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## Effects of growth hormone on the ultrastructure of bovine preimplantation embryos

Received: 24 February 2004 / Accepted: 6 April 2004 / Published online: 29 May 2004  
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**Abstract** Growth hormone (GH) has recently been shown to promote the development of preimplantation embryos. The aim of our study was therefore to analyze the effects of GH on the morphology and ultrastructure of the cells of bovine preimplantation embryos produced by in vitro fertilization (IVF). In order to determine the physiologically optimal morphology of blastocysts, ex vivo embryos obtained by uterine flushing were also included in the study. As shown by transmission electron microscopy, treatment with GH induced the elimination of glycogen storage in cells of the inner cell mass of 7-day-old embryos. GH also stimulated the exocytosis of lipid vesicles in the inner cell mass and trophoblast cells of these embryos. Quantitative analysis of micrographs demonstrated a higher volume density of embryonic mitochondria in 7-day-old embryos cultured with GH than in control embryos. Treatment with GH regularly resulted in an improvement of the ultrastructural features of embryos produced in vitro, thus resembling the

morphology of ex vivo embryos. Scanning electron-microscopy studies demonstrated that GH altered the structure and the pore size of the zona pellucida of blastocysts. Our studies imply that GH can modulate carbohydrate, lipid, and energy metabolism and influence transportation processes in the early IVF embryo.

**Keywords** Preimplantation embryo · Mechanisms of hormone action · Growth hormone · Transmission electron microscopy · Scanning electron microscopy · Bovine (Deutsches Fleckvieh)

This work was supported by the Deutsche Forschungsgemeinschaft (FOR 478/1)

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### Introduction

Pituitary growth hormone (GH) is known to modulate growth, differentiation, and metabolism after birth. The role of this hormone during early embryogenesis, however, has been controversial for a long time. As hypophysectomized mouse, rat, and pig fetuses display almost normal intrauterine growth, embryonic and fetal development was considered independent of GH (Gluckman et al. 1981). Recent experiments, however, have provided increasing evidence that GH and its receptor (GHR) are involved in embryonic development. Thus, the growth of embryonic and fetal cells in cultures in vitro is stimulated by the application of GH (Strain et al. 1987; Swenne et al. 1987; Scheven and Hamilton 1991). In addition, the developmental capacity of bovine embryos produced in vitro (IVP) is significantly improved by GH treatment (Fukaya et al. 1998; Izadyar et al. 2000; Moreira et al. 2002; Mtango et al. 2003). Both the mRNA encoding GHR and the GHR protein have been demonstrated in early embryos in mice, rats, and bovine. GHR transcripts have been reported in mice in eight-cell-stage embryos (Terada et al. 1996), blastocysts (Pantaleon et al. 1997) and embryonic stem cells (Ohlsson et al. 1993). GHR transcripts have also been demonstrated in 8-day-old bovine blastocysts (Kölle et al. 1998) and in 12-day-old rat embryos (Garcia-Aragon et al. 1992). Real-time reverse transcription/polymerase chain reaction (real-time RT-

PCR) has shown that, in the bovine, GHR mRNA is synthesized from day 2 of embryogenesis onwards and increases 5.9-fold in 6-day-old embryos compared with 2-day-old embryos (Kölle et al. 2001). The GHR protein can first be visualized 3 days after fertilization in the blastomeres of the early bovine embryo (Kölle et al. 2001) suggesting a role for GH and its receptor early after fertilization. The mRNA encoding GH itself has been demonstrated in bovine blastocysts from day 8 onward (Kölle et al. 2001), indicating that the transcription of the GH gene starts at a later stage of embryonic development than does the transcription of GHR. Thus, the early stimulation of the GHR is probably attributable to maternal GH, whereas the later activation of GHR may be the result of autocrine or paracrine GH action within the embryo.

The mechanisms of GH action in the early embryo are still unknown. Therefore, the purpose of our study was to determine the effects of GH on the ultrastructure of bovine preimplantation embryos. For this purpose, 7-day-old embryos cultured *in vitro* with and without supplementation of GH were analyzed by transmission electron microscopy and scanning electron microscopy and compared with 7-day-old and 8-day-old *ex vivo* embryos obtained by uterine flushing.

## Material and methods

### Collection of oocytes, maturation, fertilization and culture

Ovaries of cows (breed: Deutsches Fleckvieh) collected from a local slaughterhouse were transported to the laboratory in phosphate-buffered saline (PBS) at 30°C. Oocytes were aspirated from 2-mm to 8-mm follicles by using a micro/macro suction apparatus (Labotect, Göttingen, Germany) and a 20-g needle. Under microscopic control, only oocytes with a multilayered compact cumulus oophorus and a dark evenly granulated cytoplasm were selected for further maturation.

Maturation was performed in tissue culture medium 199 (TCM 199; Seromed, Germany) supplemented with 2 mM sodium pyruvate, 2.92 mM calcium lactate, 0.01 U bovine follicle-stimulating hormone, 0.01 U bovine luteinizing hormone (Sioux Biochemicals, Sioux Center, Iowa), gentamicin (60 µg/ml; Sigma, St Louis, Mo.), and 10% (v/v) estrous cow serum (ECS) in a humidified atmosphere of 5% CO<sub>2</sub> in air at 39°C for 24 h.

