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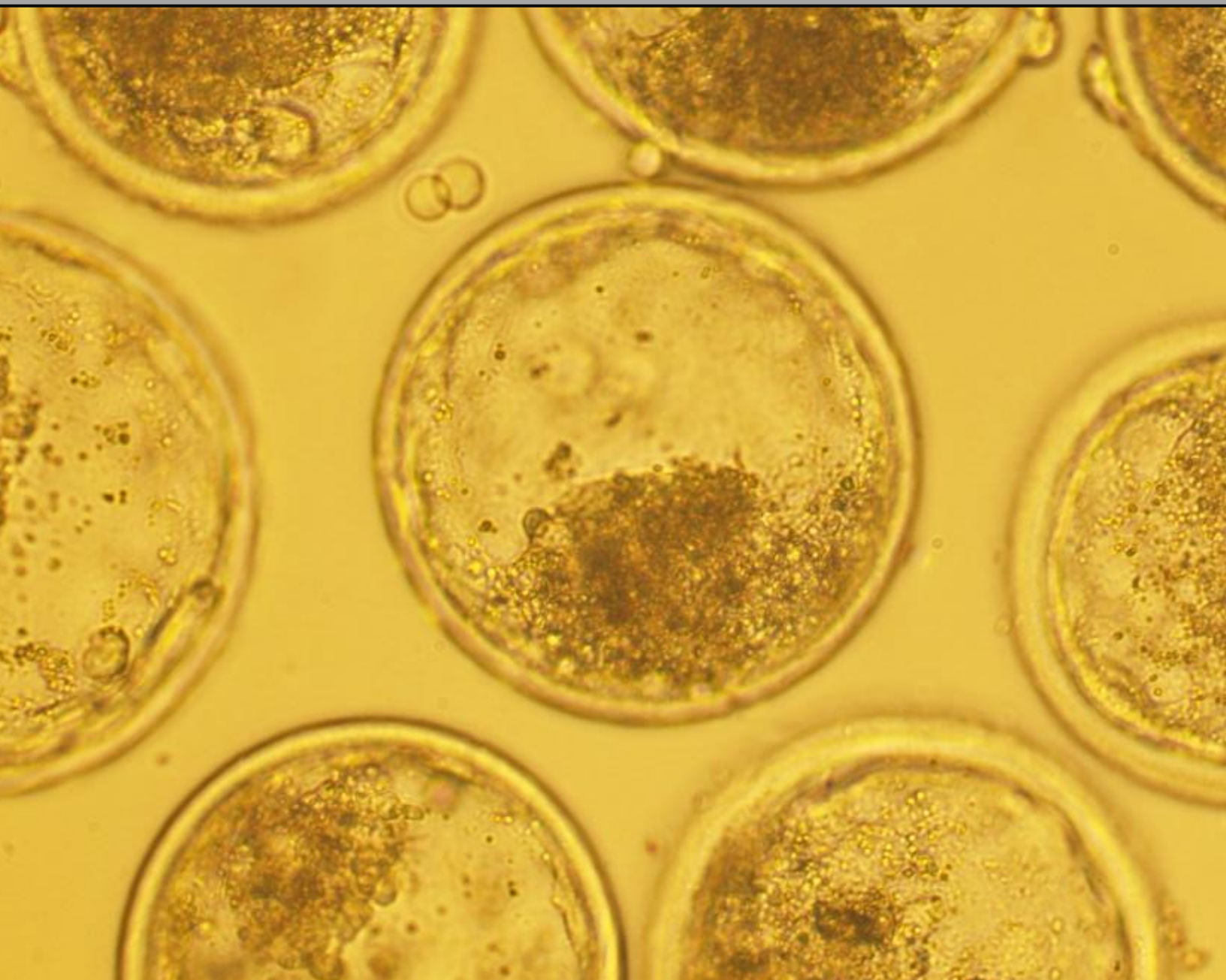

Lipid modifications to improve cryopreservation of bovine *in vitro* produced oocytes and embryos

Impact of enrichment of unsaturated fatty acids during *in vitro* maturation of bovine oocytes and *in vitro* culture of embryos on blastocyst formation, phospholipid composition and cryoresistance

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Abbreviations

BSA	bovine serum albumin
COCs	cumulus oocyte complexes
IVC	*in vitro* culture
IVM	*in vitro* maturation
IVP	*in vitro* produced
LA	linoleic acid
LAOA	linoleic acid and oleic acid
LF	lipid free
NaCl	natrium chloride
OA	oleic acid
OPU	ovum pickup
PA	phosphatidic acid
PBS	phosphate buffered saline
PBS-PVP	phosphate buffered saline with polyvinylpyrrolidone
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PS	phosphatidylserine
SD	standard deviation
SM	sphingomyelin
SOF	synthetical oviductal fluid

Abstract

The use of cryopreservation of *in vitro* produced (IVP) bovine embryos is limited because IVP embryos have a lower cryo-tolerance than in vivo embryos. In general the cryo-tolerance of cells is influenced by the lipid phase behavior: when the lipid gel phase is formed at a lower lipid phase transition temperature this will allow better cryo-survival. The lipid phase transition temperature can be influenced by adaptation of the fatty acids linked to the membrane phospholipids. The lipid phase transition temperature is lower when the fatty acids have a higher unsaturation level and a shorter chain length and when the double bonds have a more central position. This study aimed to increase the unsaturation level of phospholipids of IVP embryos by the addition of unsaturated fatty acids to *in vitro* maturation (IVM) and *in vitro* culture (IVC) media with the final goal to improve membrane properties and hence cryo-tolerance of IVP embryos. The study determined the effects of the addition of unsaturated fatty acids in IVM or IVC media influenced *in vitro* developmental competence, phospholipid acyl composition and cryo-survival properties of oocytes and blastocysts. Oleic acid (OA) and linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid; LA) were complexed to lipid free bovine serum albumin (BSA) and added to lipid free IVM or IVC media. In experiment 1, cumulus oocyte complexes (COCs) were exposed to either no fatty acids (control), 700 µM OA, 700 µM LA or a combination of 350 µM LA and 350 µM OA during the entire IVM period before routine embryo culture. In experiment 2, presumptive zygotes were exposed to either control BSA (final concentration of fatty acids 350 µM), ≥96% lipid free BSA, or BSA complexed with either 350 µM OA, 350 µM LA or a combination of 175 µM LA and 175 µM OA during the entire IVC period. The presence of LA, OA or a combination of LA and OA during IVM did not affect blastocysts rates. In contrast, the presence of LA during IVC, whether or not in combination with OA, resulted in significantly lower blastocyst rates. The largest drop in blastocyst rates was observed when IVC was performed with lipid free BSA. Lipid analysis was performed on oocytes and day 8 blastocysts of all experimental conditions. Interestingly, the overall effect of the addition of the unsaturated fatty acids LA, OA or a combination of LA and OA was a decrease in the number of double bonds in fatty acid complexed to phospholipids while no clear pattern in the changes in fatty acid chain length were observed in blastocysts. Therefore, the presence of LA, OA or a combination of LA and OA during IVM or IVC probably did not lower the lipid phase transition temperature of IVP blastocysts. Indeed, the cryo-survival rates after slow freezing of blastocysts exposed to OA or a combination of LA and OA during IVC were not affected. In oocytes, the results of this study indicate that maturation in presence of LA might improve the cryo-tolerance of oocytes due to a higher number of double bonds and a shorter chain length in phosphatidylcholine (PC).

Introduction

Assisted reproductive technologies are widely used in the bovine industry. These technologies include artificial insemination, superovulation, ovum pickup (OPU), *in vitro* embryo production, embryo cryopreservation and embryo transfer. Since the first embryo transfer pregnancy in 1949[1] and the first life born embryo transfer calf in 1951[2], embryo transfer techniques have improved, and by 2013, according to the International Embryo Technology Society, 986,983 embryos had been transferred globally. This number included 575,785 *in vivo* derived embryos, 393,625 OPU *in vitro* produced (IVP) embryos and 17,573 abattoir derived IVP embryos. The reported total annual embryo production is much higher: 1,275,874 embryos (729,246 *in vivo* embryos, 517,587 OPU IVP embryos and 29,041 abattoir derived IVP embryos).[3] As Figure 1 shows, between 1997 and 2013, globally *in vitro* embryo production has become increasingly important as a reproductive technology in bovine[3,4]

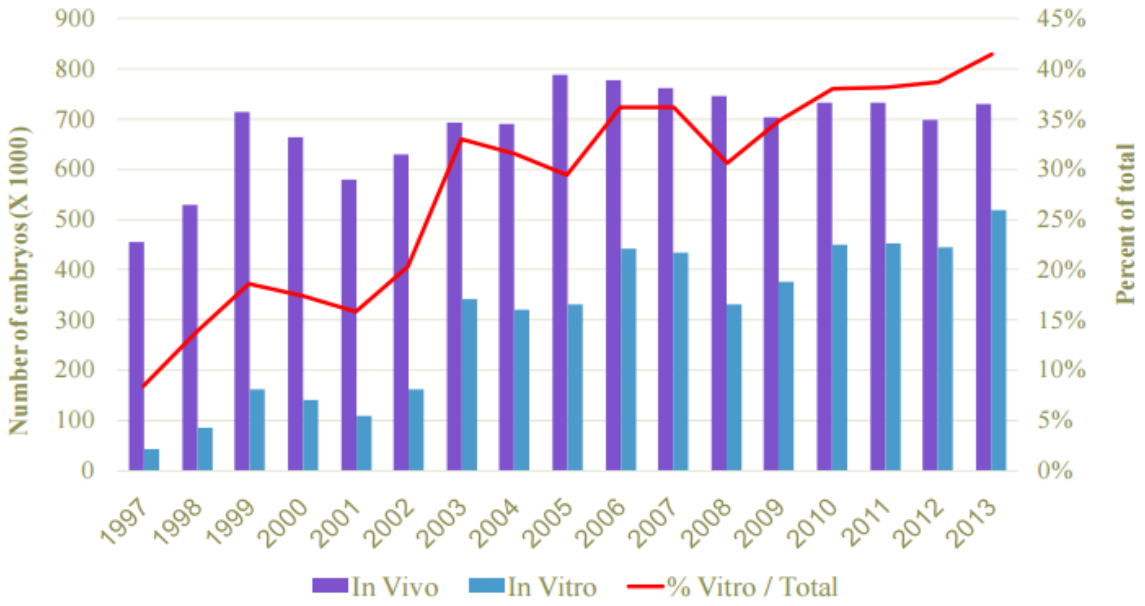

Figure 1 Worldwide *in vivo* and *in vitro* bovine embryo production between 1997 and 2013. From Blondin (2015).[4]

