 1.38$	3.09 ± 0.27	1.51 ± 0.36	1.76 ± 0.27	$60.63^b \pm 1.37$

Values within the same column with different superscripts (letters a, b) differ significantly ($p < 0.001$; Student's *t*-test). L, lipids; V, vacuoles; M, mitochondria; G, Golgi apparatus; IB (inclusion bodies), cell inclusions; C, cytoplasm; n, number of embryos.

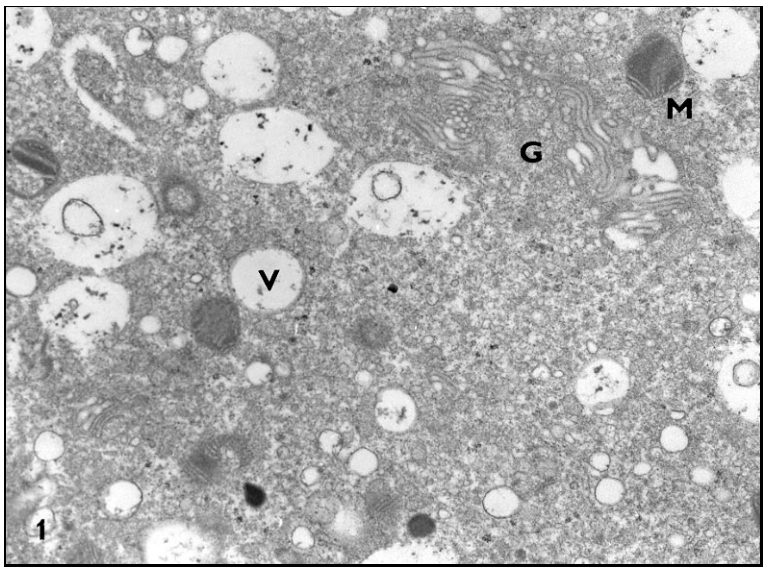

Figure 1. Electron micrograph of normal 2-cell bovine embryo obtained *in vivo* and prepared for ultrastructural morphometry evaluation as Durcupan ACM thin (silver) sections. Micrograph shows large area of Golgi apparatus (G), vacuoles (V) and immature mitochondria (M). Magnification: $\times$ 15,500.

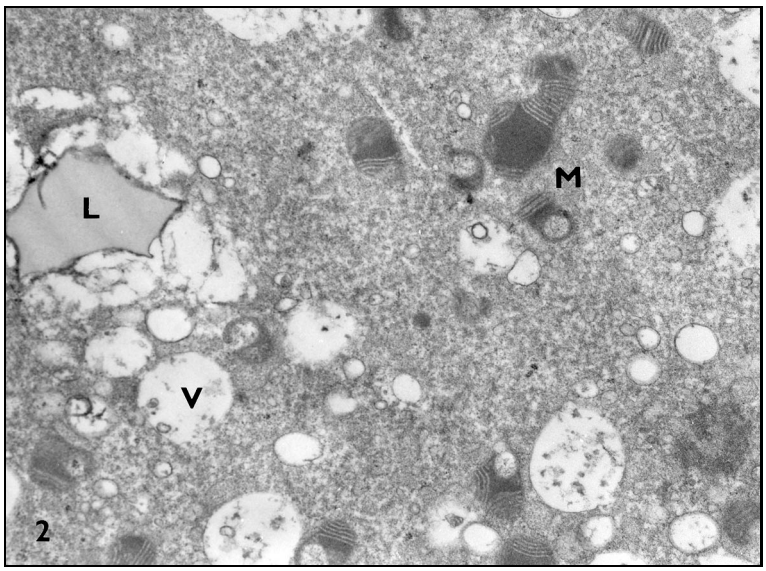

Figure 2. Electron micrograph of 4-cell bovine embryo produced *in vivo* with hooded mitochondria (M), degraded lipid droplets (L) and vacuoles (V). Magnification: $\times$15,550.

