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EMBRYO BIOLOGY

# Does serum cause lipid-droplet accumulation in bovine embryos produced *in vitro*, during developmental days 1 to 4?

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**Summary**
*Purpose* Serum supplementation has shown to have beneficial effects on in vitro bovine embryo development. However, it is often assumed that serum supplementation may produce mitochondrial damage and this damage would generate lipid accumulation, a major obstacle for cryopreservation. The aim of the present study is to investigate the previous assumptions in early embryonic stages.
*Methods* We considered in vitro produced bovine embryos from day 1 to 4 of development, which were grown in presence of serum from days 1, 2 or 3 or in absence of it. Electron transmission micrographs allowed us to quantify the area occupied by lipid droplets and by the different mitochondrial types to evaluate serum effect. Using confocal microscopy we analyzed mitochondrial activity and location.
*Results* We found no evidence of lipid droplets accumulation or mitochondrial degeneration or reduction of mitochondrial area in serum supplemented media. Further, our results suggest that events of mitochondrial proliferation are taking place even in serum supplemented media.
*Conclusions* Serum does not produce lipid accumulation or mitochondrial damage in bovine embryos from 2 to 16 cells. When serum was added to embryo culture medium on day 3 of development, there were ultrastructural signs of a beneficial effect for embryo development. The lack of serum until day 3 may also avoid the unnecessary exposure to potentially inhibitory factors present on it.

**Keywords** Embryo · Serum · Lipid droplet · Mitochondria · Ultrastructure

*Capsule* The present work is an experimental presentation that studies the effects of in vitro culture, on mammal's embryo structure. It explains why better culture could be obtained by adding serum to the medium to a strict "in vitro" embryo age.

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## Introduction

Since the beginning of assisted reproductive technologies in cattle breeding, serum has regularly been used during part or throughout total bovine embryo in vitro development as source of energetic substrates and growth factors [29,38]. However, its complex undefined and variable composition may be associated with several subcellular changes such as lipid accumulation [2,51,52,57].

Cytoplasmic lipids are not only important nutrients for the developing embryo but they also modify the physical and functional properties of the membranes. When exposed to freezing temperatures, physical changes take place in lipids, which could cause significant cell damage [65]. In embryos cultured in medium supplemented with serum, excess lipids in the cytoplasm would be responsible for the high sensitivity to cooling and low tolerance to cryopreservation [32]. Omission of the serum during the culture [50] and removal of cytoplasmic lipid droplets [17,59] improve cryopreservation tolerance increasing survival rates of in vitro produced bovine blastocysts.

Also, there are changes in mitochondrial structure associated with the culture of embryos on serum supplemented media [18,54] that may produce an impaired metabolic function. Crosier et al. [15,16], Abe et al. [3] and Abe and Hoshi

[1] based on observations in morulae and blastocysts produced in vitro proposed that the presence of serum would have adverse effects on mitochondria structure, promoting lipid accumulation, thus reducing the quality and viability of the embryo. Frequently this hypothesis is used to explain survival`s decrease of embryos after cryopreservation treatments though it has not yet been proven.

In the present study we analyze the effect of serum on the area covered by lipid droplets and the area covered by mitochondrial types associated to different functional states [25,36]. We also aim to identify mitochondrial forms that may be related to a detrimental effect of serum during embryo culture. In assessing the relationship between both effects, we examined the hypothesis that serum produces mitochondrial degeneration followed by lipid accumulation in bovine embryos during cleavage stages. Finally, we analyze the best moment to add the supplement to the culture between days 1 and 3. This knowledge will be useful to develop new protocols for in vitro embryo production.

## Materials and methods

### In vitro embryo production

The experimental work was conducted at the Laboratory of Reproductive Biotechnology, Balcarce Experimental Station, National Institute of Agricultural Technology of Argentina, from March to May 2005 and July to Setember 2008. All the chemicals used were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Ovaries were obtained from a local abattoir, placed in saline solution containing gentamycin at 20–25 °C and used within the first 4 h after slaughter. Cumulus-oocyte complexes (COCs) were collected from follicles, 3 to 8 mm in diameter using an 18-g needle. COCs were selected using stereoscopic microscopy, retaining only those containing several layers of compact cumulus cells and homogeneous cytoplasm (COCs Grade 1; [33]).

Groups of 50 COCs were gathered and placed on four-well plates containing 400 μl of Modified Parker Medium (TCM 199, Hepes, gentamycin, $Ca^{2+}$ 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, for maturation. These plates were kept at 38.5 ° C, under 5 % $CO_2$ in air and humidified atmosphere for 22 to 24 h.

For fertilization, frozen-thawed semen from a single bull was centrifuged on a Percoll (30–60–90 % density) gradient. Mature COCs were placed in a new well with 400 μl of TALP solution (Tirodes, albumin, lactate and pyruvate) and 50 μg/ml of heparin. COCs and motile spermatozoa (2 million/ml) were co-incubated in previously equilibrated medium during 24 h.

Putative zygotes were released from the cumulus cells through mechanical aspiration and cultured in 400 μl Charles Ronsenkrans Medium (CR1), 3 mg/ml BSA free of fatty acids, amino acids and glutamine, either with or without the addition of serum supplement. Each well was covered with mineral oil and the plates were maintained during 4 days at 38.5 °C under 5 % $O_2$, 5 % $CO_2$, 90 % $N_2$, and humidity at saturation. Every 24 h the media were renovated and interventions were performed according to the experimental design described below.

