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Effect of serum on the mitochondrial active area on developmental days 1 to 4 in *in vitro*-produced bovine embryos

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Summary

Certain morphological changes at the subcellular level caused by the current techniques for *in vitro* embryo production seem to affect mitochondria. Many of these, including dysfunctional changes, have been associated with the presence of serum in the culture medium. Thus, the aim of the present work was to assess the mitochondrial dynamics occurring in embryos during the first 4 days of development, in order to analyze the most appropriate time for adding the serum. We used transmission electron microscopy (TEM) micrographs to calculate the embryo area occupied by the different morphological types of mitochondria, and analyzed them with Image Pro Plus analyzer. The results showed hooded mitochondria as the most representative type in 1- to 4-day-old embryos. Swollen, on-fusion, orthodox and vacuolated types were also present. When analyzed in embryos cultured without serum, the dynamics of the different mitochondrial types appeared to be similar, a fact that may provide evidence that the developmental changes control the mitochondrial dynamics, and that swollen mitochondria may not be completely inactive. In contrast, in culture medium supplemented with serum from estrous cows, we observed an increased area of hooded mitochondria by developmental day 4, a fact that may indicate an increased production of energy compared with previous days. According to these results, the bovine serum added to the culture medium seems not to be responsible for the functional changes in mitochondria.

Keywords: Bovine, Embryo, Mitochondria, Serum, Ultrastructure

Introduction

In embryo production, the growing conditions, such as the medium and supplements, are able to induce significant morphological, physiological and biochemical changes in the embryos produced *in vitro* when compared with those produced *in vivo*. The characterization of these conditions is important for expansion of the supply of embryos suitable for implantation.

The methodology used at present for *in vitro* embryo production uses some ill-defined components for culture media, such as serum, both for the maturation and for partial or total development. By studying maturation in the absolute absence of serum and the presence of tissue culture medium (TCM) (Palma, 2001), embryos are observed to develop successfully. In addition, in post-fertilization media, serum has been successfully replaced by bovine serum albumin (BSA), polyvinyl alcohol and amino acids (Krisher & Bavister, 1998). Also, some authors have been able to obtain the complete development of blastocysts in serum-free maturation and culture media (Watson *et al*., 2000).

Both fetal and adult cow sera provide the embryo with energetic substrates, amino acids, vitamins and growth factors that stimulate development. When mammal embryos are cultured *in vitro*, whole serum and BSA are used as the protein source. The negative effects that whole serum can impose over embryo development are reduced to a minimum by the addition of BSA to the culture medium (Maurer, 1992).

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The effect of serum on different developmental stages is dissimilar. Stimulating properties can be observed in blastocyst development, while inhibitory effects have been shown in 2-cell embryos (Pinyopummintr & Bavister, 1991). According to these results, it seems that a serum supplement should be added to the culture medium only in pre-determined developmental periods.

Certain morphological changes at the subcellular level caused by the current techniques for *in vitro* embryo production seem to affect mitochondria (Dorland *et al.*, 1994; Abe *et al.*, 1999; Fair *et al.*, 2001). Embryo mitochondria in mammals are provided by the oocyte; they consist of a double membrane, specific mDNA, and transference and ribosomal RNA, which are taken as a fair proof of their endo-symbiotic origin. Mitochondrial DNA encodes sequences that are highly conserved among mammals and that have a non-coding regulatory region (Smith *et al.*, 2000). Nevertheless, most polypeptides within mitochondria follow a nuclear DNA code, are synthesized in the cytosol, and enter the organelle once they have been transcribed. Although mitochondrial DNA does not duplicate during early development (Pikó & Taylor, 1987), the genes transcribe the components of the respiratory chain from the 2-cell embryo onward. This transcription rate increases 30-fold when reaching the blastocyst stage (Taylor & Pikó, 1995).

Most oxidative reactions responsible for the energy supply take place in mitochondria, which are the greatest ATP generators at preimplantation stages. In addition, the reactions necessary for the production of lipid membranes, cell growth, and production of the vital antioxidants and reactive oxygen necessary for the maintenance of apoptosis in vertebrates also take place in mitochondria (Chi *et al.*, 2002). The internal structure of this organelle is complex and dynamic, and as their maturation is individual, several differentiation stages are found during development.

