CSIRO PUBLISHING

Reproduction, Fertility and Development
http://dx.doi.org/10.1071/RD15067

Influence of bovine serum albumin and fetal bovine serum supplementation during *in vitro* maturation on lipid and mitochondrial behaviour in oocytes and lipid accumulation in bovine embryos

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Abstract. Proper oocyte maturation is crucial for subsequent embryo development; however, oocyte mitochondrial and lipid-droplet behaviour are still poorly understood. Although excessive lipid accumulation during *in vitro* production (IVP) of bovine embryos has been linked with impaired cryotolerance, lipid oxidation is essential for adequate energy supply. Fetal bovine serum (FBS) and bovine serum albumin (BSA) are supplements used during IVP, containing high and low lipid content, respectively. This study aimed to understand how these supplements influence oocyte mitochondrial and lipid behaviour during *in vitro* maturation (IVM) in comparison to *in vivo* maturation, as well as their influence on development rates and embryo lipid accumulation during IVP. We demonstrate that only *in vivo*-matured oocytes maintained correlation between lipid content and active mitochondria. IVM media containing FBS increased total lipid content 18-fold and resulted in higher lipid accumulation in oocytes when compared with media with BSA. IVM using a lower FBS concentration combined with BSA resulted in satisfactory maturation and embryo development and also reduced lipid accumulation in blastocysts. In conclusion, IVM causes changes in mitochondrial and lipid dynamics, which may have negative effects on oocyte development rates and embryo lipid accumulation. Moreover, decreasing FBS concentrations during IVM may reduce embryo lipid accumulation without affecting production rates.

Additional keywords: *in vitro* production (IVP), lipid droplet, mitochondria, reproduction.

Received 19 February 2015, accepted 2 April 2015, published online 19 May 2015

Introduction

Oocyte maturation is one of the most precise determinants of subsequent embryo quality. During this phase, the oocyte undergoes processes that are crucial not only to activation of the embryonic genome, but also throughout development to the blastocyst stage, given that the female gamete is the sole cytoplasmic donor during the formation of the zygote (Gilbert 2003).

In vitro production (IVP) of cattle embryos has increased considerably in recent years. According to the International Embryo Transfer Society (IETS 2012), in 2011 global rates of *in vivo* embryo transfers remained stable, while transfers of

in vitro-produced bovine embryos increased for the sixth consecutive year, placing this reproductive biotechnology as an important player in the cattle industry. However, there are still many limitations to the technology, where only 40–50% of oocytes, matured and fertilised *in vitro*, reach the blastocyst stage (Rizos *et al.* 2002). Furthermore, excessive lipid accumulation occurs following IVP in bovine embryos when compared with *in vivo* production (Abd El Razek *et al.* 2000; Rizos *et al.* 2002), mainly following the use of fetal bovine serum (FBS) in the culture media, which has been associated with low embryo cryotolerance (Rizos *et al.* 2002, 2003).

The most abundant lipid in oocytes and embryos is triacylglyceride (TG; Ferguson and Leese 1999), which is stored in cytoplasmic lipid droplets. TG is synthesised in endoplasmic reticulum (ER) where the enzyme diacylglyceride acyltransferase 2 (DGAT2) is responsible for the formation of TG from diacylglycerol and acyl-CoA, during the final step of synthesis (Stone *et al.* 2009). Once synthesised, TGs can be metabolised or accumulated in lipid droplets, and DGAT2 is associated with entry of TG into the formed droplets (Kuerschner *et al.* 2008). Pirilipin 2 (PLIN2) is a structural protein found on the surface of lipid droplets that is involved in limiting the interaction between lipase and the droplets and, therefore, is positively correlated with their number and size (Bickel *et al.* 2009). Despite the known deleterious effects on cryopreservation (Rizos *et al.* 2002), lipids are required by all cells, given that the metabolism of fatty acids in mitochondrial β-oxidation is one of the most important pathways supplying energy to the cell, in the form of ATP (Cummin 2004).

The importance of mitochondrial oxidation in embryo development is well established and is associated with meiosis reactivation in oocytes, as well as embryo development to the blastocyst stage (Dunning *et al.* 2010). Although the increase of mitochondrial activity during bovine embryo development was previously described (Tarazona *et al.* 2006), it is believed that mitochondria of oocyte origin are responsible for energy supply during development, since there is no synthesis of new organelles until the blastocyst stage (Van Blerkom 2011). Although there is a consensus regarding the increase in mitochondrial activity during *in vitro* maturation (IVM) in different species (Tarazona *et al.* 2006; Romek *et al.* 2011), lipid behaviour and the interaction with mitochondria, at this stage, remain poorly understood. Recently, studies have shown an increase of lipid, particularly TG, in the oocyte during bovine IVM, with or without FBS in the maturation media (Ferreira *et al.* 2008; Aardema *et al.* 2011; González-Serrano *et al.* 2013). Migration patterns for lipid and mitochondria have not been intensively investigated, and current knowledge, based on earlier studies with electron microscopy data, shows that during *in vivo* maturation of bovine oocytes, mitochondrial and lipid migration occur concurrently from the periphery of the cytoplasm to a more dispersed distribution (Kruip *et al.* 1983; Hyttel *et al.* 1986).

Considering the variations observed between *in vivo*- and *in vitro*-matured bovine oocytes as well as differences in development rates between *in vivo*- and *in vitro*-produced blastocysts, a thorough characterisation of the maturation process, both *in vivo* and *in vitro*, is warranted. We hypothesised that IVM leads to different lipid and mitochondrial behaviour patterns in matured oocytes compared with the *in vivo* maturation process, which can, in turn, alter lipid metabolism in *in vitro*-produced bovine embryos. Based on this hypothesis, the present study aimed to investigate how supplements, with either a high or low lipids content, affect lipid and mitochondrial behaviour in *in vitro*-matured oocytes, while also making comparisons with the patterns observed during *in vivo* maturation, and the consequences of this on *in vitro* embryo development and lipid quantity. To our knowledge, this study is the first to describe the differences between bovine *in vitro* and *in vivo* maturation in relation to lipid and mitochondrial migration and quantity. We also demonstrate that it is possible to decrease embryo lipid accumulation by reducing the FBS concentration currently used during IVM in cattle.

Materials and methods

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

Ethics

All animal handling was approved by the ethics committee for animal welfare of the Faculdade de Ciências Agrárias e Veterinárias of the Universidade Estadual Paulista 'Júlio de Mesquita Filho' (FCAV/UNESP).

Experimental design

Three experimental groups with different protein supplements in IVM medium were utilised. The first group was supplemented with 8 mg mL^{-1} bovine serum albumin (BSA)–fatty acid-free (BSA-FAF; BSA group), the second with 10% FBS (Cripion; Industria Brasileira, Andradina, Brazil; FBS group) and a third group, called B+F, contained lower concentrations of each supplement: 6 mg mL^{-1} BSA-FAF+5% FBS.