The same batch of serum was used throughout the experiment. The supplement consisted of 5 % v/v serum obtained from a single pool of estrous cows, inactivated at 56 ° C for 30 min, filtered and frozen until used.

### Experimental design

We named “manipulation” or “replica” the process that began at each COCs collection and ended when embryos were cultured in different conditions until day 4 (Fig. 1a). Figure 1a shows an schematic picture of the experimental design from development day 1 to 4, in serum free (SF) or serum supplemented culture (SS) for 24, 48 or 72 h, when embryos were collected for analysis (marked with an ***X*** in Fig. 1).

**Day 0:** COCs were randomly distributed into 4-well plates, at a rate of 50 per well. There they were matured and inseminated.

**Day 1:** Presumptive zygotes of the 4 wells were placed into one tube and cumulus cells were removed. One embryo was then collected (1SS). The remaining embryos were placed to cultivate in new 4 wells distributed in equal amounts. Serum was added into one well.

**Day 2:** From each of 2 conditions, an embryo was collected: 2SF and 2SS24. Then the whole medium was changed and serum was added to another well creating a new condition.

**Day 3:** From each of the 3 conditions, an embryo was collected: 3SF, 3SS48 and 3SS24 corresponding to day 3 samples. Media were changed again and serum was added to another well.

**Day 4:** An embryo was collected from each of 4 conditions: 4SF, 4SS24, 4SS48 and 4SS72.

Embryos retrieved from the culture plates, were selected based on the number of cells more prevalent among those expected for the corresponding development day: Day 1: two-cell embryos; Day 2: four- to six-cell embryos; Day 3: seven- to eight-cell embryos, Day 4: nine- to sixteen-cell embryos. For each condition (day and hours) 5 embryos from different replicas of the experimental design were studied.

### Optical and electron microscopy

Six replicas of the previously described experimental design (Fig. 1a) were performed. Immediately after collection from

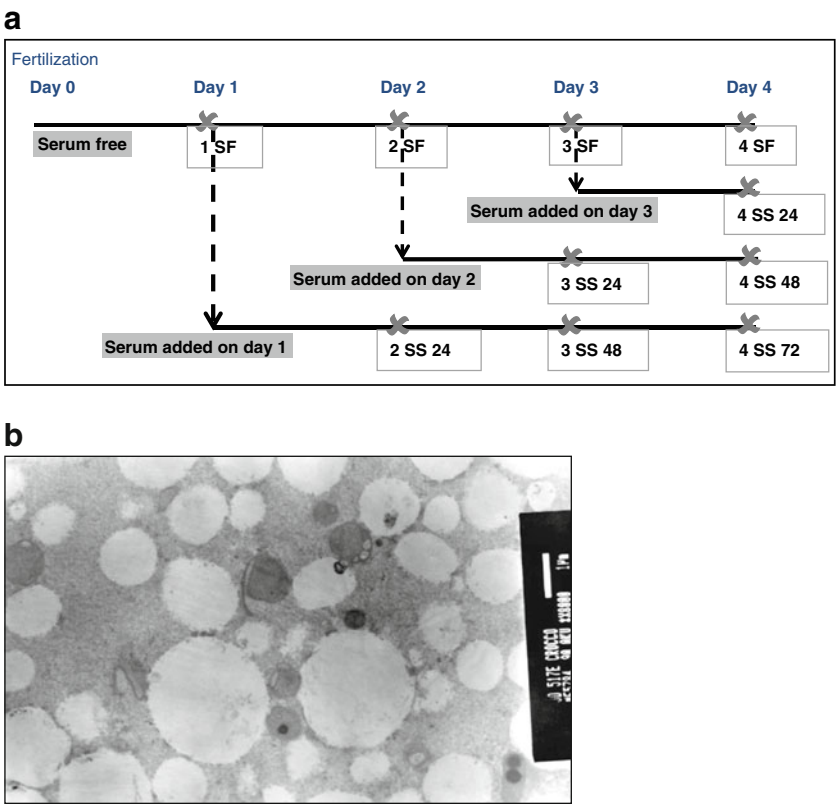


**Fig. 1** Display of the different stages of manipulation for bovine embryos production (**a**). TEM micrograph, where lipid and mitochondrial covered areas were measured (**b**)

the culture, embryos were fixed in 2 % glutaraldehyde in cacodylate buffer at 4 °C for 2 h, post fixed in 1 % osmium tetra oxide in the same buffer, and dehydrated in an ethyl alcohol series and propylene oxide. Next, they were embedded in a polybed-araldite mixture following Mollenhauer procedure [40]. 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 [48] and examined on Zeiss and Jaol transmission electron microscopes. Each set of micrographs corresponding to a single embryo constituted a block analysis.

A total of 50 embryos were analyzed. Five embryos, one from each replica, were used to assess each culture condition (combination of development days and hours of culture with serum, Fig. 1a).

## Morphometric analysis

TEM micrographs (reproduction scale about 8000:1, Fig. 1b) were used to quantify the area occupied by the different mitochondrial morphological types and lipid droplets using the Image Pro Plus analyzer. The measurements were made on five to seven micrographs in selected areas regardless of their content, one micrograph in each blastomere, or 2 micrographs on separate sectors of the same blastomere, when the embryos had few blastomeres. Each micrograph was copied on paper and converted to TIFF digital format. The total and mean covered areas of each structure were expressed as a percentage of the total area of the micrograph.

