Fatty Acid Supplementation During in vitro Embryo Production Determines Cryosurvival Characteristics of Bovine Blastocysts

H. Aardema†, I. Bertijn†, HTA. van Tol, A. Rijneveld, JCM. Vernooij, B. M. Gadella* and PLAM Vos

Department of Population Health Sciences - Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands

In vitro production (IVP) embryos have a reduced quality and poor cryotolerance in comparison to in vivo embryos. This study investigated whether free fatty acid (FFA) conditions, fatty acid free (FAF)- synthetic oviduct fluid (SOF) without or with 25 μM of saturated stearic (C_{18:0}) or unsaturated oleic (C_{18:1}) acid during the first 5 IVP days, relate to quality and cryosurvival of day 8 blastocysts. Apart from the blastocyst scores, both 1) number and size of lipid droplets of fresh blastocysts and 2) total number and apoptotic and necrotic cells, before and after freezing-thawing, were scored by confocal microscopy. Blastocyst rates were significantly lower in the FAF SOF condition in comparison to other groups. Interestingly, blastocysts originating from the C_{18:1} group, with a significantly higher lipid content, and blastocysts from the FAF SOF group demonstrated a high cryosurvival rate (70.1 and 67.4%, respectively) comparable with in vivo blastocysts (68%), in contrast to the poor cryosurvival of C_{18:0} exposed embryos (17.6%). In all freeze-thawed embryos the average amount of apoptotic and necrotic cells increased albeit that the C_{18:0} condition rates were higher (43.2%) when compared to C_{18:1} (26.0%) and FAF SOF conditions (26.5%). The current data show that FFA administered during early embryonic development significantly affect the cryotolerance of blastocysts.

Keywords: free fatty acid, embryo, blastocyst, cryosurvival, lipid, apoptose, slow freezing, cryopreservation

1 INTRODUCTION

Worldwide, the number of in vitro production (IVP) bovine embryos has dramatically increased during the last years, until more than 1 million in 2019 with a concomitant decrease in the number of in vivo derived embryos (378,769 in 2019; (Viana, 2020)). Genomic selection technologies performed by breeding companies have increased the demand for IVP produced embryos, derived from genetically desired animals, to shorten the generation intervals and to increase the genomic gain. Harvesting oocytes by ovum pick up (OPU) offers the possibility to retrieve a higher number of IVP embryos per individual animal over time in comparison to the technology of multiple ovulation embryo transfer (MOET), which is related with high donor variability producing unpredictable numbers of in vivo embryos (Boland et al., 1991; Hasler et al., 2003; Ferré et al., 2020). To this end, it is not surprising that in 2016 worldwide the number of IVP embryos for the first time exceeded the number of in vivo derived embryos (Viana, 2020). In order to manage these high numbers of embryos, cryopreservation is an ideal tool for logistical reasons because breeding companies can
optimally time the transfer of the embryo with the reproductive cycle of the recipient and transfer the embryo over long distances, but unfortunately the cryosurvival rates of IVP embryos are low in comparison to in vivo derived embryos (Moore and Hasler 2017; Viana, 2020; Ferré et al., 2020). However, the currently still reduced cryosurvival of IVP embryos versus in vivo derived embryos is costly and puts a significant burden on bovine industry. Therefore, optimization of the IVP conditions in order to increase the cryosurvival of IVP embryos is highly desirable.

It is well known that the potential of an oocyte to develop into a blastocyst after in vitro fertilization and subsequent embryo culture is largely depending on the quality of the oocyte, with only 30–40% of the in vitro matured oocytes reaching the blastocyst stage in contrast to 80% of the in vivo matured oocytes (Hendriksen et al., 2000; Dieleman et al., 2002; Rizos et al., 2002; Knijn et al., 2003; Tesfaye et al., 2007). However, the quality of the embryo itself and thus the options for cryosurvival can be improved by optimizing the post-fertilization conditions of IVP embryos (Rizos et al., 2002; Lonergan 2007; Lonergan and Fair 2008; Gad et al., 2012).

A higher lipid content in IVP embryos has been suggested as one of the factors for decreased quality and cryosurvival rates when compared to in vivo derived embryos (Abe et al., 2002; Hara et al., 2005; Reis et al., 2005; Pereira et al., 2007; Sudano et al., 2011; Abe et al., 2017). Moreover, the lipid composition in embryos has also been described as one of the possible factors causing a reduction in the cryosurvival of IVP embryos. In fact high quality oocytes and embryos were shown to contain relatively high proportions of unsaturated fatty acids, linoleic and oleic acid, and low proportions of saturated fatty acids in comparison to oocytes and embryos of lower quality (Hochi et al., 1999; Kim et al., 2001; Haggarty et al., 2006). Furthermore, embryos have been shown to take up exogenous free fatty acid (FFA) during IVP and are able to incorporate these fatty acids in neutral lipid stored in lipid droplets (Flynn and Hillman 1980; Wang and Tsuji 1998; Khandoker and Tsuji 1999; Haggarty et al., 2006; Sturmey et al., 2009; Hughes et al., 2011; McKeegan and Sturmey 2012). Thus in the current study the aim was to investigate whether the quality of IVP embryos and their cryosurvival rates is influenced by supplementation of FFA during the first 5 days of embryonic development, i.e. at the physiological period that the embryo in vivo would reside in the oviduct. To this end, presumed zygotes were exposed to stearic acid (C18:0) or oleic acid (C18:1) during day 1 till 5 of IVP (day 0 = IVF). In literature, both cytoplasm lipid content and cell apoptosis rates have been considered to be important factors that relate to embryo cryosurvival (Sudano et al., 2014). Therefore, the number and size of lipid droplets was investigated in day 8 blastocysts before freezing and the number of apoptotic and necrotic cells in blastocysts before and after freeze/thawing.

### 2 MATERIALS AND METHODS

#### 2.1 Chemicals and Media

All chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Corp., St. Louis, MO, United States) unless otherwise stated. All culture media were prepared manually, corrected for pH (pH = 7.35 ± 0.05) and filter sterilized through a 0.22 µm Millex GV filter (Merck, Darmstadt, Germany).