Serum is often associated with lipid accumulation. It has been suggested that insufficient mitochondrial metabolism, caused either by the presence of degenerated forms or by the scarce number of mature mitochondria in *in vitro*-produced embryos cells, can lead to a poor use of lipids as energy source (Crosier *et al.*, 2001). Although the rates between blastocyst production and total number of cleaved oocytes when embryos grow in a medium without serum are similar to those when embryos grow in a medium supplemented with serum from the third day of culture onwards (Mucci *et al.*, 2006), the characterization of the culture medium conditions that can minimize the intracellular alterations might optimise the metabolic function, and thus improve embryo survival. This study was designed to evaluate the effect of the serum added to the culture medium at different times after fertilization on the characteristics of embryonic mitochondria. To this end, we analyzed the mitochondrial morph types of embryos produced during the first 4 days of development in the culture medium with or without addition of serum.

Materials and methods

All the chemicals used were from Sigma-Aldrich unless otherwise indicated.

In-vitro embryo production

The production process was carried out between March to May 2005, at the Laboratory of Reproductive Biotechnology, Balcarce Experimental Station, National Institute of Agricultural Technology of Argentina. The research complied with institutional protocols and the usual legal requirements.

The ovaries were collected from bovines from a local slaughterhouse, placed in saline solution with gentamycin added (20–25 °C), and used within the first 4 h after collection. The cumulus–oocyte complexes (COCs), 3 to 8 mm in diameter, were extracted through an 18-g needle puncture using a vacuum. Those with several layers of compact cumulus cells and homogeneous cytoplasm (oocytes Grade 1; Lindner & Wright, 1983) were retained. The COCs were cultured in maturation modified Parker medium (TCM199, HEPES, gentamycin, Ca lactate, Na pyruvate, $NaHCO_3$) supplemented with 10 mg/ml follicle stimulating hormone (rhFSH, Gonal F-75, Serono, UK), 10 mg/ml epidermal growth factor (EGF) and 100 μM cysteamine. Groups of 50 COCs were gathered and placed on 4-well plates for culture with 400 μl medium at 38.5 °C, under 5% CO_2 and a humidity-saturated atmosphere for 22 to 24 h.

For fertilization, frozen–thawed semen was subjected to centrifugation on a Percoll (30–60–90% density) gradient column. Mature COCs were placed in a new well with 400 μl of TALP solution (Tyrodes, albumin, lactate and pyruvate) and 50 μg/ml of heparin. COCs and spermatozoa (2 million/ml) were co-incubated at the previously settled environment.

Presumptive zygotes were released from the cumulus through mechanical agitation and then placed in the same plates in which maturation was induced with 400 μl of final culture medium, Charles Rosenkrans medium (CR1), 3 mg/ml BSA free of fatty acids, amino acids and glutamine, either with or without the addition of supplement. The supplement consisted of 5% serum obtained from a single pool of estrous cows, inactivated at 56 °C for 30 min and frozen until used. Each well was covered with mineral oil and

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maintained at 38.5 °C under 5% O_2, 5% CO_2, 90% N_2, and humidity to saturation.

Experimental design

Following maturation and fertilization (day 0), the embryos were cultured in serum-free medium. After 48 h of culture (day 2), a group of embryos was placed into the serum-supplemented medium (5% estrous cow serum) and after 72 h of culture (day 3), a second group of embryos was placed into the serum-supplemented medium. Six replicas were performed following the same outline.

For each of the replicas, embryos grown in serum-free medium were collected on days 1 to 4; embryos grown in medium supplemented with serum during the last 24 h of growth were collected on day 3; and embryos grown in medium supplemented with serum either for the last 24 or 48 h were collected on day 4.

Five embryos, one from each replica, were used to create each group of analysis which characterized a developmental stage and treatment. For each group of analysis, embryos representing the most common stage among those expected for that developmental day were recovered: day 1: 2-cell embryos; day 2: 4- to 6-cell embryos; day 3: 7- to 8-cell embryos; day 4: 9- to 16-cell embryos. A total of 35 embryos were analysed.