## Statistical analysis

On each TEM micrograph (Fig. 1b) four variables were studied in order to detect differences due to the development day and serum addition: total mitochondrial covered area, total lipid covered area, mean mitochondrial area and mean lipid droplets area. Next, we describe the multiple regressions fitted for each variable using replica, development day and hours of culture in serum as co-control variables.

In order to characterize the differences between the total mitochondrial covered areas among days of development, two regression models were adjusted: one for embryos cultured without serum, and another for those cultured with serum. Both models used day of development and replica as explanatory variables. Replica was included to control differences between them. A similar analysis was performed to characterize the area covered by lipids.

To characterize differences in the total mitochondrial covered area due to serum, a model for each day was adjusted, using replica and hours of culture in serum as explanatory variables. A similar analysis was conducted to characterize differences in the area covered by lipids.

To characterize differences in the mean mitochondrial area and the mean lipid droplets area, two multiple regression models were adjusted with the mean area as the dependent variable and day of development, hours of culture with serum and replica were used as explanatory variables.

To determine whether the total mitochondrial area was associated with the organelle number on each micrograph and the mean mitochondrial area, multiple regressions were employed using the total mitochondrial area as a dependent variable.

Statistical analyses were performed employing the R software from Development Core Team [46].

### Cytochemistry and confocal microscopy

Three replicas of the above described experimental design (Fig. 1) were performed. Immediately after collection from culture, the embryos were incubated with MitoTracker ® Deep Red 633 (MTDR, Molecular Probes, USA), a cell-permeant probe, which passively diffuses across the plasma membrane and accumulates in metabolically active mitochondria, where it reacts with peptides to form a fluorescent conjugate. The embryos were exposed to a 150 nm concentration of MTDR in dim ethyl sulfoxide (DMSO) at 37 ° C for 15 min in the dark under culture conditions. Fixation was performed in 4 % formaldehyde in phosphate buffer during 1 h. To avoid possible fluorescence reactions which were not due to MTDR, simultaneous controls were performed by treating other embryos with DMSO only. To avoid fluorescent decay during storage, the embryos were placed onto slides in Vectashield medium (Vector Labs, USA).

Embryo analysis was performed with a confocal microscope Olympus FV300 linked to a fluorescence microscope Olympus BX61 and serial optical sections were taken every 5 μm. A red helium neon laser with a wavelength of 633 nm was used to excite the fluorochrome. Fluorescence emission was captured by a long pass filter with a wavelength of cut-off of 660 nm. All images were taken keeping the same acquisition parameters. A total of 40 embryos were analyzed.

## Results

We compare bovine embryos from day-1 to −4, which were cultured in serum free (SF) or serum supplemented (SS) media for different periods (24, 48 or 72 h).

### Lipids

#### *Mean droplet size*

Lipid droplets are distinguished for their lack of double-membrane (characteristic of aqueous vesicles) and their electron dense content. Comparing the mean droplet size in each micrograph, controlling for the hours of culture with serum and the effect of manipulation, we can see that:

- There is no significant effect due to the presence of serum in the culture medium
- There is a significant effect ($P=0.0009$) regarding the days of development. Moreover, the lipid average area triplicates each development day (Table 1).

#### *Lipid total area*

Evaluating embryos in serum-free cultures (Fig. 2a) we observed that total lipid droplets area was similar between days 1 to 3. By day 4 (4SF), the total area increased significantly in relation to day 1 (1SF, $t=3.184$, $df=91$, $P=0.002$) and 3 (3SF, $t=2.611$, $df=91$, $P=0.011$).

Cultures supplemented from day 1 (Fig. 2b), presented a significant decrease of the area covered by lipid droplets on day 3 (3SS48), regarding days 1 (1SF, $t=2.269$, $df=85$, $P=0.026$) and 2 (2SS24, $t=2.054$, $df=85$, $P=0.043$).

Assessing day-3 embryos (Fig. 2c), those cultured with serum for 48 h (3SS48) showed a significant decrease in the total lipid droplets area compared with both 24 h serum (3SS24, $t=-3.532$, $df=66$, $P=0.001$), and without serum (3SF, $t=-2.621$, $df=66$, $P=0.011$).

Day-4 embryos (Fig. 2d), presented significantly higher total areas in serum-free cultures (4SF) than both 24 h serum (4SS24, $t=-2.906$, $df=89$, $P=0.005$) and 72 h serum (4SS72, $t=-3.064$, $df=89$, $P=0.003$).

In Fig. 2a and b, although the total area studied (bar height) increased for the condition 3SS48 regarding the condition 1SF, the lipid areas were reduced. This shows that the increase in the area is not associated to the area covered by lipid.