#### 2.2 Experimental Design

In experiment 1, the effect of fatty acid supplementation during the first 5 days of in vitro embryonic development on bovine embryo lipid content, cell damage and cryosurvival was investigated. After standard in vitro maturation of cumulus-oocyte-complexes (COCs; day -1), and fertilization (day 0; (Aardema et al., 2011)), presumed zygotes were cultured from day 1 until day 5 in SOF with 1) bovine serum albumin (BSA) (standard SOF) or 2) delipidified BSA (>96% fatty acid free (FAF; FAF SOF)) (iii), FAF BSA complexed with 25 µM saturated C18:0 or 4) with unsaturated C18:1. From day 5 onwards, SOF was refreshed and embryos were cultured in standard embryo culture conditions with BSA (standard SOF) or with delipidified BSA (FAF SOF). At day 8 post-fertilization, fresh and frozen-thawed blastocysts were stained for apoptosis (TUNEL; Roche Cell Death Detection Kit, Sigma Chemical Co., St Louis, MO, United States), necrosis (Ethidium-homodimer 1; EthD-1, Invitrogen, Waltham, MA, United States) and DNA (Hoechst 33342). Another set of fresh blastocysts was fixed in 1% PFA and stained with LD540 (a dye based on the Bodipy fluorophore, fabricated by (Spandl et al., 2009) for lipid droplet (LD) staining. Scheme 1.

#### 2.3 In vitro Embryo Production

##### 2.3.1 Maturation

In vitro embryo production was performed as described previously (Aardema et al., 2011). In short, bovine ovaries were collected from a slaughterhouse and transported to the laboratory within 2 h. Ethical approval was not necessary, as the ovaries were a waste product from regular slaughter. COCs were aspirated from antral follicles (2–8 mm diameter) using a winged infusion set (18 gauge needle) connected to a vacuum suction system (~0.6 bar), pooled in a conical tube and allowed to sediment for 30 min at room temperature. COCs with a minimum of two cumulus cell layers were selected and washed 3x in HEPES buffered M199 cell culture medium with 2.2 mg/ml NaHCO3 at room temperature. 50–55 COCs were matured in 500 µl M199 cell culture medium per well with 2.2 mg/ml NaHCO3, 0.05 IU/ml Human recombinant follicle-stimulating hormone, 0.1 µM cysteamine, 10 ng/ml epidermal growth factor in 0.1% (w/v) FAF BSA and 1% (v/v) penicillin-streptomycin (Gibco BRL, United States), and no coverage of oil, in four-well culture plates (Nunc A/S, Roskilde, Denmark) for 23 h at 38.5°C in a humidified atmosphere of 5% (v/v) CO2 in air, according to our standard protocol (Aardema et al., 2011).
2.3.2 Fertilization
After 23 h of maturation (day -1), in vitro fertilization (day 0) was performed with $1 \times 10^6$ sperm from a bull with proven fertility. Procedures for in vitro fertilization were performed as described by Parrish et al. (Parrish et al., 1988) with minor modifications (Izadyar et al., 1996). Briefly, sperm cells were added to the fertilization medium (modified Tyrode’s medium also called Fert-TALP (Parrish et al., 1988), without glucose and 1% (v/v) penicillin-streptomycin instead of gentamycin (Izadyar et al., 1996) to a final concentration of $1 \times 10^6$ sperm cells/mL in the presence of 10 µg/mL heparin, 20 µM d-penicillamine, 10 µM hypotaurine, and 1 µM epinephrine. Fertilization lasted for 18–20 h at 38.5°C in a humidified atmosphere of 5% (v/v) CO$_2$ and 7% (v/v) O$_2$. C$_{18:0}$ or C$_{18:1}$ were complexed to >99.9% delipidified bovine serum albumin (BSA), according to our protocol with a fatty acid:BSA stoichiometry of 5:1 (Aardema et al., 2011). On day 5 post insemination, cleavage rates were determined and non-cleaved embryos were discarded. The cleaved embryos were transferred to fresh standard SOF or FAF SOF medium. Blastocyst rates and quality, according to the International Embryo Technology Society (IETS) identification system (Stringfellow and Givens, 2009), were determined at day 7 and 8 post insemination. Blastocysts were collected on day 8 post fertilization for further analysis.

2.3.3 Embryo Culture
At 18–20 h after sperm addition, cumulus cells of presumptive zygotes were removed by vortexing for 3 min. Denuded zygotes were transferred in groups of 50–55 to wells with 500 µL standard SOF or FAF SOF without or with supplementation of 25 µM C$_{18:0}$ or C$_{18:1}$ and cultured at 38.5°C in a humidified atmosphere of 5% (v/v) CO$_2$ and 7% (v/v) O$_2$. C$_{18:0}$ or C$_{18:1}$ were complexed to >99.9% delipidified bovine serum albumin (BSA), according to our protocol with a fatty acid:BSA stoichiometry of 5:1 (Aardema et al., 2011). On day 5 post insemination, cleavage rates were determined and non-cleaved embryos were discarded. The cleaved embryos were transferred to fresh standard SOF or FAF SOF medium. Blastocyst rates and quality, according to the International Embryo Technology Society (IETS) identification system (Stringfellow and Givens, 2009), were determined at day 7 and 8 post insemination. Blastocysts were collected on day 8 post fertilization for further analysis.

2.4 Cryopreservation
2.4.1 Slow Freezing
On day 8 post insemination, excellent (grade 1) and fair (grade 2) quality blastocysts were selected for the slow freezing procedure, collected in small droplets of standard SOF or FAF SOF and allowed to cool to room temperature in 5 min. Subsequently, they were washed 5 times in EMCARE™ holding solution (ICPbio Reproduction, Glenfield, New Zealand), supplemented with 20 ml/L of 20% (w/v) albumin solution (ICPbio Reproduction,
Glenfield, New Zealand), submerged in ViGRO™ Ethylene Glycol Freeze Plus sucrose medium (Vetoquinol SA, Lure, France), loaded onto 0.25 ml embryo transfer straws, and dropped into a -7°C alcohol bath slow freezer (Julabo FP40-ME, Boven-Leeuwen, Netherlands) within 10-20 min after introduction into the freezing medium. Each straw was loaded with 1-2 blastocysts. Straws were seeded after 5 min and held at -7°C for 10 min to complete ice crystal formation. The blastocysts were cooled with a rate of 0.5°C/min until −35°C and plunged into liquid N2 at −196°C.