For *in vitro* fertilization (IVF), frozen semen of bulls with proven fertility was used. Each straw was thawed in a water bath at 39°C for 10 s. Motile spermatozoa were separated by a modified swim-up technique (Parrish et al. 1986) with bovine serum albumin (BSA) fraction V (6 mg/ml, Sigma), sodium pyruvate (0.1 mg/ml; Sigma), and gentamicin (50 µl/ml; Seromed, Germany). After being washed and centrifuged, spermatozoa were resuspended to a final concentration of 1.0 × 10<sup>6</sup> sperm/ml. Following maturation the cumulus-oocyte-complexes (COCs) were transferred to Tyrode's albumin-lactate-pyruvate (TALP) medium (Bavister and Yanagimachi 1977) supplemented with BSA (6 mg/ml; Sigma) and heparin (10 µg/ml; Sigma). IVF was performed in a humidified atmosphere (5% CO<sub>2</sub>, 95% air) at 39°C. After 18 h, the cumulus cells were removed by vortexing for 2 min in 0.8 ml PBS supplemented with 6 mg/ml BSA. Presumptive zygotes were washed three times in synthetic oviduct fluid (SOF; Sigma) supplemented with MEM essential and non-essential amino acids (Sigma) and 10% ECS. *In vitro* culture was performed according to Stojkovic et al. (1999). Briefly, groups of 30 presumptive zygotes were placed in 400 µl SOF medium covered by

water-saturated paraffin oil (Merck, Darmstadt, Germany) and were cultured at 39°C in a humidified atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>.

The effects of GH on the ultrastructure of the early embryo were investigated as follows: in three experiments, groups of 30 presumptive zygotes were cultured in 400 µl SOF supplemented with 3 mg/ml polyvinylalcohol (PVA) and 100 ng/ml recombinant bovine GH (rbGH; Elanco, Greenfield, Ind.) for 7 days. Control embryos were cultured in SOF supplemented with PVA. For the determination of the volume density of mitochondria, embryos cultured in the routinely used standard medium SOF/10% ECS were used as an additional control group. Embryo culture was regularly performed at 39°C in a humidified atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub>. All experiments were repeated three times. For all experiments, only high quality embryos selected by light microscopy according to the morphological criteria described by Kennedy et al. (1983) were used. In each experiment, sets of 12–15 blastocysts were investigated.

### Collection of blastocysts *ex vivo* by superovulation and uterine flushing

Twelve cows (breed: Deutsches Fleckvieh) were synchronized on days 9–13 of the estrous cycle by intramuscular injection of 500 µg prostaglandin F 2α-analog (cloprostenol, Estrumate<sup>R</sup>; Essex, Munich, Germany). Cows revealing a functional corpus luteum on day 8 of the cycle were superovulated by application of 2,500 U pregnant mare serum gonadotropin (PMSG; Pregmagon<sup>R</sup>, IDT, Roßlau, Germany) between days 10 and 14 of the cycle. Three days later, 500 µg Estrumate<sup>R</sup> was injected again. As soon as clinical signs of estrus occurred, cows were inseminated two to three times with frozen-thawed semen of bulls of proven fertility. On day 7 after insemination, 44 embryos were obtained by uterine flushing under epidural anesthesia. As embryos *in vivo* generally develop more slowly compared with embryos *in vitro* (Walker et al. 1992), 30 embryos were additionally obtained on day 8 after insemination. Only high quality blastocysts were included in the studies.

### Transmission electron microscopy

Seven-day-old embryos cultured with or without GH were removed from the medium and washed twice in cacodylate buffer (0.2 M sodium cacodylate, pH 7.2). Embryos obtained *ex vivo* were removed from the uterine flushing fluid and washed. After fixation in Karnovsky's fluid (2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer), embryos were post-fixed in 1% OsO<sub>4</sub> and 1.5% KFe(CN)<sub>6</sub>. To optimize diffusion conditions for embedding, a small incision was cut into the zona pellucida (ZP) by using a small needle. Embryos were then transferred to a drop of 20% BSA in cacodylate buffer. By adding 25% glutaraldehyde, the BSA was polymerized to a pellet containing the embryo. The pellet was dehydrated in a graded series of ethanol and embedded in Epon (Polysciences, Eppelheim, Germany). In order to assess the cellular compartments of the embryos, semithin sections (1 µm) were cut and stained with methylene blue. Ultrathin sections (50 nm) were mounted on grids, post-stained with OsO<sub>4</sub> and examined with a Zeiss electron microscope TEM 902 at magnifications from 3,000× to 25,000×.