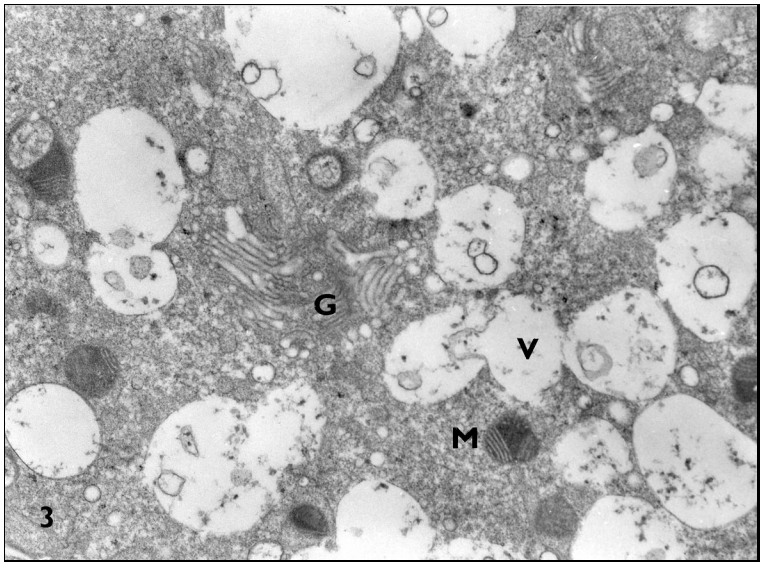

Figure 3. Electron micrograph of 8-cell bovine embryo obtained *in vivo* with Golgi apparatus (G), small vacuoles (V) connected in large vacuoles and mitochondria (M). Magnification: × 15,550.

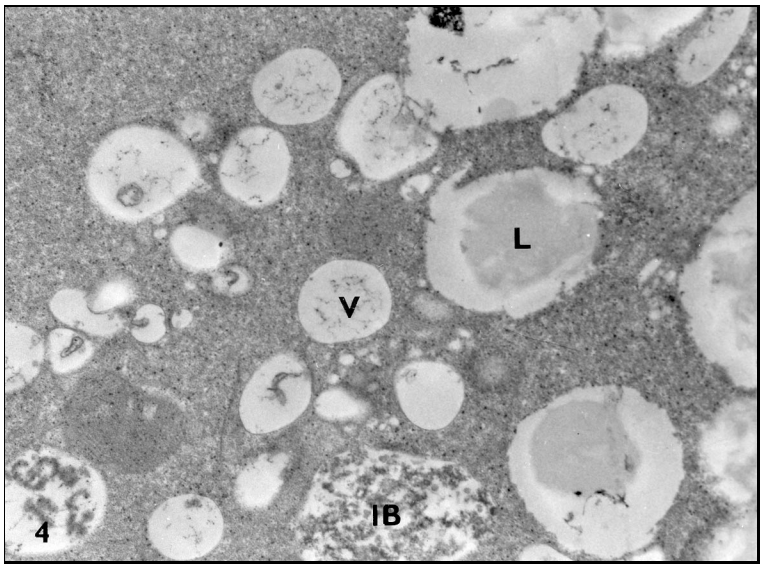

Figure 4. Electron micrograph of 2-cell bovine parthenogenotes obtained *in vitro* after AC/DC stimulation and prepared for ultrastructural morphometry as Durcupan ACM thin (silver) sections. Micrograph shows lipids (L), vacuoles (V) and large cell inclusions (inclusion bodies, IB). Magnification: × 15,550.

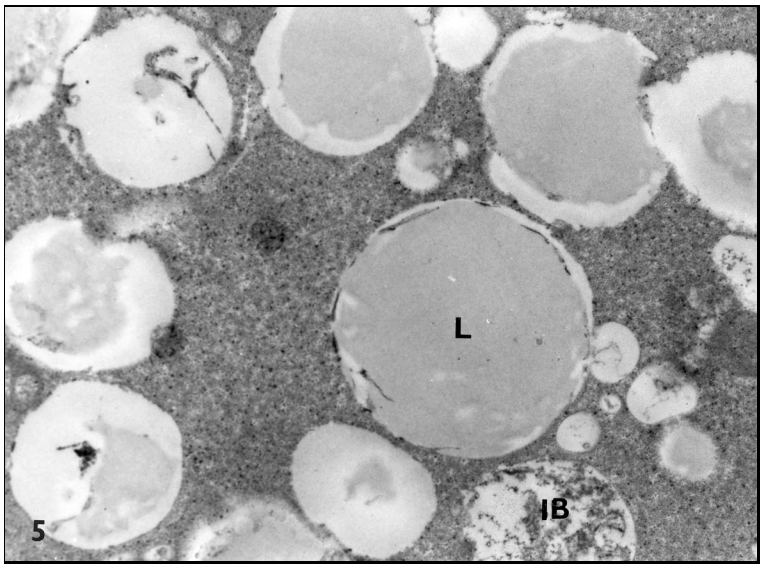

Figure 5. Electron micrograph of 4-cell embryo obtained *in vitro* after AC/DC activation. Micrograph shows large lipid droplets (L), several of which are degraded, and cell inclusions (inclusion bodies, IB). Magnification: × 15,550.