In order to characterise the influence of each maturation medium on oocyte and embryo lipid accumulation during the *in vitro* process, we calculated the total lipid content of the media used, based on the lipid content of BSA-FAF provided by the manufacturer and by analysing that of FBS using gas chromatography.

Next, we assessed the effects of the different supplements used during IVM on nuclear maturation and migration of cortical granules. Subsequently, we performed a comparative study of the lipid–mitochondrial behaviour between IVM groups and an *in vivo*-matured group. We also evaluated the effects of IVM treatments on cleavage and blastocyst rates and embryo lipid accumulation. Lastly, to better understand the effects of protein supplementation on lipid metabolism, we investigated the expression of two genes involved in lipid synthesis, *PLIN2* and *DGAT2*, in cumulus cells, in immature and mature denuded oocytes, as well as in embryos.

Quantification and analysis of lipid profile in FBS and calculation of lipid content in IVM media

The lipid quantification and fatty-acid profile of FBS were performed by gas chromatography. The results of the lipid

content and fatty-acid percentages were determined by averaging five replicates from samples of 5 mL of FBS. Following lipid extraction with acid hydrolysis and transformation of acids into fatty-acid methyl esters, the samples were injected into a gas chromatograph (GC Schimadzu 17 A/Class CG 10; GC Shimadzu 17A/Class CG 10, Kyoto, Japan) in a chromatographic column of SP-2560 fused silica 100 m and 0.25 mm internal diameter (i.d.). The results of fatty-acid profiles for the samples are expressed in mg per 100 mL and % of area, and the determination of total lipids, calculated from the triglyceride tridecanoic acid (internal standard), is shown in g per 100 mL.

After determination of lipid quantity for FBS (g per 100 mL), and taking into account the manufacturer's indication of the maximum lipid content of BSA-FAF (0.02% fatty acids), we calculated the amount of lipid present in each IVM medium, according to the concentration of each supplement used.

Obtaining immature and in vitro- *and* in vivo-*matured oocytes*

We collected cumulus–oocyte complexes (COCs) by follicular aspiration from slaughterhouse ovaries to obtain immature and also to produce *in vitro* matured oocytes, following IVM. Grade I and II COCs, following the classification described by Leibfried-Rutledge *et al.* (1987), were selected. A portion of the COCs was randomly allocated to evaluations involving immature oocytes and the remainder were used for IVM in the different maturation media (treatment groups: BSA, FBS and B+F). The stock for IVM media comprised TCM199 medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 25 mM sodium bicarbonate, 1 μg mL^{-1} FSH (Folltropin; Bioniche Animal Health, Belleville, ON, Canada), 50 μg mL^{-1} human chorionic gonadotrophin (hCG, Vetecorn; Intervet, Itagaçaba Cruzeiro, Brazil), 1 μg mL^{-1} oestradiol, 83.4 mg mL^{-1} amikacin (Biochimico Institute, Rio de Janeiro, Brazil) and 0.2 mM sodium pyruvate. For IVM, we maintained COCs at 38.5°C and 5% CO_2 in air and high humidity for 24 h.

To collect *in vivo*-matured oocytes, six crossbred cows were synchronised and overstimulated according to the protocol described in Fig. 1. On the Day 1 of the protocol, we performed transvaginal ultrasound-guided ovum pick-up (OPU) for synchronisation of follicular waves and inserted a progesterone implant (Sincrogest; Ourofino Agribusiness, Cravinhos, Brazil) that remained in place for 7 days. On the Day 4 we began follicular overstimulation by injecting decreasing doses of FSH (Pluset; Laboratorio Calier SA, Barcelona, Spain) for a total of 250 IU FSH per animal by the Day 7. We applied a single dose of 2 mL prostaglandin F2alpha (PGF2a) (Sincrocio; Ourofino) on the afternoon of Day 6 and 2 mL of gonadotrophin-releasing hormone 1 (GnRHl, Sincroforte; Ourofino) to each animal 24 h before oocyte collection on Day 9.

In vitro *production of blastocysts from oocytes undergoing IVM*

Matured oocytes from all three experimental groups (total of 1232, 844 and 959 oocytes for the groups BSA, FBS and B+F, respectively) were used for *in vitro* fertilisation (IVF) in order to produce blastocysts from the different maturation media. After IVF on Tyrode/albumin/sodium lactate/sodium pyruvate (IVF-TALP) medium supplemented with 0.6% BSA, zygotes from all experimental groups were cultured *in vitro* (IVC) for 8 days in synthetic oviduct fluid (SOF) medium supplemented with 8 mg mL^{-1} BSA-FAF, 0.2 mM sodium pyruvate and 84.3 mg mL^{-1} amikacin in the absence of FBS in a controlled atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

Evaluation of nuclear maturation and cortical granule distribution

We assessed nuclear maturation of oocytes based on meiotic progression to metaphase II (MII) and extrusion of the first polar body. Cytoplasmic maturation was inferred by cortical granule (CG) migration to the cortical region of the oocyte, following the methodology described by Cherr *et al.* (1988) with modifications. Briefly, we used ~112 oocytes per group for evaluation. For cytoplasmic maturation analyses, denuded oocytes, following the removal of the zona pellucida by 0.5% pronase in phosphate-buffered saline (PBS), were permeabilised with 0.1% Triton X-100 for 5 min at 38°C and incubated with 10 μg mL^{-1} *Lens culinaris* agglutinin conjugated to fluorescein isothiocyanate (FITC-LCA) for 15 min. For nuclear assessment, denuded oocytes were stained with 10 μg mL^{-1} Hoechst 33342 followed by PBS washes. To visualise the structures, we used an epifluorescence microscope (Olympus IX-FLA-70; Olympus, Tokyo, Japan) on the 40× objective, using filters with excitation of 330–385 nm and 420–490 nm emissions to view the nuclear stage and a filter with 460–490 nm and 515 nm excitation and emission, respectively, for the cortical granules. Oocytes at MII with the presence of the first polar body were considered positive for nuclear maturation

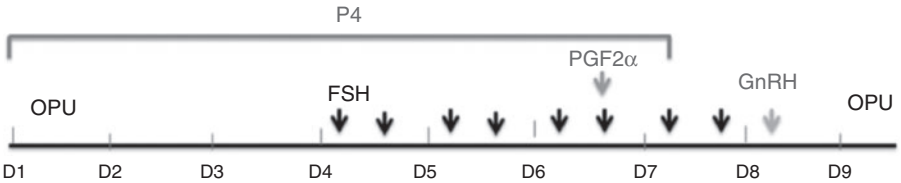


Fig. 1. Synchronisation and overstimulation protocol of *in vivo*-matured oocyte donors. Cows were synchronised after dominant follicle aspiration by OPU and overstimulated by administration of decreasing doses of FSH and a dose of GnRH 24 h before OPU for collection.