2.4.2 Thawing
Straws with frozen blastocysts were collected from liquid N2 and held for 8 s in air, before being plunged into a 22°C water bath for 20 s. Thawed blastocysts were collected in a Petri dish. They were guided through five washing steps at room temperature, consisting of standard SOF or FAF SOF mixed with subsequently 80, 60, 40, 20 and 0% freezing medium, each washing step lasted 5 min. Blastocysts were cultured in standard SOF or FAF SOF to determine the cryosurvival, defined as re-inflation of the blastocoele 24 h post-thawing (Dinnys et al., 1999; Sudano et al., 2011). In vivo derived blastocysts, cryopreserved via a comparable slow freezing procedure, were generously donated by the breeding company CRV (CRV, Arnhem, Netherlands). The in vivo derived blastocysts were thawed and cultured for 24 h in standard SOF according to a comparable protocol as described above, to compare the cryosurvival rate of in vivo derived blastocysts with the in vitro blastocysts derived from the different embryo culture conditions in the current study.

2.5 Apoptosis and Necrosis Staining
Necrosis and late-stage apoptosis were detected with EthD1 and a TUNEL assay, respectively, as described by Pomar et al. (Pomar et al., 2004), with minor modifications. Briefly, blastocysts were incubated in 4 µM EthD-1 in PBS for 5 min in the dark at room temperature and fixed in 4% w/v paraformaldehyde (PFA) overnight (J.T. Baker Chemicals Co., Phillipsburg, NJ, United States). Thereafter, blastocysts were permeabilized in PBS with 0.1 vol% Triton X-100 and 0.1% w/v sodium citrate for 5 min on ice, incubated in TUNEL reaction mixture for 1 h at 37°C in the dark and subsequently stained with 10 µM Hoechst 33342 in PBS for 30 min. Blastocysts were mounted on glass slides between 120 µm spacers in 100% Vectashield (Vector Laboratories Inc., Burlingame, CA, United States). Before and after staining, blastocysts were washed 3x in 0.3% (w/v) PVP-PBS for 3 min and subsequently mounted on glass slides between 120 µm spacers in 100% Vectashield (Vector Laboratories Inc., Burlingame, CA, United States).

2.7 Fluorescent Imaging
Confocal microscopy was performed using a Leica SPE-II–DMI14000 laser scanning microscope (Leica microsystems, Wetzlar, Germany), using a ×20 magnification oil immersion objective combined with 1.5 digital zoom, resulting in ×30 magnification. Leica LAS software was used for acquiring images (LAS-AF, HCS Basic Module, Leica microsystems, Wetzlar, Germany). Blastocysts were imaged using z-stack serial sections with 10 µm steps and were excited sequentially with 405 nm, 488 and 561 nm at 7–14% laser strength for the detection of Hoechst 33342, TUNEL and EthD-1, respectively. Emission was detected at 420–490 nm, 500–550 nm and 580–650 nm, respectively. The LD540 stain was imaged using 488 nm excitation and 500–550 nm emission. Differential interference contrast (DIC) images were made using 488 nm excitation and 500–550 nm emission. The 8-bit z-stack series were analyzed using FIJI (Schindelin et al., 2012); ImageJ 1.48t, Java 6.0_24). LD540 stained lipid droplets were counted using a macro with the following steps: subtract background (“rolling = 10”), set threshold (55–125 over 255) per run, watershed, count particles (>0.3 µm to avoid counting background noise). The total number of droplets, average droplet area (in µm²) and standard deviation per blastocyst were documented. The number of nuclei per blastocyst (Hoechst 33342 staining) was counted according to the following procedure; 1) enhance contrast (0.5%), 2) set threshold (45 over 255), 3) fill holes, 4) watershed, 5) count particles (>50 µm with a minimal circularity of 0.2). Necrotic and apoptotic positive cells were counted manually in each separate z-stack image using the LAS AF Lite software (LAS AF lite, version 2.6.3, Leica microsystems, Wetzlar, Germany). A nucleus was scored as necrotic when the EthD-1 stain was visible and corresponded to an Hoechst 33342 stained nucleus in the overlay image. Apoptotic cells were counted similarly, using the TUNEL stain.

2.8 Statistical Analysis
Cleavage score (total cleaved/oocytes), blastocyst score day 7 and 8 (total blastocysts/oocytes), and cryosurvival, defined as expansion of the blastocoele 24 h post-thawing (re-expanded blastocysts/total thawed), were analyzed by mixed effect grouped binomial logistic regression models with experimental group as explanatory variable. For cryosurvival, the explanatory variable blastocyst development stage and its interaction with the experimental group was also tested. The number of lipid droplets and nuclei, respectively, were analyzed by a mixed effect regression model with a Poisson link. Average lipid droplet
size, number and total lipid (average lipid droplet size * number of lipid droplets) were analyzed with a mixed effect linear regression model. Residuals of the fitted model assumptions were visually inspected and therefore both outcome variables were log transformed to meet the assumptions. Experimental group was added as explanatory variable. For the model that analyses the number of nuclei per blastocyst, explanatory variable blastocyst development stage and its interaction with the experimental group was tested. The proportion of apoptotic-, necrotic- and double stained cells per blastocyst (stained with TUNEL and/or EthD) were analyzed like binomial logistic regression models. Besides the explanatory variable of experimental group also the variable thaw/fresh and the interaction between group and thaw/fresh was added to the model. In all models, run (replicate number of the experiment) was added as random effect to account for the correlation of observations within each run. A second random effect was added in the models testing cleavage score, day 7 and 8 blastocyst score and cryosurvival to account for the grouping of oocytes or blastocysts in these models. The group and blastocyst development stage effects were presented as odds ratio (OR) with 95% confidence interval (CI) for the binomial logistic regression models, ratio’s for the models with Poisson link and ratio’s between means for the linear regression models due to the log-transformation of the outcome variables. Pairwise comparison between groups were made using Tukey HSD. Blastocyst developmental stages were compared within each experimental group using a reference developmental stage. The Akaike’s Information Criterion (AIC) was used to select the best fitting model but variable experiment group remained in the model at all times as this answered the research questions. The models were fitted in statistical software R version 4.0.5 (R Core Team, 2021, (R Core Team 2021)) with library lme4 (Bates et al., 2015) and multcomp (Hothorn et al., 2008). Graphs were made using library ggplot2 (Wickham 2016) and dplyr (Wickham et al., 2020). Group differences were based on the 95% CI. When the 95% CI of the groups overlapped there was no significant difference (OR = 1). When the 95% CI were different, a higher or lower value gave information about the direction of the difference (increased or decreased). Furthermore, the width of the 95% CI shows the range and precision of the group estimate.