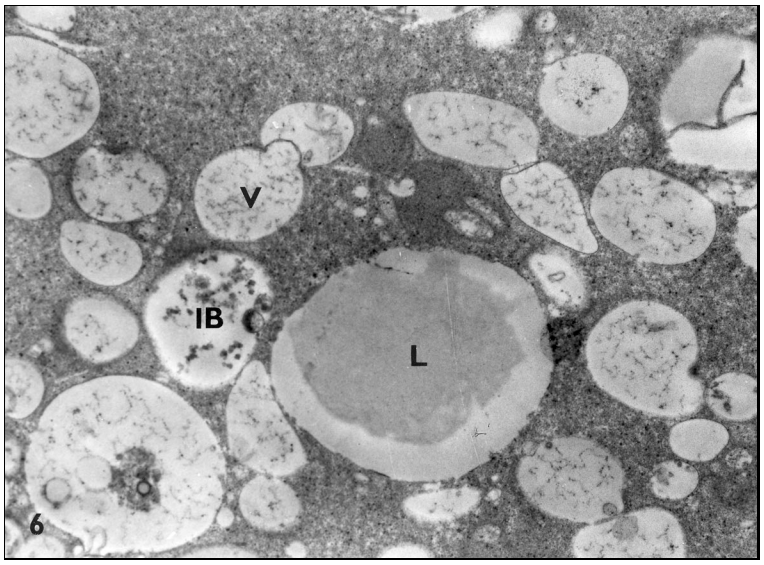

Figure 6. Electron micrograph of 8-cell embryo obtained *in vitro* after AC/DC activation. Micrograph shows large lipid droplets (L), vacuoles (V) and cell inclusions (inclusion bodies, IB). Magnification: × 15,550.

normal blastomeres was occupied by vacuoles with electron dense granular material and large lipid droplets. Most of the lysosomes contained cell inclusions. Many of recognizable mitochondria looked hood-like, what is characteristic for early stages of normal bovine embryo development. Golgi apparatus showed normal structural pattern. Relative volume occupied by cytoplasm (60.63 %) was lower than in *in vivo* embryos (82.93 %). The volume occupied by vacuoles and lipids was relatively high (18.68 and 14.33 %, respectively). Relative volume of cell inclusions was minimal (1.51 %) (Table 1, Figs. 4–6).

Comparison of *in vivo* and parthenogenetic embryos showed that the latter ones had an altered ultrastructural morphology. The relative volume of vacuoles and lipids in parthenogenetic embryos was significantly higher ($p < 0.001$) when compared to *in vivo* embryos, because the reduction in relative volume of cytoplasm ($p < 0.001$) caused an increase in the relative volume of lipids ($p < 0.001$) and vacuoles ($p < 0.001$).

Discussion

In our experiments an effective activation of oocytes was made using method published by Landa and Hájková (1989), but in comparison with Landa and Kopečný (1995), who obtained the development of parthenogenotes upon 2- to 16-cells, we observed the development of parthenogenotes only until 2–8 blastomeres after 72 h of *in vitro* culture. Although parthenogenesis represents a crucial step for understanding the basic mechanisms underlying oocyte activation and embryo development, up to date, despite the large number of studies on oocyte activation, a little is known about the electrical events induced by different activators or the nature of the ion channels responsible for this process (Tosti et al. 2002).

We observed an increased volume density of lipids in parthenogenetic embryos compared with *in vivo* embryos. Significant differences ($p < 0.001$) in relative volume of lipids between pronuclear (PN) development stage and 2-, 4- and 8-blastomere stages were determined. Lipids are very important factor influencing embryonal development and have potent effects on cell-cell interactions, cell proliferation and regulation of maturation (Stubbs and Smith 1984). The importance of lipids in maintainance of normal cell functions is therefore unequivocal. The increase in lipid volume in our experiments may be affected by the method used for production of embryos.