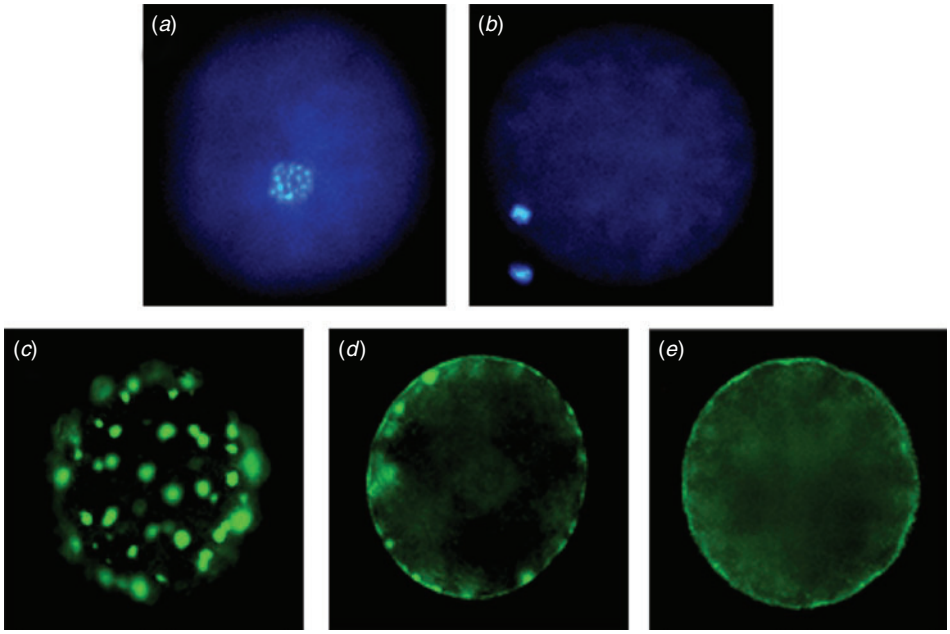


Fig. 2. Photomicrograph of nuclear progression and cortical granule distribution in bovine oocytes. Regarding the evaluation of nuclear progression, (*b*) oocytes in MII and with the presence of the first polar body were considered mature, while (*a*) those in other stages were considered immature. Regarding cortical granule migration, oocytes with cortical granules at (*e*) the periphery forming a halo were considered mature and oocytes with the presence of (*c*) 'clusters' or (*d*) in transition were considered immature. Images were captured on 40× objective.

(Fig. 2*b*), while other nuclear stages were considered immature (Fig. 2*a*). For evaluation of cytoplasmic maturation, oocytes with cortical granules arranged at the periphery, forming a halo (Fig. 2*e*), were considered matured, whereas oocytes with the presence of 'clusters' (Fig. 2*c*) or in transition (Fig. 2*d*), were considered immature.

Comparative analysis of lipid-droplet and mitochondrial behaviour during in vitro *and* in vivo *maturation*

We evaluated immature and *in vitro*-matured oocytes as well as oocytes collected *in vivo* following the treatment for follicular overstimulation–maturation described above, for distribution and quantity of lipid droplets and mitochondria. For *in vivo*-matured oocytes, collected by OPU, meiotic progression was evaluated as described above for *in vitro*-matured oocytes. We evaluated all oocytes (~60 per group) regarding the distribution and quantity of lipid droplets and mitochondria simultaneously. After being stripped, we first stained oocytes for visualisation of mitochondria with 0.5 mM Mitotracker Red CMXRos (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C, which stains active mitochondria in living cells. Afterwards, oocytes were fixed in 4% formaldehyde for 30 min. For analysis of lipid droplets, we permeabilised the fixed oocytes with 0.1% saponin for 30 min and stained with 5× HCS LipidTOX Green Neutral Lipid Stain (Molecular Probes) in Ca^{2+}- and Mg^{2+}- free PBS. Next, oocytes were analysed by confocal microscopy using a TCS-SP5 AOBS (Leica, Soims, Germany). We evaluated the distribution of structures in ~60 oocytes per group and captured images of 36 oocytes per group for quantification, obtained in five separate routines. For lipid-droplet distribution, we classified oocytes based on droplet location on the cytoplasm, as 'periphery' or 'dispersed' and, for mitochondria, we included a 'transition' category (Figs 3*a–e*). All images were captured under the same parameters, performing sequential acquisition. We used an argon laser for visualisation of lipid droplets and the excitation and emission were set to 488 nm and 500–537 nm, respectively. For mitochondria, we used a HeNe laser with excitation and emission set at 543 nm and 580–650 nm, respectively. We captured three images of each oocyte: one in the middle of the oocyte (the image with largest diameter) and the other two in the middle of the resulting halves. We used a 40× objective at a resolution of 1024 × 1024 and images were analysed with the ImageJ program (NIH; http://rsb.info.nih.gov/ij/). For mitochondrial activity, fluorescence intensity in the cytoplasm (Fig. 3*f*) was measured, averaging three sections per oocyte, with software assignment of intensity values between 0 and 255 for each pixel. For lipid quantification, we used the 'nucleus counter' tool, set to detect, distinguish and quantify droplet areas (Fig. 3*g*). The quantity of lipid in oocytes was obtained by analysis of the total area of lipids in each slide and obtaining the average of three sections (μm^2 of lipid per oocyte). In oocytes where all lipid droplets could be totally individualised (10 oocytes per group, 30 images per group), we also evaluated