### Mitochondria

#### *Activity and distribution*

Mitochondrial activity and distribution in the blastomeres were shown by MTDR reagent fluorescence in confocal microscope images (Fig. 3). In cultured embryos from day-

**Table 1** Estimated changes in lipids mean area of bovine embryos regarding day 1

| Day | Estimation | Standard error | t statistic test | *p*-value (>\|t\|) |
|---|---|---|---|---|
| 2 | 0.06364 | 0.19218 | 0.331 | 0.740811 |
| 3 | 0.20904 | 0.18425 | 1.134 | 0.257694 |
| 4 | 0.62165 | 0.18595 | 3.343 | 0.000958 |

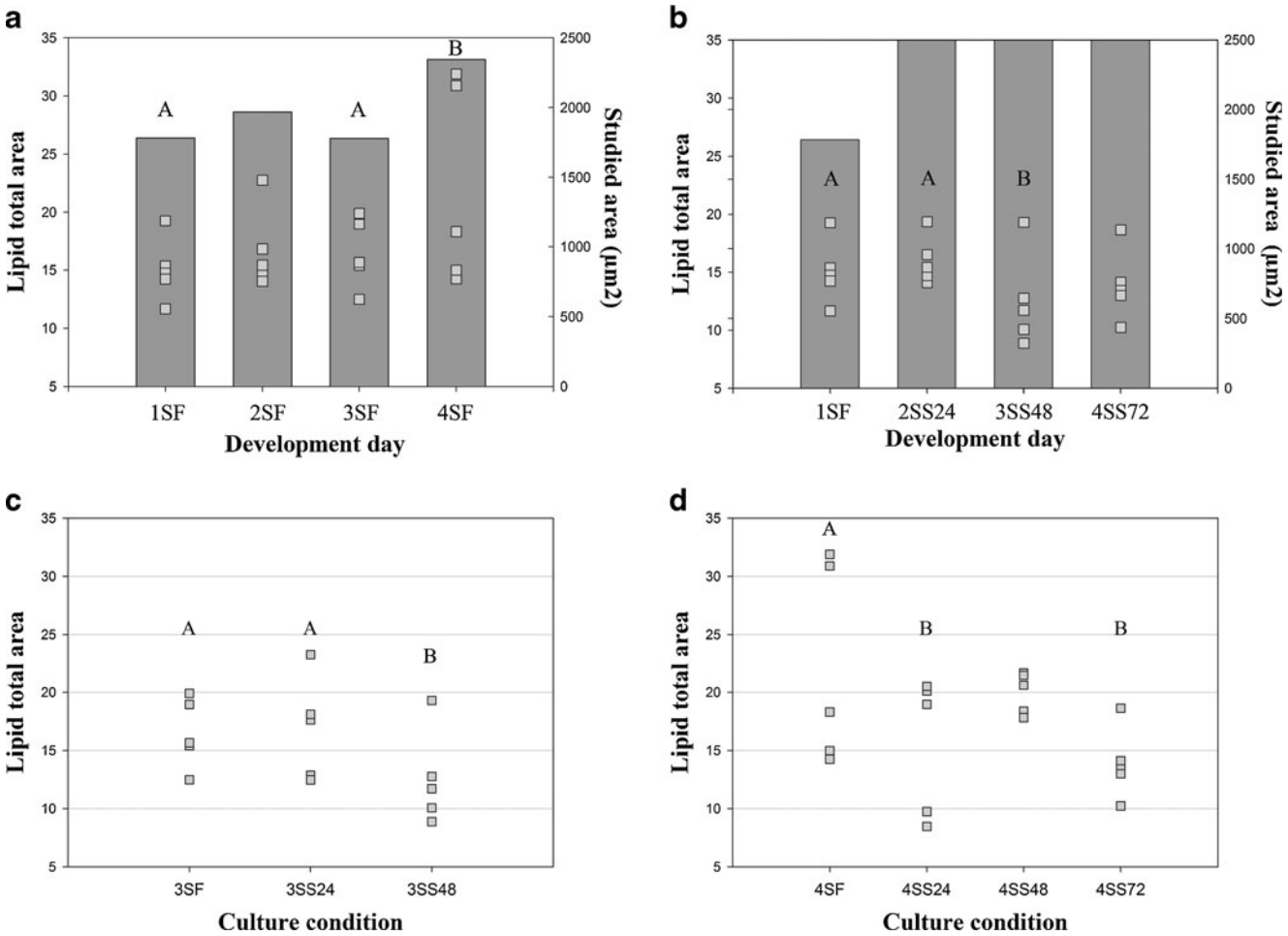


**Fig. 2** Area covered by lipids on bovine embryos of 1 to 4 days cultured without serum and with serum since day 1 (**a**, **b**). For each embryo, *empty squares* show the average of lipid droplets area. The height of each bar represents the studied area of the 5 embryos. *Bars* greater than 2,500 µm2 were cut. Area covered by lipids on bovine embryos of 3 and 4 days of development (**c**, **d**). Different letters on the display indicate significant differences

1 to −3, a peri-nuclear distribution of active mitochondria was observed. However, on day 4 mitochondrial distributions showed a more diffuse pattern.

*Ultrastructure and mitochondrial types*

All embryos analyzed presented a well preserved cell structure regardless of the culture conditions. Mitochondrial types were those already identified in [14] for bovine embryos from 2 to 16 cells.

- **Hooded:** Previously described by Senger and Saacke [53], have a hooded end, visible as a hook or as an internal vesicle, according to the cutting plane. Cristae retracted at the periphery following a concentric arrangement.
- **Orthodox:** Round or elongated forms, with cristae perpendicular to the axis, distributed either only in part or in the whole organelle.
- **Swollen:** Rounded and swollen forms where the matrix is less electron dense than the cristae area. Cristae are very scarce, retracted towards the periphery.
- **On-fusion-state**: The shape suggests the adhesion of several mitochondria, including the hooded type.

*Mitochondrial total area*

Hooded mitochondria prevailed in embryos from day-1 to −4 of development, regardless of the culture condition. Swollen, orthodox, and on-fusion types were observed in a smaller proportion and were assembled under the name of "other types" in Fig. 4.