In the bovine industry, genomic selection became increasingly important in order to produce genetically superior cows. The genomic selection technology has significantly increased the demand of IVP embryos to accelerate genetic gain since *in vitro* production of embryos can increase the genetic improvement on a large scale[5]. The annual genetic improvement is determined by multiple factors: the genetic standard deviation, the generation interval, the selection intensity and the selection accuracy[6]. All of these factors can be positively affected by *in vitro* production. First, *in vitro* production can increase the genetic standard deviation, which depends on genetic variability. Each cow is born with hundreds of thousands oocytes in each ovary and thus carries a huge variability of genetic material[7]. However, almost all oocytes are lost during the lifetime of a cow due to atresia and as a consequence a huge loss of potentially superior

genetic material occurs. OPU in combination with *in vitro* production can reduce the loss of genetic material. Using the OPU technology, multiple immature oocytes can be retrieved from one genetically superior cow at the same point in time. These immature OPU oocytes can then be maturated, fertilized and cultured *in vitro* and the produced viable embryos can be transferred to recipient cows[8]. OPU combined with *in vitro* production therefore increases the number of embryos retrieved from genetically superior cows[9] and accelerates the genetic improvement[6]. Second, *in vitro* production can increase the genetic improvement by reducing the generation interval[5], the period between the birth of an animal and the birth of its offspring, when used in juvenile cows[8,10] and in pregnant cows during the first three months of pregnancy[8]. Finally, *in vitro* production can accelerate genetic gain by increasing selection intensity and accuracy[6,8]. *In vitro* production results in a higher selection intensity in cows because fewer donor cows can produce more offspring per time unit. It also results in a higher selection accuracy because of an increase in full and half sibling information[8].

Although the use of IVP embryos is attractive for genetic improvement[5] and the genetic selection technology, both immediate transfer and cryopreservation of IVP embryos have disadvantages compared to *in vivo* embryos. For immediate transfer, large recipient herds are necessary because recipient cows that are not in the exact right phase of the hormonal cycle do not possess a perceptive uterine environment. Management of large recipient herds is costly and time consuming. When cryopreservation is used, less management procedures are necessary. Unfortunately, the cryo-survival rates of IVP embryos are significantly lower than those of in vivo embryos[11-13]. The eventual conception rates of cryopreserved IVP embryos are considered to be too low for commercial use. This problem is reflected in the global embryo transfer numbers: only 9% of the transferred IVP embryos are cryopreserved before transfer, versus 60% of the transferred in vivo embryos[3].

Cryopreservation is a process in which cells are cooled to temperatures far below 0ºC and are stored for a long time without loss of viability. Currently, there are two common methods for embryo cryopreservation: slow freezing and vitrification.[14] The main differences between slow freezing and vitrification are the cooling rates and the level of cryo-protectant agents used.[14] Slow freezing uses a relatively low level of cryo-protectant with a concomitant low level of toxicity. The cells are exposed to cryo-protectants at room temperature when the cell metabolism is still active. Despite the active cell metabolism, the toxicity of the cryo-protectant agents is limited because of the low levels used.[14] After initial dehydration with the cryo-protectant agents, the temperature is lowered to -4^0C to -9^0C. In this temperature range, seeding is performed to induce controlled extracellular ice formation. The volume of the unfrozen fraction will decrease and the osmotic gradient will increase, which induces further water extraction from the cells.[14,15] At this point, the level of cryo-protectant agents is relatively high but the metabolism of the cells is minimal active thus the toxic effects are limited.[14] After seeding and equilibration, the temperature is lowered further at a slow cooling rate of 0.3ºC per minute[14,15]. This cooling rate is used until the temperature is lowered to -32^0C[14] and then the embryos are plunged into liquid nitrogen. If vitrification is used, embryos are plunged directly into liquid nitrogen because the used levels of cryo-protectants are higher than the

levels used for slow freezing. Embryos can only be exposed to high levels of cryo-protectants for a very limited period of time at room temperature.[14] During vitrification, high cooling rates prevent ice crystal formation and have to be followed by high warming rates, to prevent ice crystal formation during thawing. Thus both slow frozen and vitrified embryos are usually stored in liquid nitrogen thus at a temperature of -196°C.[14,15] This temperature lies below the glass transition temperature and therefore prevents glass formation during storage[15]. Furthermore, at this temperature, no biological changes take place, making the actual storage relatively safe[14]. Most of the chilling injuries do occur during cooling from body temperature to -196°C or during warming from -196°C to body temperature[14,15].

During the cooling, the temperature will be lowered below the freezing point of water and the formed ice crystals, because of their lower density, have a larger volume than the water. This increases the pressure on the cell and its membranes, which can cause chilling injuries. Because of that, ice crystal formation is unwanted during cryopreservation and several cryopreservation techniques are designed to limit ice crystal formation. The main preventive approach is to add membrane permeating cryo-protectants which can limit ice crystallization by forming hydrogen bonds with water molecules[14]. In addition to chilling injuries caused by ice crystals, a decrease in the volume of the unfrozen fraction can also lead to cell damage. When the volume of the unfrozen fraction decreases, the salt concentration and the osmotic pressure increases. A higher salt concentration can be toxic and even lethal for cells, and so membrane permeating cryo-protectants are employed to avoid high salt concentrations[14,16]. Differences in osmotic pressure can induce significant volume changes in cells due to movement of water and cryo-protectants and can result in membrane damage and osmotic shock. During thawing, differences in osmotic pressure can also result in chilling injuries. A rapid decrease in extracellular osmotic pressure occurs during the transformation of water from ice to liquid. Water enters the cell quicker than permeating cryo-protectants leave the cell and this phenomenon may cause the cells to swell or even rupture. To avoid this, non-permeating cryo-protectants like sucrose are added to the thawing solutions. Sucrose reduces the osmotic gradient between the inside and the outside of the cell because of its high molecular weight and thus reduces the water influx, cellular tremendous swelling and concomitant cell lysis.[14,15] The risk of tremendous swelling can also be minimized by choosing another cryo-protectant agent, for example replacing glycerol with more permeating ethylene glycol. Furthermore, the use of ethylene glycol enables direct transfers of cryopreserved embryos, eliminating the need for several washing steps after the initial thawing. Therefore, the contents of an embryo straw can be placed in the uterus of a recipient cow during embryo transfer directly after the initial thawing.[7]

The major goal of all cryopreservation techniques is avoiding the mentioned risks during cryopreservation[14]. However, these risks cannot be excluded completely and cryopreserved embryos do suffer from chilling injuries. The severity of chilling injuries is determined by different factors such as species, developmental stage of embryos, origin (*in vivo* or *in vitro*), the used cryo-protectants and the used cryopreservation method.[17] Attempts to improve cryopreservation can be classified broadly into two major approaches: adaptation of the cryopreservation techniques or adaptation of the cells itself.

This study examines the latter approach and focuses on the main site of chilling injuries: the cytoplasmic membrane and in particular its lipid content[17,18]. In vivo, oocytes and embryos are exposed to fatty acids during their development in follicular, oviductal and uterine fluid[19]. These fatty acids are actively taken up by oocytes from their environment and incorporated in phospholipids and neutral lipids[20-22]. In bovine, the four most abundant fatty acids in the follicular, oviductal and uterine fluid are palmitic, stearic, oleic (OA) and linoleic acid (LA).[19,23,24] Hence, these fatty acids are also the most abundant in bovine oocytes[19,20,25,26] and in cumulus cells (unpublished data from our group). Interestingly, in high quality oocytes, unsaturated fatty acids appear in greater quantities than in lower quality oocytes, in particular OA, LA and arachidonic acid[20,27]. In line with this, the results of the study of Aardema et al. (2011) suggest that a lower saturated/unsaturated fatty acid ratio in the oocyte relates to a higher developmental competence of maturating oocytes[22]. Furthermore, as several studies have shown, saturated fatty acids tend to have a negative effect on not only oocyte and embryo development but also embryo cryopreservation properties, while unsaturated fatty acids tend to have a positive effect[22,27-34]. This positive effect of unsaturated fatty acids on embryo cryopreservation may be explained by incorporation of those unsaturated fatty acids in phospholipids and neutral lipids[20-22]. Both the lipid composition and the amount of lipid have an impact on the cryo-tolerance of cells [17,35] and therefore adaptation of the lipid composition may improve cryo-survival properties of IVP embryos[20,21,36].