3 RESULTS

3.1 A Fatty Acid Free Condition Results in Reduced Embryo Development

Presumed zygotes were exposed to either C18:1 or C18:0 from day 1 until day 5 of embryo culture (day 0 = IVF), in order to investigate whether these FFA affect embryonic development. The control group was cultured in the same fatty acid free synthetic oviduct fluid (FAF SOF) medium without the addition of FFA. The standard (non-FAF) SOF was used as an internal control for the embryo culture procedure. The cleavage rates at day 5 were determined from day 8 blastocysts in each exposure group. The proportion of apoptotic- (stained with TUNEL and/or EthD) were analyzed like binomial logistic regression models. The AIC was used to select the best fitted model assumptions. On day 7 there was a significantly lower blastocyst rate of embryos exposed to FAF SOF (23.7 ± 7.2%) in comparison to those exposed to a standard (non-FAF) SOF (31.7 ± 5.9%; OR 0.66, 95% CI 0.48–0.90) and C18:1 condition (30.8 ± 8.4%; OR 0.73, 95% CI 0.54–0.98). On day 8, the blastocyst yield of embryos exposed to FAF SOF was comparable with the C18:0 condition (27.9 ± 8.6%; OR 0.77, 95% CI 0.57–1.04, Figure 1C). Note that FAF SOF medium is a chemical defined medium for embryo culture which yields lower blastocyst rates at day 8 when compared to SOF with the addition of fetal calf serum (FCS). Experimentally, the effects of addition of FA to SOF can only be tested in chemical defined medium without FCS and compared to FAF SOF.

3.2 Free Fatty Acid Exposure has Minor Effects on the Cell Number in Blastocysts

Likewise, the total cell number of blastocysts was scored for all IVP embryo groups at day 8. There was a significant difference among the number of cells per blastocyst between FAF SOF and the other groups, but biological differences in the total number of cells were small (OR 0.96 CI 0.94–0.98 and OR 1.03 CI 1.01–1.04, for C18:0 and C18:1, respectively; data not shown). This finding indicates that the different FFA exposures had a minor effect on the blastocyst size at day 8 of IVP. In contrast, the blastocyst stage did have a clear effect on the number of cells. Within all groups, non-expanded blastocysts had a significantly lower nuclei number compared to expanded blastocysts (ratio 0.75, 0.76, 0.68 and 0.71, 95% CI 0.73–0.77, 0.74–0.78, 0.67–0.70 and 0.70–0.73 for respectively standard SOF, FAF SOF, C18:0 and C18:1), and hatched blastocysts (ratio 0.69, 0.74, 0.65 and 0.73, 95% CI 0.67–0.72, 0.71–0.77, 0.62–0.67 and 0.71–0.76 for respectively standard SOF, FAF SOF, C18:0 and C18:1). Figure 2.

3.3 C18:1 Exposure Results in Higher Lipid Content of Blastocysts

In order to test whether the lipid storage of IVP embryos can be influenced by FFA exposure during the first 5 days of embryo culture, the amount and size of cytoplasmic lipid droplets was determined from day 8 blastocysts in each exposure group. The number of lipid droplets in embryos exposed to C18:0 was significantly lower (1043.79 ± 406.70) than in the FAF SOF and C18:1 group (ratio 0.83 and 0.86, 95% CI 0.83–0.84 and 0.86–0.87, resp.; Figure 3E). Interestingly, blastocysts of embryos exposed to C18:1 demonstrated a larger lipid droplet size (3.96 ± 1.62 μm²), measured two dimensionally by confocal microscopy, than the FAF SOF (3.11 ± 1.44 μm², ratio 1.33, 95% CI 1.15–1.54) and the C18:0 (2.70 ± 2.16 μm², ratio 1.63 95% CI 1.41–1.88) group (Figure 3F). Over all, blastocysts exposed to C18:1 contained significantly more lipid (lipid droplet number x size) than blastocysts from the other groups (ratio 1.27 and 1.93 and 95% CI 1.05–1.54 and 1.60–2.32, compared to FAF SOF and C18:0 resp.; Figure 3G). In contrast, blastocysts of the C18:0...
exposed embryos had a lower total lipid amount than those of the FAF SOF group (ratio 0.76, 95% CI 0.62–0.93).