Hooded mitochondria dynamics were similar for embryos grown without serum (2SF, 3SF, 4SF, Fig. 4a) and grown with serum from day 1 (2SS24, 3SS48, 4SS72, Fig. 4b). In both instances areas decreased towards day 2, increased in day 3, and decreased again on day 4.

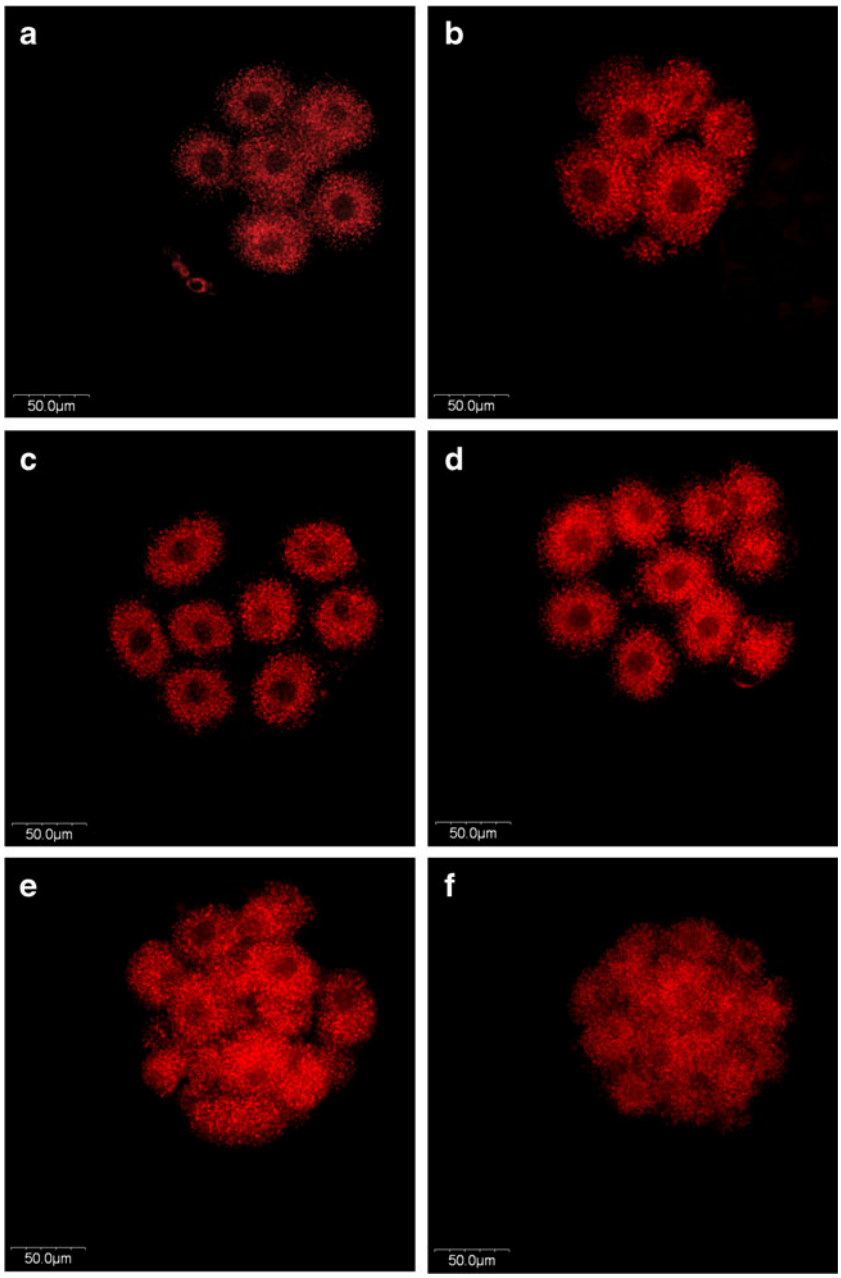


**Fig. 3** Active mitochondria in bovine embryos of 2 (**a**, **b**), 3 (**c**, **d**) and 4 (**e**, **f**) days of development, cultured respectively without serum (*left side*) and with serum since the day 1 (*right side*). Mitochondria can be observed fluorescing in *red*

Towards day 2 in serum-free medium (2SF) we found a significant decline with respect to day 1 (1SF, t statistic value ($t$)=−2.758, degree of freedom ($df$)=100, p-value ($P$)=0.007) and day 3 (3SF, $t$=−2.754, $df$=100, $P$=0.007). However, this reduction was not significant on day 2 embryos cultured in supplemented medium (2SS24) compared to day 1(1SF) or day 3 (3SS48).

Towards day 4, in supplemented cultures (4SS72), the hooded mitochondria covered area was reduced compared to day 1 (1SF, $t$=−2.079, $df$=93, $P$=0.040) and day 3 (3SS48, $t$=−2.286, $df$=93, $P$=0.025, Fig. 4b). A similar but non significant decrease was observed in cultures without serum (4SF)

Assessing day-3 embryos and comparing culture conditions with each other (3SF, 3SS24, 3SS48, Fig. 4c) we found no differences between the areas covered by the hooded mitochondria.