All mammalian cell membranes consist of lipid bilayers that mainly contain phospholipids. Each phospholipid has two fatty acid tails that can differ in length and in saturation level. The properties of the fatty acid tails are important for the resistance of a cell for cryopreservation. Cryopreservation can lead to modifications of het structure and the integrity of membranes, thereby causing membrane chilling injuries[17,36]. Membrane chilling injuries occur less often in more fluid membranes[21,37]. This observation suggests that increasing membrane fluidity may improve cryoresistance of cells. Membrane fluidity is influenced by the amount of cholesterol present in the membrane and the unsaturation degree of the fatty acid tails of the phospholipids[38,39]. In other words, membranes are more fluid when the unsaturation level of phospholipids is higher[40]. During cryopreservation, membranes transform from a liquid crystalline phase to a crystal gel phase. The temperature at which this transformation occurs is called the lipid phase transition temperature.[41] The lipid phase transition has been suggested to be predominantly due to changes in membrane phospholipids and not to changes in neutral lipids[36]. Oocytes exposed to the lipid phase transition temperature show more membrane damage than oocytes exposed to lower temperatures[36,42]. A beneficial effect of lowering the lipid phase temperature is expected since membrane damage occurs during the lipid phase transition temperature[41-43]. At a lower temperature, biological processes are slower and injuries will occur at a slower rate. With lower phase transition temperatures, cells will experience less cryodamage.[41] The lipid phase transition temperature can be lowered by alteration of the phospholipid composition; raising the unsaturation level[26,44-46], shortening the chain length and placing the double bonds central in the fatty acids (for a review see Quinn (1985)[44]). The hypothesis that a higher unsaturation level corresponds with a higher cryo-tolerance is supported with preliminary data from our group concerning the phospholipid composition of the more cryotolerant *in vivo* embryos

and the less cryotolerant IVP embryos. Lipid analysis of *in vivo* and IVP embryos showed that *in vivo* embryos contain more of the most abundant phospholipid class phosphatidylcholine (PC) and that the unsaturation level of PC is higher than in IVP embryos. This difference in unsaturation level may explain the differences in cryo-tolerance of these embryo types[11-13]. In short, adaptation of fatty acids linked to phospholipids seems to be a potential useful tool to improve IVP embryo cryoresistance. Adaptation of fatty acids linked to phospholipids may be accomplished by addition of fatty acids to *in vitro* maturation (IVM) or *in vitro* culture (IVC) media. Although multiple studies have reported positive effects of the addition of LA on oocyte and embryo cryopreservation[28,29,31,34], limited information about the effects of the addition of LA on oocyte and embryo phospholipid composition is available. These effects may explain the observed differences in cryo-tolerance and can offer new insights into treatments that could further improve cryo-tolerance. Hence, this study aimed to increase the unsaturation level of phospholipids of IVP embryos by the addition of unsaturated fatty acids to IVM or IVC media with the final goal to improve membrane properties and hence cryo-tolerance of IVP embryos. Therefore, the developmental competence and phospholipid composition of both IVP oocytes and embryos, under different fatty acid conditions with LA and OA was assessed. In addition, the phospholipid composition of blastocysts from different cultures was determined to assess the variability of phospholipids of blastocysts. Furthermore, the cryo-tolerance of IVP embryos cultured in presence of LA or a combination of LA and OA. Finally, an additional experiment was performed to assess the lipid raft distribution in bovine oocytes as a potential marker for cryoresistance.

Embedding in the faculty research program

This study is embedded in the 'Fertility and Reproduction' program of the faculty of Veterinary Medicine in Utrecht. The 'Fertility and Reproduction' program focusses on studying reproductive performance of individual animals and animal populations. One of the key objectives of the program is to improve techniques for germ cell preservation and this study meets this key objective with its focus on improving of the cryopreservation properties of bovine IVP embryos. This study uses biochemical knowledge to meet practical needs in the veterinary field: improving a widely employed technique, bovine embryo cryopreservation. This project was used to establish an working cryopreservation protocol for bovine embryos at the faculty that enables further research on bovine embryo cryopreservation.

Materials and methods

Reagents

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

Oocyte collection

Bovine ovaries were collected from a local abattoir and were transported at 30ºC to the laboratory within two hours. In the laboratory, the ovaries were washed once with tap water of 30^0C. Ovaries were collected in beakers containing 0.9% natrium chloride (NaCl; B. Braun Melsungen AG, Melsungen, Germany) and 0.1% penicillin-streptomycin (Gibco BRL, Life Technologies, Cergy Pontoise, France) at 30^0C. Follicular fluid was collected by aspiration of follicles ranging from a diameter of 2 to 8 mm with a winged infusion set (Vasuflo, Gelnhausen, Germany) to a vacuum suction system and a 50 ml plastic centrifuge tube (Greiner Bio-One B.V., Alphen aan den Rijn, The Netherlands). The tubes were left for at least ten minutes to achieve sedimentation of the follicular fluid. The sediment was then transferred to a Petri dish (Greiner Bio-One B.V.) and cumulus oocyte complexes (COCs) were selected under the microscope. Only COCs with a minimum of three layers of cumulus cells were used for the experiments.

In vitro maturation

Selected COCs were washed twice in HEPES Medium 199 (Thermo Fisher Scientific, Bremen, Germany) and once in maturation medium. The maturation medium consisted of LAL reagent water (Lonza, Walkersville, USA) supplemented with 9.5 g/l M199 with Earle's salts and glutamine (Thermo Fisher Scientific), 2.2 mg/ml $NaHCO_3$ (Merck, Darmstadt, Germany), 1% pencillin-streptomycin (Gibco BRL), 0.01U/ml follicle-stimulating hormone (Sioux Biochemical Inc., Sioux Center, IA, USA), 0.01U/ml luteinizing hormone (Sioux Biochemical Inc.), 7,72 ng/ml cysteamine and 10 ng/ml epidermal growth factor. For maturation, groups of 50-70 COCs were placed in 500 µl maturation medium in four-well culture plates (Thermo Fisher Scientific) with 1 ml sterile water in the middle of the plate. COCs were incubated for 22-24 hours under a humidified atmosphere of 5% CO_2 in air at 39^0C.

In vitro fertilization and *in vitro* embryo culture

After maturation groups of 50-70 COCs were fertilized according to the procedure described by Parrish et al. (1988)[47] with minor modifications[48]. In short, 50-70 COCs were placed in 430 µl fertilization medium (Fert-Talp[47]) and in addition 20 µl heparin (final concentration 10µg/ml in 0.9% NaCl), 20 µl PHE (containing 20 µM d-penicillamine, 10 µM hypotaurine and 1 µM epinephrine in 0.9% NaCl) and $1.0x10^6$ spermatozoa/ml were added. Fertilization lasted for 18-22 hours under a humidified atmosphere of 5% CO_2 in air at 39^0C. After fertilization, cumulus cells were removed from presumptive zygotes by vortexing for 3 minutes. The presumptive zygotes were placed in 500 µl synthetical oviductal fluid (SOF) supplemented with essential and nonessential amino acids and 0.1% (w/v) bovine serum albumin (BSA)[49]

under a humidified atmosphere of 7% O_2 and 5% CO_2 in air at 39^0C for 7 days. On day 5 post fertilization, the number of non-cleaved presumptive zygotes, 2-8 cell embryos and ≥8 cell embryos were scored and all cleaved embryos were transferred to fresh SOF medium. On day 7 and 8 the number of blastocysts was scored.

Embryo cryopreservation and thawing

For embryo cryopreservation, it was decided to test exclusively control blastocysts and blastocysts from conditions in experiment 2 that did not significantly reduce blastocyst rates during the first four cultures (± 532 COCs). Therefore, control blastocysts and blastocysts cultured in presence of OA or a combination of LA and OA were used. The embryo cryopreservation was performed with a slow freezing protocol. Day 7 and day 8 expanded blastocysts were washed five times in dishes containing EMCARE Holding Solution (Bodinco, Alkmaar, The Netherlands) with 20 ml/L 20% w/v albumin solution (Bodinco), two times in dishes containing 0.25% Trypsin phenol red (Thermo Fisher Scientific) and again five times in dishes containing EMCARE™ Holding solution (ICPbio Reproduction, Glenfield, New Zealand) with 20 ml/L 20% w/v albumin solution (ICPbio Reproduction). After the washing steps, embryos were immediately transferred to the cryopreservation medium ViGRO™ Ethylene Glycol Freeze Plus 1/01M sucrose (NIFA Technologies, Leeuwarden, The Netherlands). The embryos were loaded in 133 mm flexible straws (NIFA Technologies). The straws contained three columns of air and four columns of cryopreservation medium, with in the third and largest column two expanded blastocysts. The straws were closed with 48 mm plugging rods (NIFA Technologies). In 10-20 minutes after transferring the embryos into the cryopreservation medium, the straws were placed in a Kryo 10 series III planer (Cryotech Benelux, Schagen, The Netherlands) at -7°C. After 5 minutes, seeding was performed by touching the first column of the straw with a metal object that was held in liquid nitrogen. 10 minutes after seeding, the temperature was lowered at a rate of -0.5°C per minute. At -32°C, the straws were plunged into liquid nitrogen. Cryopreserved embryos were stored in liquid nitrogen for at least 4 days and for a maximum of 18 days. For thawing, straws were held in the air for 8 seconds and then plunged in water of 22°C for 20 seconds. Plugging rods were removed and straws were attached to a syringe. The other ends of the straws were cut and the different columns were blown out on a petridish (Greiner Bio-One B.V.). Embryos were transferred immediately transferred to the first washing medium. Five different washing steps were used: SOF mixed met respectively 80%, 60%, 40%, 20% and 0% ViGRO™ Ethylene Glycol Freeze Plus 1/01M sucrose. Each washing step was performed for five minutes. After the last washing step, the embryos were transferred to 500 µl SOF in groups of 8-11 embryos and incubated in a humidified atmosphere of 7% O_2 and 5% CO_2 in air at 39^0C. Re-expansion and hatching rates were assessed 24, 48 and 72 hours post-thawing.