### Morphometry

The relative volume density of mitochondria in 7-day-old embryos was determined utilizing the point-count-method according to Weibel (1979). Three types of mitochondria were analyzed: (1) mature mitochondria characterized by well-developed, evenly stacked cristae, (2) immature (embryonic) mitochondria characterized by few peripheral cristae or a hooded appearance, (3)

vacuolated mitochondria containing a membrane-bound vesicle (Crosier et al. 2000). For determining the volume density of mitochondria, a transparent grid consisting of 1,024 fine and 64 coarse test-points was laid over each micrograph. The number of test points falling on the various types of mitochondria was recorded. The volume density of mitochondria was equivalent to the proportion of points falling on mitochondria divided by the total number of test-points available on the test grid. Micrographs of ten different embryos were analyzed in each of the following groups: (1) embryos cultured in SOF-PVA (control), (2) embryos cultured in SOF-PVA-GH, (3) embryos cultured in SOF-ECS, (4) embryos obtained ex vivo. Ex vivo embryos flushed from the uterus were included in the study to obtain an idea of the physiological distribution of the three types of mitochondria. As we wanted to determine the relevance of the results for the developmental capacity of embryos in vitro, blastocysts cultured in the routinely used standard medium SOF-ECS were additionally analyzed. All micrographs were taken at a magnification of 6,000 $\times$ . In each embryo, an area of 351  $\mu\text{m}^2$  was analyzed. The volume density of mitochondria was regularly determined in the cells of the inner cell mass (ICM).

#### Statistical analysis

The volume density of mitochondria in the various groups was compared by using an analysis of variance followed by the post hoc Bonferoni test. Data are presented as the mean  $\pm$  SEM.  $P < 0.05$  was considered significant.

#### Scanning electron microscopy

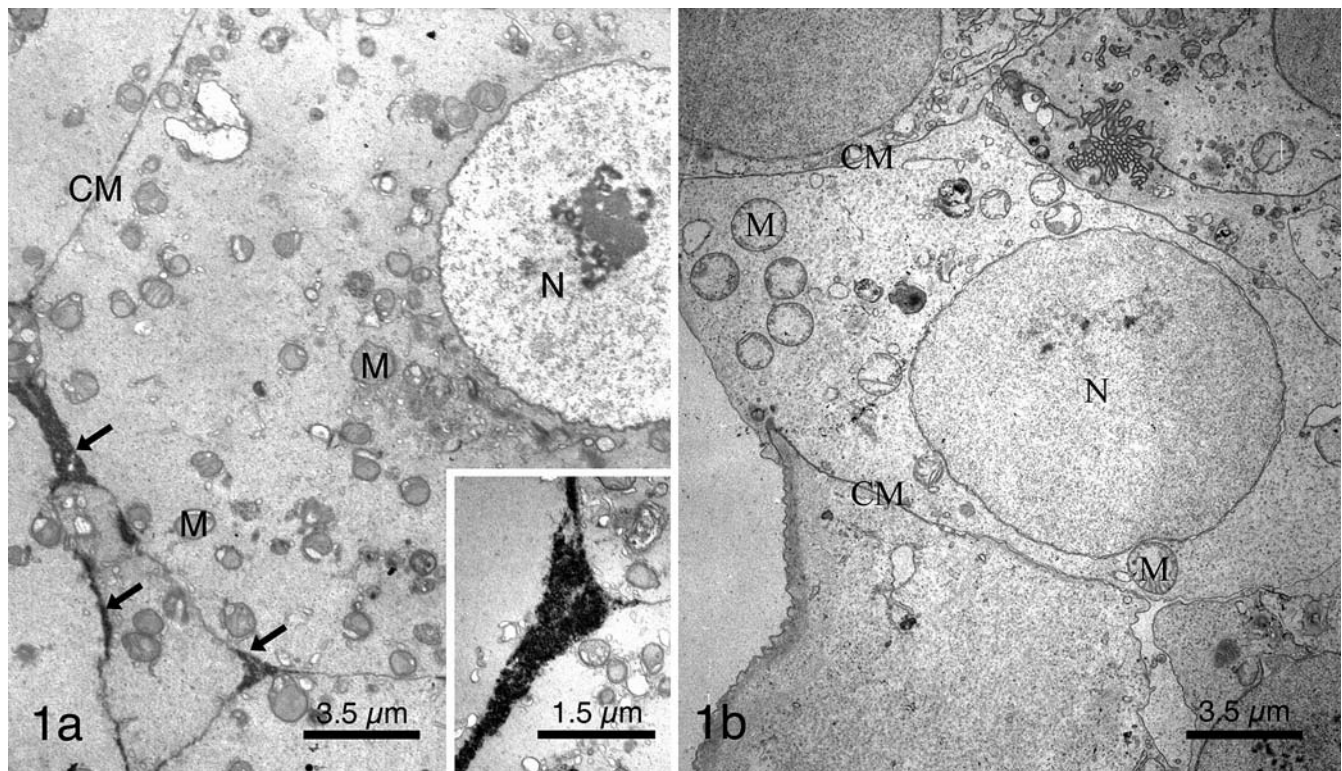
Seven-day-old embryos cultured with and without GH were removed from the medium; embryos obtained ex vivo were