3.4 Cryopreservation Results in High Level of Damaged Cells in C₁₈:₀ Blastocysts
To investigate the impact of cryopreservation on cell survival, the amount of apoptotic (TUNEL) and necrotic (EthD-1) cells in fresh and frozen-thawed blastocysts was scored. Fresh, non-frozen, day 8 blastocysts from the different groups demonstrated a low level of cell damage. The highest level of damaged cells was observed in the group of C₁₈:₁ (14.92 ± 11.52%) versus the standard SOF (11.56 ± 5.48%; OR 1.15, 95% CI 1.07–1.24), C₁₈:₀ (13.93 ± 10.88%; OR 1.16, 95% CI 1.09–1.25) and control FAF SOF (13.03 ± 6.73%, OR 1.07, 95% CI 1.00–1.16), resulting in small differences among the groups and significant changes, but these were not considered to be biologically relevant (Figure 4A). The analysis of the damaged...
cells post freeze-thawing (Figure 4B) showed for all groups a significant increase in the percentage of apoptotic and necrotic cells compared to fresh blastocysts (Figure 4A). Remarkable was the significantly higher percentage of damaged cells in surviving blastocysts of the C18:0 group (apoptotic and necrotic; 43.2 ± 20.3%) in comparison to C18:1 (26.0 ± 12.5%, ratio 2.27, 95% CI 2.08–2.56) and FAF SOF (26.5 ± 10.7%, ratio 2.17, 95% CI 1.96–2.38; Figure 4B).

3.5 Early Embryos Exposed to C18:1 Show Increased Cryosurvival of Blastocysts

The cryosurvival of day 8 IVP blastocysts, originating from the different FFA conditions during day 1–5 of culture, was investigated by scoring the re-inflation of the blastocoel after a 24 h revitalization culture period post-thawing. After freeze-thawing IVP blastocysts of the different groups demonstrated a striking difference in the cryosurvival rates. Blastocysts from the C18:0 group had a cryosurvival rate of only 17.6% (0–38.0%), which was significantly lower compared to embryos cultured in FAF SOF and C18:1 (OR 0.12 and 0.12, 95% CI 0.05–0.28 and 0.05–0.29, resp.; Figure 4G). Interestingly, day 8 blastocysts originating from the group exposed to FAF SOF and C18:1 showed a cryosurvival rate of on average 67.4% (54.6–75%) and 70.1% (33.3–100%), comparable to the cryosurvival rate of the in vivo derived frozen and thawed blastocysts (68.4%, Figure 4G). Furthermore, the stage of the blastocyst scored before freezing, non-expanded or expanded, had an effect on the cryosurvival rates. Expanded blastocysts cultured with C18:1 showed a significantly higher cryosurvival rate of 83.3% (66.7–100%), when compared to their non-expanded counterparts with a cryosurvival of 56.94% (33.3–100%; OR 5.431, 95% CI 2.06–31.53, Figure 4H). In the other groups, a similar trend was observed with the exception of blastocysts cultured in C18:0.

Taken together, C18:0 exposure during day 1–5 of IVP results in blastocysts with a low cryotolerance, demonstrated by a significantly lower cryosurvival after freeze/thawing and higher degree of cell deterioration in surviving blastocysts when compared to the other exposure groups. In contrast, embryos exposed during day 1–5 of IVP to C18:1 demonstrated cryosurvival rates comparable to those of in vivo embryos.

4 DISCUSSION

A major difference between the quality of in vivo and in vitro embryos is reflected in the significantly lower cryosurvival rates of IVP embryos (Sudano et al., 2014; Moore and Hasler 2017; Viana, 2020; Ferré et al., 2020). The current study investigated the effect

FIGURE 3 Lipid droplet number and size per blastocyst. Bovine embryos were exposed to standard SOF (A), control FAFSOF (B), 25 μM C18:0 (C) or 25 μM C18:1 (D) during day 1 until 5 of in vitro culture. Panel A–D shows representative images for the different groups with in blue DNA (Hoechst 33342) and in green lipid droplets (LD540), Bar = 50 μm. Lipid droplet number (E), size (F) and total lipid in μm² (G) are shown for blastocysts of each group. Different superscripts differ significantly. For runs and total number see Scheme 1 (in the Materials and Methods section).
of low concentrations of FFA during IVP on the cryosurvival, defined as reinflation of the blastocoel after a 24 h revitalization culture period post-thawing (Dinnyés et al., 1999; Sudano et al., 2011), of IVP blastocysts. To this end, bovine embryos were in vitro exposed to 25 μM saturated C18:0 or unsaturated C18:1 during the first 5 days of embryo culture, resembling the physiological period that the embryo resides in the oviduct. The absence of FFA during the first 5 days of development, as
reflected in the FAF SOF group, resulted in a tendency for a lower blastocyst rate on day 8 and demonstrates the importance of FFAs during this period of preimplantation development. Interestingly, blastocysts derived from the C18:1 group, with a significantly higher lipid content, showed a dramatically reduced cryosurvival rate comparable with in vivo production MOET embryos. The cryosurvival rate of both the C18:0 and FAF SOF group were significantly higher than those of the C18:1 and standard SOF group. Embryos exposed to C18:0 demonstrated not only a dramatically reduced cryosurvival rate in comparison to the other groups, but the blastocysts that survived the freezing and thawing procedure also showed a significantly higher percentage of damaged cells. The current data demonstrate that exposure of embryos to unsaturated C18:1 significantly improved the cryosurvival rate of blastocysts, in contrast to exposure to C18:0. Embryo culture in FAF SOF also improved cryosurvival, but resulted in a decreased developmental potential as shown by the significantly reduced blastocyst percentage. Our results indicate that the FFA condition to which in vitro embryos are exposed during the first 5 days of embryo culture can have a determining role in the outcome of cryopreservation.

In analogy to the finding in this manuscript, previous studies demonstrated a clear difference in the impact of saturated and unsaturated FFAs on oocyte developmental competence. The predominate saturated FFAs in blood and follicular fluid, palmitic (C16:0) and C18:0, had a dose-dependent negative impact on the developmental competence of maturing oocytes (Leroy et al., 2005; Aardema et al., 2011; Aardema et al., 2013). Interestingly, the dominating mono-unsaturated FFA in blood and follicular fluid, oleic acid (C18:1), was harmless for the oocyte and could even counteract the negative impact of saturated FFAs (Aardema et al., 2011; Aardema et al., 2013). Given our new data in this study one may speculate that a similar protective effect of C18:1 is involved for bovine IVP embryos during day 1–5 of culture resulting in the cryoresistance properties of day 8 blastocysts.