Lipid extraction and analysis

For lipid analysis, denuded oocytes and day 8 blastocysts were used. Cumulus cells were removed of oocytes by vortexing for three minutes. Oocytes and embryos were washed three times in phosphate buffered saline (PBS) (B. Braun Melsungen AG) with 0,05% polyvinyl alcohol.1 ml inserts (Grace & Co., Columbia, Maryland, USA) were placed in 1.5 ml screw neck vials (Grace & Co.). 5 oocytes or embryos were placed in a minimal amount of fluid in one insert and screw neck vials were closed with short thread caps (Grace & Co.) and were stored at -20^{0}C. For lipid extraction, samples were thawed and inserts were transferred to Eppendorf tubes. 200 µL chloroform:methanol (2:1) solution (chloroform from Carl Roth GmbH & Co., Karlsruhe, Germany and methanol from Biosolve Chimie SARL, Dieuze, France) with internal referents was added to each insert. Per insert 10 pmol triacylglycerol 15:0/15:0 (Larodan Fine Chemicals, Malmö, Sweden) 5 pmol PC 14:0/14:0 (Avanti, Alabaster, AL, USA), 50 pmol sitosterol and 10 pmol palmitate [H7,7,8,8-^{2}H4] (Cambridge Isotope Laboratories, Andover, MA USA) were added as internal referents. Note that for phospholipid analysis, only PC 14:0/14:0 was used as internal referent. Samples were left on room temperature for 60 minutes and were centrifuged in an Eppendorf centrifuge for 5 minutes on full speed. The upper 95% of the supernatant was pipetted with a Pasteurs pipette to clean inserts in the original screw neck vials and lipid extracts were evaporated with N_2. Screw neck vials were filled with N_2, closed with screw neck vials and stored at -20^{0}C. For mass spectrometry, 100 µL chloroform:methanol solution (1:1) was added to the insert and 50 µL was transferred to a second insert. The first sample was used for phospholipid analysis and the second for neutral lipid analysis but since the lipid analysis expert did not have time to convert both the phospholipid and the neutral lipid data, data presented in this study is solely phospholipid data. For phospholipid analysis, lipid class separation was performed on a LTQ-XL mass spectrometer (Thermo Fisher Scientific) on a hydrophilic interaction liquid chromatography Kinetex 2.6µ 50x4.6 mm column (Phenomenex, Torrance, CA, USA). 25µl of each sample was injected with a CTC-PAL auto sampler and eluted with a gradient from mass spectrometry solvents (A) acetonitrile/acetone (9:1, Biosolve, Valkenswaard, The Netherlands) with 0.1% formic acid (Biosolve) and (B) acetonitrile/water (7:3, Biosolve) with 0.1% formic acid (Biosolve). The gradient was set at 100% solvent A at time of injection, followed by 4 minutes 50% solvent A with 50% solvent B and 7 minutes and 6 seconds 100% solvent B. The flow rate was set at 1 ml/min. The column outlet was connected to a heated electrospray ionization II (HESI-II) source (Thermo Fisher Scientific) and full scan spectra were collected. The chain length and the number double bonds of the fatty acid of the phospholipids phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS) and sphingomyelin (SM) were assessed.

Experiment 1 *in vitro* maturation in presence of unsaturated fatty acids

In experiment 1, COCs were exposed either (i) 0 µM fatty acids (control), (ii) 700 µM *cis*-9,*cis*-12-octadecadienoic acid (from now on referred to as linoleic acid (LA)) complexed to BSA, (iii) 700 µM oleic

acid (OA) complexed to BSA or (iv) a combination of 350 µM LA and 350 µM OA complexed to BSA during the entire maturation period (Figure 2). Levels of fatty acids were based on measurements of physiological levels of fatty acids in follicular fluid[50]. Fatty acids were complexed to ≥96% lipid free BSA that had undergone charcoal treatment according to the procedure described by Chen (1967)[51] to eliminate all fatty acids and most contaminating metabolic components[52]. Fatty acids were complexed to the lipid free BSA according to the procedure described by Aardema et al. (2011) with a fatty acid:BSA ratio of 5:1[22]. Maturation with or without fatty acids was followed by routine IVF and IVC. A total of 1,468 COCs was used to assess oocyte developmental competence. 364 COCs were exposed to 0 µM fatty acids (4 replicates), 356 COCs were exposed to LA (3 replicates), 404 COCs were exposed to OA (4 replicates) and 344 COCs were exposed to both LA and OA (3 replicates). For lipid analysis, a total of 230 denuded oocytes was used: 50 oocytes that had been exposed 0 µM fatty acids (10 replicates), 100 oocytes that had been exposed to LA (20 replicates), 25 oocytes that had been exposed to OA (5 replicates) and 55 oocytes that had been exposed to both LA and OA (11 replicates). Furthermore, a total of 200 blastocysts was used for lipid analysis: 60 blastocysts that were exposed to 0 µM fatty acids (12 replicates), 35 blastocysts that were exposed to LA (7 replicates), 60 blastocysts that were exposed to OA (12 replicates) and 45 blastocysts that were exposed to both LA and OA (9 replicates). Each replicate consisted of 89-133 oocytes.

Experiment 2 *in vitro* culture in presence of unsaturated fatty acids

For experiment 2, presumptive zygotes derived from IVM with 0 µM fatty acids and routine IVF procedure were used. For the control groups Probumin® BSA (Celliance Corporation, Norcross, GA, USA) was used (final concentration 280-560 µM fatty acids[53]). The Probumin® BSA was replaced in the experimental groups by the same amount of BSA, either (i) ≥96% lipid free BSA, (ii) 350 µM LA complexed to BSA, (iii) 350 µM OA complexed to BSA or (iv) 175 µM LA and 175 µM OA complexed to BSA during the entire IVC period (Figure 2). For this experiment, the fatty acids were complexed directly to ≥96% lipid free BSA. Fatty acids were added to a 16% lipid free BSA solution with a fatty acid:BSA ratio of 1.25:1. In total, 3,594 presumptive zygotes were used to assess embryo rates. 932 presumptive zygotes were exposed to control BSA (7 replicates), 380 presumptive zygotes were exposed to lipid free BSA (3 replicates), 538 presumptive zygotes were exposed to BSA with LA (4 replicates), 867 presumptive zygotes were exposed to BSA with OA (7 replicates) and 877 presumptive zygotes were exposed to both BSA with both LA and OA (7 replicates). For lipid analysis, a total of 220 blastocysts was used: 45 blastocysts that were exposed to 0 µM fatty acids (9 replicates), 40 blastocysts that were exposed to lipid free BSA (8 replicates), 45 blastocysts that were exposed to LA (9 replicates), 45 blastocysts that were exposed to OA (9 replicates) and 45 blastocysts that were exposed to both LA and OA (9 replicates). For testing the cryopreservation properties, a total of 182 blastocysts was used: 74 control blastocysts (8 replicates), 56 blastocysts that were cultured in presence of OA (6 replicates) and 52 blastocysts that were cultured in presence of both LA and OA (6 replicates). Each replicate consisted of 120-140 presumptive zygotes.

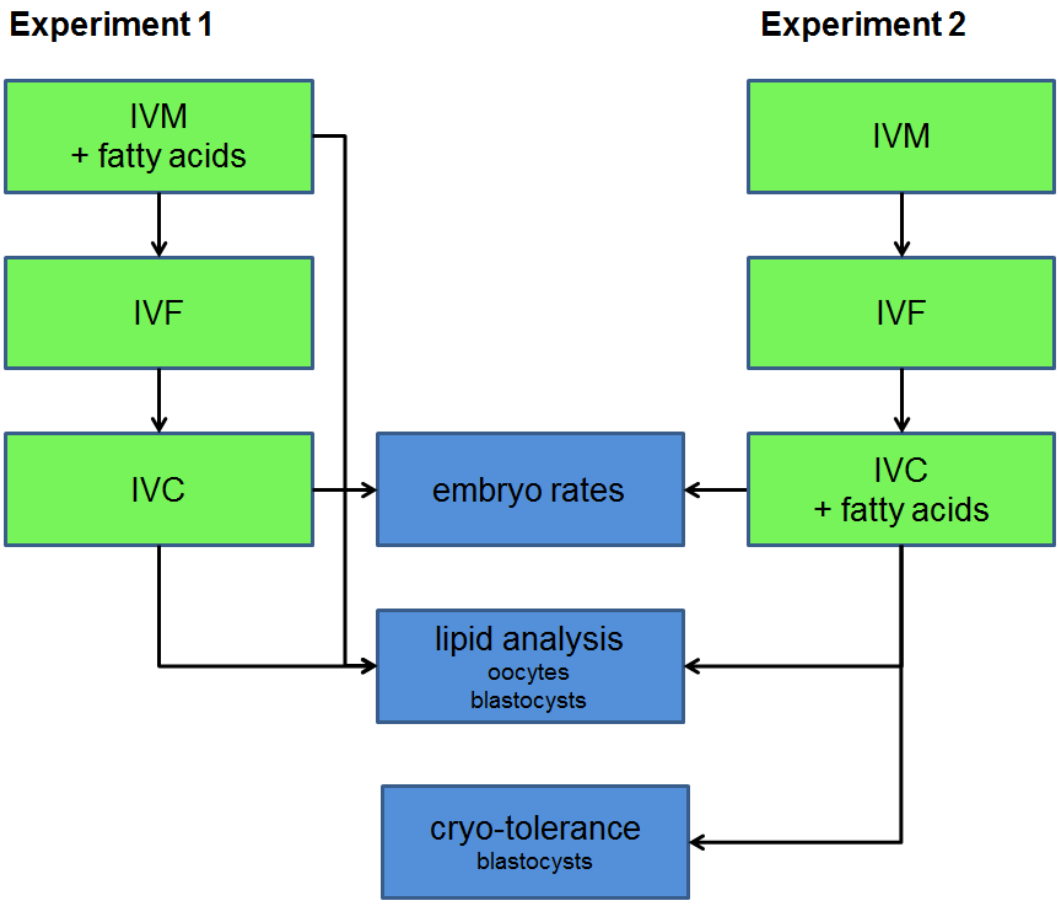

Figure 2 Experimental design of the study. In experiment 1, the effects of LA, OA or a combination of LA and OA during IVM on oocyte developmental competence and oocyte and blastocyst lipid composition were determined. In experiment 2, the effects of LA, OA or a combination of LA and OA during IVC on embryo developmental competence, blastocyst lipid composition and blastocyst cryo-tolerance (for OA or a combination of LA and OA) were determined.

