Modulation of long-chain Acyl-CoA synthetase on the development, lipid deposit and cryosurvival of in vitro produced bovine embryos

Roniele Santana Valente1,2☯, Tamie Guibu de Almeida3☯, Mayra Fernanda Alves4☯, Janine de Camargo1☯, Andrea Cristina Basso4☯, Katia Roberta Anacleto Belaz5☯, Marcos Nogueira Eberlin6☯, Fernanda da Cruz Landim-Alvarenga7☯, Patrícia Kubo Fontes8☯, Marcelo Fábio Gouveia Nogueira9☯, Mateus José Sudano1,2*1

1 School of Veterinary Medicine, Federal University of Pampa, Uruguai na, RS, Brazil, 2 Center for Natural and Human Sciences, Federal University of ABC, Santo André, SP, Brazil, 3 Department of Animal Reproduction, University of São Paulo, São Paulo, SP, Brazil, 4 In Vitro Brasil, ABS Pecplan, Mogi Mirim, SP, Brazil, 5 Department of Chemistry, Federal University of Uberlândia, Uberlândia, MG, Brazil, 6 School of Engineering, Mackenzie Presbyterian University, São Paulo, SP, Brazil, 7 Department of Animal Reproduction, São Paulo State University, Botucatu, SP, Brazil, 8 Department of Biological Sciences, São Paulo State University, Assis, SP, Brazil

☯ These authors contributed equally to this work.
* mjsudano@gmail.com

Abstract

In this study, we evaluated the modulation effect of long-chain Acyl-CoA synthetase during early embryo development. Bovine embryos were cultured in four groups: positive modulation (ACS+) with GW3965 hydrochloride, negative modulation (ACS-) with Triacsin C, association of both modulators (ACS±), and control. Embryo development rates were not altered (P>0.05) by treatments. Embryonic cytoplasmic lipid content increased in ACS+ but reduced in ACS- compared to the control (P<0.05), whereas the membrane phospholipids profile was not altered by treatments. The total number of blastomeres did not differ (P>0.05) between groups; however, an increased apoptotic cells percentage was found in ACS- compared to control. Twenty-four hours after warming, ACS+ and control grade I embryos presented the best hatching rates, whereas the ACS+ group equaled the hatching rates between their embryos of grades I, II and III 48 hours after warming. The relative abundance of transcripts for genes associated with lipid metabolism (ACSL3, ACSL6, ACAT1, SCD, and AUH), heatshock (HSP90AA1 and HSF1), oxidative stress (GPX4), and angiogenesis (VEGF), among other important genes for embryo development were affected by at least one of the treatments. The treatments were effective in modulating the level of transcripts for ACSL3 and the cytoplasmic lipid content. The ACS- was not effective in increasing embryonic cryosurvival, whereas ACS+ restored survival rates after vitrification of embryos with low quality, making them equivalent to embryos of excellent quality.
Introduction

Over the past several decades, increased demand for in vitro produced (IVP) bovine embryos has triggered a constant search for improvements of the technique, aiming at the production of embryos of better quality that result in higher pregnancy rates. Despite being considered an already established biotechnology, the high sensitivity after cryopreservation still represents one of the greatest challenges to be overcome for the wide dissemination of IVP embryo transfer. In view of the satisfactory results obtained with embryos produced in vivo, the reduced cryosurvival of IVP may be directly related to sub-optimal in vitro culture conditions during development, leading to structural and functional modifications that compromise embryo viability.

In recent years, the existence of morphological and metabolic differences in IVP embryos has been clearly demonstrated in relation to in vivo-produced ones. Among these differences is the higher cytoplasmic lipid content, commonly associated with lower rates of embryonic survival after freezing [1] and deviations in the relative abundance of transcripts of important genes for embryonic development and establishment of gestation [2].

The mechanisms through which IVP embryos accumulate more lipids are not yet fully elucidated; however, these embryos are known to be less resistant to cryopreservation when compared to those produced in vivo. Alternatives such as changes in media culture conditions, like the addition of lipolytic chemicals [3] or the reduction or removal of fetal calf serum [4–6] were proposed to decrease the amount of lipid droplets within the embryonic cytoplasm.

The participation of fatty acids in most metabolic pathways, including β-oxidation and biosynthesis of complex lipids (such as triacylglycerols and phospholipids), requires their initial activation by the addition of a CoA group [7] resulting in the formation of Acyl-CoA, a reaction catalyzed by Acyl-CoA synthetase (ACS) [8]. Three ACS subfamilies are expressed in mammals: long chain acyl-CoA synthetase (ACSL), fatty acid transport proteins (FATP) and Acyl-CoA synthetase bubblegum (ACSBG) [9]. Five genes of the ACSL family were identified based on sequence homology, tissue and intracellular distribution, and termed ACSL1 and ACSL3 to ACSL6.

Liver nuclear X receptors α and β (LXRα and LXRβ) are regulators of lipid metabolism in many tissues, acting on the expression of multiple genes involved in the cholesterol efflux, transport and excretion, fatty acid biosynthesis, and lipoprotein metabolism in different tissues [10]. Triacsin C is a fungal metabolic regulator that acts as a competitive selective inhibitor of ACLS 1, 3 and 4 [11] almost completely inhibiting the de novo synthesis of triacylglycerol and phospholipids from glycerol in human fibroblasts [12]. GW3965 hydrochloride is an agonist specific for liver X receptors (LXRα and LXRβ), and is capable of inducing an increase in the expression of hepatic ACSL3 in hamsters [13] and placental trophoblast cells [7]. Apparently, to date there are no reports of the action of these compounds on embryonic development.

The objective of the present work was to evaluate the effects of the addition of GW3965 hydrochloride and Triacsin C, positive (ACS+) and negative (ACS-) modulators of ACSL, respectively, on development, cryosurvival, lipid accumulation and profile, gene expression, and viability of bovine IVP embryos.

Material and methods

Reagents

All materials were acquired from Sigma (Sigma-Aldrich Corp.) except when specified.

Experimental design

The positive and negative modulators of ACSL, GW3965 hydrochloride (ACS+) and Triacsin C (ACS-), were added to the in vitro culture medium of bovine embryos. On the fourth day of
culture (96 hpi) the embryos were randomly distributed into four groups: ACS+, ACS-, association between modulators (ACS±) or control group. The procedure consisted of the withdrawal of 2.5 μl of culture medium and addition of 2.5 μl of the modulator in each group. Day four was selected to start the treatment because is the moment that precede: i) the highest lipid content of the total embryo area at morula stage (not normalized by cell number), ii) the drop of lipid content from morula to blastocyst (normalize or not by the embryo cell number), and iii) the shift in membrane lipid profile during early embryo development [14, 15].

Production rates and embryo quality were recorded. Embryo re-expansion and hatching rates were evaluated at 12, 24, and 48 hours after warming. The total number of cells and the apoptotic cells index were measured for evaluation of embryonic viability. In addition, the cytoplasmic lipid droplets content (Sudan Black B) and membrane phospholipid profiles (MALDI-MS) were determined. Finally, the expression of genes related to lipid metabolism and embryonic quality were investigated.