removed from the flushing fluid and were washed twice in Soerensen buffer at pH 7.4 (1:5 solution of 0.07 M  $\text{KH}_2\text{PO}_4$  and 0.07 M  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ). The embryos were then fixed in 1% glutaraldehyde in Soerensen buffer at 4°C for 24 h. After further washes in Soerensen buffer, the embryos were dehydrated in an ascending series of acetone (10%, 20%, 30%, 40%, 50%, and 60%: twice, 5 min each; 70%, 80% and 90%: 1 h each; 100%: 12 h). Following dehydration, the embryos were dried in a Union Point Dryer CPD 030 (Bal-Tec, Walluf, Germany) by using liquid  $\text{CO}_2$  as the transitional fluid. After being dried, specimens were coated with 12-nm gold-palladium by a Union SCD 040 sputtering device (Bal-Tec, Walluf, Germany). Scanning electron-microscopic observations were made with the Zeiss scanning electron microscope DSM 950 at magnifications of 200 $\times$  to 10,000 $\times$ .

## Results

### Transmission electron microscopy

Like the ex vivo embryos, the 7-day-old embryos cultured with or without GH revealed a clear differentiation of ICM cells and trophoblast cells surrounding the blastocoele. The polyhedral ICM cells were characterized by a spherical nucleus with large nucleoli, whereas the flattened trophoblast cells regularly revealed an oval nucleus. In all groups, the microvilli of the apical membrane of the trophoblast cells were well developed. The most abundant organelles in the cytoplasm of the embryonic cells were embryonic mitochondria characterized by their round to



**Fig. 1a, b** Localization of glycogen in 7-day-old embryos cultured with and without GH (*M* mitochondria, *N* nucleus, *CM* cell membrane). **a** Embryos cultured in vitro generally reveal accumulations of glycogen in the intercellular spaces of the embryonic cells

(*arrows*). *Inset*: Accumulation of glycogen crystals at higher magnification. **b** Glycogen storage is below detection in embryos cultured with GH

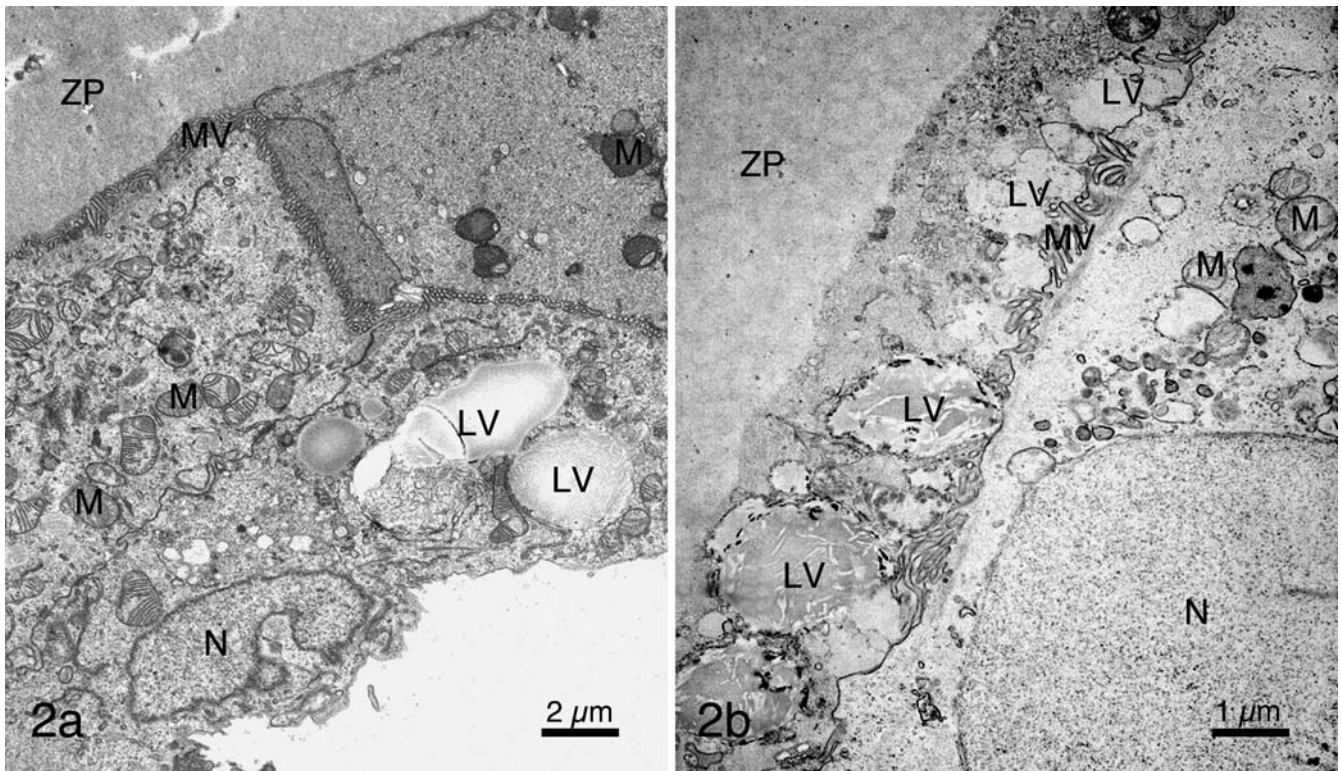
elongated shape and single transverse cristae. Golgi-apparatus and rough endoplasmic reticulum were well developed in the embryos of all groups. Distinct differences between embryos cultured with or without GH were seen in the accumulation of glycogen in the embryonic cells. Seven-day-old embryos cultured without GH possessed considerable amounts of glycogen in the cytoplasm of the embryonic cells and in the intercellular spaces (Fig. 1a, arrows). In embryos cultured with GH, however, glycogen storage was below detection (Fig. 1b). Small accumulations of glycogen were infrequently seen in the intercellular spaces in *ex vivo* embryos.