Additional experiment immunofluorescence staining of lipid rafts in oocytes

For immunofluorescence staining oocytes maturated for 20-26 hours were selected. Cells were rinsed 3 times in PBS (B. Braun Melsungen AG) with 3 mg/ml polyvinylpyrrolidone (PBS-PVP), fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) in PBS for 30 minutes at room temperature and stored for a maximum of 5 days at 4^0C. Oocytes were washed 3 times in PBS-PVP, placed in 0.25% Triton X for 30 minutes and placed in 5% donkey serum in PBS-PVP for 1 hour. Incubation with the primary antibodies (1:50 dilution, see Table 1) took place in 5% donkey serum in PBS-PVP overnight in a humidified chamber at 4^0C. Oocytes were washed 3 times in PBS-PVP and incubated with the secondary antibodies (1:200 dilution, see Table 1) in 5% donkey serum in PBS-PVP for 1 hour. Again, oocytes were washed 3 times in PBS-PVP and were incubated with 5 mg/ml Hoechst for 20 minutes. Cells were mounted between a glass slide and a coverslip in 8 µl Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Confocal microscopy was performed by using a model TCS SPE-II setup (Leica Microsystems GmbH, Wetzlar, Germany) attached to an inverted semi-automated DMI4000 microscope (Leica Microsystems GmbH) with a 40x magnification oil immersion objective.

Table 1 Primary and secondary antibodies used for lipid raft staining in bovine oocytes.

Primary antibody	Manufacturer	Secondary antibody	Manufacturer
Flottilin-1 goat polyclonal IgG 200 µg/ml	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	Donkey anti goat Alexa 647	Jackson Immunoresearch Lab, West Grove, PA, USA
Caveolin-1 rabbit polyclonal IgG 200 µg/ml	Santa Cruz Biotechnology Inc.	Donkey anti rabbit Alexa 594	Thermo Fisher Scientific

Statistical analysis

Statistical analysis was performed using Rstudio statistical software (R Core Team (2015). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/). Statistical analysis was performed using general linear models and each group was compared to the other groups. All SD-values were based on the number of replicates. For the embryo and cryo-survival rates, a logistic regression was used. Used factors were number of culture, group and day. The interaction between different factors was also taken into account. For the lipid data, the phospholipids that could be annotated were analyzed. Statistical analysis was performed on the average chain length and number of double bonds for each sample per phospholipid class. Quantitative analysis could not be performed because the internal referent PC 14:0/14:0 was not detected in the samples. Instead of quantitative analysis, phospholipid classes as percentage of the total amount of annotated phospholipids were compared between the different experimental conditions.

Results

Experiment 1 *in vitro* maturation in presence of unsaturated fatty acids

Effects of unsaturated fatty acids during *in vitro* maturation on embryo development

In experiment 1, COCs were exposed to either 0 µM fatty acids, 700 µM LA, 700 µM OA or a combination of 350 µM LA and 350 µM OA[50] during IVM to assess the effects of unsaturated fatty acids during IVM on oocyte developmental competence. In all conditions, approximately 80% oocytes had cleaved on day 5 (P>0.062) and >50% of the oocytes reached a ≥ 8 cell stage on day 5. After correction for culture and interactions of experiments a minimal but significant positive effect on 8 cell stage 5 on day 5 was noted for addition of LA (P<0.025) and LA in combination with OA (P<0.009). The eventual blastocyst rates on day 7 and day 8 did not differ between the different conditions (Figure 3A, 3B; P>0.151).

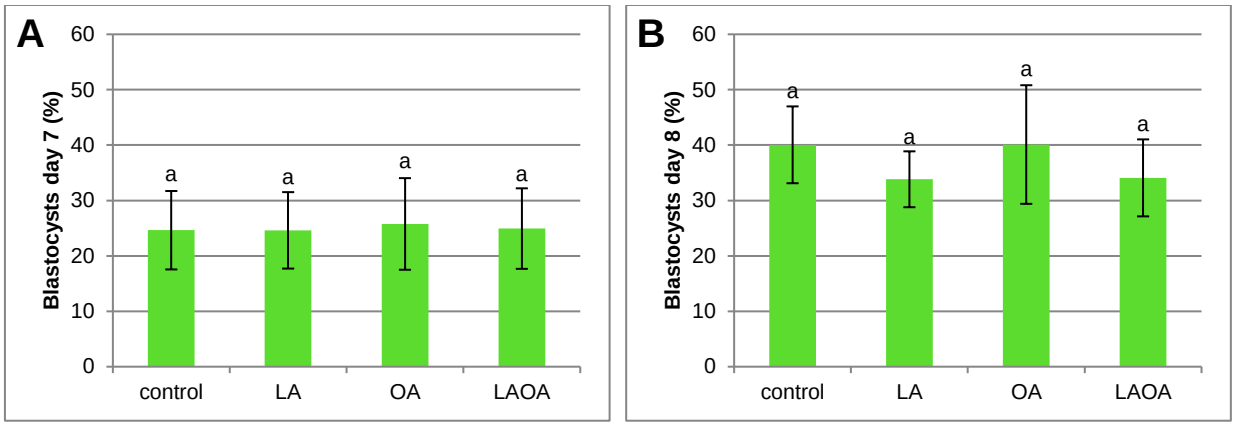

Figure 3 Effects of unsaturated fatty acids during *in vitro* maturation on embryo development. Percentage of blastocysts on day 7 (A) and percentage of blastocysts on day 8 (B) of oocytes exposed to either 0 µM fatty acids (control), 700 µM LA (LA), 700 µM OA (OA) or a combination of 350 µM LA and 350 µM OA (LAOA) during IVM (3-4 replicates per condition). Data are presented as mean ± SD. Note these graphs represent raw data that are not corrected for culture and interaction effects. Different characters indicate that mean values are significantly different (P<0.05) between the different groups.

Effects of unsaturated fatty acids during *in vitro* maturation on the unsaturation level of phospholipids

In oocytes, addition of unsaturated fatty acids to IVM media affected the number of double bonds in fatty acid tails of all phospholipid classes except PA (Table 2). The main effect was a reduction in the number of double bonds. LA reduced the number of double bonds in the least number of phospholipid classes: PI, PS and SM. Combining LA with OA did not only reduce the number of double bonds in PI, PS and SM but also in PE, while OA alone did not only reduce the number of double bonds in PI, PS and SM but also in PC. The number of double bonds increased only in PG for all three treatments and in PC for treatment with LA. In blastocysts derived from these oocytes, effects from the addition of LA, OA or a combination of LA and OA were still visible in the number of double bonds (Table 3). In the blastocysts, less differences in double bonds were observed than in oocytes but the observed differences followed the same pattern. In oocytes, each treatment affected the number of double bonds in at least five phospholipid class while in blastocysts, only treatment with OA affected more than one phospholipid class. Treatment with OA decreased the number of double bonds in PE, PI and PS and increased the number of double bonds in PC. Treatment with both LA and OA showed a decrease in the number of double bonds in only PS while treatment with LA alone increased the number of double bonds only in PC.

Table 2 Effects of unsaturated fatty acids during *in vitro* maturation on the unsaturation level of phospholipids in oocytes. Double bonds in fatty acids per phospholipid class in oocytes maturated in presence of 0 µM fatty acids (control), 700 µM LA (LA), 700 µM OA (OA) or 350 µM LA and 350 µM OA (LAOA).*

phospholipid class	control	LA	OA	LAOA
PA	1.24 ± 0.30^{a}	1.17 ± 0.09^{a}	1.23 ± 0.11^{a}	1.27 ± 0.15^{a}
PC	3.27 ± 0.09^{a}	3.41 ± 0.06^{b}	3.12 ± 0.03^{c}	3.30 ± 0.03^{a}
PE	3.07 ± 0.09^{a}	3.11 ± 0.06^{a}	2.88 ± 0.05^{b}	2.93 ± 0.09^{b}
PG	1.24 ± 0.09^{a}	1.32 ± 0.04^{b}	1.47 ± 0.02^{c}	1.39 ± 0.06^{d}
PI	3.60 ± 0.09^{a}	3.37 ± 0.04^{b}	2.79 ± 0.02^{c}	3.17 ± 0.06^{d}
PS	3.09 ± 0.06^{a}	2.81 ± 0.05^{b}	2.62 ± 0.08^{c}	2.65 ± 0.08^{c}
SM	3.11 ± 0.15^{a}	2.71 ± 0.06^{b}	2.72 ± 0.05^{b}	2.60 ± 0.08^{b}

*5-20 replicates per condition. Data are presented as mean ± SD. Different characters indicate that mean values within a line are significantly different ($P<0.05$).

Table 3 Effects of unsaturated fatty acids during *in vitro* maturation on the unsaturation level of phospholipids in blastocysts. Double bonds in fatty acids per phospholipid class in blastocysts derived from oocytes maturated in presence of 0 µM fatty acids (control), 700 µM LA (LA), 700 µM OA (OA) or 350 µM LA and 350 µM OA (LAOA).*

phospholipid class	control	LA	OA	LAOA
PA	1.23 ± 0.23[a]	1.25 ± 0.09[a]	1.29 ± 0.12[a]	1.17 ± 0.06[a]
PC	3.09 ± 0.10[a]	3.22 ± 0.09[b]	3.02 ± 0.10[a]	3.09 ± 0.09[a]
PE	3.18 ± 0.09[ac]	3.26 ± 0.06[a]	3.02 ± 0.08[b]	3.14 ± 0.07[c]
PG	1.33 ± 0.09[a]	1.40 ± 0.03[ab]	1.44 ± 0.09[b]	1.40 ± 0.09[ab]
PI	2.96 ± 0.14[ac]	3.04 ± 0.18[a]	2.81 ± 0.18[b]	2.87 ± 0.10[bc]
PS	2.59 ± 0.09[a]	2.59 ± 0.11[a]	2.38 ± 0.15[b]	2.35 ± 0.11[b]
SM	3.21 ± 0.29[a]	3.11 ± 0.32[a]	3.03 ± 0.29[a]	3.15 ± 0.22[a]

*7-12 replicates per condition. Data are presented as mean ± SD. Different characters indicate that mean values within a line are significantly different (P<0.05).