Optical and electron microscopy

Embryos were fixed in 2% glutaraldehyde in cacodylate buffer at 4 °C for 2 h, post-fixed in 1% osmium tetraoxide in the same buffer, and dehydrated with ethyl alcohol series and propylene oxide. They were then embedded in a polybed–araldite mixture, following Mollenhauer (1964). Sections for light microscopy were stained with methylene blue. Thin sections (about 60 nm) for electron microscopy were placed in either copper mesh grids or single-hole grids treated with Formvar membrane, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined on Zeiss and Jaol transmission electron microscopes.

Morphometric and statistical analysis

TEM micrographs (whose reproduction scale was about 8000:1) were used to quantify the area occupied by the different mitochondrial morphological types, using the ImagePro Plus analyzer. The measurements were made on five random micrographs of different cells per embryo, and the results were presented as proportions of covered area. These five micrographs constituted a block analysis. For each day and treatment there were five embryos and five blocks.

Data were statistically treated through a regression analysis with categorical explanatory variables ('unbalanced ANOVA', XLSTAT software).

Results

Characterization

According to our observations, and based on their ultra structure, different mitochondrial morphological populations were found on days 1 to 4 of bovine embryo development, (Fig. 1).

Hooded mitochondria

Round to triangular and trapezoidal sections, with a hooded end that appear in the section open to the cytoplasm, and appear like internal vesicles on other levels. Their cristae are retracted at the periphery following a concentric arrangement and cover either the entire organelle or only the periphery (Fig. 2*A*).

Hooded forms sometimes had either more than one hood or, in cross-section, more than one circular area. These are usually smaller than typical hoods and coexist with them. This variation is often accompanied by loss of the typical shape (round or triangular) and increased matrix electron density (Fig. 2*B*).

Orthodox mitochondria

Round or elongated forms, with cristae perpendicular to the axis, distributed either only in part (immature) or the whole (mature) of the organelle. They present a uniform electron density (Fig. 2*C*).

Swollen mitochondria

Rounded and swollen forms where the matrix is less electron dense than the cristae area. Cristae are very scarce and appear retracted towards the periphery (Fig. 2*D*, *E*).

Mitochondria also appeared in on-fusion state (Fig. 2*F*–*H*), at which they maintain the cristae arrangement of the hooded type and often have fingerings.

Dynamics and quantification

Hooded mitochondria prevailed in all the embryonic developmental days studied (days 1 to 4), while swollen, orthodox and on-fusion types (other mitochondrial types) were observed in a smaller proportion.

When embryos were cultured in serum-free medium from days 1 to 4, the area covered by hooded mitochondria decreased on day 2 ($F = 2.350$; $t_{91,100} = -2.057$; $P = 0.043$), but increased on day 3 ($t_{91,100} = -2.692$; $P = 0.008$). Then, the area covered by hooded mitochondria decreased on day 4. The dynamics