The second conspicuous difference between embryos cultured with or without GH was seen in lipid exocytosis. Embryos cultured under both conditions revealed a considerable number of lipid droplets in the cytoplasm of the embryonic cells (Fig. 2). However, the excretion of lipid droplets was increased in embryos treated with GH (Fig. 2b). Thus, these embryos displayed numerous lipid vesicles between the plasmalemma of the trophectoderm cells and the ZP (Fig. 2b); this was not seen in control embryos (Fig. 2a). In contrast to the embryos cultured *in vitro*, the embryos obtained *ex vivo* merely showed single lipid droplets in the cytoplasm of the embryonic cells. Exocytosis of lipids was rarely seen in single embryonic cells.

## Morphometry

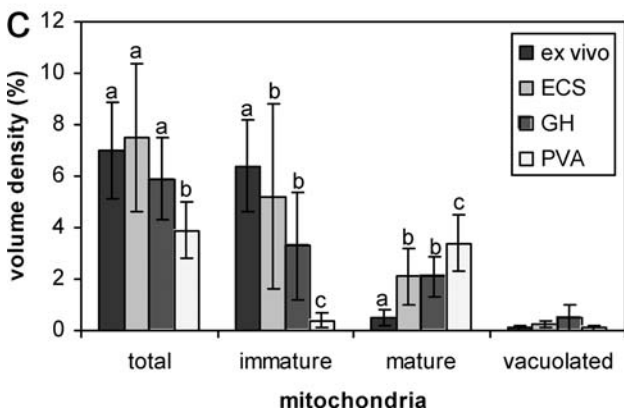
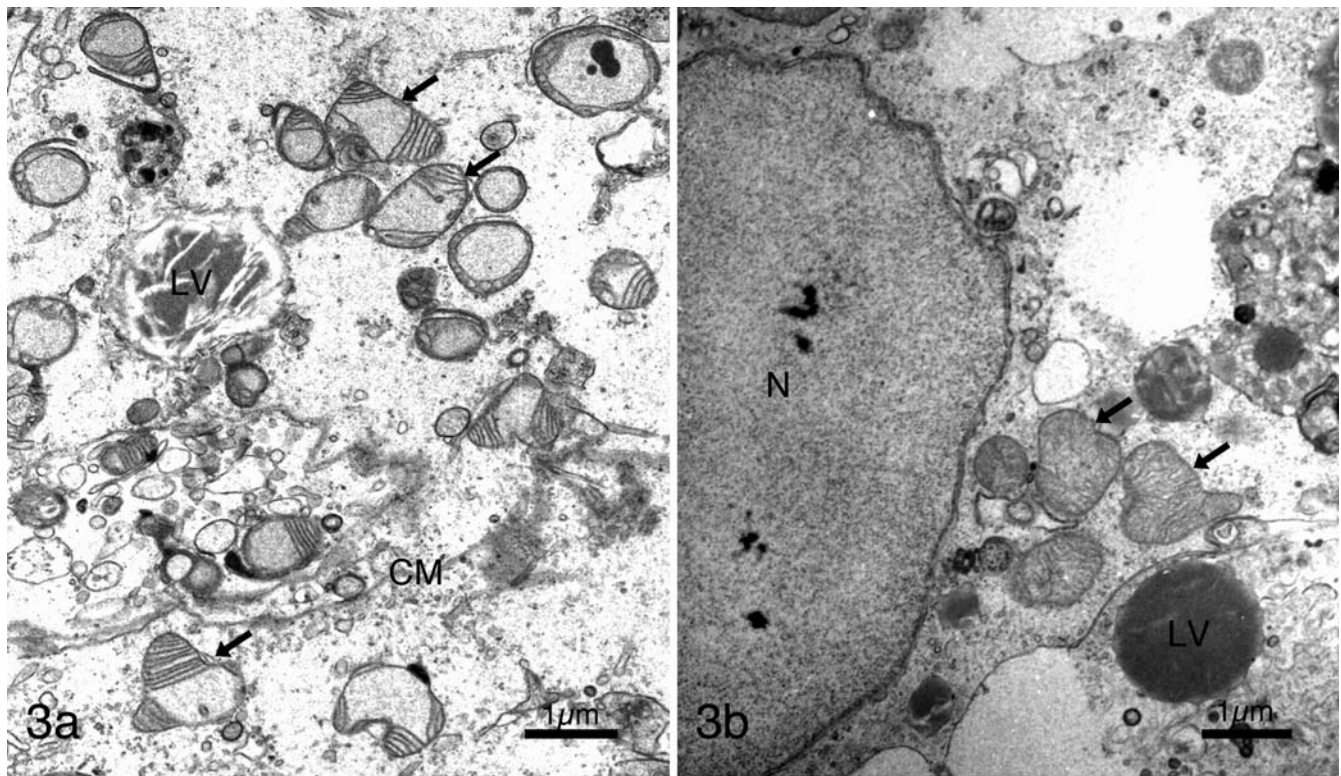
The total mitochondrial volume density and the volume densities of immature (embryonic; Fig. 3a), mature (Fig. 3b), and vacuolated mitochondria were investigated in all groups of embryos. Whereas the total volume density of mitochondria did not significantly differ between embryos obtained *ex vivo* and embryos cultured with supplementation of ECS or GH, it was significantly smaller in embryos cultured with PVA (Fig. 3c).