Effects of unsaturated fatty acids during *in vitro* maturation on the fatty acid chain length of phospholipids

The fatty acid chain length of phospholipids in oocytes and blastocysts was affected by unsaturated fatty acids present in IVM media. In oocytes, each condition affected the fatty acid chain length in every phospholipid class (Table 4). Treatment with LA resulted in a longer chain length in PA and PE and in a shorter chain length in PC, PI and PS. Treatment with OA resulted in a longer chain length in PE and PG and in a shorter chain length in PI, PS and SM. Treatment with a combination of LA and OA showed a shorter chain length in PC and PG and a longer chain length in PI, PS and SM. Most of the induced differences in chain length in oocytes were not visible in blastocysts (Table 5) except for a longer chain length in PG and a shorter chain length in PI when respectively OA or a combination of LA and OA was used.

Table 4 Effects of unsaturated fatty acids during *in vitro* maturation on the fatty acid chain length of phospholipids in oocytes. Fatty acid chain length per phospholipid class in oocytes maturated in presence of 0 µM fatty acids (control), 700 µM LA (LA), 700 µM OA (OA) or 350 µM LA and 350 µM OA (LAOA).*

phospholipid class	control	LA	OA	LAOA
PA	33.18 ± 0.16^{a}	33.29 ± 0.12^{b}	33.18 ± 0.13^{ab}	33.25 ± 0.10^{ab}
PC	37.11 ± 0.19^{a}	36.79 ± 0.13^{b}	37.14 ± 0.16^{a}	37.14 ± 0.10^{a}
PE	38.68 ± 0.25^{a}	38.87 ± 0.12^{b}	38.85 ± 0.12^{b}	38.79 ± 0.09^{ab}
PG	34.88 ± 0.30^{a}	34.86 ± 0.13^{a}	35.15 ± 0.09^{b}	35.11 ± 0.07^{b}
PI	37.79 ± 0.14^{a}	37.27 ± 0.08^{b}	37.26 ± 0.14^{b}	37.39 ± 0.10^{c}
PS	37.68 ± 0.14^{a}	37.43 ± 0.08^{b}	37.33 ± 0.12^{c}	37.27 ± 0.07^{c}
SM	38.88 ± 0.43^{a}	38.80 ± 0.16^{a}	38.43 ± 0.30^{b}	38.51 ± 0.23^{b}

*5-20 replicates per condition. Data are presented as mean ± SD. Different characters indicate that mean values within a line are significantly different (P<0.05).

Table 5 Effects of unsaturated fatty acids during *in vitro* maturation on the fatty acid chain length of phospholipids in blastocysts. Fatty acid chain length per phospholipid class in blastocysts derived from oocytes maturated in presence of 0 µM fatty acids (control), 700 µM LA (LA), 700 µM OA (OA) or 350 µM LA and 350 µM OA (LAOA).*

phospholipid class	control	LA	OA	LAOA
PA	33.31 ± 0.23^{a}	33.29 ± 0.09^{a}	33.24 ± 0.12^{a}	33.37 ± 0.06^{a}
PC	37.21 ± 0.26^{ab}	37.00 ± 0.31^{a}	37.29 ± 0.28^{b}	37.26 ± 0.21^{ab}
PE	39.10 ± 0.11^{a}	39.13 ± 0.19^{a}	39.16 ± 0.42^{a}	39.04 ± 0.24^{a}
PG	35.25 ± 0.17^{a}	35.28 ± 0.07^{ab}	35.39 ± 0.10^{b}	35.36 ± 0.15^{ab}
PI	37.93 ± 0.31^{a}	37.71 ± 0.12^{ab}	37.74 ± 0.20^{ab}	37.68 ± 0.23^{b}
PS	37.47 ± 0.09^{a}	37.37 ± 0.09^{ab}	37.28 ± 0.15^{bc}	37.18 ± 0.13^{c}
SM	38.81 ± 0.35^{a}	38.82 ± 0.30^{a}	38.71 ± 0.38^{a}	38.75 ± 0.17^{a}

*7-12 replicates per condition. Data are presented as mean ± SD. Different characters indicate that mean values within a line are significantly different (P<0.05).

Effects of unsaturated fatty acids during *in vitro* maturation on phospholipid class distribution

Quantitative analysis of phospholipid classes was not possible and therefore data presented here are representing relative levels of each phospholipid class as percentage for the total of phospholipids. In oocytes, treatment with OA did not result in a shift in phospholipid classes except for SM, while treatment with LA or a combination of LA and OA resulted in a shift in almost all phospholipid classes but especially in PC (Table 6). In blastocysts derived from these oocytes, no shift was observed when oocytes were treated with LA or a combination of LA and OA (Table 7). In contrast, blastocysts derived from oocytes maturated in presence of OA a shift in the majority phospholipid classes was observed.

Table 6 Effects of unsaturated fatty acids during *in vitro* maturation on phospholipid class distribution in oocytes.
Percentage of phospholipid classes of total phospholipids of oocytes maturated in presence of 0 µM fatty acids (control), 700 µM LA (LA), 700 µM OA (OA) or 350 µM LA and 350 µM OA (LAOA) .*

phospholipid class	control	LA	OA	LAOA
PA	12.14 ± 5.68[a]	8.19 ± 2.04[b]	12.53 ± 1.75[a]	8.36 ± 2.20[b]
PC	14.18 ± 2.12[a]	27.55 ± 2.92[b]	14.05 ± 0.35[a]	21.10 ± 2.73[c]
PE	40.05 ± 3.64[a]	33.78 ± 1.79[b]	40.44 ± 1.28[a]	36.97 ± 1.63[c]
PG	2.00 ± 0.77[a]	0.99 ± 0.22[b]	1.59 ± 0.09[ac]	1.39 ± 0.28[c]
PI	18.33 ± 1.44[a]	17.07 ± 1.06[b]	17.39 ± 0.73[ab]	19.08 ± 1.16[a]
PS	11.80 ± 1.58[a]	10.73 ± 0.94[b]	12.07 ± 1.14[a]	11.22 ± 1.26[ab]
SM	1.49 ± 0.26[a]	1.69 ± 0.18[b]	1.94 ± 0.43[c]	1.89 ± 0.23[c]

*5-20 replicates per condition. Data are presented as mean percentage ± SD. Different characters indicate that mean values within a line are significantly different (P<0.05).

Table 7 Effects of unsaturated fatty acids during *in vitro* maturation on phospholipid class distribution in blastocysts.
Percentage of phospholipid classes of total phospholipids of blastocysts derived from oocytes maturated in presence of 0 µM fatty acids (control), 700 µM LA (LA), 700 µM OA (OA) or 350 µM LA and 350 µM OA (LAOA).*

phospholipid class	control	LA	OA	LAOA
PA	24.51 ± 4.02[a]	24.55 ± 3.58[a]	20.28 ± 3.95[b]	27.09 ± 2.71[a]
PC	11.92 ± 1.63[ab]	12.71 ± 0.98[a]	11.16 ± 1.13[b]	11.43 ± 1.42[ab]
PE	34.72 ± 2.76[a]	35.62 ± 1.99[a]	38.97 ± 3.05[b]	34.47 ± 1.59[a]
PG	2.01 ± 0.34[a]	1.71 ± 0.31[a]	2.38 ± 0.55[b]	2.01 ± 0.18[a]
PI	13.43 ± 1.82[a]	12.76 ± 1.22[a]	14.14 ± 1.35[a]	12.78 ± 1.96[a]
PS	11.91 ± 1.58[a]	11.27 ± 1.12[a]	11.68 ± 1.69[a]	10.74 ± 1.71[a]
SM	1.51 ± 0.37[a]	1.38 ± 0.29[a]	1.40 ± 0.30[a]	1.47 ± 0.12[a]

*7-12 replicates per condition. Data are presented as mean percentage ± SD. Different characters indicate that mean values within a line are significantly different (P<0.05).

Changes in phospholipid composition during development of mature oocytes into blastocysts

The phospholipid composition of mature oocytes was compared to the phospholipid composition of blastocysts derived from these oocytes to assess the effect of early embryo development on phospholipid composition. A comparison between oocytes maturated in the presence of 0 µM fatty acids, 700 µM LA, 700 µM OA or 350 µM LA and 350 µM OA and blastocysts derived from oocytes maturated under these conditions was made (Table 8 and 9). In all conditions, differences between the fatty acid chain length and double bonds in fatty acids per phospholipid class were observed between oocytes and blastocysts. Overall, a longer fatty acid chain length was observed in the majority of the phospholipid classes after oocyte development into blastocysts. As regards to the double bonds, the effects of early embryo

development seem to depend on the phospholipid class. Overall, an increase in the number of double bonds was observed in PE for all conditions, in PG for the control and the treatment with LA and in SM for treatment with LA or a combination of LA and OA. A decrease in the number of double bonds was observed in PC and PS under all conditions and in PI for the control condition and for treatment with LA or a combination of LA and OA.

Table 8 Changes in unsaturation level per phospholipid class during development of mature oocytes into blastocysts. Double bonds per phospholipid class in oocytes compared to blastocysts from experiment 1. Oocytes were maturated in presence of 0 µM fatty acids (control), 700 µM LA (LA), 700 µM OA (OA) or 350 µM LA and 350 µM OA (LAOA) and blastocysts were derived from oocytes maturated under the mentioned conditions.*

phospholipid class	control	LA	OA	LAOA
PA	=	=	=	=
PC	↓	↓	↓	↓
PE	↑	↑	↑	↑
PG	↑	↑	=	=
PI	↓	↓	=	↓
PS	↓	↓	↓	↓
SM	=	↑	=	↑

*5-20 replicates per condition. Data are presented as changes in the number of double bonds per phospholipid class (no significant difference (=), a significant increase (↑) or a significant decrease (↓) in the number of double bonds). Arrows indicate that values within a line are significantly different ($P<0.05$).