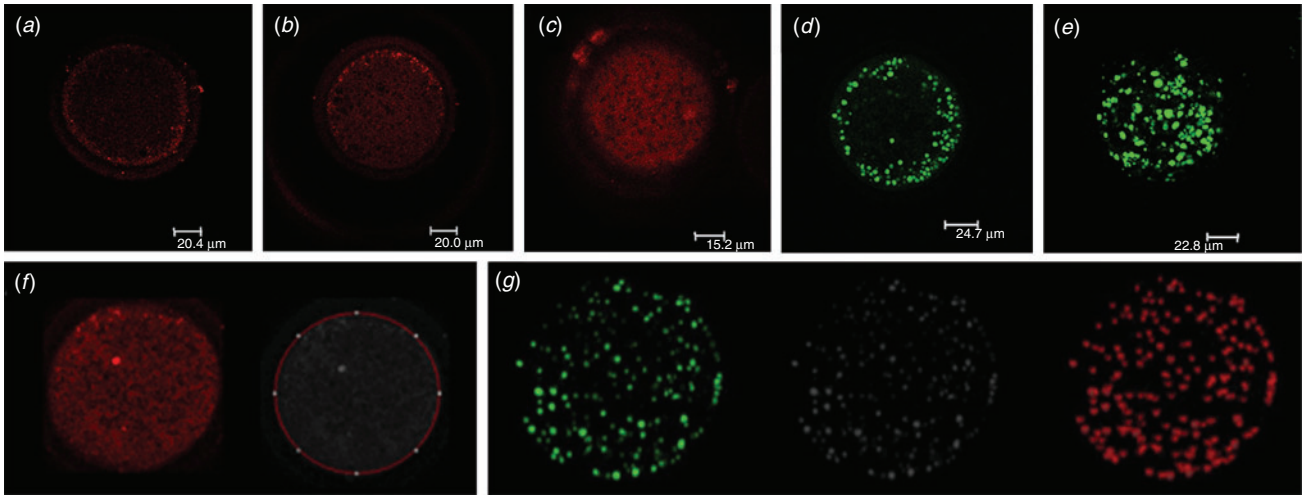


Fig. 3. Photomicrograph illustrating different categories of mitochondrial and lipid-droplet distribution in oocytes, and processing for mitochondrial and lipid quantification. Regarding mitochondrial distribution, they were characterised as (*a*) periphery, (*b*) transition, where most mitochondria were dispersed in the cytoplasm but with some accumulated in a minor part of the periphery or (*c*) dispersed, where mitochondria were located homogeneously distributed throughout the cytoplasm. Regarding the distribution of lipid droplets, they were ranked as being (*d*) in the periphery or (*e*) scattered. ImageJ software was used to quantify the structures, which for (*f*) mitochondria was performed measuring the fluorescence intensity after conversion of the image to 8 bits and delimitation of cytoplasm. (*g*) For lipids the droplets were identified by the 'nucleus counter' tool of ImageJ. After conversion of the images to 8 bits, the tool located and individualised each droplet.

droplet sizes and classified into <2 µm, 2–6 µm and >6 µm in diameter (Abe *et al.* 2002).

Embryo development rates and lipid quantification

We evaluated cleavage and blastocyst rates on the Day 2 and Day 8 of culture, respectively. The overall methodology for quantification and analysis of lipid-droplet size in embryos was performed as previously explained for oocytes, with minor modifications. For blastocysts, lipid quantity was corrected by area, to account for varying embryo sizes. After verification of a significant correlation ($r^2 = 0.76$ and $P < 0.0001$ by Pearson's correlation test) between lipid quantity of three sections in 30 embryos (10 per group) we chose the section with the largest area per embryo to be analysed. We analysed 62, 64 and 82 embryos for groups BSA, FBS and B+F, respectively. For the analysis of droplet size, we chose 15 sections of 15 embryos per group.

PLIN2 and DGAT2 expression in immature and in vitro*-matured oocytes and IVP embryos*

For reverse transcription polymerase chain reaction (RT-PCR) we used cumulus cells from three pools of 50 immature COCs and COCs matured in different media (BSA, FBS and B+F), three pools from 90 denuded oocytes of each group (immature and matured *in vitro*) and three pools of 30 embryos from the three IVM groups. RNA extraction of samples was performed using the RNeasy Protect Micro kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and reverse transcription was performed using the oligodT primer and Superscript III kit (Invitrogen, Carlsbad, CA, USA). Using the Quantitect SyberGreen kit (Qiagen) according to the manufacturer's recommendations, we performed real-time PCR reactions in a Mx3005P QPCR System from Stratagene (La Jolla, CA, USA). PCR reactions were conducted in triplicate and expression was determined based on the ratio of each gene over the control, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). A gene-specific standard curve was utilised at each run for quantification. *PLIN2* and *DGAT2* primers were designed, based on the bovine sequences available on the genome browser of University of California Santa Cruz (UCSC) (http://genome.ucsc.edu, accessed December 2013), using the program Primer3 (Primer3web, version 4.0.0: http://primer3.ut.ee), and synthesised by Sigma Aldrich. The *GAPDH* primers, used as endogenous control gene, were obtained from the company Promidol (Alere, Belo Horizonte, Brazil). The primer sequences were: *PLIN2* (product size: 149 bp) F 5′ TGCACTCACC AAATCAGAGC 3′, R 5′ GCAGCTTGGTGGACAGAGAT 3′; *DGAT2* (product size: 152 bp): F 5′ TTGGCTCAATAGG TCCAAGG 3′, R 5′ TGAAGTAGAGCACGGCAATG 3′; *GAPDH* (NM_001034034; product size: 76 bp) F 5′ AAGG CCATCACCATCTTCCA 3′, R 5′ CCACTACATACTCAGCA CCAGCAT 3′.

Statistical analysis

We evaluated nuclear maturation and cortical-granule migration rates, as well as embryo development rates by chi-square ($\chi 2$) test or, when appropriate, the Fisher exact test. Lipid quantification in oocytes and embryos, as well as mitochondrial activity quantification, were assessed by non-parametric Mann–Whitney test. The correlation between the structures in the oocytes was determined by linear regression r2 test and significance of the correlation by F test. For gene-expression

evaluation, we used analysis of variance (ANOVA) and Duncan's multiple-range test. The results were analysed with the GraphPad Instat 1.6 program (GraphPad Software, Inc., La Jolla, CA, USA), with the exception of gene-expression data, which were evaluated using the XLSTAT (2012) program (XLSTAT, New York, NY, USA).

Results

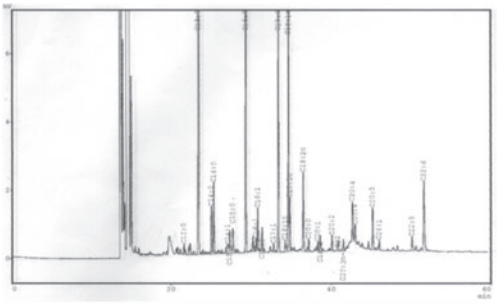
FBS lipid content

Aliquots of FBS were subjected to chromatography for the separation of fatty acids (Fig. 4*a*). The graph generated allowed for FBS lipid content determination (0.029 g per 100 mL) and fatty-acid profiling. Based on this result, we estimated lipid quantity for each IVM medium (Fig. 4*b*), revealing that medium containing FBS has eighteen times more fatty acids (0.029 mg mL^{-1}) than medium containing BSA only (0.0016 mg mL^{-1}) and nearly twice as much as medium using the combination of the two protein sources, B+F (0.0157 mg mL^{-1}). Of the fatty acids present in FBS, almost half (47.89%) are saturated fatty acids (Fig. 4*c*), with palmitic acid making up 23.4% of the total, followed by stearic acid (17.71%) and by the unsaturated oleic acid (15.24%); Fig. 4*d*.

Presence of FBS during IVM results in higher rates of nuclear maturation and migration of cortical granules (CG)

Routinely, the rates of meiotic progression and migration of cortical granules are used as indicators of oocyte maturation. In the present work, we demonstrated the limitations of BSA-FAF in this process. BSA had lower rates of oocytes in MII with first polar body extrusion and oocytes with cortical granules in the periphery, compared with groups containing FBS (FBS and B+F) (Table 1).