Pilot study

To test the most appropriate dose of each modulator to be used, a pilot study was performed using 1100 oocytes in which the concentrations of 100, 10, and 1 x (literature reference dose), $10^{-1}$ x and $10^{-2}$ x of each drug were tested in relation to the control group. On the fourth day of culture, embryos were randomly distributed among the groups that would receive each of the doses of ACS+, ACS-, or to the control group. The highest dose of each modulator that did not significantly affect the production and quality of the embryos in relation to the control group was selected (GW3965 hydrochloride $10^{-1}$ x (10 μM) and Triacsin C $10^{-2}$ x (0.1 μM) and used in the next replicas. The protocol for the embryos production is described below.

**In vitro recovery and maturation (IVM) of cumulus-oocyte complexes**

Bovine ovaries (predominantly *Bos taurus indicus*) were collected at a local slaughterhouse and kept in saline solution heated to 35˚C until use. Antral follicles (2 to 8 mm in diameter) were aspirated using an 18G needle and 10 mL syringe. A total of 3,393 cumulus-oocyte complexes (COCs) containing homogeneous cytoplasm and at least three layers of cumulus cells were selected and incubated at 38.5˚C in 5% CO₂ atmosphere and 100% humidity for 24 h. The maturation process was carried out in groups of 20–25 COCs, in drops containing 90 μL of serum-containing (10% v/v) IVM medium (*In Vitro Brazil—IVB | ABS Pecplan*), placed in Petri dishes, and covered with mineral oil.

**In vitro fertilization (IVF) of oocytes**

At the end of the maturation period, the COCs (20–25) were transferred to new Petri dishes containing 90 μL of fertilization medium (*In Vitro Brazil—IVB | ABS Pecplan*). For *in vitro* fertilization (IVF), a sperm pool from the commercial semen of four different bulls (two Nellore, one Gyr and one Holstein bulls) with proven fertility was used. Viable sperm was selected using a Percoll gradient [16] and IVF was performed with $2 \times 10^6$ spermatozoa/mL. Co-culture was maintained at a temperature of 38.5˚C in 5% CO₂ atmosphere and 100% humidity for 18 h. In total, four batches of IVF were performed.

**In vitro culture (IVC) of embryos**

After the IVF period, the presumptive zygotes were denuded by successive pipetting and then transferred to Petri dishes containing 100 μL of mSOF culture medium (*In Vitro Brazil—IVB | ABS Pecplan*) supplemented with 5 mg/mL of BSA and 2.5% (v/v) of fetal calf serum, covered
with mineral oil and maintained at a temperature of 38.5°C in an atmosphere of 5% CO₂, 5% O₂, and balance of N₂ with 100% of humidity. A serum-supplemented medium was used because: i) it is the standard in vitro culture media used for majority in vitro production of bovine embryos worldwide; ii) there is evidences that increased activity of ACSL3 is associated with the fatty acid uptake [7]; and iii) to try to pharmacology modulate lipid content and cryo-survival of the IVP embryos in serum containing medium. On day four (D4–96hpi), prior to the treatment with the modulators and in order to avoid an additional petri dish handling, 50% of the culture medium was replaced with the same amount of new mSOF plus glucose [1 μg/mL]. The embryos were then randomly distributed between the groups that received the treatments with individual modulators (ACS+, 10 μM), (ACS-, 0.1 μM), association of both modulators (ACS±; ACS+ 10 μM and ACS- 0.1 μM)) or the control group. Each modulator used was diluted in dimethylsulfoxide (DMSO) and rediluted in PBS, according manufacturer’s instructions. The procedure consisted of the removal of 2.5 μL of culture medium and addition of 2.5 μL of the respective modulator in each of the groups. The vehicle used to dilute the modulators was added to the control group (2.5 μL of PBS). Cleavage and blastocyst rates were recorded on day two and day 8. For all analyses, embryos were collected at the expanded blastocyst stage on D6, D7 and D8. Expanded blastocyst quality was evaluated based on embryonic mass symmetry, size, color, and uniformity of the blastomeres and classified morphologically as grade I, II and III, according to methodology proposed by Stringfellow and Seidel [17].

Vitrification of embryos
All embryos in the expanded blastocyst stage (n = 293) were cryopreserved by the Cryotop vitrification method, as described by Sanches, Marinho [18]. Briefly, grade I, II and III embryos were equilibrated in base medium (TCM-HEPES + 20% fetal calf serum) and then transferred to base medium supplemented with 10% ethylene glycol and 10% DMSO for 1 minute. The embryos were then transferred to a vitrification solution consisting of 20% ethylene glycol, 20% DMSO, and 0.5 M sucrose in base medium, and incubated for 20 seconds. They were then deposited on the top of a polypropylene strip of a Cryotop [19] (three to five embryos) with a minimal amount of vitrification solution, and then immediately immersed in liquid nitrogen (N₂).

Warming and re-culture of embryos
For warming, cryotops containing the embryos were withdrawn from the N₂, held for four seconds at room temperature and immediately immersed in TCM-HEPES medium + 20% fetal calf serum + 0.5M sucrose at 35°C where they remained for one minute. Then, the embryos were transferred to TCM-HEPES media + 20% SFB + 0.3M sucrose and TCM-HEPES + 20% FBS + 0.15M sucrose, at room temperature, where they remained for five minutes for each solution. The embryos were washed in mSOF medium and cultured under the same conditions described above during a period of 48 h. Embryo cryosurvival was defined based on the blastocoel re-expansion after 12 hours, followed by total hatching of the zona pellucida evaluated at 24 and 48 hours of incubation.

Semiquantitative and semimorphometric evaluation of cytoplasmic lipid droplets content
Expanded blastocysts (N = 18 per group) were initially fixed in 10% formaldehyde solution for 2 hours at room temperature. Subsequently, they were transferred to 50% ethanol droplets and then placed in drops of 1% Sudan-Black B (cytoplasmic lipophilic dye) diluted in 70% ethanol for 2 min. The embryos were then washed in 50% ethanol drops and finally placed on slides containing glycerol and covered with coverslips. The analysis was performed under an optical
microscope at a magnification of 200 x. The semiquantitative lipid droplets content was estimated using Image J 1.4 software. Images captured of the embryos were converted to a gray scale. The gray intensity mean was recorded and the area of the embryo was calculated with the use of the freehand selection tool. The semiquantitative lipid content data is presented as gray intensity mean per area (gray intensity/μm²).

Semimorphometric evaluation was conducted in order to investigate the total number, total area, and the area per drop of small, large and giant lipid droplets (<2 μm, 2–6 μm, and >6 μm; respectively). Each image of embryos was converted to gray scale, following threshold adjustment, and using the particle analyzer tool of the Image J 1.4 software, the aforementioned variables were calculated based on the area of each drop size (0 to 3.14, 3.14 to 28.27, and >28.27 μm²; respectively for small, large, and giant lipid drops). The following formula of area (Area = π. r²) was used to calculate the area of each drop size based on the ray of each drop (ray = diameter/2). Slighted corrections were conducted when the software merged different drops. A representative panel of each step of the semimorphometric evaluation methodology is presented in the Fig 1.