Table 9 Changes in fatty acid chain length per phospholipid class during development of mature oocytes into blastocysts. Fatty acid chain length per phospholipid class in oocytes compared to blastocysts from experiment 1. Oocytes were maturated in presence of 0 µM fatty acids (control), 700 µM LA (LA), 700 µM OA (OA) or 350 µM LA and 350 µM OA (LAOA) and blastocysts were derived from oocytes maturated under the mentioned conditions.*

phospholipid class	control	LA	OA	LAOA
PA	=	=	=	↑
PC	=	↑	=	=
PE	↑	↑	=	↑
PG	↑	↑	↑	↑
PI	=	↑	↑	↑
PS	↓	=	=	=
SM	=	=	=	↑

*5-20 replicates per condition. Data are presented as changes in fatty acid chain length per phospholipid class (no significant difference (=), a significant increase (↑) or a significant decrease (↓) in the fatty acid chain length). Arrows indicate that values within a line are significantly different ($P<0.05$).

Experiment 2 *in vitro* culture in presence of unsaturated fatty acids

Effects of unsaturated fatty acids during *in vitro* culture on embryo development

The effects of unsaturated fatty acids during IVC on embryo developmental competence were determined by exposing presumptive zygotes to either BSA extracted from blood (final concentration 350µM fatty acids[53]; control), ≥96% lipid free BSA (LF), BSA with 350 µM LA (LA), BSA with 350 µM OA (OA), or BSA with 175 µM LA and 175 µM OA (LAOA) respectively. None of the treatments affected the day 5 cleavage rate of approximately 80% (all P values >0.135). After correction for culture and interaction a minimal but significant increase was found for the percentage of oocytes that developed into ≥8 cell stage when OA ($P<0.004$) or a combination of LA and OA ($P<0.049$) was used. Day 7 blastocyst rates were lower for all treatment groups as compared to the control (Figure 4A) ($P<0.001$, $P<0.001$, $P<0.007$ and $P<0.002$ respectively). Day 8 blastocyst rates were lower for treatment with lipid free BSA, LA or a combination of LA and OA ($P<0.001$, $P<0.001$ and $P<0.017$ respectively) while treatment with OA showed no effect on day 8 blastocyst rates ($P>0.059$) (Figure 4B).

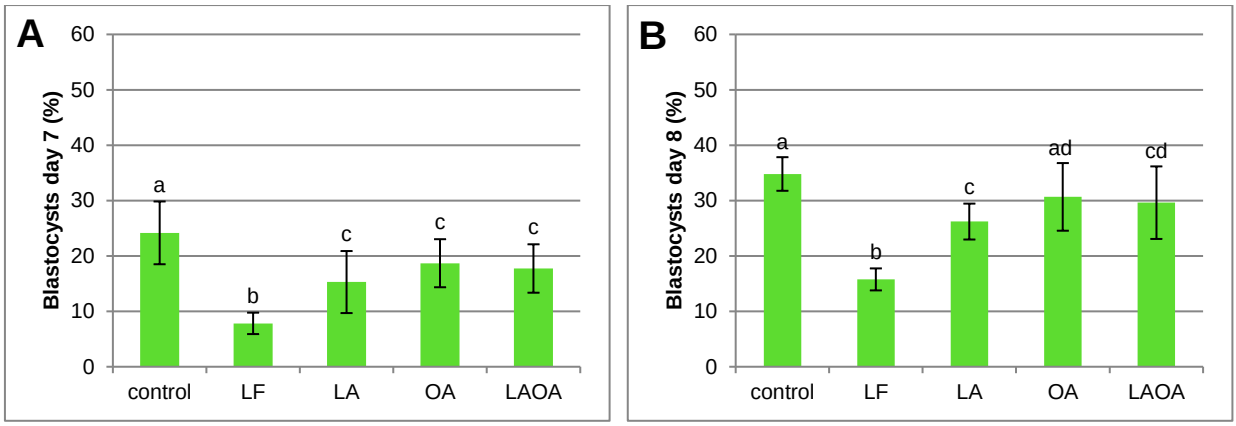

Figure 4 Effects of unsaturated fatty acids during *in vitro* culture on embryo development. Percentage of blastocysts on day 7 (A) and percentage of blastocysts on day 8 (B) of presumptive zygotes exposed to either control BSA (control), ≥96% lipid free BSA (LF), BSA with 350 µM LA (LA), BSA with 350 µM OA (OA) or 175 µM LA and 175 µM OA (LAOA) during IVC (3-7 replicates per condition). Data are presented as mean ± SD. Note these graphs represent raw data that are not corrected for culture and interaction effects. Different characters indicate that mean values are significantly different ($P<0.05$) between the different groups

Effects of unsaturated fatty acids during *in vitro* culture on the unsaturation level of phospholipids

The use of lipid free BSA, LA, OA or a combination of LA and OA during IVC increased the number of double bonds only in SM as compared to the control (Table 10). In the remaining phospholipid classes none of the conditions resulted in an increased number of double bonds. In PA, PC and PG, no effect on the number of double bonds was observed. In PE, PI and PS, the fatty acid conditions resulted in a decrease in the number of double bonds.

Table 10 Effects of unsaturated fatty acids during *in vitro* culture on the unsaturation level of phospholipids. Double bonds in fatty acids per phospholipid class in blastocysts derived from presumptive zygotes exposed to either control BSA (control), ≥96% lipid free BSA (LF), BSA with 350 µM LA (LA), BSA with 350 µM OA (OA) or 175 µM LA and 175 µM OA (LAOA) during IVC.*

phospholipid class	control	LF	LA	OA	LAOA
PA	1.71 ± 0.19^{ab}	1.88 ± 0.19^{a}	1.74 ± 0.29^{ab}	1.74 ± 0.08^{ab}	1.61 ± 0.32^{b}
PC	3.72 ± 0.28^{a}	3.65 ± 0.21^{a}	3.72 ± 0.37^{a}	3.66 ± 0.19^{a}	3.74 ± 0.47^{a}
PE	3.51 ± 0.12^{a}	3.51 ± 0.09^{a}	3.11 ± 0.42^{b}	3.18 ± 0.11^{b}	2.95 ± 0.42^{b}
PG	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
PI	3.03 ± 0.23^{a}	2.90 ± 0.08^{ab}	2.56 ± 0.31^{bc}	2.50 ± 0.08^{c}	2.41 ± 0.76^{c}
PS	2.19 ± 0.09^{a}	2.10 ± 0.10^{ab}	2.00 ± 0.11^{b}	1.87 ± 0.07^{c}	1.81 ± 0.20^{c}
SM	3.68 ± 0.21^{a}	3.83 ± 0.09^{b}	3.84 ± 0.11^{b}	3.78 ± 0.12^{ab}	3.85 ± 0.13^{b}

*8-9 replicates per condition. Data are presented as mean ± SD. Different characters indicate that mean values within a line are significantly different (P<0.05).

Effects of unsaturated fatty acids during *in vitro* culture on the fatty acid chain length of phospholipids

Fatty acid chain lengths of phospholipids of blastocysts were affected by the different treatments during IVC (Table 11). The lipid free BSA treatment resulted in a longer chain length in both PA and PC. For the other treatments, mainly a shorter chain length was observed. Treatment with LA resulted in a shorter chain length in PS. Treatment with OA resulted in a shorter chain length in both PS and SM, however, it resulted in a longer chain length in PG. Treatment with OA and LA shortened the chain length in the majority of the phospholipid classes: PA, PE, PS and SM.

Table 11 Effects of unsaturated fatty acids during *in vitro* culture on the fatty acid chain length of phospholipids. Fatty acid chain length per phospholipid class in blastocysts derived from presumptive zygotes exposed to either control BSA (control), ≥96% lipid free BSA (LF), BSA with 350 µM LA (LA), BSA with 350 µM OA (OA) or 175 µM LA and 175 µM OA (LAOA) during IVC.*

phospholipid class	control	LF	LA	OA	LAOA
PA	34.42 ± 0.13^{a}	34.69 ± 0.28^{b}	34.35 ± 0.16^{ac}	34.43 ± 0.19^{a}	34.22 ± 0.13^{c}
PC	33.49 ± 0.82^{a}	34.15 ± 0.71^{a}	33.74 ± 0.60^{a}	33.52 ± 0.52^{a}	33.50 ± 1.11^{a}
PE	36.96 ± 0.22^{a}	37.24 ± 0.07^{b}	36.78 ± 0.26^{ac}	36.91 ± 0.12^{a}	36.68 ± 0.33^{c}
PG	34.73 ± 0.12^{a}	34.75 ± 0.13^{a}	34.82 ± 0.18^{ab}	34.96 ± 0.17^{b}	34.77 ± 0.22^{a}
PI	37.50 ± 0.22^{a}	37.54 ± 0.22^{a}	37.18 ± 0.36^{a}	37.28 ± 0.05^{a}	37.44 ± 0.83^{a}
PS	37.13 ± 0.09^{a}	37.10 ± 0.09^{a}	36.83 ± 0.15^{b}	36.81 ± 0.13^{bc}	36.69 ± 0.22^{c}
SM	37.00 ± 0.53^{a}	36.79 ± 0.33^{ab}	36.55 ± 0.41^{ab}	36.54 ± 0.56^{b}	36.37 ± 0.49^{b}

*8-9 replicates per condition. Data are presented as mean ± SD. Different characters indicate that mean values within a line are significantly different (P<0.05).

Effects of unsaturated fatty acids during *in vitro* culture on phospholipid class distribution

The addition of LA or a combination of LA and OA resulted in shifts in more phospholipid classes than OA alone (Table 12). The shifts observed when LA or a combination of LA and OA was added, were predominantly due to an increase in the percentage of PC. The addition of OA affected only the percentage of PG, a decrease in the percentage of PG was observed. Furthermore, the addition of lipid free BSA resulted in shifts in the phospholipid classes PE, PG and PS, predominantly due to an increase in the percentage of PE and a decrease of the percentage of PS.