The highest volume density of immature mitochondria (6.4%) was seen in embryos obtained *ex vivo* (Fig. 3c). In 7-day-old embryos cultured with GH, the volume density of embryonic mitochondria was significantly higher compared with the controls cultured without GH (Fig. 3c). Similarly, embryos cultured in the regularly used standard TCM 199 medium supplemented with ECS revealed a significantly increased volume density of embryonic mitochondria compared with controls cultured with PVA (Fig. 3c). However, in all embryos cultured *in vitro*, the volume density of embryonic mitochondria was lower compared with that of embryos obtained *ex vivo*. In the embryos cultured with supplementation by GH or ECS, the immature mitochondria represented the greatest part (56% and 69%, respectively) of the mitochondrial volume fraction (Fig. 3d). In the controls, however, mature mitochondria (87%) clearly predominated (Fig. 3c, d). Consequently, the proportion of immature (embryonic) to



**Fig. 2a, b** Localization of lipids in 7-day-old embryos cultured with and without GH (*M* mitochondria, *MV* microvilli, *N* nucleus, *ZP* zona pellucida). **a** Embryos cultured without GH possessed a large number of lipid vesicles (*LV*) in the cytoplasm of the embryonic

cells. **b** Embryos cultured with GH display an increased excretion of lipid vesicles (*LV*) between the plasmalemma of the trophectoderm cells and the zona pellucida (*ZP*)



**Fig. 3a-d** Volume densities of immature, mature, and vacuolated mitochondria in ICM cells of 7-day-old embryos (*CM* cell membrane, *LV* lipid vesicles, *arrows* mitochondria). **a** Immature (embryonic) mitochondria are characterized by a few peripheral cristae or a hooded appearance. **b** Mature mitochondria have well-developed, evenly stacked cristae (*N* nucleus). **c** Volume densities of

immature, mature, and vacuolated mitochondria in 8-day-old ex vivo embryos and in 7-day-old IVF embryos cultured in SOF supplemented with ECS, GH, or PVA. **d** Volume density ratio of immature, mature, and vacuolated mitochondria in 8-day-old ex vivo embryos and in 7-day-old IVF embryos cultured in SOF supplemented with ECS, GH or PVA