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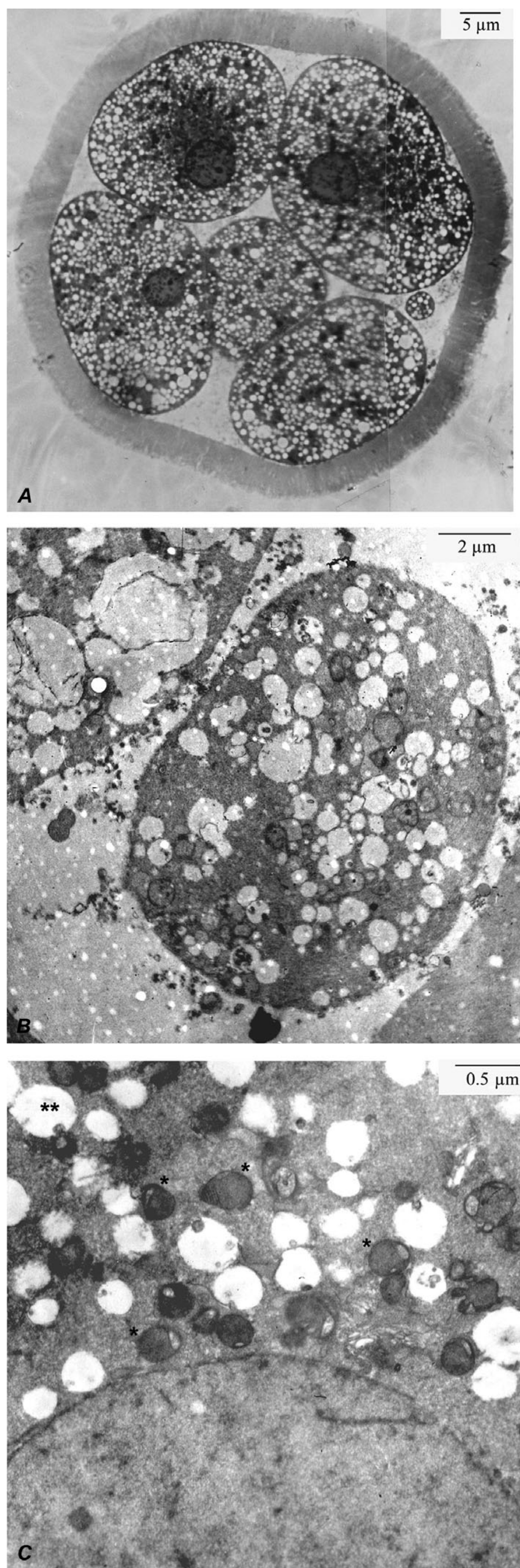

Figure 1 Bovine embryo and cells. (*A*) Embryo on day 3 of development (sections of six cells). (*B*, *C*) Detail of cell. Hooded mitochondria (*) and vesicles (**) are indicated.

observed for the other mitochondrial types was similar (Fig. 3, Table 1).

For the embryos cultured for 3 days in serum-free medium and the embryos cultured during the last 24 h in serum-supplemented medium, the area covered by hooded mitochondria showed no significant difference. The same result was observed for the other mitochondrial types (Table 1).

For the embryos cultured for 4 days, the area covered by hooded mitochondria increased when serum was added (serum 24 h, F = 2.025; $t_{67,75}$ = 2.956; $P = 0.004$; serum 48 h, $t_{67,75}$ = 2.157; $P = 0.035$; Fig. 4) as compared with embryos cultured without the serum treatment. The highest percentage of covered area on day 4 was obtained by keeping embryos in serum for 24 h (serum from day 3 onwards); however, these observations were not significantly different from those obtained with embryos kept in serum for 48 h (serum from day 2 onwards).

On day 4, no significant difference was found regarding the developmental time, for the other mitochondrial types, which can be ascribed to the faint proportions found.

Discussion

Mitochondrial proliferation

The shape, size and number of mitochondria are controlled by fission and fusion GTP-dependent processes (for review see Logan, 2003, 2006; Okamoto & Shaw, 2005). In the human cell cycle, the fusion process gives rise to a network that carries half the number of individual organelles. Then, the number of mitochondria increases again by fragmentation of the network (Barni *et al.* 1996; Karbowski *et al.* 2001; Margineantu *et al.* 2002). The fusion might fulfil a role in the redistribution of mitochondrial DNA (Sheahan *et al.*, 2005), as well as in the membrane and matrix exchange, by helping to maintain and/or restore functions (Chen *et al.*, 2003). The fission process seems to isolate network areas that present mitochondrial damage (Skulachev *et al.*, 2004).

Mitochondrial types and their possible functional relations

According to the mitochondrial types found in the present work, it seems that the hooded type is the main form in all embryos from days 1 to 4, independently of the culture medium.