**Lipid profiles of matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS)**

Expanded blastocysts (N = 15 per group) were randomly collected for establishment of embryonic lipid profiles after each treatment. The MALDI-MS technique with 2,5-dihydroxybenzoic
Modulation of Acyl-coa synthetase in bovine embryos

RNA extraction, reverse transcription, and transcript level determination by real-time PCR

Expanded blastocysts (n = 20 per group) were individually collected and stored in small volume of PBS-PVP at -80°C until RNA extraction. The embryos were combined to form pools of five blastocysts, defined as biological replicates and submitted to RNA extraction (n = 4 per group). Total RNA from each blastocyst group was extracted with the PicoPure RNA isolation kit (Life Technologies, Foster City, CA, USA), following the manufacturer’s instructions. DNase treatment was performed on all samples during RNA isolation according to the manufacturer’s instructions. The extracted RNA was stored at -80°C. RNA concentration was checked using a Nanodrop instrument (ThermoFischer Scientific, MA, USA), and RNA integrity was evaluated using a Bioanalyzer 2100 (Agilent Technologies, CA, USA) with the use of RICH Pico Chips (Agilent Technologies). All samples analyzed had an RNA integrity number (RIN) ≥ 7. The samples were reverse transcribed for preparation of the cDNA using the High Capacity cDNA Reverse transcription Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer’s instructions. Gene expression analysis was performed using TaqMan Applied Biosystems assays specific for *Bos taurus taurus*. We analyzed the abundance of transcripts for target genes associated with biological processes related to lipid metabolism and embryo quality (for a complete list of gene abbreviations, nomenclature and more information, see S1 Table). Prior to qPCR assays, each sample was subjected to a sequence-specific pre-amplification procedure as follows: 1.25 μL assay mixture (the TaqMan Assay was combined to a final concentration of 0.2 × for each of 96 assays), 2.5 μL TaqMan PreAmp Master Mix (Applied Biosystems, # 4391128) and 1.25 μL cDNA (5 ng/μL). Reactions underwent activation at 95°C for 10 minutes, denaturation at 95°C for 15 seconds, and annealing and amplification at 60°C for 4 minutes for 12 cycles. These pre-amplified products were diluted 5-fold prior to qPCR analysis. The assays and preamplified samples were transferred to an integrated fluidic circuit (IFC) plate. For analysis of gene expression, the prepared sample solution consisted of 2.25 μL of cDNA (pre-amplified products), 2.5 μL of TaqMan Universal PCR Master Mix (2 ×, Applied Biosystems) and 0.25 μL of 20 × GE Sample Loading Reagent (Fluidigm); and assay solution: 2.5 μL of 20 × TaqMan Gene Expression Assay (Applied Biosystems) and 2.5 μL of 2 × Assay Loading Reagent (Fluidigm). The 96.96 Dynamic Array Integrated Fluid Circuit (Fluidigm) chip was used for data collection. After initiation, the chip was loaded with 5 μL of each assay solution and 5 μL of each sample solution and loaded into an automated controller that prepares reactions at nanoliter scales. The thermal cycling qPCR was performed on a Biomark HD System (Fluidigm, South San Francisco, CA, USA) using the TaqMan GE 96 × 96 standard protocol, which consisted of a thermal mixing stage (50°C for 2 minutes, 70°C for 20 minutes and 25°C during 10 minutes), followed by one activation stage (50°C for 2 minutes and 95°C for 10 minutes), followed by 40 cycles of denaturation (95°C for 15 seconds), annealing and primer extension (60°C for 60 seconds). The data were analyzed using the $2^{ΔCq}$ method and
using the median sample from the control group as a calibrator [21]. The geometric mean of the Cq values obtained for the ACTB, GAPDH, and PPIA genes was used as a reference.

TUNEL (Terminal deoxynucleotyl transferase dUTP nick end-labeling) analysis

Expanded blastocysts (N = 15–18 per group) were fixed in 4% paraformaldehyde for 1 hour at room temperature. After this, they were permeabilized in 0.5% Triton X-100 solution, with 0.1% sodium citrate in PBS for 1 hour. Groups of fixed and permeabilized embryos were subdivided into: positive, negative controls and experimental samples. The positive control was treated with 3U/mL DNase (FPLCpure, Amersham Biosciences) in solution with 400 mM Tris-HCL, 50 mM MgCl₂ and ultrapure water for 1 hour at 37˚C. After washing, the positive control and the samples were incubated in microdroplets of the TUNEL reagent mixture—Kit in Situ Cell Death Detection Kit Fluorescein (Roche, Germany), containing 10% enzyme solution (terminal deoxynucleotide transferase enzyme) with 90% marker solution (dUTP fluorescent conjugate) for 1 hour at 37˚C in the dark in a humid camera. The negative control was incubated in microdroplets with marker solution only, in the absence of the enzyme solution. After washing in PBS/PVP solution, controls and samples were mounted on slides containing Hoechst 33342 dye (10 μg/μL) diluted in glycerol for DNA visualization, and for counting the total number of blastomeres for each embryo. This analysis was performed under fluorescence microscopy. Cells that presented FITC-labeled nuclei (green) were considered TUNEL positive cells, that is, they exhibited fragmented DNA. Apoptotic percentage was calculated dividing the number of apoptotic cells by the total number of cells.

Statistical analysis

Embryo production, cryosurvival, cytoplasmic lipid droplet content, lipid droplets morphometric evaluation, transcripts abundance evaluation, and cell viability data were analyzed by ANOVA using the generalized linear mixed model (GLIMMIX) procedure with the SAS statistical software package (SAS Inst. Inc., Cary, NC, USA), after confirming that data were distributed normally and variances were homogeneous. Treatment, embryo quality (for cryosurvival) and first order interactions were considered fixed effects, whereas replicate was considered to be a random effect. If the results of ANOVA were significant, means were analyzed using the probability of individual differences (PDIFF) test. Logarithmic transformation was applied to qPCR data to improve normality. The data are reported as untransformed least-squares means ± SEMs. Principal component analysis was also used for the qPCR data, and hierarchical clustering of transcript levels was performed using Euclidean distances and Ward linkage to evaluate relationships between samples and features.

For mass spectrometric lipid profile analysis, multivariate and univariate statistical models were used. Ion peak intensities were normalized using the total ion current (TIC) for the spectrum. Missing values were replaced by half of the minimum positive value in the data obtained from the preprocessing procedure. The intensity values of each ion peak across multiple spectra were auto-scaled (mean-centered and divided by the standard deviation of each variable). Principal component analysis (PCA) was performed using MetaboAnalyst 2.0 [22] to identify relationships between variance in the data and differences among different treatment samples. For analyses, a significance level of 5% (P < 0.05) was used.