(*a*)



(*b*)

Groups	BSA	FBS	B+F
mg of lipid/mL	0.0016	0.029	0.0157

(*c*)

Name	mg/100 mL	% area
Saturated	14.12 ± 2.23	47.89 ± 4.89
Monounsaturated	7.3 ± 0.79	22.4 ± 1.58
Polyunsaturated	4.99 ± 1.67	16.57 ± 3.48
Trans	0.46	1.50 ± 0.73
Not identified		11.64 ± 3.11

(*d*)

Formula	Name	FBS	
		mg/100 mL	%Area
12:0	Lauric	0.11 ± 0.07	0.32 ± 0.28
14:0	Myristic	0.68 ± 0.35	2.59 ± 1.02
15:0	Pentadecanoic	0.52 ± 0.46	1.07 ± 0.52
16:0	Palmitic	6.82 ± 1.08	23.40 ± 1.74
16:1 (*n*-7)	Palmitoleic	0.64 ± 0.14	2.27 ± 0.22
17:0	Margaric	0.42 ± 0.05	1.43 ± 0.07
17:1 (*n*-7)	Heptadecenoic	0.24 ± 0.03	0.72 ± 0.18
18:0	Estearic	5.16 ± 0.77	17.71 ± 3.51
18:1 9t	Elaidic	0.46 ± 0.18	1.50 ± 0.73
18:1 (*n*-9)	Oleic	5.01 ± 0.74	15.24 ± 1.01
18:1 (*n*-7)	Vaccenic	1.09 ± 0.05	3.51 ± 0.47
18:2 (*n*-6)	Linoleic	1.51 ± 0.48	5.13 ± 1.12
20:0	Eicosanoic	0.14 ± 0.07	0.49 ± 0.17
20:1 (*n*-9)	Eicosenoic	—	—
18:3 (*n*-3)	α-Linolenic	0.17 ± 0.16	0.67 ± 0.42
22:0	Docosanoic	0.28 ± 0.05	0.88 ± 0.17
22:1	Docosenoic	—	—
20:3 (*n*-6)	Eicotrienoic	0.40 ± 0.08	1.24 ± 0.41
20:4 (*n*-6)	Arachidonic	1.00 ± 0.09	3.10 ± 0.56
24:0	Lignoceric	—	—
20:5 (*n*-3)	Eicosapentaenoic	0.42 ± 0.40	1.56 ± 1.17
24:1	Nervonic	0.33 ± 0.12	0.65 ± 0.38
22:5 (*n*-3)	Docosapentaenoic	0.50 ± 0.08	1.45 ± 0.31
22:6 (*n*-3)	Docosahexaenoic	0.99 ± 0.81	3.42 ± 2.37

Fig. 4. FBS gas chromatography. (*a*) Graphic illustration of chromatography results, where each peak refers to each fatty-acid area and is used to determine the acid ratio in the sample. (*b*) Table with lipid quantity in each IVM medium. (*c*, *d*) Tables with each fatty-acid proportion in the sample.

Table 1. Rates of bovine oocytes in MII with cortical granules (CG) arranged peripherally after 24 h of *in vitro* maturation with the different supplements

BSA, 8 mg mL^{-1} BSA-FAF; FBS, 10% FBS; B+F, 6 mg mL^{-1} BSA-FAF + 5% FBS. [a,b]Values in the same column with different superscripts differ by $\chi 2$ test ($P < 0.05$)

Group	Oocytes in MII *n* (%)	Oocytes with CG in periphery *n* (%)
BSA	69/92 (75.0)[a]	54/123 (43.9)[a]
FBS	104/117 (88.9)[b]	78/125 (62.4)[b]
B+F	95/106 (89.6)[b]	58/113 (51.3)[a,b]

IVM affects distribution and quantity of lipids and active mitochondria

Following the observation of a high lipid content in FBS and its significance on nuclear maturation and the migration of cortical granules, we sought to test if this supplement affected the structures responsible for the β-oxidation process, such as lipid droplets and mitochondria, during IVM. We compared lipid–mitochondrial behaviour between *in vivo* and *in vitro* processes. Given that the *in vivo* nuclear maturation rate was 65.5%, we concluded that not all oocytes from animals undergoing hormone treatment completed lipid-droplet and mitochondrial migration, which was demonstrated by the asynchrony of migration of these structures in some oocytes (Fig. 5). We observed the peripheral category as predominant for both structures in immature oocytes (Table 2). However, there were differences during *in vivo* and *in vitro* oocyte maturation. In regards to lipid migration, we observed lower migration rates in the BSA group when compared with the groups containing FBS (FBS and B+F groups). However, the lipid migration rate in the *in vivo* group was lower than expected. Regarding mitochondrial migration, although none

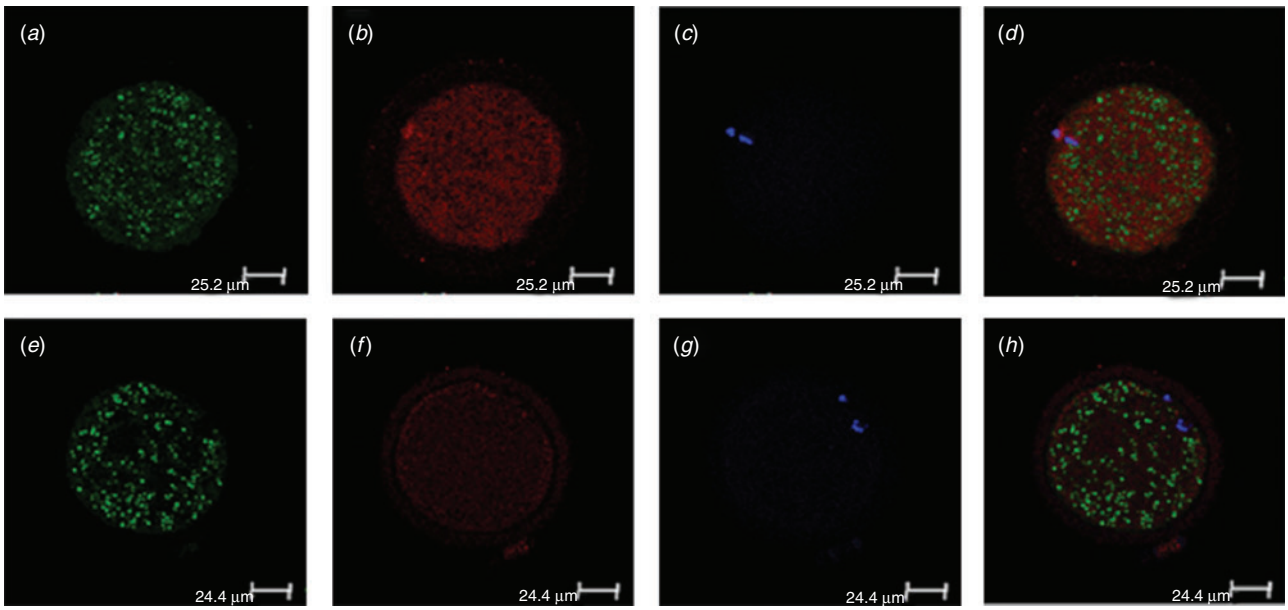


Fig. 5. Lipid and mitochondrial behaviour during *in vivo* maturation in MII oocytes; examples of asynchronous and synchronous migration of structures in *in vivo* MII oocytes. Locations of (*a*, *e*) lipid droplets, (*b*, *f*) mitochondria and (*c*, *g*) nucleus at MII. (*d*, *h*) Merged images. (*a–d*) Oocyte showing synchronous migration of lipid droplets and mitochondria. (*e–h*) Oocyte representing asynchronous migration of these structures.