Hooded mitochondria have been previously considered either as an unusual or a transition type toward mature mitochondria, which appear in early embryos obtained either *in vivo* or *in vitro* (Mohr & Trounson, 1981; Betteridge & Fléchon 1988; Albihn

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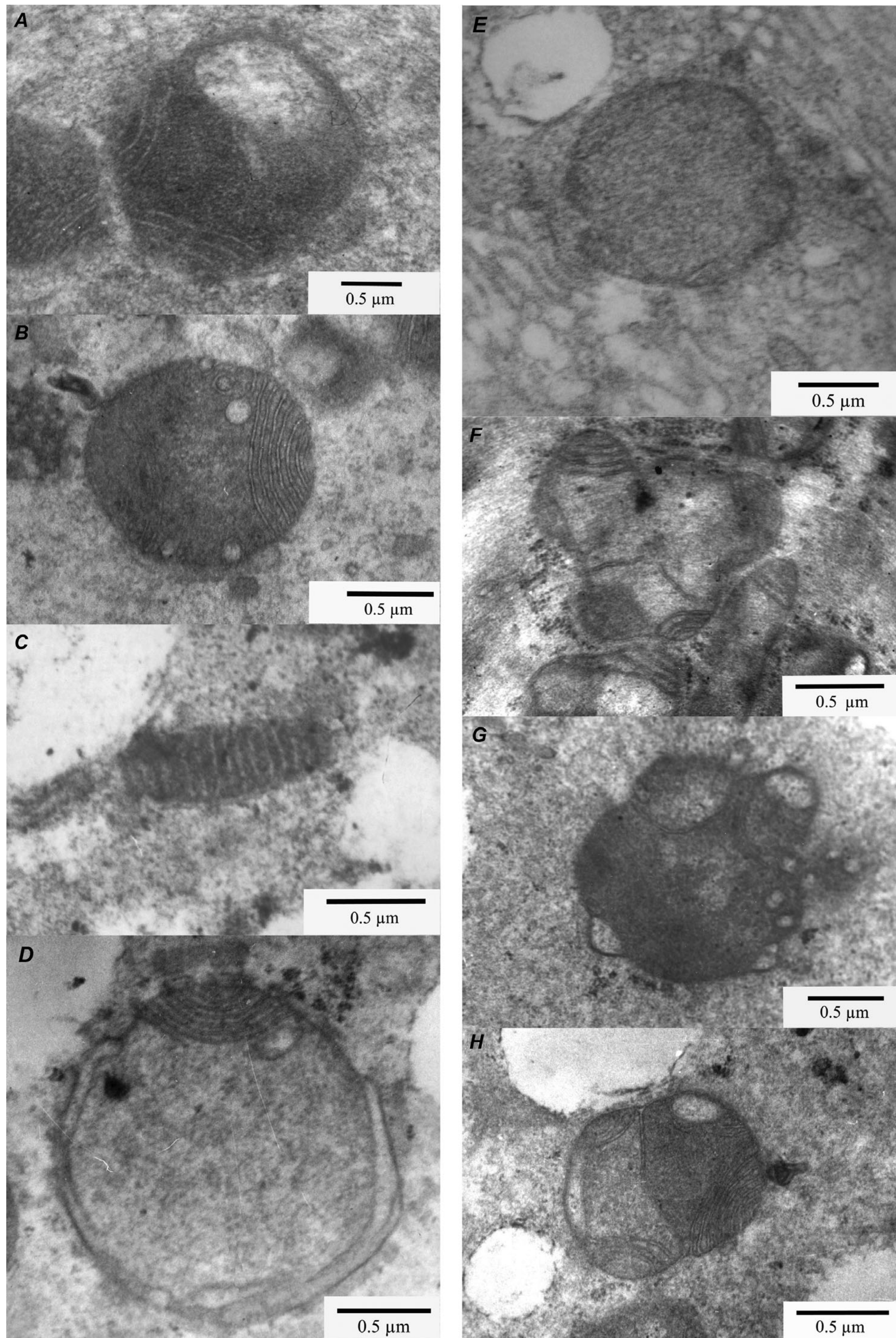

Figure 2 Mitochondrial morphological types of embryos on days 1 to 4. (*A*, *B*) Hooded type; (*C*) orthodox type; (*D*, *E*) swollen type; (*F*–*H*) on-fusion state mitochondria.