Evaluating day-4 embryos exposed to different culture conditions (4SF, 4SS24, 4SS48, 4SS72, Fig. 4d), we found that the area covered by hooded mitochondria was significantly higher in medium that was supplemented with serum for 24 h (4SS24) compared with serum-free medium (4SF, $t$=−2.452, $df$=90, $P$=0.016), and medium supplemented for 72 h (4SS72, $t$=2.332, $df$=90, $P$=0.022).

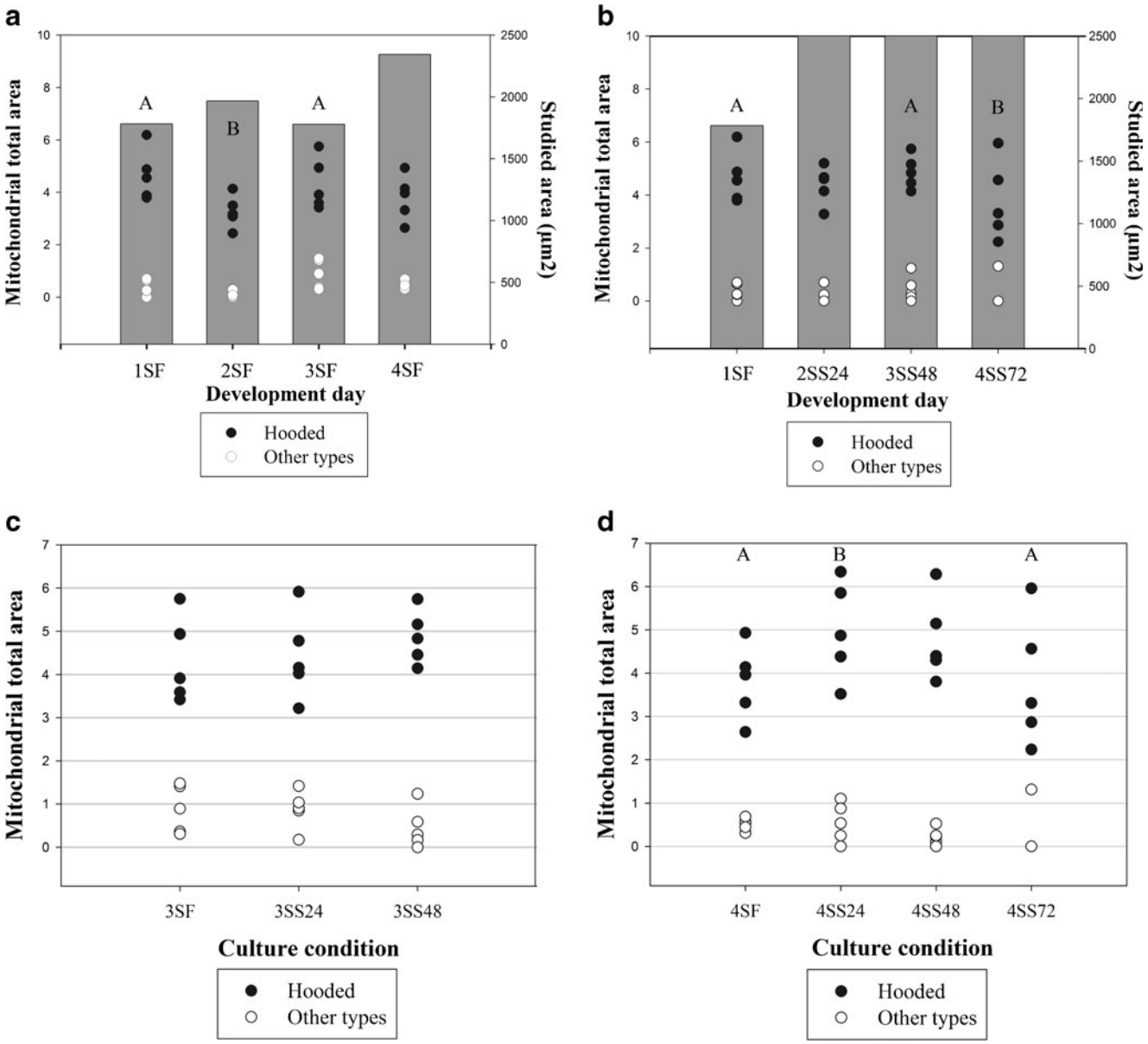


**Fig. 4** Area covered by mitochondria on bovine embryos of 1 to 4 days cultured without serum (**a**, [14] Reprinted with permission), and with serum since day 1 (**b**). For each embryo, filled and empty dots show respectively the average of hooded and "other types" mitochondrial areas. The height of each bar represents the studied area of the 5 embryos. Bars greater than 2,500 $\mu m^2$ were cut. Area covered by mitochondria on bovine embryos of 3 and 4 days of development (**c, d**). Different letters on the display indicate significant differences

The "other types" mitochondria reached very small areas and presented a high relative variability. That may be the reason why no statistical differences were found between the mitochondrial areas among different days of development or different culture conditions.

In Fig. 4a, although the total area studied (bar height) increased for the condition 4SF regarding 3SF, the mitochondrial areas were reduced. This shows that the increase in the area is not associated to the area covered by mitochondria

*Relation between the area, number and mitochondrial size*

The hooded mitochondria covered area presented a positive association with both the number of mitochondria (correlation coefficient ($r$)=0.59, $P<0.00001$) and the mean mitochondrial area ($r=0.26$, $P<0.00001$). This association is stronger in the first case. As previous associations may have been affected by confounding effects due to differences in the developmental days, hours of culture in serum and manipulation, multiple regression models were adjusted using

these variables as control co-variables. These models showed that 47 % of the variability observed in the total hooded area can be explained by the number of mitochondria and 18 % of the variability can be explained by the mean area.