Results

Production of embryos

In our experiments, we tested the effects of negative and positive modulators of ACSL on the development and quality of bovine IVP embryos. 3,393 oocytes were used for the production
of 1464 blastocysts. There were no differences (P > 0.05) in the rates of cleavage, embryonic production, and morphological evaluation between the different groups, because of this, were presented just viable blastocysts (grade I and II), as shown in Table 1.

### Vitrification of embryos

The vitrified and subsequently warmed embryos were evaluated for blastocoel re-expansion capacity after 12 hours of further culture. There were no differences (P > 0.05) among treatments or among quality grade in re-expansion rates (Table 2). Embryo hatching rates were evaluated 24 hours and 48 hours after warming. The main effect of treatment and the main effect of embryo quality were observed (P < 0.05) at 24 hours, whereas only the main effect of embryo quality was identified (P < 0.05) at 48 hours after warming. There was no interaction (P > 0.05) effect of treatment and embryo quality in both endpoints (24 and 48 hours), however because of the relevance of the biological results and in order to avoid losing information, the data was not presented and discussed as main effects.

When we analyzed the rating rates of grade I blastocysts at 24 hours, the best results were found in the control and ACS+ groups, which were higher (P < 0.05) than in the ACS- and ACS± groups. In addition, the greatest (P < 0.05) hatching rate at 24 hours of grade II blastocysts was observed on the ACS+ treatment. At 24 hours, embryos of grades I, II and III of the ACS± group presented similar (P > 0.05) hatching rates. However, grade III embryos of the other groups presented lower (P < 0.05) hatching rates compared to those grades I or II.

After 48 hours of incubation, grade III blastocysts treated with ACS-, ACS±, and controls had lower (P < 0.05) hatching rates compared to grade I embryos of their respective groups. Unlike the other treatments, grade III embryos of the ACS+ group presented similar

---

### Table 1. Effect of long chain Acyl-CoA synthetase modulators during the development of in vitro produced bovine embryos: positive (ACS), negative (ACS-), both (ACS±) or none (control).

| Group | Oocyte | Cleaved | Cleavage (%) | Blastocysts | Blastocysts/Oocytes (%) | Blastocysts/Cleaved (%) |
|-------|--------|---------|-------------|-------------|-------------------------|-------------------------|
| ACS+  | 829    | 579     | 69.8        | 199         | 24.0                    | 34.4                    |
| ACS-  | 831    | 594     | 71.5        | 198         | 23.8                    | 33.3                    |
| ACS±  | 815    | 581     | 71.3        | 190         | 23.3                    | 32.7                    |
| Control | 826   | 600     | 72.6        | 221         | 26.8                    | 36.8                    |

P > 0.05

https://doi.org/10.1371/journal.pone.0220731.t001

---

### Table 2. Cryosurvival of in vitro produced bovine embryos treated with long chain Acyl-CoA synthetase modulators: Positive (ACS+), negative (ACS-), both (ACS±) or none (control). Re-expansion rates were recorded with 12 hours after warming while the cumulative hatching rates were recorded with 24 and 48 hours after warming.

| Group | Grade I | Grade II | Grade III | Re-expansion 12h (%) | Hatched 24h (%) | Hatched 48h (%)* |
|-------|---------|----------|-----------|----------------------|----------------|----------------|
| ACS+  | 100 (15/15) | 95.6 (22/23) | 91.3 (21/23) | 73.3 (11/15)Aa | 65.2 (15/23)Aa | 93.3 (14/15)Aa |
| ACS-  | 90.3 (28/31) | 100 (13/13) | 92.8 (26/28) | 51.6 (16/31)Ab | 38.4 (5/13)Ab | 83.8 (26/31)Ab |
| ACS±  | 95.6 (22/23) | 95.6 (22/23) | 96.5 (28/29) | 47.8 (11/23)Ab | 34.7 (8/23)Ab | 86.9 (20/23)Ab |
| Control | 96.7 (30/31) | 96.0 (24/25) | 86.2 (25/29) | 77.4 (24/31)Aa | 60 (15/25)Ab | 96.7 (30/31)Aa |

A, B, C: Within the same column, uncommon lowercase letters differ (P < 0.05)

Aa, Ab, Ac: Within the same row for each endpoint (re-expansion 12 hours, hatched 24 hours, and hatched 48 hours), uncommon uppercase letters differ (P < 0.05)

* Hatching rates at 48 hours are cumulative

https://doi.org/10.1371/journal.pone.0220731.t002
(P > 0.05) hatching rate to embryos of grades I and II of their group and higher (P < 0.05) compared to the grade III embryos of other treatments. Additionally, ACS+ treatment increased the hatching rates of grade II and grade III blastocysts compared to control following 48 hours after warming.

**Lipid droplets content and phospholipid membrane profiles**

Analysis with the lipophilic dye Sudan Black B showed that, when compared to the control group, embryos treated with ACS+ showed higher (P < 0.05) cytoplasmic lipid content, and the embryos treated with ACS- had a reduction (P < 0.05) in lipid content (Fig 2). Additionally, we examined if the positive modulation of Acyl-CoA synthetase through direct regulation of ACSL3, mediated by GW3965, accounted for the increase of the lipid content by incubating the embryos also in the presence of Triacsin C. Triacsin C inhibits the acyl-CoA synthetase activity of the ACSL1, 3, and 4 proteins family member [11, 23]. As the ACSL3 is the only Acyl-CoA synthetase family member that is differentially expressed in both groups and induced by the positive modulation of GW3965, the co-incubation with this inhibitor (Triacsin C) allowed us to separate the direct regulation of ACSL3 in increasing cytoplasmic lipid content from other Acyl-CoA synthetase family member (ACSL1, 4, 5 and 6). The embryos of the group that received both modulators (ACS±) did not differ (P > 0.05) from control group.

![Figure 2](https://doi.org/10.1371/journal.pone.0220731.g002)

Fig 2. Semiquantitative cytoplasmic lipid content of *in vitro* produced bovine embryos with long chain Acyl-CoA synthetase modulators: positive (ACS+), negative (ACS-), both (ACS±) or none (control). Columns with different letters indicate difference (P < 0.05). Sudan Black B staining. N = 18 per group.
The same results were observed in the semimorphometric evaluation of lipid droplets based in the total number of small, large and giant drops (Table 3 and Fig 1). Total area of the drop and the area per drop of small and giant lipid droplets were similar (P > 0.05) among groups. ACS+ blastocysts presented an increased (P < 0.05) total area and area per drop in the large droplets compared to control (Table 3).

In the lipid profile analysis, there was no remarkable change in the profiles of membrane phospholipids caused by the different treatments. In the three-dimensional plot of the PCA analysis, sample overlapping was observed with no group individualization among different treatments when the samples were grouped according the identified lipid species abundances (S1 Fig).

**Transcriptional profiles**

No marked individualization of the transcriptional profiles of the gene panel evaluated in the different treatments was observed in the two- and three-dimensional PCA plots (Fig 3A and 3B). Among the 96 genes investigated, 36 genes presented different transcription levels (P < 0.05) compared to the control group in at least one of the treatments, of which 18 genes were differentially expressed (P < 0.05) only in the ACS± group (Fig 3C).