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Table 1 Area (%) covered by mitochondria in *in vitro*-produced embryos

	Day 1 sf		Day 2 sf		Day 3 sf		Day 3 serum 24 h		Day 4 sf		Day 4 serum 24 h		Day 4 serum 48 h	
	%	σ	%	σ	%	σ	%	σ	%	σ	%	σ	%	σ
Hooded mitochondria	3.79	1.73	3.49	1.02	3.59	2.87	4.02	0.48	3.32	0.97	4.87	1.24	4.30	1.77
	4.88	0.76	3.17	1.46	5.75	2.53	4.78	1.95	2.64	0.62	4.38	2.18	6.28	1.07
	4.55	1.85	2.43	0.70	3.42	2.92	3.21	1.81	3.96	1.46	5.85	1.80	5.15	0.46
	6.19	1.84	3.08	1.09	3.91	1.75	5.91	2.73	4.14	1.79	6.34	1.48	4.40	1.83
	3.88	1.69	4.14	0.92	4.93	2.50	4.16	2.21	4.93	1.78	3.52	0.79	3.81	0.34
Area (μm^2)	1783		1968		1779		1611		2344		2845		1694	

Data represent the average values of the five observations made in each embryo, taking into account the time at which the serum was added during development.
As the minor mitochondrial types (orthodox, swollen and on-fusion state) covered a small area, they could not be well studied and are thus not presented in the table.
Day 0: fertilization; %: Percentage of area covered respect to total area; σ: standard deviation; sf: serum-free medium; serum 24 h: in serum for the last 24 h; serum 48 h: in serum for the last 48 h.

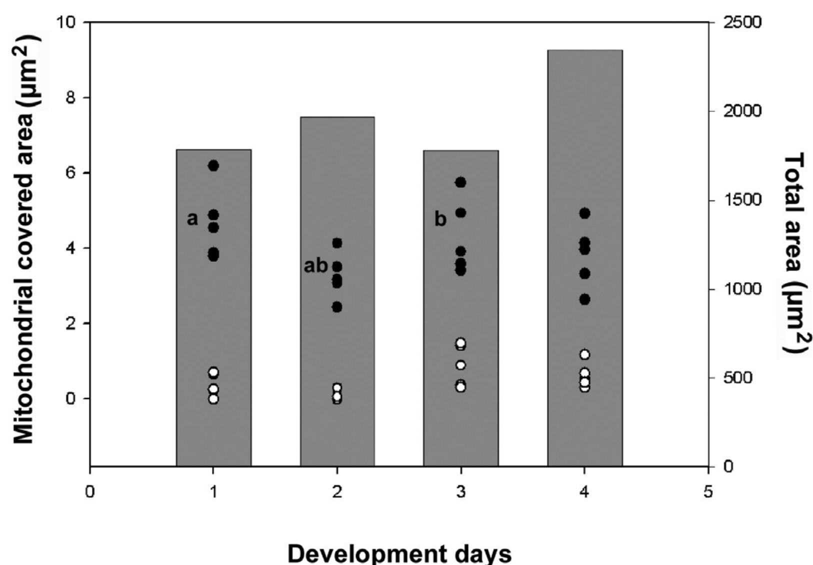

Figure 3 Mitochondrial morph types of embryos cultured in serum-free medium on days 1 to 4. Each datum represents the average values of the five observations made in each embryo. Area covered by hooded mitochondria (black circles), and swollen, orthodox, and on-fusion state mitochondria (other types, white circles) on days 1 to 4. Grey bars indicate the total area studied. [a, b]The same letter indicates significant differences.

et al., 1990; Shamsuddin & Rodriguez-Martinez, 1994). This form appears in *in vitro*-produced mammal embryos, including human embryos, and persists through cleavage and late morulae (Van Blerkom, 2004). The hooded type can be called 'underdeveloped' because it seems to be the useful mitochondrial form in the regulation of mitochondrial activities, limiting the ability of this organelle to oxidative phosphorylation while reducing the generation of reactive oxygen species (ROS; Van Blerkom, 2004). This finding is consistent with previous observations, where pre-compaction mitochondria have shown low metabolic activity (Van Blerkom *et al.*, 1995), low respiratory rate (Trimarchi *et al.*, 2000) and low glucose metabolism (Gardner, 1998).