An important key to improve the quality of IVP embryos and thus cryosurvival outcomes appears to be hidden in the oviduct, based on the outcome of the current study. Former studies have also demonstrated that embryo quality and thus the options for successfull cryopreservation, were related to the post-fertilization conditions in which embryos reside during the early steps of development (Rizos et al., 2002; Lonergan 2007; Lonergan and Fair 2008; Gad et al., 2012). In contrast to the blastocyst yield itself, which appears to depend primarily on oocyte quality (Hendriksen et al., 2000; Lonergan et al., 2001; Dieleman et al., 2002; Rizos et al., 2002; Knijn et al., 2003; Tefsaye et al., 2007; Lonergan and Fair 2016). It is important to consider the potential impact of the addition of FFA on the genetic imprint of the embryo. The periconception period is a highly sensible period were epigenetic modifications can result in a lifelong impact on offspring, which was clearly demonstrated in the Dutch Hungerwinterstudies (Heijmans et al., 2008). In bovine, the relation between large offspring syndrome (LOS) and IVP embryos appears to be the most well-known example for an undesired epigenetic impact on offspring (Farin et al., 2006; Mangiavacchi et al., 2021). However, LOS and genetic aberrations in IVP embryos have primarily been related to embryo cultures with FCS. Embryo culture media without FCS, like we used in the current study, were demonstrated to result in embryos with a gene expression pattern closer to in vivo derived embryos (Niemann and Wrenzycki 2000; Heras et al., 2016). In the current study we exposed embryos to levels of FFA, complexed to albumin for a physiological presentation of the FFA, far below the FFA levels present in the oviduct. In an ex-vivo study of Jordaens et al. (Jordaens et al., 2017a), the total levels of FFA in the oviduct appeared to be comparable to the total FFA levels in blood. Based on the currently available information, we therefore expect that the low level of FFA used in the current study will have an impact on offspring like demonstrated for FCS. Nevertheless, the potential impact of FFA on the (epi)genetic imprint of the embryo certainly needs further attention in future studies.

In general, unsaturated fatty acids like C18:1 are shown to be less toxic on somatic cells due to their routing towards lipid droplets for storage and mitochondria for fatty acid breakdown (Cnop et al., 2001; Listenberger et al., 2001; Maedler et al., 2001; Listenberger et al., 2003; Maedler et al., 2003; Mishra and Simonson 2005). As mentioned above, in vitro exposure to C18:1 prevents the potentially toxic impact of saturated FFA on oocyte developmental competence and increased lipid uptake in cumulus cells after exposure of C18:1 to COCs (Aardema et al., 2011; Aardema et al., 2013; Aardema et al., 2017). Likewise the current study shows that blastocysts originating from the C18:1 group have a higher lipid content. Moreover, a shift towards a more unsaturated fatty acid content, like C18:1 and linoleic acid, in oocytes and embryos has been reported to be related to an improved quality of embryos (Kim et al., 2001; Abe et al., 2002; Haggarty et al., 2006). Therefore, the relationship between a higher lipid content in the C18:1 group and successfull cryopreservation is tempting. In particular, since a high lipid content in embryos, induced by the addition of FCS, has been linked to a reduced cryopreservation and even resulted in attempts to completely remove lipid from the embryo in order to improve cryosurvival rates (Nagashima et al., 1994; Nagashima et al., 1995; Abe et al., 2002; Hara et al., 2005; Pereira et al., 2007; Sudano et al., 2011; Abe et al., 2017). Nevertheless, our current data clearly show that although an increased lipid content of embryos exposed to C18:1 was present, the blastocysts demonstrated significantly improved cryosurvival, i.e., comparable to the percentages of in vivo developed blastocysts.

In sharp contrast to the observed effect of 25 μM C18:1 exposure during the first 5 days of IVP on cryosurvival, C18:0 exposure resulted in a very low cryosurvival, and dramatically increased numbers of damaged, apoptotic and necrotic, cells in the blastocysts that survived the freezing and thawing procedures. These contrasting outcomes of saturated and unsaturated FFA are in line with previous studies in maturing oocytes (Leroy et al., 2005; Aardema et al., 2011; Aardema et al., 2017). The higher level of damaged, apoptotic and necrotic, cells in blastocysts after exposure to C18:0 appears to hint in the direction of apoptotic pathways. Indeed, the distinct impact of saturated and unsaturated FFA on somatic cells has for a large extent been
attributed to the different routing of the fatty acids in cells. Saturated fatty acids appear to be directed towards apoptotic pathways via reactive oxygen species (ROS) and ceramide formation, and can induce mitochondrial damage and endoplasmatic reticulum (ER) stress (Listenstein et al., 2001; Mu et al., 2001; Leroy et al., 2005; Wu et al., 2012; Lolicato et al., 2015; Sutton-Mc Dowall et al., 2016; Aardema et al., 2017). Remarkably, in fresh (non-frozen) blastocysts of all groups the levels of damaged cells were low, indicating that the effect of the cryopreservation and subsequent thawing must have induced the high number of damaged cells in blastocysts from the C18:0 group. This finding indicates that exposure to C18:0 results in blastocysts that are less cryotolerant, which may ultimately result in increased embryonic death. This phenomenon certainly needs further investigation.

Besides, the toxic effects of FFA and their derived metabolites and the different incorporation into neutral lipids stored in lipid droplets of the embryo, FFA can also be incorporated into the phospholipids that reside in the membranes of the embryo. Like all biomembranes that are composed of a complex of phospholipid molecular species, gametes and embryos are also extremely sensitive to changes in temperatures (Polge 1977; Quinn 1985; Sieme et al., 2015). Lipid phase transitions have been depicted to play a major role in the cell damage that occurs in response to the chilling procedure during slow freezing, and controlling the lipid content is therefore a major step to improve the cryotolerance of embryos (Amstislavsky et al., 2019). A higher level of unsaturation and a shorter chain length of fatty acids, results in a reduced melting point and increases the fluidity and therefore flexibility of cell membranes. To this end, different fatty acids can have a very distinct effect on the cryotolerance of cells. The melting point of C18:0 is for example 69.6°C, and 63.1°C for acids can have a very distinct effect on the cryotolerance of cells.