### Relations between mitochondrial and lipids total areas

Using a simple linear regression between mitochondrial and lipids total areas we estimated a correlation coefficient of −0.29 ($P<0.00001$).

Considering this correlation could be affected by confounding effects due to differences among the developmental days, hours of culture in serum and manipulation, we adjusted a multiple regression model where these variables were included as covariates of control. We obtained a coefficient of −0.30 for the area covered by lipids ($P<0.00001$). The results indicate a clear negative association so that if there is an increase in the area covered by hooded mitochondria there is a reduction in the area covered by lipids in each category, and vice versa. This is independent of culture with or without serum.

## Discussion

### Lipids

As the embryo develops, lipids can be transported into the mitochondrial matrix, decomposed by successive cycles of beta-oxidation, and used to increase the ATP production. The lipid β-oxidation would be essential for embryonic development, as demonstrated by Dunning et al. [19]. According to our observations, there is a negative association between the area covered by lipids and hooded mitochondria. This negative association may indicate that the increase in mitochondrial area enhances the ability to perform embryonic beta-oxidation of fatty acids.

From day 1 through 4, i.e. 2–16 cells embryos, we observe an increase of the average size of the lipid droplets independent of the culture condition (Table 1). During cryopreservation, larger droplets lead to a reduction of the organization of the cytoplasm and produce irreversible damage to the embryo [24,26]. Thus we do not expect that 2–16 cells embryos tolerance to cryopreservation will be different between embryos cultured with or without serum, as occurs in blastocysts [50]. In contrast, because the average sizes of lipid droplets increase with time we consider that the tolerance to this procedure may be lower on day 4 embryos.

In embryos cultured in serum-free medium, according to Ferguson and Leese [21], triglycerides the main content of lipid droplets, remain constant between 2-cell stage and blastocysts. In our study, lipids increase at day 4 due to a couple of embryos (Fig. 2a). Those embryos may reflect intrinsic biological variability and/or alterations due to laboratory handling of embryos independent of serum. Without them, the observed differences regarding day 4 embryos cultured with serum for 24 and 72 h (Fig. 2d) would vanish. However, excluding these 2 embryos of the previous analyzes, the associations between droplet size and days of development do not change (Table 1).

The embryos cultured in the presence of serum since day 1 reduced on day 3 lipid areas (Fig. 2b), this could indicate an increase in the use of lipids on the block onset of development.

Looking at the embryos of 3 (Fig. 2c) and 4 days of development (Fig. 2d) we did not find evidence that the culture medium containing serum increases the area covered by lipid droplets. Neither did Crosier et al. [15], with the same kind of serum and a similar technique used by us to quantify, studying the morula stage. However, on day 4 of embryos cultured with serum since day 2 (9–16 cells), Ferguson and Leese [21], observed that the triglyceride content increases in regard to the previous days and to the same stage without serum. Although the technique used by us to quantify lipids might not have been sensitive enough to detect the accumulation such as the one used by Ferguson and Leese [21], it is possible that 10 % v/v of fetal serum used by them generate earlier accumulation than the 5 % v/v estrous serum used by us and Crosier et al. [15].

### Mitochondria

Confocal microscopy showed that a high proportion of the embryo is covered by active mitochondria (hooded, orthodox and possible on-fusion state types) where most oxidative reactions responsible for the supply of energy are settled [12]. On our study they are arranged in perinuclear zones of the blastomeres during the first 3 days of development, in agreement with the observations of Krisher and Bavister [28]. The perinuclear distribution would be a strategy to adjust mitochondrial density to a region where high concentrations of ATP are required [61]. By day 4 we found them homogeneously distributed, this change in distribution could be attributed to the culture environment [6], or to changes in the embryo energy needs. These suggestions may be the aim of further studies.

Electron microscopy showed very high percentages of hooded-type mitochondria the most prevalent type in bovine embryos from 2 to 16 cells [14]. According to Van Blerkom [60] they regulate the activities of the organelle and therefore they control the oxidative functions in the blastomere. This was the only type that allowed us to detect changes in the covered area among the culture conditions studied. We take the total area covered by hooded mitochondria as the sole indicator of the embryo's activity, and assume that larger areas correspond to greater potential for metabolic activity.

When comparing the areas covered by hooded mitochondria differences were found attributable to the supplement's effects. The embryos cultured in supplemented medium since day 1 (Fig. 4a and b), show maintenance of mitochondrial area between days 1 and 3 of development. This may be useful for sustaining the high energy demand caused by the activation of embryonic genome on day 3 (8 cells, [7]).

On day 3 begins the "blocks to development" characteristic of mammalian in vitro development (8 a16 cells in cattle, [47]). It is possible that the in-vitro block to development occurs because of the high energy demands unsupported by the culture medium [5]. Our results on day 3 (Fig. 4c) indicates that serum added from day 1 or 2 does not promote the increase of mitochondrial areas.

Comparing all treatments on day 4 (Fig. 4d), serum supplement from day 3 when the embryo depends on its own energy production (4SS24), seems advantageous. Serum from day 3 appears to induce an increase in mitochondrial area in these embryos. This increase in potential for mitochondrial activity at day 4 would favor the change to a glucosidic metabolism and facilitate the resumption of development (16 cells in cattle, [7,22]).