Compared to the control group, genes associated with lipid metabolism were overexpressed (P < 0.05) in the ACS+ (ACSL3, ACAT1, and AUH; Fig 4A and 4B) and in the ACS- (SREBF1) group (Fig 4C), or underexpressed (P < 0.05) in the ACS- (ACSL3, ACSL6, AUH, and SCD), whereas there were no differences (P > 0.05) in the expression of ACST1 and ELOVL (1–5).

The expression profiles of genes associated with heatshock were increased (P < 0.05) in both ACS+ (HSP90AA1) and ACS- (HSF1) compared to the control group. In addition, in the ACS- group, the abundance of gene transcripts associated with oxidative stress (GPX4), pregnancy signaling (IFNT2), angiogenesis (VEGFA), energy metabolism (SLC2A5) and transcriptional regulation (REST and RPLPO) were increased (P < 0.05) compared to the control group, whereas levels of the regulatory genes AQP3 and FOX03 were decreased (P < 0.05).

**Embryo cell viability**

The total number of blastomeres counted after nuclear staining with Hoechst 33342 did not differ (P > 0.05) among treatments (Fig 5A). In comparison to the control group, the

| Drop Size | Treatment | Drops (N) | Total area (μm²) | Area per drop (μm²) |
|-----------|-----------|-----------|------------------|-------------------|
| Small (< 2 μm) | ACS+ | 154.6±10.2a | 122.1±11.9 | 0.86±0.06 |
| | ACS- | 98.2±9.3b | 105.4±11.9 | 0.76±0.06 |
| | ACS± | 123.4±7.2c | 104.6±11.9 | 0.83±0.06 |
| | Control | 126.6±7.6c | 102.7±11.9 | 0.81±0.06 |
| Large (2–6 μm) | ACS+ | 49.8±3.5a | 467.5±36.2a | 9.4±0.3a |
| | ACS- | 27.0±4.4b | 244.1±45.3b | 8.8±0.3b |
| | ACS± | 37.4±3.5c | 325.2±34.2c | 8.7±0.3c |
| | Control | 39.8±3.2c | 347.0±33.3c | 8.6±0.3c |
| Giant (> 6μm) | ACS+ | 16.0±1.2a | 9257.8±742.4 | 581.5±94.9 |
| | ACS- | 9.2±1.1b | 9098.5±664.1 | 822.8±94.9 |
| | ACS± | 13.4±1.5c | 9659.2±664.1 | 728.8±84.9 |
| | Control | 12.6±1.4c | 9858.7±664.1 | 813.1±84.9 |

abc Within the same column for each drop size, uncommon lowercase letters differ (P < 0.05). N = 18 per group.

https://doi.org/10.1371/journal.pone.0220731.t003
Fig 3. Two-dimensional (A) and three-dimensional (B) principal component plots and hierarchical clustering of each data set (C) showing transcriptional profiles abundance of in vitro–produced bovine embryos with positive (ACS+), negative (ACS–), both (ACS±) or none (control) modulators of long-chain Acyl-CoA synthetases. N = 4 per group.

https://doi.org/10.1371/journal.pone.0220731.g003
percentage of apoptotic cells with DNA fragmentation was higher (P < 0.05) in the ACS- group (Fig 5B). The other two groups, ACS+ and ACS±, did not differ (P > 0.05) from the control group.

Discussion

In the present work, we tested the effects of addition of GW3965 hydrochloride (ACS+) and Triacsin C (ACS-), positive and negative modulators of long chain Acyl-CoA synthetase (ACSL), respectively, during the development of IVP bovine embryos. We identified the effectiveness of the treatments in modulating the level of ACSL3 transcripts, and also the clear impact of this modulation on cytoplasmic lipid droplets content. However, despite the effectiveness of ACS- in reducing cellular lipid deposit, this treatment was not effective in increasing embryonic cryosurvival. Conversely, the use of ACS+ restored the survival rates after vitrification of low quality embryos, making them equivalent to embryos of excellent quality.

Embryo production rates are commonly used as a control endpoint when modifications are made in the culture medium. In our study, the rates of cleavage and production of blastocysts did not differ between groups, evidencing that there were no detrimental or beneficial effects attributed to the use of the modulators on fresh embryo development. The achieved development rates are similar to those commonly described in the literature [3, 24, 25]. Additionally, after
morphological evaluation, our results demonstrated that the treatments were not able to significantly alter the degree of quality of the embryos produced when compared to the control group.

To verify the effect of ACS+ and ACS- on total lipid content of the embryos, the lipophilic staining technique of Sudan Black B was used. The results validate the effect initially proposed for both modulators, i.e., the addition of ACS+ to the embryo culture medium was effective in increasing the accumulation of cytoplasmic lipid droplets, whereas a reduction in lipid content was observed in embryos produced with ACS- treatment. The co-incubation of embryos with GW3965 and Triacsin C revealed the direct association between the regulation of ACSL3 and the cytoplasmic lipid content, i.e. the positive modulation of ACSL3 accounted for the observed increased cytoplasmic lipid droplets content, by not other Acyl-CoA synthetase family member. Indeed, ACSL3 protein has already been associated with triacylglycerol accumulation and fatty acid uptake in many other cell types [7].

Phospholipids are the most abundant lipids in the membrane of eukaryotic cells, their composition determines crucial physicochemical properties applied in the cryopreservation field, such as fluidity and permeability [26]. At the present work, the lipid profile of the embryos was not altered by the treatments. In this trial only the fatty acid activation was modulated, through ACSL3 regulation (ACSL3 has a highest affinity for laurate, myristate, arachidonate and eicosapentaenoic [27]), not involving other fatty acid elongases and desaturases that also have great importance on the determination of fatty acids and phospholipids composition. The distinct intracellular location (tissue and intracellular distribution) of each Acyl-CoA synthetases family member has been hypothesized to channel fatty acids at different metabolic fates by activating fatty acids at different subcellular compartments [28]. There are evidences that increased activity of ACSL3 protein is associated with the fatty acid uptake in the placental trophoblasts [7]. These findings also could explain the reason for phospholipid membrane profile remain unaltered by modulators at the present work, i.e., the fatty acids could be directed for triglyceride synthesis and not for altering phospholipid membrane composition. However, an investigation of the regulation of other ACSL family member with its respectively preferred substrate should be conducted to discard their involvement in the determination of the phospholipid membrane composition.