Mitochondrial swelling appears to be indicative of open pores (mitochondrial permeability transition pore MPTP; Peng & Jou, 2004). Reactive oxygen super-oxides normally synthesized in mitochondria are eliminated through MPTP. High amounts of ROS can facilitate the opening of pores in the mitochondrial membrane and mitochondria with open pores cannot keep potential or imported proteins synthesized in the cytosol (Skulachev *et al.*, 2004). The mitochondrion morphological type that is present when the highest amount of ROS is synthesized by photoradiation, and

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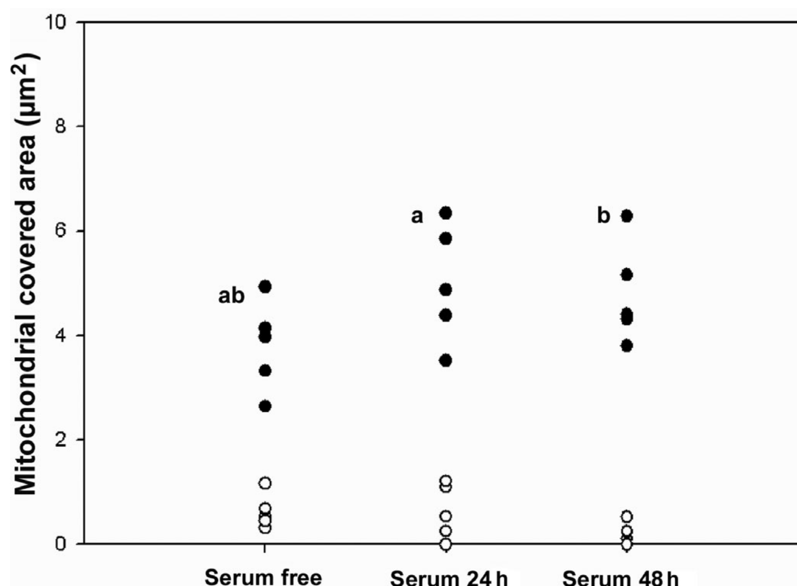

Figure 4 Serum effects on mitochondrial morph types on day 4. Each datum represents the average values of the five observations made in each embryo. Area covered by hooded mitochondria (black circles), and swollen, orthodox, and on-fusion-state mitochondria (other types, white circles) on day 4: serum-free, serum 24 h and serum 48 h treatments. [a, b]The same letter indicates significant differences.

no membrane potential exists, is the swollen one (Peng & Jou, 2004).

Mitochondrial swelling, which is considered degenerative, is common in many pathological conditions (Ferreirinha *et al.*, 2004). In bovines, this mitochondrial morphological type is characteristic on day 13 in frozen–thawed embryos (Mohr & Trounson, 1981). This mitochondrial swelling has been observed both in 2-cell parthenogenetic mouse embryos and in 2-cell fertilized embryos. In the latter, the swelling is observed together with concentric lamellar membranes (Han *et al.*, 2008).

Our results show that this mitochondrial type, associated with high amounts of ROS, is present in proportions less than 15%, depending on the day and treatment. Also, the proportion of the area covered by the swollen type tends to decrease on days 3 and 4 in embryos cultured in serum-supplemented medium, compared with embryos cultured in serum-free medium (day 3: 15% on serum-free medium, 13.5% in serum for 24 h; day 4: 10.2% in serum-free medium, 5.5% in serum for 24 h, 2.8% in serum for 48 h). These results allow us to speculate that the swollen type cannot be endorsed as degeneration produced by the serum-supplemented medium.

On-fusion state mitochondria, which cover a rather scarce area, are present between developmental days 1 and 4 and have a striking similarity with the hooded type. This situation might be related to an incomplete fission and/or mitochondrial fusion process, which, due to the increase in the number of cristae, might become functionally more efficient. These phenomena, although not fully described, have been previously observed in embryos obtained from superovulated cows between days 5 and 13 of development (Mohr & Trounson, 1981).