The mitochondrial proliferation is due to its fusion and fission processes [11,34,35,37,43,55]. We questioned ourselves whether the differences detected in the mitochondrial covered area were due to proliferation or changes in the size of individual mitochondrion. We observed that variability in the total area covered by mitochondria could be explained mainly by the increased number of organelles in each category, i.e. by the fission process (Section 3.2.4). In this sense, the results of Chiaratti et al. [13] are consistent with our observations: 1-cell embryos that were able to recover the high mtDNA content removed by centrifugation and advanced to the blastocyst stage, must maintain functional mechanisms of fusion and fission required for the distribution of genetic material. This may also explain on the mouse oocyte model, in which with only 4,000 copies of mtDNA, embryos were able to progress until the blastocyst stage [62].

### Best time for the addition of serum

To evaluate the best time to add the serum to the culture we recall that none of with-serum treatments produced lipid accumulation in the blastomeres. Respect to the area covered by hooded mitochondria the favorable effects were observed in embryos cultured with serum from day 1 and from day 3. However, when the serum is added on day 1, the mitochondrial covered areas are reduced on day 4 compared to the embryos cultured with serum since day 3 (Fig. 4d). Serum since day 3 is beneficial regarding serum from day 1 not only on day 4 but also in day 6 embryos [15]. In this morulae stage the effect was also due to the hooded mitochondrial type.

In previous reports, the addition of serum on days 1 or 2 doesn't seem to produce improvements in the percentage of blastocysts compared to cultures without-serum [10, 20, 50]. Also, addition of serum to the culture before completing day 1 of development, significantly reduced the proportion of embryos reaching the morula and blastocyst stages compared to the ones cultured with serum since day 3 [45]. Another study conducted in the same laboratory as ours but in embryos co-cultured with granulosa cells, showed that total blastocyst yield and cryopreservation tolerance did not differ among embryos cultured without serum and with serum since day 3 [42]. Our results indicate that day-4 embryos grown without co-cultured but with serum since day 3 are better prepared to sustain the energy demand of subsequent processes than embryos grown without serum or with serum from day 1.

Serum introduced in the culture media provides variable amounts of factors that lead to variations in the media`s final composition, produce diverse effects on the development and mask the understanding of nutritional requirements. However, defined and sequential media [23,30,41,44,56] allow reproducible results. In this study the restriction on the use of the serum until day 3, in the presence of BSA, would be advantageous also by avoiding unnecessary exposure to potential inhibitory factors. Restricting the use of serum will increase the odds of understanding the unique physiology of the embryo during in vitro development [39] in order to reduce damage caused by the technique.

### Alternative mechanism for lipid accumulation

Lipid accumulation was found and quantified from day 4 of development until the blastocyst stage of embryos cultured with serum [21]. Further, in the blastocyst stage of bovine embryos, the relative abundance of mRNA for lipidogenics genes increased regarding the previous stages [4]. Camargo et al. [9] described a link between the level of metabolized glucose through the pentose phosphate pathway and the expression of some lipidogenics genes that would explain lipid accumulation in the embryo's cytoplasm. In this link, the glucose 6-phosphate dehydrogenase (G6PD) is the limiting enzyme which controls the pathway's flow. The use of G6PD inhibitors in the culture medium increases the proportion of embryos that survive the cryopreservation process [27].

On preimplantation embryo both aerobic and anaerobic respiration are actively contributing to development [61,63,64]. On stages previous to the embryonic genome activation (8 cells), part of the scarce glucose used would be metabolized through the pentose phosphate pathway [49], involved in lipid accumulation. Then, from 16 cells stage, the consumption of glucose increases and appears to be essential for further development [7,22]. Through the mitochondrial pathway glucose is metabolized giving a small contribution to the energy requirements in cleavage stage [58] increasing to the blastocyst stage [8]. However, it would be the pentose phosphate pathway which leads to

the accumulation of lipids obtained by de novo lipidogenesis [9]. This hypothesis based on the use of glucose as substrate, explains why in the early cleavage stages embryos analyzed in this study, which employ pyruvate, lactate and glutamine as preferred substrates (which have numerous, overlapping, metabolic roles -[31]), there is no evidence of lipid accumulation. Also, glucose is one of the most important components of serum, and even more important in fetal than in adult serum [45]. It is possible that this difference would lead to Ferguson and Leese [21] to find early lipids accumulation in embryos of 4 days of development.

## Conclusions

1) Regarding the hypothesis that attributes to serum lipid accumulation through mitochondrial damage, we can only conclude that serum does not produce lipid accumulation nor mitochondrial damage in bovine embryos from 2 to 16 cells:

- Intracellular lipids are not increased in embryos cultured in supplemented medium regarding the serum-free medium.
- We have no evidence of the reduction of hooded mitochondria in medium containing serum with regard to the serum-free one. Instead, by treating embryos for 24 h with serum, the areas covered by hooded mitochondria increased.
- We found no mitochondria with evidence of degeneration due to serum. Also, there is no evidence in other studies of different mitochondrial types than those described here. The swollen, the only type associated with mitochondrial function degeneration, was present in cultures with and without serum.

2) Regarding the best moment to add the supplement to the culture between days 1 and 3, day 3 shows signs of being advantageous.

3) Evidence emerged to assume that events of mitochondrial proliferation are taking place in cleavage stage embryos.

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