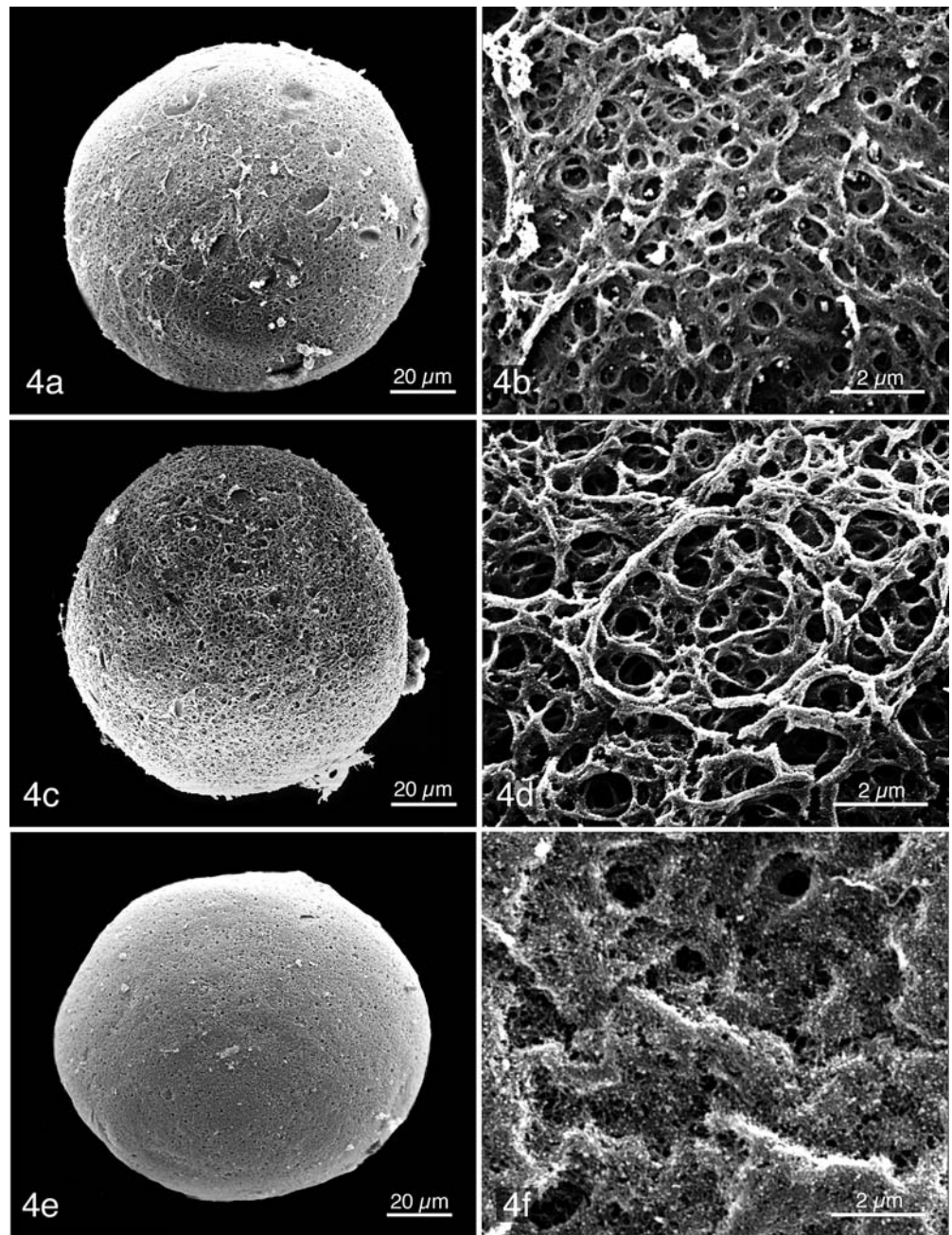
mature mitochondria decreased from ex vivo embryos to embryos cultured with supplementation by ECS or GH and was lowest in the control embryos cultured with PVA (Fig. 3d). The volume density of vacuolated mitochondria did not significantly differ between the different groups (Fig. 3c).

#### Scanning electron microscopy

The scanning electron-microscopic studies demonstrated that the surface of the control embryos was smoother with distinctly fewer pores compared with GH-treated embryos

(Fig. 4a, c). The ZP of the embryos treated with GH had a spongy appearance with numerous circular or elliptical pores (Fig. 4c, d). In control embryos, however, the zona surface appeared smooth with only a few pores (Fig. 4b). In both groups, the pores showed a centripetal narrowing architecture, as the diameter of the pores decreased toward the inner layer of the ZP. The ZP of embryos obtained ex vivo clearly differed from that of embryos cultured in vitro (Fig. 4e, f). The pores of the ZP, which were regularly distributed all over the embryo (Fig. 4e), were only partly visible, as the ZP was densely covered with extracellular matrix revealing many small secretory granules on the surface (Fig. 4f).

**Fig. 4a–f** Structure of the zona pellucida in IVF embryos cultured with and without GH and in embryos obtained ex vivo. **a** Control IVF embryos cultured without GH possess a moderate number of regularly distributed small pores. **b** The zona surface of IVF embryos cultured without GH has a smooth melted appearance with flat pores. **c** After culture with GH, the embryonic ZP has a spongy appearance and reveals numerous deep pores. **d** The ZP of embryos cultured with GH contains numerous circular or elliptical pores with a centripetal narrowing architecture. **e** In 7-day-old embryos obtained ex vivo, the regularly distributed pores are only partly visible. **f** The zona surface of ex vivo embryos is covered by extracellular matrix



## Discussion

In the present study, we have demonstrated that GH is able to modulate glycogen metabolism and lipid exocytosis in the bovine preimplantation embryo. In the adult, the complex effects of GH on protein, carbohydrate, and lipid metabolism have been classified as short-term insulin-like effects, such as enhanced glucose utilization and antilipolysis, and long-term insulin-antagonistic effects, such as the impairment of glucose utilization and stimulation of lipolysis (Davidson 1987). Our transmission electron-microscopic studies have shown that, in IVF blastocysts, the application of GH results in the elimination of glycogen accumulations in the ICM and trophoblast

cells. Possible mechanisms for the elimination of glycogen in the embryonic cells are the impairment of glucose transport, the inhibition of glycogen synthesis, or an increase in glycogenolysis. As treatment with GH has been shown to promote glucose transport in mouse blastocysts (Pantaleon et al. 1997), the elimination of glycogen is probably attributable to a decrease in glycogen synthase activity or an activation of the glycogenolysis pathway. From the morula onwards, glucose is the preferred energy substrate during in vitro embryogenesis (Rieger et al. 1992a, 1992b). During preimplantation development, glucose consumption is at its highest at the blastocyst stage (Sturmeijer and Leese 2003). Thus, the reported effects of GH during in vitro culture, such as the improvement of embryonic developmental capacity (Moreira et al. 2002;