Not only the sources of the artificial stimules, but also culture conditions influence embryo quality and viability *in vitro* (Rizos et al. 2002; Galli et al. 2001), what can result in embryos with varying morphology compared with those produced *in vivo* (Van Langendonckt et al. 1997; Van Soom 1997). It has been suggested, that the addition of sera to the culture medium increased the occurance of lipid droplets (Thompson et al. 1995) and changed the structure of ovine embryos (Dorland et al. 1994). On the other hand, we found out that the increased supply of lipids in embryos cultivated *in vitro* may result from membrane breakdown in response to *in vitro* environment rather than from the uptake of lipids from the fetal serum in

culture medium. Alternatively, lipids may have been accumulated also as a result of insufficient metabolism by mitochondria presented in early embryos produced *in vitro* (Dorland et al. 1994). The latter mechanism may be supported by the observed reduction in volume density of mitochondria in embryos produced by AC/DC activation. The conversion of lipid substrates into the citric acid cycle occurs in the mitochondria, which contain the enzymes directly responsible for the production of ATP. Therefore, a reduction in the volume density of mitochondria may contribute to the accumulation of nonmetabolized lipid. We assume, that increased volume of lipids in early parthenogenetic AC/DC-activated embryos may be caused also by opening of pores in cytoplasmic membrane after application of the electric pulse, as has previously been noted by Procházka (1992). Weak accumulation of lipids in the cells has no influence on the viability of cells, whilst large lipid droplets can make a pressure on the organels and, after an excessive proliferation, can cause a disruption of cells and fusion of more cells together.

The presence of cytoplasmic vacuoles has been noted in primate (Enders et al. 1990) and mouse (Batten et al. 1987) embryos produced *in vivo* as well as in bovine embryos produced *in vitro* (Shamsuddin and Rodrigues-Martinez 1994). Vacuoles usually contain electrondense components and interproducts of metabolism (cellular debris resulting from autophagy or ingestion of embryonic cell fragments), which are indicators of abnormal or delayed differentiation (Enders et al. 1990). Many from these components, after their increased accumulation in cytoplasm, can offer a toxic influence on the cell metabolism. Vacuoles in the cells are formed by the deposition of fluid in the basal cytoplasm, but their formation is influenced by the changes in mitochondria, endoplasmic reticulum, lysosomes and also by deep incisions in the cytoplasmic membrane. We detected significant differences ($p < 0.001$) in the volume density of embryo vacuoles between PN and 2- and 8-blastomere developmental stages ($p < 0.01$).

Since the AC/DC-derived parthenogenetic embryos had an increased relative volume of vacuoles than *in vivo* developed embryos, they can be more compromised in their further development. Vacuoles presented in blastomeres of early embryos play an important role in the osmotic processes of the cell. Its shape, size and chemical composition in early bovine embryos cultivated *in vitro* can be altered depending on the intensity of metabolic processes. Vacuoles reflect the physiological state in blastomeres during anabolic and catabolic processes. We detected an increased number of these cytoplasmic structures in the AC/DC-activated embryos what may evidence for the changes in the internal metabolism. From the electronograms we have detected a distinct processes of fusion of small vacuoles into bigger ones and also the changes of their content. They often contain swelling structures of the membrane, amorphic matter and dark osmothic granules.

Relative volume of vacuoles was significantly higher and the relative volume of the cytoplasm decreased in the parthenogenetically AC/DC-activated embryos in comparison with the *in vivo* embryos. This can be explained by higher occurrence of vacuoles and lipids in the cytoplasm of blastomeres, what corresponds with the results of other authors, who observed a higher degree of vacuolization in *in vitro*

produced embryos compared to *in vivo* embryos (Crosier et al. 2000).

In this paper, using morphometric technique, we demonstrated on the ultrastructural level that parthenogenetic embryos have morphometric characteristics distinct from *in vivo* embryos. This finding is in agreement with the observation of Plante and King (1994). Laurinčík et al. (2003) reported a delay in ribosomal RNA gene activation of bovine parthenogenetic embryos (nucleolar proteins on ultrastructural level) compared to *in vivo* or *in vitro* produced bovine embryos. On the other hand, Ferrandi et al. (2002) did not find difference in metabolic changes (main metabolic enzymes) between parthenogenetic and *in vitro* fertilized embryos. It seems that ultrastructural studies enable to detect morphological changes in embryos prior to their expression on metabolic level.

In conclusion, electron microscopic morphometry showed that early bovine parthenogenetic embryos (1- to 8-cells) have an altered morphology in comparison with *in vivo* embryos. Vacuoles and lipids occupied a relatively large area within blastomeres of early parthenogenetically AC/DC-activated embryos. These results support findings of other authors concerning differences in development between the embryos produced *in vivo* and after parthenogenetic activation.

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