Fig 5. Cell viability analysis of in vitro produced bovine embryos with positive (ACS+), negative (ACS-), both (ACS±) or none (control) modulators of long chain Acyl-CoA synthetase. (A) Total number of blastomeres stained with Hoechst 33342 dye. (B) Apoptosis percentage. Columns with different letters differ (P < 0.05). N = 15–18 per group.

https://doi.org/10.1371/journal.pone.0220731.g005
Twelve hours after warming, no differences were found in the rates of re-expansion of the blastocoel in the vitrified embryos, indicating that use of the modulators did not alter either the survival capacity, or the resumption of embryonic metabolic activity, in the initial period after cryopreservation. After 24 hours, however, we surprisingly observed that, grade I embryos, considered as those of high quality that favored pregnancy success after transfer to recipients, presented the best hatching rates in the control and ACS+ groups. Perhaps the resumption of development after cryopreservation in a timely manner could be used as indicative of a greater embryo quality, as frequently used during early embryo (fresh) development [29]. After 48 hours of re-culture, ACS+ was able to restore the hatching rate of grade III blastocysts as like as the results of grade I embryos, resulting in higher hatching rates than the control group. But these findings should be validated with pregnancy rate results, which is considerate the gold standard phenotype, and also on a slow freezing cryopreservation technique in order to verify if the results remain the same.

Although the cytoplasmic accumulation of lipids is commonly associated with a reduction of embryo cryosurvival [1, 30], a cellular and molecular mechanism describing a direct correlation between them is still lacking. In a previous study of our group [20], we have already indicated that just not only the amount lipids affect embryo cryosurvival. When we analyze only ACS+ and ACS- groups, the negative modulation of ACSL3 reduced lipid deposit and do not improve cryosurvival, whereas the positive modulation of ACSL3 resulted in embryos with higher lipid deposit and greater cryosurvival, especially in lower quality embryos. A plausible reason for this contradict result can be explained by the fact that the treatments did not altered total area and the area per drop of giant lipid droplets, especially when larger lipid droplets are considered to be more detrimental for embryo post-cryopreservation survival [1, 31].

Additionally, it is fair to speculate that ACS+ treatment provided beneficial effects for the lower quality embryos by pathways other than lipid metabolism such as energetic metabolism (improving glucose tolerance and insulin resistance by regulating genes involved in glucose metabolism [32]) and inflammatory pathway (inhibiting inflammatory mediators [33]). Unfortunately, we did not identify any mRNA transcripts level for genes associated with these pathways in our microfluidic gene panel (the panel was not setup for this pathways).

GW3965 hydrochloride is an agonist specific for LXRα and LXRβ receptors through which it is capable of raising the level of ACSL3 expression. According to Patel, Oza [34] activation of LXR ligands inhibits the induction of NF-κB, an inducible transcription factor. Upon its activation, NF-κB can induce the transcription of multiple genes involved in the cascade pathway of inflammation. The regulation of NF-κB acts not only on the increase of the production of inflammatory cytokines, chemokines and adhesion molecules, but also cell proliferation, apoptosis, morphogenesis and differentiation [35]. It is possible that the embryos of the ACS+ group suffered an inactivation of this pathway, especially grade III embryos, which resulted in a higher rate of cell survival and, consequently, greater survival capacity after cryopreservation. This results also shed light to pathways other than lipid accumulation interfering with embryo survival capacity after cryopreservation.

The various methods of production and culture systems can affect blastocysts cryotolerance and the transcripts expression, which can serve as a sensitive indicator of embryonic quality, and as a marker of developmental competence [36]. In an enzymatic assay conducted by Van Horn, Caviglia [11] in the presence of Triacsin C, there was an inhibition of ACSL1, 3, and 4 isoform activity, but not ACSL5 and 6. In our trials, embryos produced in the ACS- group showed a reduction in the relative abundance of ACSL3 transcripts and, unlike previous work, we did not find alterations in ACSL1 levels, whereas there was a reduction in ACSL6 levels, including in the group treated with both modulators (ACS±). In addition to the reduction in the ACS- group, ACSL3 expression was stimulated in the ACS+ group, in accordance with results obtained in other studies [7, 13].
the group that received the addition of both modulators (ACS±), we observed a reduction in ACSL3 expression compared to the control group. In addition, ACS+ also caused increased expression of other genes related to lipid metabolism, such as ACAT1, which converts free intracellular cholesterol to a storage form in cholesterol esters [37], SCD, involved in the synthesis of unsaturated fatty acids [38] and AUH, more precisely associated with the beta oxidation activity of enoyl-CoA hydratase [39]. Moreover, Bos taurus taurus embryos that showed higher cryotolerance also had super-expressed levels of AUH, a behavior similar to that identified in the ACS+ group [2].

Induction of HSPs involves a family of heat shock transcription factors (HSF) that bind to the heat shock elements of HSP genes and mediate their transcription [40]. The mechanisms by which HSF1 is triggered by stress are not entirely understood. It is believed that HSF1 is repressed by its products—the heatshock proteins—through a mechanism of feedback inhibition [41]. In the ACS- and ACS± groups, there was overexpression of HSF1 transcripts, which could probably be explained by the low levels of HSP90AA1 in both groups, unlike in ACS+.

The total number of cells, and the incidence of apoptosis, have been suggested as additional criteria for the evaluation of embryos in order to evaluate the quality and effectively predict their viability [42]. From our results, the total number of blastomeres did not differ between groups. However, embryos from the ACS- group showed higher numbers of cells with DNA fragmentation than the control group. Although it did not affect the rate of production, the use of Triacsin C may have caused embryonic toxicity, since the choice of modulator dosage in the pilot experiment was based only on production rates and morphology of the embryos. In another study, Triacsin C has already demonstrated a dose dependent cell growth inhibitory effect in animal cells [43]. In addition, embryos from the ACS- group showed increased GPX4 expression and decreased VEGF transcript abundance, indicating a possible increase in cellular oxidative stress [44] with consequent developmental impairment [45].

Lipids have different turnover rates in the body. They can be broken down through a series of mitochondrial β-oxidation processes involving Acetyl-CoA, which then enters the tricarboxylic acid cycle to assist the generation of ATP, or alternatively, can be incorporated into triacylglycerols, phospholipids or cholesterol esters [46]. Both distinct pathways require a common initial stage known as activation of fatty acids by ACS. In animal cells the reaction of long chain Acyl-CoA synthetase is the main (probably only) route to supply Acyl-CoA [43]. Thus, we can speculate that the negative modulation of ACSL can lead to the blockade of an important embryonic metabolic pathway, triggering adverse effects that may compromise embryo viability and survival still in the initial period of development.

Conclusion

In conclusion, the use of GW3965 hydrochloride and Triacsin C during embryonic development effectively modulated ACSL3 and regulated cytoplasmic lipid content without any detrimental effect for embryo yield. Positive modulation of ACSL3 increased the cryosurvival of low quality embryos when submitted to vitrification, such finding could be considered as an alternative for cryopreservation of the larger number of IVP embryos, allowing the cryopreservation of grade I, II and III expanded blastocysts.

Supporting information

S1 Table. Microfluidic panel gene list. Complete list of gene abbreviations, nomenclature and function.
(XLSX)
S1 Fig. Three-dimensional PCA plot of lipid profiles of in vitro produced bovine embryos with long chain Acyl-CoA synthetase modulators: positive (ACS+), negative (ACS-), both (ACS±) or none (control). N = 15 per group.