Table 2. Rates of oocytes in different categories for the location of mitochondria (peripheral, transition and dispersed) and lipid droplets (periphery and dispersed) in immature, *in vitro*-matured (BSA, FBS and B+F) and *in vivo*-matured oocytes

BSA, 8 mg mL^{-1} BSA-FAF; FBS, 10% FBS; B+F, 6 mg mL^{-1} BSA-FAF + 5% FBS. [a,b,c]Values in the same column with different superscripts differ by $\chi 2$ test ($P < 0.05$)

Group	Mitochondria			Lipid droplets	
	Periphery *n* (%)	Transition *n* (%)	Dispersed *n* (%)	Periphery *n* (%)	Dispersed *n* (%)
Immature	55/59 (93.2)[a]	0/59[a]	4/59 (6.8)[a]	36/59 (61)[a]	23/59 (39)[a]
BSA	42/61 (68.8)[b]	12/61 (19.7)[b,c]	7/61 (11.5)[a]	17/61 (27.9)[b]	44/61 (72.1)[b]
FBS	25/65 (38.5)[c]	19/65 (29.23)[b]	21/65 (32.3)[b]	2/65 (3.1)[c]	63/65 (96.9)[c]
B+F	33/66 (50)[b]	23/66 (34.8)[b]	10/66 (15.1)[a]	3/66 (4.5)[c]	63/66 (95.4)[c]
In vivo	22/58 (37.9)[c]	6/58 (10.3)[c]	30/58 (51.7)[c]	12/58 (20.7)[b]	46/58 (79.3)[b]

of the IVM groups achieved similar migration rates to those seen *in vivo*, the rates observed for the FBS group were the closest to those *in vivo* (Table 2).

When we analysed lipid and active mitochondria values of immature, *in vivo*- and *in vitro*-matured oocytes, we noticed higher levels of lipid accumulation during IVM in comparison to those oocytes that underwent hormonal stimulation in the *in vivo* group. Furthermore, the FBS group had higher accumulation compared with the group supplemented only with BSA (Fig. 6*a*). Supplementation during IVM did not affect oocyte lipid-droplet sizes (Fig. 6*b*). However, as illustrated in Fig. 6*c*, the only group able to increase mitochondrial activity during maturation was the BSA group, showing significantly higher values than those observed in the groups containing serum (FBS and B+F). Interestingly, we observed a decrease in mitochondrial activity in the *in vivo* group (Fig. 6*c*). Notwithstanding, we analysed the correlation between active mitochondria and lipid droplets within each group, and the only group able to maintain a significant correlation between the structures was the *in vivo* group (Fig. 6*d*).

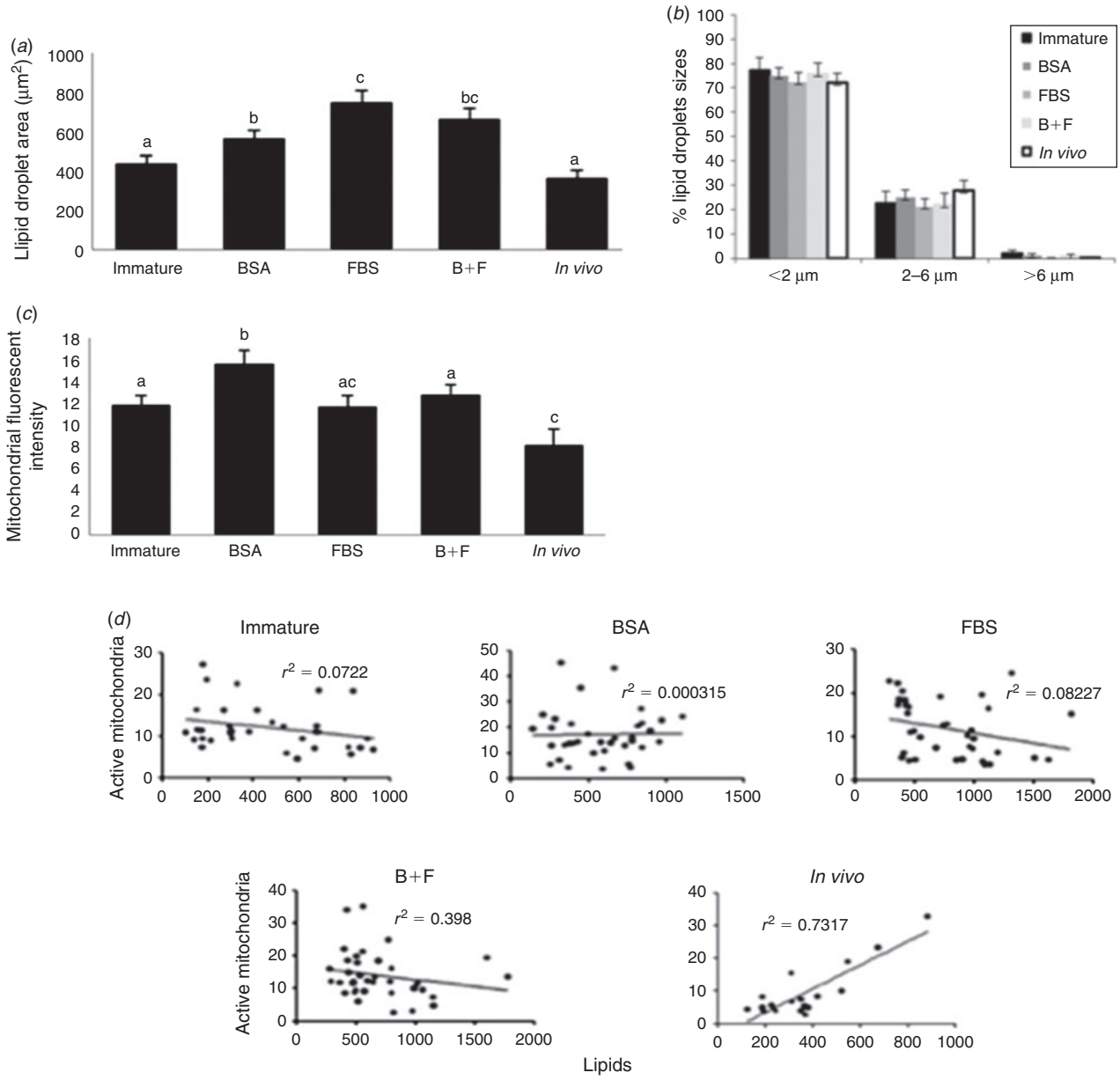


Fig. 6. Behaviour and quantity of lipid droplets and active mitochondria during IVM and *in vivo* maturation. (*a*, *c*) Amount of lipid and numbers of active mitochondria in immature ($n = 41$), BSA ($n = 39$), FBS ($n = 41$), B+F ($n = 40$) and *in vivo*-matured ($n = 20$) oocytes. (*b*) Lipid-droplet size classification of oocyte groups. (*d*) Correlation between mitochondria and lipid droplets in the different groups.