The promising results that are achieved via the freezing is still routine in practice (Loutradi et al., 2008; Ferré et al., 2020). The promising results that are achieved via the method using a high amount of cryoprotectants which prevents ice crystal formation, while in the bovine species slow freezing is still routine in practice (Loutradi et al., 2008; Ferré et al., 2020). The promising results that are achieved via the vitrification process did not result in a wide use under farm conditions thus far, possibly due to the rather complicated warming procedure by an in-straw dilution (open-straw technique and potential spread of infections is not an option in bovine industry), which is required to remove the high levels of cryoprotectants before embryo transfer (Caamaño et al., 2015; Ferré et al., 2020).

To bridge the gap between in vivo and in vitro embryos, several attempts have been undertaken to mimic more closely the in vivo circumstances during in vitro embryo culture by introducing 2D and 3D culture systems with oviductal cells (Chen et al., 2017; Ferraz et al., 2017; Ferraz et al., 2018; Hamdi et al., 2019; Jordaens et al., 2020). Until now the exact lipid composition of the oviductal cells and fluid during early embryo development, which presumably has a major impact on embryo quality and thus the expected cryosurvival, has not been completely unraveled (Saint-Dizier et al., 2020). The oviductal fluid composition originates from both oviductal cell scresction (de novo synthesis) and blood, and includes several lipid categories; e.g., extracellular vesicles rich in phospholipids, lipoproteins, both high-density lipoproteins (HDL) and low-density (LDL) and thus cholesterol and triglycerides, and FFA (Belaz et al., 2016; Jordaens et al., 2017a; Banliat et al., 2019; Saint-Dizier et al., 2020). Apart from the total FFA levels present in the oviductal fluid that appear to reflect the total FFA composition from blood (Jordaens et al., 2017a) there is, as far as we know, no information on the composition of the lipids in the bovine ovudt.

In the current study, embryos were exposed to FFA concentrations 4–10 times below the levels that COCs are able to cope with (Leroy et al., 2005; Aardema et al., 2011; Aardema et al., 2013; Aardema et al., 2017). Embryos exposed to levels of 50 μM C18:0 showed a significant drop in blastocyst yield (data not shown), which suggests a high sensitivity of embryos for FFA in comparison to oocytes. This finding is in line with the results of a study in mice wherein a dramatic drop in blastocyst yield after exposing embryos to low concentrations of saturated C16:0 was demonstrated (Yousif et al., 2020). Moreover, Jordaens et al. showed that hardly any of the bovine embryos exposed to high levels of FFA, based on the metabolic stress levels in blood, developed into a blastocyst (respectively around 6 and 3%) in an insert culture system with or without the presence of oviductal cells (Jordaens et al., 2020). Interestingly, embryos appear to be more prone to the effects of FFA than the by cumulus cells enclosed oocytes, which can be explained by the protective role of cumulus cells that surround the oocyte during in vitro maturation, whereas embryos in conventional IVP systems lack potential protection and stabilization of the culture environment by oviductal cells. Future studies should also take into account the potential role that oviductal cells may have to protect the embryo against FFA exposure. Oviductal cells may control the exposure of metabolites to the embryo in vivo, in analogy with the protective and regulatory role of cumulus cells for the oocyte (Fatehi et al., 2005; Gilchrist et al., 2008; Dunning et al., 2012; Aardema et al., 2013; Lolicato et al., 2015; Aardema et al., 2017). Indeed, oviductal cells are capable to store lipids, when exposed at the apical site of the oviduct, and express the
enzyme stearoyl-CoA desaturase, like cumulus cells (Aardema et al., 2017; Jordaens et al., 2017b; Aardema et al., 2018). Oviductal cells may filter and regulate the exposure of embryos to fatty acids. Thus far, it is unclear how the here used concentrations of FFA relate to the physiological concentrations of individual FFA in the oviduct during the first 5 days post-ovulation. To the best of our knowledge, there is currently no information on the specific FFA composition in the oviduct. However, the proven distinct impact of saturated versus unsaturated FFA under IVP conditions strongly suggests that the molecular species composition and total levels of FFA in the oviduct must be regulated to allow optimal embryogenesis. Likely, the FFA composition in the oviduct has a major impact on early embryonic development and information on the oviductal FFA composition could be used to optimize in vitro embryo culture conditions. The importance of the oviduct for early embryo development has clearly been demonstrated in studies where presumed zygotes were partly in vivo cultured after IVM and IVF, which resulted in increased quality and cryosurvival rates of blastocysts (Havlíček et al., 2005; Havlíček et al., 2007; Havlíček et al., 2010; Besenfelder et al., 2012). To this end, the development of systems that integrate oviductal cells during embryo culture to approach more closely the in vivo conditions appears to be a vital move to further improve embryo quality and cryosurvival rates (Chen et al., 2017; Ferraz et al., 2017; Ferraz et al., 2018; Hamdi et al., 2019; Jordaens et al., 2020). However, the current study lacks information on the impact of the in vitro FFA conditions on pregnancy outcomes after embryo transfer and offspring. Future experiments should unravel the defined physiological oviductal FFA composition, via lipidomic analysis, to which early developing embryos are exposed and the specific role that oviductal cells may play in composing the lipid profile and environment for the embryo and follow up in vitro experiments to investigate their impact on embryo quality and cryosurvival.

This study shows that saturated and unsaturated FFA have a very distinct impact on embryo quality and the cryotolerance of IVP blastocysts. Saturated C18:0 resulted in a high level of damaged cells and a low cryosurvival of blastocysts. In contrast to the high cryosurvival of embryos exposed to unsaturated C18:1, comparable to in vivo derived blastocysts. Future studies should be designed to validate these results before they can become applicable in commerciak in vitro bovine embryo production systems.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, on request.

ETHICS STATEMENT

Ethical review and approval were waived for this study, as the bovine ovaries used for the current project were a rest product of the regular slaughter process in a commercial slaughterhouse.