TIFF

Acknowledgments

The present work was carried out with the support of CNPq, National Council of Scientific and Technological Development–Brazil, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–Brasil (CAPES)—financial code 1, and grant 2012/50533-2 and 2019/16239-9, São Paulo Research Foundation (FAPESP).

Author Contributions

Conceptualization: Mateus José Sudano.
Data curation: Mateus José Sudano.
Funding acquisition: Mateus José Sudano.
Investigation: Roniele Santana Valente, Mateus José Sudano.
Methodology: Roniele Santana Valente, Tamie Guibu de Almeida, Mayra Fernanda Alves, Janine de Camargo, Andrea Cristina Basso, Katia Roberta Anacleto Belaz, Marcos Nogueira Eberlin, Fernanda da Cruz Landim-Alvarenga, Patricia Kubo Fontes, Marcelo Fábio Gouveia Nogueira.
Project administration: Mateus José Sudano.
Resources: Andrea Cristina Basso, Katia Roberta Anacleto Belaz, Marcos Nogueira Eberlin, Fernanda da Cruz Landim-Alvarenga, Patricia Kubo Fontes, Marcelo Fábio Gouveia Nogueira.
Supervision: Mateus José Sudano.
Validation: Janine de Camargo.
Writing – original draft: Roniele Santana Valente.
Writing – review & editing: Katia Roberta Anacleto Belaz, Marcelo Fábio Gouveia Nogueira, Mateus José Sudano.

References

1. Abe H, Yamashita S, Satoh T, Hoshi H. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. Molecular reproduction and development. 2002; 61(1):57–66. https://doi.org/10.1002/mrd.1131 PMID: 11774376

2. Sudano MJ, Caixeta ES, Paschoal DM, Martins A, Machado R, Buratini J, et al. Cryotolerance and global gene-expression patterns of Bos taurus indicus and Bos taurus taurus in vitro-and in vivo-produced blastocysts. Reproduction, Fertility and Development. 2014; 26(8):1129–41.

3. Panyaboribnan S, Tharasani T, Chankitisakul V, Swangchan-Uthai T, Techakumphu M. Treatment with chemical delipidation forskolin prior to cryopreservation improves the survival rates of swamp buffalo (Bubalus bubalis) and bovine (Bos indicus) in vitro produced embryos. Cryobiology. 2018; 84:46–51. https://doi.org/10.1016/j.cryobiol.2018.08.003 PMID: 30092172

4. Murillo A, Muñoz M, Martín-González D, Carrocera S, Martínez-Nistal A, Gómez E. Low serum concentration in bovine embryo culture enhances early blastocyst rates on Day-6 with quality traits in the expanded blastocyst stage similar to BSA-cultured embryos. Reproductive biology. 2017; 17(2):162–71. https://doi.org/10.1016/j.reproto.2017.04.002 PMID: 28479126
5. Sudano M, Paschoal D, Maziero R, Rascado T, Guastali M, Crocomo L, et al. Improving postcryopreservation survival capacity: an embryo-focused approach. Animal Reproduction. 2013:160–7.

6. Sudano MJ, Paschoal DM, da Silva Rascado T, Magalhães LCO, Crocomo LF, de Lima-Neto JF, et al. Lipid content and apoptosis of in vitro-produced bovine embryos as determinants of susceptibility to vitrification. Theriogenology. 2011; 75(7):1211–20. https://doi.org/10.1016/j.theriogenology.2010.11.033 PMID: 21247620

7. Weedon-Fekjaer MS, Dalen KT, Solaas K, Staff AC, Duttaroy AK, Nebb HI. Activation of LXR increases acyl-CoA synthetase activity through direct regulation of ACSL3 in human placental trophoblasts. Journal of lipid research. 2010; Jr. M004978.

8. Soupene E, Kuppers FA. Mammalian long-chain acyl-CoA synthetases. Experimental biology and medicine. 2008; 233(5):507–21. https://doi.org/10.3181/0710-MR-287 PMID: 18375835

9. Mashek DG, Li LO, Coleman RA. Long-chain acyl-CoA synthetases and fatty acid channeling. Future lipidology. 2007; 2(4):465–76. https://doi.org/10.1021/fl060432l PMID: 20354580

10. Ulven SM, Dalen KT, Gustafsson J-A, Nebb HI. LXR is crucial in lipid metabolism. Prostaglandins, leukotrienes and essential fatty acids. 2005; 73(1):59–63.

11. Van Horn CG, Caviglia JM, Li LO, Wang S, Granger DA, Coleman RA. Characterization of recombinant long-chain rat acyl-CoA synthetase isoforms 3 and 6: identification of a novel variant of isoform 6. Biochemistry. 2005; 44(15):6353–42. https://doi.org/10.1021/bi047721l PMID: 15683247

12. Igal RA, Ping W, Coleman RA. Triacsin C blocks de novo synthesis of glycerolipids and cholesterol esters but not recycling of fatty acid into phospholipid: evidence for functionally separate pools of acyl-CoA. Biochemical Journal. 1997; 324(2):529–34.

13. Dong B, Kan CFK, Singh AB, Liu J. High fructose diet downregulates long-chain acyl-CoA synthetase 3 expression in liver of hamsters via impairing LXR/RXR signaling pathway. Journal of lipid research. 2013; Jr. M032599.

14. Gómez E, Carrocera S, Uzbekova S, Martin D, Murillo A, Alonso-Guervos M, et al. Protein in culture and endogenous lipid interact with embryonic stages in vitro to alter calf birthweight after embryo vitrification and warming. Reproduction, Fertility and Development. 2017; 29(10):1932–43.

15. Sudano MJ, Rascado TD, Tata A, Belaz KR, Santos VG, Valente RS, et al. Lipidome signatures in early bovine embryo development. Theriogenology. 2016; 86(2):472–84. e1. https://doi.org/10.1016/j.theriogenology.2016.03.025 PMID: 27107972

16. Parrish J, Susko-Parrish J, Winer M, First N. Capacitation of bovine sperm by heparin. Biology of reproduction. 1988; 38(5):1171–80. https://doi.org/10.1095/biolreprod.38.5.1171 PMID: 3408784

17. Stringfellow D, Seidel S. Manual da sociedade internacional de transferência de embriões: um guia de procedimento e informações gerais para uso em tecnologia de transferência de embriões enfatizando procedimentos sanitários. Illinois: Savoy. 1998; 3:180.

18. Sanches B, Marinho L, Pontes J, Basso A, Meirinhos M, Silva-Santos K, et al. Phosphatidylcholine and sphingomyelin profiles vary in Bos taurus indicus and Bos taurus taurus in vitro-and in vivo-produced blastocysts. Biology of reproduction. 2012; 87(6).

19. Pflaff MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic acids research. 2001; 29(9):e45–e. https://doi.org/10.1093/nar/29.9.e45 PMID: 11328886

20. Xia J, Mandal R, Sinelnikov IV, Broadhurst D, Wishart DS. MetaboAnalyst 2.0—a comprehensive server for metabolomic data analysis. Nucleic acids research. 2012; 40(W1):W127–W33.