Mtango et al. 2003), higher blastocyst rates (Izadyar et al. 2000; Fukaya et al. 1998), and higher implantation rates in IVF programs (Fukaya et al. 1998) may partly be attributable to a higher availability of glucose both from increased uptake and increased glycogen utilization. This is supported by the finding that GH has no effects on the developmental rate of embryos in the absence of glucose (Iwata et al. 2003). As shown by our comparative studies with embryos obtained ex vivo, the excessive storage of glycogen in the intercellular spaces of the embryonic cells is a typical feature of embryos cultured in vitro and does not occur in ex vivo embryos. Hence, GH is able to improve the carbohydrate metabolism of cultured embryos, which thus resembles that of ex vivo embryos. A second typical morphological difference between IVP and ex vivo embryos is the accumulation of lipid droplets in IVP embryos, a feature that occurs independently of the culture system (Thompson 1997; Abe et al. 1999a; Farin et al. 2001). Addition of serum to the culture medium results in a further increase in the number and size of the lipid droplets (Ferguson and Leese 1999; Abe et al. 1999b). As shown in our studies, the addition of GH to the medium leads to increased exocytosis of lipids, thereby reducing excessive lipid accumulation in the embryonic cells. The metabolic changes caused by increased lipid accumulation in the IVP embryo cannot be exactly defined because the role of intracellular lipids in cellular metabolism is still unclear (Thompson 2000). In each case, the accumulation of lipids in the embryonic cells is correlated with decreased cryotolerance (Abe et al. 2002). Therefore, GH might also be able to increase the viability of IVP blastocysts after freezing and thawing.

Mitochondria are essential for the provision of energy for the developing embryo. Our morphometric studies show, for the first time, that not only the total mitochondrial volume density, but also the volume density of specifically embryonic (immature) mitochondria is strongly correlated with the developmental capacity of the embryo. Thus, ex vivo embryos have a significantly higher volume density of embryonic mitochondria than embryos cultured in vitro. With regard to IVP embryos, the culture medium significantly influences the volume density of embryonic mitochondria. Addition of GH results in a significant increase of the volume density of embryonic mitochondria. As the mitochondrial volume density distinctly increases during embryogenesis in vivo but as a rule not in vitro (Farin 2001), GH may be able to improve the embryonic supply of energy within in vitro culture systems. Consideration of the proportion of immature to mature mitochondria makes it obvious that media known to produce embryos with a high developmental capacity (such as the routinely used standard TCM 199/ECS medium) are characterized by a high proportion of immature mitochondria. On the other hand, embryos cultured in SOF-PVA possess predominantly mature mitochondria in their cytoplasm. Embryos cultured in this medium are characterized by significantly lower blastocyst rates and decreased survival after vitrification (Eckert et al. 1998; Kuran et al. 2001). These results imply

that the so-called immature mitochondria are essential for the energy metabolism of embryonic cells. They seem to have special functions that cannot be fulfilled by mature mitochondria. Consequently, these mitochondria should not be called immature, but embryonic. In bovine oocytes, mitochondrial ATP levels have been shown to be different between morphologically good and poor oocytes (Stojkovic et al. 2001) suggesting that functional embryonic mitochondria are crucial for effective energy production. This is an essential prerequisite for development, as the demand for energy distinctly increases during embryogenesis (Devreker and Englert 2000).

Not only the demand for energy, but also the demand for nutrients increases during development. Our scanning electron-microscopic studies show that GH is additionally able to modulate the structure of the ZP. Thus, the ZP of embryos treated with GH have a spongy appearance with numerous circular or elliptical deep pores. In contrast, embryos cultured with SOF-PVA, which is known to produce low blastocyst yields, have a zona surface with a smooth melted appearance and only a few pores. This suggests that the transportation of nutrients from the oviduct or the uterus to the embryo via the ZP is facilitated after GH treatment. Therefore, improved nutrition may be involved in the increased developmental capacity of embryos cultured with GH (Mtango et al. 2003). Embryos obtained ex vivo possess numerous deep pores that are distributed all over the blastocyst. The pores, however, are only partly visible as the ZP of these embryos is densely covered with extracellular matrix. The matrix is probably derived from the oviduct and the uterus and is a sign of embryo-maternal interaction, which is not present in the embryo cultured in vitro.

In summary, our studies have shown that GH is able to modulate carbohydrate and energy metabolism and to affect lipid transportation in the preimplantation embryo. In assisted reproduction techniques, GH might promote the development of IVP embryos by improving the transportation and metabolism of nutrients.

**Acknowledgements** We thank Mrs. Christine Neumüller, Mrs. Katrin Berger, und Mrs. Gudrun Boie for excellent technical assistance. The help of Mrs. Heidrun School, Department of Parasitology, University of Munich with the scanning electron microscopy is gratefully acknowledged.

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