AUTHOR CONTRIBUTIONS

Conceptualization, IB, PV, BG, and HA; methodology, IB, PV, LT AR, BG, and HA; validation, IB, PV, BG, and HA; formal analysis, IB and HA; statistical analysis IB, HA, and HV; investigation HA, IB, BG, and PV; resources, PV, BG, and HA; data curation, IB and HA; writing—original draft preparation, IB, PV, BG, and HA; writing—review and editing, IB, PV, LT HV, BG, and HA; visualization, IB and HA; supervision, PV, BG, and HA; project administration, IB and HA; funding acquisition, not applicable. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

Imaging of the embryos was performed at the Centre for Cellular Imaging of the faculty of Veterinary Medicine of Utrecht University (the Netherlands) under the supervision of R. Wubbolts and Ing. E. van’t Veld. The LD540 probe was produced and supplied as personal gift by Professor C. Thiele (Univ. of Bonn, Germany). We thank breeding company CRV for the generously provided frozen embryos.

REFERENCES

Aardema, H., Fontes, P. K., VosLAM, P., Gadella, B. M., Roelen, B. A. J., and Van Tol, H. T. A. (2018). Oviductal Cells Express Stearoyl-CoA Desaturase that Can Protect the Embryo against Saturated Fatty Acids. Anim. Reprod. 15, 579. doi:10.21451/1984-3143-ar2018-0063

Aardema, H., Lolicato, F., van de Lest, C. H. A., Brouwers, J. F., Vaandrager, A. B., van Tol, H. T. A., et al. (2013). Bovine Cumulus Cells Protect Maturng Oocytes from Increased Fatty Acid Levels by Massive Intracellular Lipid Storage. Biol. Reprod. 88, 164. doi:10.1095/biroleprod.112.106062

Aardema, H., van Tol, H. T. A., Wubbolts, R. W., Brouwers, J. F. H. M., Gadella, B. M., and Roelen, B. A. J. (2017). Stearoyl-CoA Desaturase Activity in Bovine Cumulus Cells Protects the Oocyte against Saturated Fatty Acid Stress. Biol. Reprod. 96, 982–992. doi:10.1095/biroleprod.116.146159

Aardema, H., Vos, P. L. A. M., Lolicato, F., Roelen, B. A. J., Knijn, H. M., Vaandrager, A. B., et al. (2011). Oleic Acid Prevents Detrimental Effects of Saturated Fatty Acids on Bovine Oocyte Developmental Competence. Biol. Reprod. 85, 62–69. doi:10.1095/biroleprod.110.088815

Abe, H., Yamashita, S., Satoh, T., and Hoshi, H. (2002). Accumulation of Cytoplasmic Lipid Droplets in Bovine Embryos and Cryotolerance of Embryos Developed in Different Culture Systems Using Serum-free or Serum-Containing media. Mol. Reprod. Dev. 61, 57–66. doi:10.1002/mrd.1131

Abe, T., Kawahara-Miki, R., Hara, T., Naguchi, T., Hayashi, T., Shirasuna, K., et al. (2017). Modification of Mitochondrial Function, Cytoplasmic Lipid Content and Cryosensitivity of Bovine Embryos by Resveratrol. J. Reprod. Dev. 63, 455–461. doi:10.1262/jrd.2016-182

Cumulus Cells Protects the Oocyte against Saturated Fatty Acid Stress.
Reticulum Stress in Cattle Cumulus Oocyte Complexes Alters Cell Metabolism and Developmental Competence. *Biol. Reprod.* 94, 23. doi:10.1095/biolreprod.115.131862

Tesfaye, D., Lonergan, P., Hoelker, M., Rings, F., Nganvongpanit, K., Havlicek, V., et al. (2007). Suppression of Connexin 43 and E-Cadherin Transcripts in *In Vitro* Derived Bovine Embryos Following Culture *In Vitro* or *In Vivo* in the Homologous Bovine Oviduct. *Mol. Reprod. Dev.* 74, 978–988. doi:10.1002/mrd.20678

Viana, J. (2020). 2019 Statistics of Embryo Production and Transfer in Domestic Farm Animals: Divergent Trends for IVD and IVP Embryos. *Embryo Technology Newsl.* 4, 7–26.

Wang, G., and Tsujii, H. (1998). Incorporation and Distribution of Both 3H-Palmitic and 14C-Oleic Acids in Lipids by Preimplantation Mouse Embryos. *J. Mamm. Ova. Res.* 15, 185–190. doi:10.1274/jmor.15.185

Wickham, H., François, R., Henry, L., and Müller, K. (2020). *A Grammar of Data Manipulation*. R Package Version 0.8 2020, 4.

Wickham, H. (2016). *Ggplot2: Elegant Graphics for Data Analysis*. New York: Springer-Verlag.

Wu, L. L., Russell, D. L., Norman, R. J., and Robker, R. L. (2012). Endoplasmic Reticulum (ER) Stress in Cumulus-Oocyte Complexes Impairs Pentraxin-3 Secretion, Mitochondrial Membrane Potential (ΔΨm), and Embryo Development. *Mol. Endocrinol.* 26, 562–573. doi:10.1210/me.2011-1362

Yousif, M. D., Calder, M. D., Du, J. T., Ruetz, K. N., Crocker, K., Urquhart, B. L., et al. (2020). Oleic Acid Counters Impaired Blastocyst Development Induced by Palmitic Acid during Mouse Preimplantation Development: Understanding Obesity-Related Declines in Fertility. *Reprod. Sci.* 27, 2038–2051. doi:10.1007/s43032-020-00223-5

Zeron, Y., Sklan, D., and Arav, A. (2002). Effect of Polyunsaturated Fatty Acid Supplementation on Biophysical Parameters and Chilling Sensitivity of Ewe Oocytes. *Mol. Reprod. Dev.* 61, 271–278. doi:10.1002/mrd.1156

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Aardema, Bertijn, van Tol, Rijneveld, Vernooij, Gadella and Vos. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.