21. Kim J-H, Lewin TM, Coleman RA. Expression and Characterization of Recombinant Rat Acal-CoA Synthetases 1, 4, and 5 Selective Inhibition By Triacsin C And Thiazolidinediones. Journal of Biological Chemistry. 2001; 276(27):24667–73. https://doi.org/10.1074/jbc.M010793200 PMID: 11319222

22. Held-Hoelker E, Klein S, Rings F, Salilaw-Wondim D, Saeed-Zidane M, Neuhoff C, et al. Cryosurvival of in vitro produced bovine embryos supplemented with l-Carnitine and concurrent reduction of fatty acids. Theriogenology. 2017; 96:145–52. https://doi.org/10.1016/j.theriogenology.2017.03.014 PMID: 28532831

23. Knitlova D, Hulinska P, Jeseta M, Hanzalova K, Kempisty B, Machatková M. Supplementation of l-carnitine during in vitro maturation improves embryo development from less competent bovine oocytes. Theriogenology. 2017; 102:16–22. https://doi.org/10.1016/j.theriogenology.2017.06.025 PMID: 28719824
26. Edidin M. Lipids on the frontier: a century of cell-membrane bilayers. Nature Reviews Molecular Cell Biology. 2003; 4(5):414. https://doi.org/10.1038/nrm1102 PMID: 12728275

27. Fujiwara T, Kanagawa H, Suzuki H, Iijima H, Yamamoto T. Molecular characterization and expression of rat acyl-CoA synthetase 3. Journal of Biological Chemistry. 1996; 271(28):16748–52. https://doi.org/10.1074/jbc.271.28.16748 PMID: 8663269

28. Lewin TM, Kim J-H, Granger DA, Vance JE, Coleman RA. Acyl-CoA synthetase isoforms 1, 4, and 5 are present in different subcellular membranes in rat liver and can be inhibited independently. Journal of Biological Chemistry. 2001; 276(27):24674–9. https://doi.org/10.1074/jbc.M102036200 PMID: 11319232

29. Gutiérrez-Adán A, White CR, Van Soom A, Mann MR. Why we should not select the faster embryo: lessons from mice and cattle. Reproduction, Fertility and Development. 2015; 27(5):765–75.

30. Rizos D, Clemente M, Bermejo-Alvarez P, de la Fuente J, Lonergan P, Gutiérrez-Adán A. Consequences of in vitro culture conditions on embryo development and quality. Reproduction in Domestic Animals. 2008; 43:44–50. https://doi.org/10.1111/j.1439-0531.2008.01230.x PMID: 18803756

31. López-Damián EP, Jiménez-Medina JA, Lammoglia MA, Pimentel JA, Agredano-Moreno LT, Wood C, et al. Lipid droplets in clusters negatively affect Bos indicus embryos during cryopreservation. Anatopia, histologia, embryologia. 2018; 47(5):435–43. https://doi.org/10.1111/ahe.12382 PMID: 29978506

32. Laffitte BA, Chao LC, Li J, Walczak R, Hummasti S, Joseph SB, et al. Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. Proceedings of the National Academy of Sciences. 2003; 100(9):5419–24.

33. Joseph SB, Castrillo A, Laffitte BA, Manglesdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. Nature medicine. 2003; 9(2):213. https://doi.org/10.1038/nmm820 PMID: 12524543

34. Patel M, Oza N, Anand I, Deshpande S, Patel C. Liver X Receptor: A novel therapeutic target. Indian journal of pharmaceutical sciences. 2008; 70(2):135. https://doi.org/10.4103/0250-474X.41445 PMID: 20046702

35. Liu T, Zhang L, Joo D, Sun S-C. NF-κB signaling in inflammation. Signal transduction and targeted therapy. 2017; 2:17023. https://doi.org/10.1038/sigtrans.2017.23 PMID: 29158945

36. Kuzmany A, Havlicek V, Wrenzycki C, Wilkening S, Brem G, Besenfelder U. Expression of mRNA, before and after freezing, in bovine blastocysts cultured under different conditions. Theriogenology. 2011; 75(3):482–94. https://doi.org/10.1016/j.theriogenology.2010.09.016 PMID: 21144573

37. Tian G-P, Chen W-J, He P-P, Tang S-L, Zhao G-J, Lv Y-C, et al. MicroRNA-467b targets LPL gene in RAW 264.7 macrophages and attenuates lipid accumulation and proinflammatory cytokine secretion. Biochimie. 2012; 94(12):2749–55. https://doi.org/10.1016/j.bioch.2012.08.018 PMID: 22963823

38. Peck B, Schug ZT, Zhang G, Dankworth B, Jones DT, Smethurst E, et al. Inhibition of fatty acid desaturation is detrimental to cancer cell survival in metabolically compromised environments. Cancer & metabolism. 2016; 4(1):6.

39. Nakagawa J, Waldner H, Meyer-Monard S, Hofsteenge J, Jené P, Moroni C. AUH: a gene encoding an AU-specific RNA binding protein with intrinsic enoyl-CoA hydratase activity. Proceedings of the National Academy of Sciences. 1995; 92(6):2051–5.

40. Zhang Y, Chou S-D, Murshed A, Prince TL, Schreiner S, Stevenson MA, et al. The role of heat shock factors in stress-induced transcription. Molecular Chaperones. Springer; 2011. p. 21–32.

41. Zou J, Guo Y, Guettouche T, Smith DF, Voellmy R. Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell. 1998; 94(4):471–80. https://doi.org/10.1016/s0092-8674(00)81588-3 PMID: 9727490

42. Pomar FR, Teerds K, Kidson A, Colenbrander B, Tharasanit T, Aguilar B, et al. Differences in the incidence of apoptosis between in vivo and in vitro produced blastocysts of farm animal species: a comparative study. Theriogenology. 2005; 63(8):2254–68. https://doi.org/10.1016/j.theriogenology.2004.10.015 PMID: 15826688

43. Komuro H, Isogai K, Cyong J-C, Omura S. Evidence for an essential role of long chain acyl-CoA synthetase in animal cell proliferation. Inhibition of long chain acyl-CoA synthetase by triacsin causes inhibition of Raji cell proliferation. Journal of Biological Chemistry. 1991; 266(7):4214–9. PMID: 1999415

44. Laffitte BA, Chao LC, Li J, Walczak R, Hummasti S, Joseph SB, et al. Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. Proceedings of the National Academy of Sciences. 2003; 100(9):5419–24.

45. Joseph SB, Castrillo A, Laffitte BA, Manglesdorf DJ, Tontonoz P. Reciprocal regulation of inflammation and lipid metabolism by liver X receptors. Nature medicine. 2003; 9(2):213. https://doi.org/10.1038/nmm820 PMID: 12524543

46. Patel M, Oza N, Anand I, Deshpande S, Patel C. Liver X Receptor: A novel therapeutic target. Indian journal of pharmaceutical sciences. 2008; 70(2):135. https://doi.org/10.4103/0250-474X.41445 PMID: 20046702