Reduction in FBS concentration during IVM lowers the amount of lipid in embryos

BSA supplementation during IVM resulted in a significant reduction in the rate of blastocyst formation after IVF and IVC (Table 3). Interestingly, the B+F group, containing half the concentration of FBS used in standard medium, allowed for a reduction in total lipid amount in the embryos, while maintaining embryo production rates similar to those obtained by FBS alone (Fig. 7*a*). IVM supplementation did not influence the size of the lipid droplets present in the embryos (Fig. 7*b*).

Table 3. Cleavage and blastocyst rates after IVM of oocytes in different media (BSA, FBS and B+F), fertilisation and *in vitro* culture for 8 days in the absence of FBS

BSA, 8 mg mL^{-1} BSA-FAF; FBS, 10% FBS; B+F, 6 mg mL^{-1} BSA-FAF + 5% FBS. [a,b]Values in the same column with different superscripts differ by $\chi 2$ test ($P < 0.05$)

Group	Cleaved *n* (%)	Blastocysts *n* (%)	Blastocysts/cleaved *n* (%)
BSA	1232/1410 (87.38)[a]	279/1410 (19.79)[a]	279/1232 (22.65)[a]
FBS	844/927 (91.05)[b]	336/927 (36.25)[b]	336/844 (39.81)[b]
B+F	959/1062 (90.30)[b]	368/1062 (34.65)[b]	368/959 (38.37)[b]

IVM causes changes in PLIN2 and DGAT2 expression in oocytes, but not in embryos

In order to understand how supplementation during IVM influences lipid metabolism of oocytes and embryos, we investigated the expression of *PLIN2* and *DAGT2* in cumulus cells, immature and *in vitro* mature denuded oocytes and in IVM-derived embryos (Fig. 8). We observed different patterns of expression for *DGAT2* and *PLIN2* in cumulus cells. Regardless of the treatment, *PLIN2* expression was increased in cumulus cells from matured oocytes, whereas *DGAT2* showed decreased expression in these cells following IVM. Among the denuded oocytes, the only significant difference found was in the FBS group, which showed a decreased expression of *DGAT2* compared with the immature group. Treatments during IVM did not affect *DGAT2* and *PLIN2* expression in blastocysts.

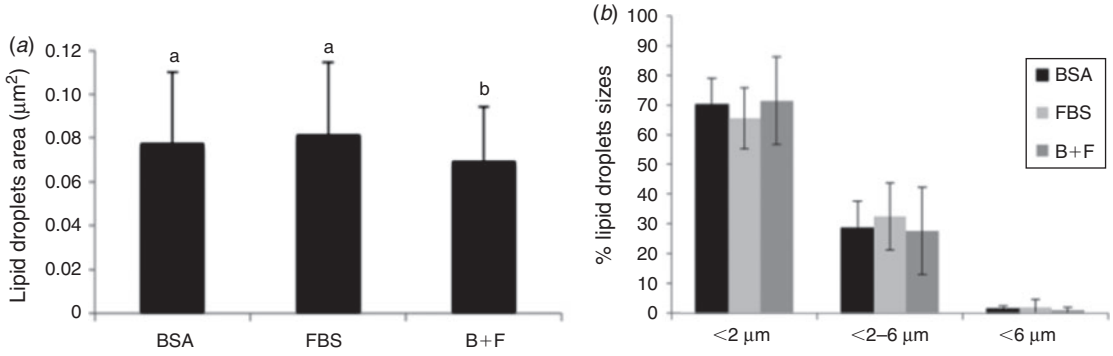


Fig. 7. Embryo lipid analysis. (*a*) Amount of lipid in blastocysts of the IVM groups: BSA, FBS and B+F. (*b*) Lipid-droplet size evaluation in embryos following IVM.

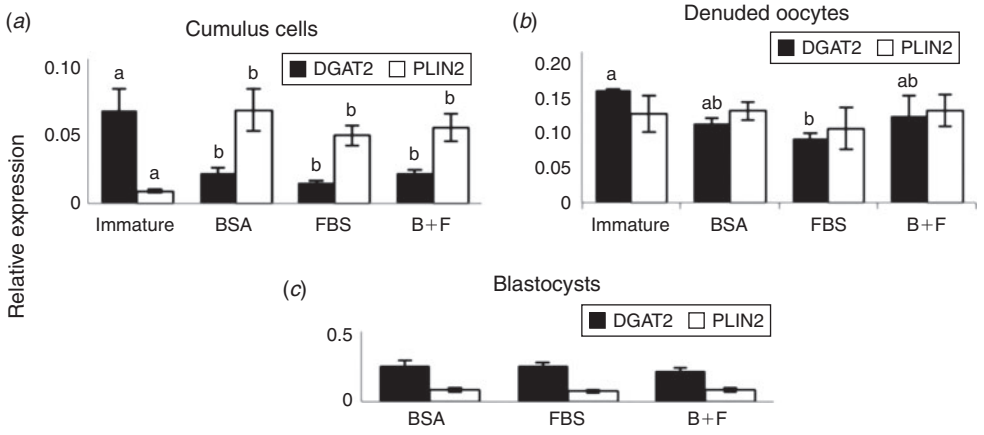


Fig. 8. Expression of *DGAT2* and *PLIN2* in (*a*) cumulus cells, (*b*) IVM oocytes (BSA, FBS and B+F) and (*c*) subsequent blastocysts. Different letters for the same gene indicate significant differences ($P < 0.05$).

Discussion

To our knowledge, the present study is the first to determine the total lipid content of FBS. We confirmed that serum has a much higher concentration of fatty acids than the other commonly used media supplement, BSA-FAF. These findings support the hypothesis that the increased lipid accumulation found in oocytes matured in the presence of FBS may be, in part, due to lipid transport from the medium to the cytoplasm of the oocyte. In fact, saturated fatty acids, prevalent in FBS, can penetrate the oocyte (Adamiak *et al.* 2006). The elevated concentration of palmitic acid found in FBS also concurs with this idea, as previous researchers have shown that oocytes and embryos, when cultured in the presence of FBS, have a high concentration of this particular fatty acid (Sata *et al.* 1999; Kim *et al.* 2001; Ferreira *et al.* 2012). In the present study, oleic acid is the third most-abundant in FBS, and it is known that this fatty acid is predominant in the follicular fluid (Tsujii *et al.* 2001; Leroy *et al.* 2005). It is possible that this change in the ratio of palmitic/oleic acid adversely affects IVP. Harmful effects of excess palmitic acid in both maturation and embryo development (Leroy *et al.* 2005), and positive effects of oleic acid in the same stages of development (Aardema *et al.* 2011), have been reported. We consider that the difference in lipid profile between follicular fluid and FBS, normally used in IVM and *in vitro* culture, could be one of the causes for the observed differences and outcomes between *in vitro* and *in vivo* embryos in cattle.