Our observations indicate that vacuolated mitochondria are found in cultures both with and without serum. Thus, such mitochondrial metabolic type cannot be attributed to serum-supplemented culture media.

The topology of the mitochondrial inner-membrane (cristae) can have a profound effect on mitochondrial activities. These morphological remodelling changes, associated with altered functional states of the mitochondrion, involve fusion and fission of this membrane and alter internal diffusion pathways (Mannella, 2008). The area covered by each mitochondrion is directly correlated with the area covered by the cristae, which is the active surface of mitochondria. Below we will describe some of the mitochondrial and cellular events that might be metabolically related.

The mitochondrial function depends on the coordinated expression of nuclear and mitochondrial genomes. When embryo development starts, the nuclear genome is not fully active; a proportional reduction of the area covered by hooded mitochondria toward day 2 of development might be in relation to the activation of the nuclear genome that starts on day 2.

The activation of the embryonic genome, together with the transcription of mtDNA in cattle, starts during day 2 (in agreement with an 8-cell stage,

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revision see Bavister 1995). This finding would explain the activity increase observed towards day 3 of development.

Early egg divisions are vulnerable and easily stopped, which results in a blocked development, which is observed in the 8- to 16-cell stage of bovine embryos (Rexroad, 1989). The decrease in mitochondrial activity observed towards day 4 in serum-free medium is consistent with the development blockage.

The dynamics observed in the less represented types was similar to the tendency presented by the hooded one; this situation may be caused by the same events: initiation of transcription and blockage of development, which regulates both dynamics and show that the swollen type is not completely inactive.

Serum

The onset of transcription may not be responsible for the blockage of development (Ram & Schultz, 1993). Instead it seems to take place when the culture environment does not provide enough energy for high-demanding processes such as development and differentiation (Barnett & Bavister, 1996). The serum accelerates blastocyst development even if the culture is maintained in serum-supplemented media for only 1 day (9- to 16-cell embryos up to morulae; Gomez & Diez, 2000; Van Langendonckt *et al.*, 1997).

According to our observations, the impact of the blockage on mitochondrial activity may be recovered for embryos cultured in media supplemented with serum for 24 to 48 h. The presence of serum increases mitochondrial activity up to day 4 (embryos about 9 to 16 cells), and the increase in the mitochondrial active area might allow cells to metabolize the predominant glucose from day 4 onwards (Bavister 1995; Gardner, 1998). Oxidative ATP production in the early bovine embryo is provided by the mitochondria from direct oxidation of external lactate and glutamine until the morulae stage (Dumollard *et al.*, 2009).

Many mitochondrial changes, including dysfunctional emergences and lipid accumulation, have been associated with the presence of serum in the culture medium (Dorland *et al.*, 1994; Crosier *et al.*, 2001). Our experimental observations indicate that many embryonic mitochondria are active (as suggested by confocal microscopy images; data not presented here) and their physiological dynamics are coherent with the cell state that should be present according with the embryo daily development. The degenerated types, like the swollen or vacuolated ones, are scarce and are found mainly in serum-free cultures. Therefore, the mitochondrial dynamics seem not to be related to the hindrance of lipid metabolism associated with the presence of serum.

It is our aim for future work to evaluate mitochondrial dynamics experimentally when embryos are fully cultured *in vitro* in serum-supplemented media. In addition, mitochondrial morphology and its correlation with the physiological state of mitochondrial populations shall be carefully evaluated.

In conclusion, from days 1 to 4 of development, the hooded mitochondrial type is the main form present in all embryos, independently of the culture media. Orthodox, swollen, on-fusion-state and vacuolated mitochondria also appear, although in a smaller proportion. The vacuolated mitochondrial type cannot be attributed to serum-supplemented culture media. The presence of serum increases the active area of mitochondria up to day 4. Our results indicate that the mitochondrial dynamics seem not to be related to the hindrance of lipid metabolism associated with the presence of serum.

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