Despite possible deleterious effects of FBS, we suggest that elements present in this supplement promote, in addition to nuclear maturation, the migration of organelles during IVM. We found that 5% FBS, half of the concentration commonly used in maturation media, resulted in satisfactory migration of cortical granules and meiotic progression (Table 1). On the other hand, the supplementation with BSA only during IVM prevented adequate nuclear maturation and CG migration rates. A decrease in nuclear maturation in oocytes cultured with BSA-FAF during IVM has been described elsewhere (Ali and Sirard 2002). This result can be explained by the effects that fatty acids, and their oxidation, have on meiotic progression (Downs *et al.* 2009). One such effect is demonstrated by the activation of mitogen-activated protein kinase (MAPK) eliciting germinal-vesicle breakdown and reactivation of meiosis (Chen *et al.* 2006). According to Downs *et al.* (2009), MAPK phosphorylates acetyl-CoA carboxylase (ACC), thus deactivating it and decreasing cellular malonyl-CoA levels, which is an oxidative pathway inhibitor. Also, the relationship between β-oxidation and nuclear maturation rates were shown in cattle, pigs and mice (Downs *et al.* 2009; Dunning *et al.* 2010; Paczkowski *et al.* 2013).

Dunning *et al.* (2010) showed the importance of oocyte oxidation for embryo development and found that, although there is increased oxidation during IVM in mice, maturation in the presence of FBS results in an even higher oxidation rate, with positive consequences in embryo development when compared with IVM supplemented with BSA only. The authors refer to a possible lack of L-carnitine from medium composed solely of BSA, as L-carnitine is responsible for transporting fatty acids into the mitochondrial membrane for β-oxidation to occur (Downs *et al.* 2009; Dunning *et al.* 2010). BSA-FAF is substantially free of fatty acids. This fact, associated with a possible limitation in the oxidation described above for murine oocytes matured in this supplement, could be a plausible explanation for the lower meiotic progression rate we have observed in oocytes matured with this supplement as the sole protein source, during the present study.

Although the rates of mitochondrial migration in the *in vivo* group were not as high as previously reported in the literature (Hyttel *et al.* 1986), mitochondrial migration appears to be negatively affected by the *in vitro* system. None of the IVM treatment groups were able to reach the values observed in oocytes matured *in vivo*, albeit FBS provided the highest rate of migration of the three IVM groups. However, despite lower rates of mitochondrial migration, the group that utilised intermediate concentrations of the two supplements showed similar rates of maturation and blastocyst formation, arguing that incomplete mitochondrial migration does not appear to affect oocyte competence and subsequent embryonic development.

The fact that lipid accumulation was present in oocytes of all three groups following IVM but was not observed in the *in vivo* group, suggests that, in addition to protein supplementation, there are other factors in the *in vitro* system affecting lipid metabolism. It is possible that during physiological maturation (*in vivo*), lipid synthesis occurs concomitantly with its metabolism by oxidation, impeding excessive lipid accumulation. This hypothesis is supported by the high correlation between lipids and mitochondria observed for the *in vivo* group. Lipid accumulation following IVM, shown in the present study, is in accordance with current literature reports (Ferreira *et al.* 2008; Aardema *et al.* 2011; González-Serrano *et al.* 2013). In the same way, an increase in lipid quantity during IVM was observed when culture media contained only BSA (Aardema *et al.* 2011). Moreover, reports have demonstrated an increase in the expression of genes and proteins of lipid metabolism during IVM in lipid-free medium (Auclair *et al.* 2013; Dunning *et al.* 2014). Those data suggest an increase in lipid accumulation not only by lipid incorporation, but also by affecting lipid metabolism during IVM.

Unlike the oocytes recovered after animal hormonal stimulation, oocytes undergoing IVM with higher concentrations of FBS failed to maintain correlation between active mitochondria and lipid content. Furthermore, the increase in lipid accumulation in this group was not accompanied by an increase in active mitochondria, which may have influenced the efficiency of oxidation of these lipids. Regarding oocytes matured in the presence of BSA-FAF, although the group was able to increase the amount of active mitochondria and lipids, the physical distance between the structures, caused by asynchrony during migration, along with a previously suggested lack of L-carnitine and consequently low levels of oxidation (Dunning *et al.* 2010), could have caused the low rates of maturation and embryo development observed in this group. All of these differences may have affected the ability to metabolise lipids accumulated during IVM in this experimental group, and possibly further during IVC, as no differences in lipids were observed in blastocysts from the BSA and FBS groups. As suggested by Dunning *et al.* (2010) the group containing 10% FBS could

have promoted higher β-oxidation rates. However, serum has detrimental effects on mitochondrial membranes (Russell *et al.* 2006), which, associated with the high lipid concentration in this supplement, may have prevented the metabolism of such large amounts of fatty acids, worsened by the high lipid intake by the embryos. In contrast, the combination of BSA-FAF with 5% FBS, analysed in this study, possibly allowed for a better balance between intake and oxidation, while providing acceptable maturation and embryo development rates and lower lipid accumulation in embryos.

Despite its effects in lipid accumulation on oocytes and embryos, IVM supplementation had no effect on lipid-droplet sizes during our study, in agreement with a previous literature report (Aardema *et al.* 2011).

Expression of *PLIN2* and *DGAT2* in cumulus cells during IVM suggests an increase in lipid synthesis and this could be related to the lipid accumulation observed in the oocyte cytoplasm. This lipid accumulation in cumulus cells likely happens to provide energy to the cells, to supply the needs of these steroidogenic cells or perform the transportation of lipids to the oocyte. For *PLIN2*, an increase in mRNA could be justified by increased demand for lipid-droplet formation, and for *DGAT2*, the decrease in mRNA may be linked to increased protein translation and production of this enzyme required for lipid synthesis. However, despite the differences observed in embryo lipid accumulation between groups, supplementation during IVM did not affect these genes involved in lipid metabolism in embryos.

In conclusion, we noted improper migration of mitochondria and lipid droplets following IVM, and the possible effects that could carry over through the *in vitro* process, affecting not only development rates but also lipid accumulation. Furthermore, we have shown that lowering the FBS concentration to 5% may be an interesting alternative, since it enabled the reduction of lipid accumulation in embryos without adversely affecting oocyte maturation and embryo development.

Acknowledgements

We are grateful to the staff of the Animal Reproduction Laboratory of the Departamento de Medicina Veterinária Preventiva e Reprodução Animal (UNESP, Jaboticabal, Brazil) for their support during this work. We thank Prof. Felipe Perecin (FZEA (Faculdade de Zootecnia e Engenharia de Alimentos- FZEA, USP, Pirassununga, Brazil), USP, Brazil) for his help with the statistical analysis. We also would like to thank Dr Daniel Robert Arnold for proofreading the manuscript for English content. Supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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