Table 12 Effects of unsaturated fatty acids during *in vitro* culture on phospholipid class distribution. Percentage of phospholipid classes of total phospholipids of blastocysts derived from presumptive zygotes exposed to either control BSA (control), ≥96% lipid free BSA (LF), BSA with 350 µM LA (LA), BSA with 350 µM OA (OA) or 175 µM LA and 175 µM OA (LAOA) during IVC.*

phospholipid class	control	LF	LA	OA	LAOA
PA	8.11 ± 3.69^{a}	7.26 ± 2.78^{a}	8.66 ± 2.46^{a}	8.19 ± 4.13^{a}	10.54 ± 6.74^{a}
PC	26.58 ± 2.49^{a}	26.51 ± 4.97^{a}	39.47 ± 8.30^{b}	28.00 ± 6.84^{a}	35.78 ± 12.56^{b}
PE	19.52 ± 2.61^{a}	28.15 ± 4.61^{b}	16.00 ± 2.31^{a}	18.61 ± 3.33^{a}	16.74 ± 7.05^{a}
PG	1.31 ± 0.51^{a}	1.78 ± 0.27^{b}	0.46 ± 0.11^{c}	0.82 ± 0.24^{d}	0.53 ± 0.24^{c}
PI	16.44 ± 1.57^{a}	16.43 ± 2.81^{a}	14.64 ± 4.31^{a}	18.31 ± 2.10^{a}	16.72 ± 10.66^{a}
PS	19.65 ± 2.77^{a}	12.59 ± 1.90^{b}	11.94 ± 3.67^{b}	17.12 ± 2.41^{a}	10.48 ± 3.41^{b}
SM	8.39 ± 5.28^{a}	7.29 ± 1.39^{a}	8.82 ± 1.68^{a}	8.96 ± 1.53^{a}	9.20 ± 2.36^{a}

*8-9 replicates of 5 blastocysts per condition. Data are presented as mean ± SD. Different characters indicate that mean values within a line are significantly different (P<0.05).

Effects of unsaturated fatty acids during *in vitro* culture on blastocyst cryo-survival

After thawing, the re-expansion and hatching rates of the cryopreserved day 7 and 8 blastocysts were assessed every 24 hours for three days (Figure 5A and 5B). Treatment with OA or a combination of LA and OA during IVC did not significantly affect re-expansion nor hatching rates at any time point analyzed (Table 13). Treatment with a combination of LA and OA tend to decrease the expansion rate after 48 and 72 hours as compared to the control but the tendency was not significant (P<0.100 and P<0.055 respectively). Furthermore, no differences were observed in the cryoresistance of Day 7 and Day 8 blastocysts (data not shown).

Table 13 Effects of unsaturated fatty acids during *in vitro* culture on blastocyst cryo-survival. Re-expansion and hatching rates of cryopreserved day 7 and 8 expanded blastocysts exposed to control BSA (control), OA (OA) or LA and OA (LAOA) during IVC, 24, 48 and 72 hours after thawing.*

	Re-expanded blastocysts			Hatched blastocysts			Total**
	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	
control	25 (33.8%[a])	27 (36.5%[a])	15 (20.3%[a])	3 (4.1%[a])	4 (5.4%[a])	2 (2.7%[a])	74
OA	21 (37.5%[a])	18 (32.1%[a])	10 (17.9%[a])	5 (8.9%[a])	3 (5.4%[a])	2 (3.6%[a])	56
LAOA	12 (23.1%[a])	12 (23.1%[a])	4 (7.7%[a])	2 (3.8%[a])	3 (5.8%[a])	1 (1.9%[a])	52

*6-8 replicates per condition. None of the values differed significantly (P<0.05) from the values from the other groups.

** Total number of embryos cryopreserved and thawed per condition.

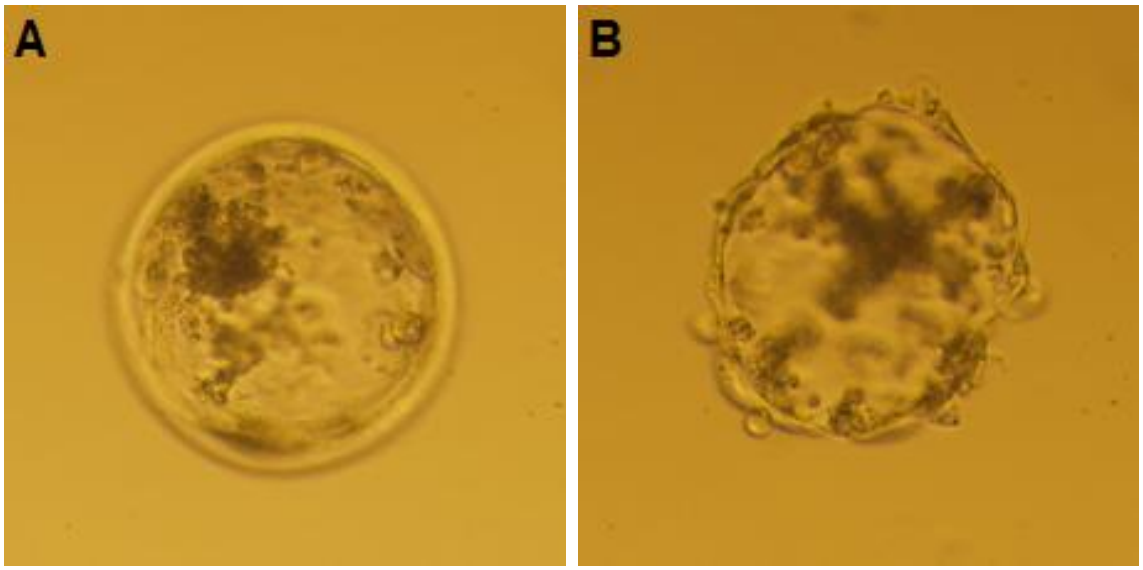

Figure 5 Re-expanded (A) and hatched (B) control blastocysts 72 hours after thawing.

Experiment 1 and 2 *in vitro* maturation or culture in presence of unsaturated fatty acids

Variability of blastocyst phospholipid composition

The variability of the phospholipid composition of blastocysts was assessed by a comparison between control blastocysts of experiment 1 and 2 (Table 14 and Table 15). The blastocysts for experiment 1 were produced in December and for experiment 2 in March (except the lipid free blastocysts were produced in June). The fatty acid chain length differed between the control blastocysts from the two experiments. Blastocysts from experiment 1 had a longer fatty acid chain length in six out of seven phospholipid classes than blastocysts from experiment 2, only in PC a shorter fatty acid chain length was observed. Furthermore, blastocysts from experiment 1 possessed a lower number of double bonds in PA, PC, PE and SM and a higher number of double bonds in PG and PS than blastocysts from experiment 2.

Table 14 Fatty acid chain length per phospholipid class in control blastocysts used in experiment 1 and 2.*

phospholipid class	experiment 1	experiment 2
PA	33.31 ± 0.23[a]	34.42 ± 0.13[b]
PC	37.21 ± 0.26[a]	33.49 ± 0.82[b]
PE	39.10 ± 0.11[a]	36.96 ± 0.22[b]
PG	35.25 ± 0.17[a]	34.73 ± 0.12[b]
PI	37.93 ± 0.31[a]	37.50 ± 0.22[b]
PS	37.47 ± 0.09[a]	37.13 ± 0.09[b]
SM	38.81 ± 0.35[a]	37.00 ± 0.53[b]

*7-12 replicates per condition. Data are presented as mean ± SD. Different characters indicate that mean values within a line are significantly different (P<0.05).

Table 15 Double bonds in fatty acids per phospholipid class in control blastocysts used in experiment 1 and 2.*

phospholipid class	experiment 1	experiment 2
PA	1.23 ± 0.23[a]	1.71 ± 0.19[b]
PC	3.09 ± 0.10[a]	3.72 ± 0.28[b]
PE	3.18 ± 0.09[a]	3.51 ± 0.12[b]
PG	1.33 ± 0.09[a]	1.00 ± 0.00[b]
PI	2.96 ± 0.14[a]	3.03 ± 0.23[a]
PS	2.59 ± 0.09[a]	2.19 ± 0.09[b]
SM	3.21 ± 0.29[a]	3.68 ± 0.21[b]

*7-12 replicates of 5 blastocysts per condition. Data are presented as mean ± SD. Different characters indicate that mean values within a line are significantly different (P<0.05).

Discussion

Embryo rates

This study aimed to increase the unsaturation level of phospholipids of IVP embryos by the addition of unsaturated fatty acids LA, OA or a combination of LA and OA to IVM or IVC media with the final goal to improve membrane properties and hence cryo-tolerance of IVP embryos. Firstly, the effects of unsaturated fatty acids complexed to BSA during IVM or IVC on blastocyst formation rates were tested. In this study, addition of LA, OA or a combination of LA and OA in a total physiological fatty acid concentration of 700 µM[50] during IVM did not affect blastocyst rates. Non-physiological concentrations of LA of 50 or 100 mM[50] did negatively affect blastocyst rates according to Marei et al. (2010)[54]. Aardema et al. (2011) tested lower concentrations of OA and found that 250 µM OA does not affect blastocyst rates while 500 µM OA increases blastocyst rates[22]. Therefore, the most optimal concentration of OA for embryo development probably lies in the range of 250 to 700 µM OA. Although in this study no increase in blastocyst rates was observed the described method is suitable to expose oocytes to unsaturated fatty acids during IVM.

Secondly, a comparable method for the addition of unsaturated fatty acids to IVC media was tested. In this method a total fatty acid concentration of 350 µM was used because BSA which is routinely used for embryo culture in our laboratory contains 280-560 µM fatty acids according to Peters (1985)[53]. The addition of OA did not affect day 8 blastocyst rates while addition of solely LA or a combination of LA and OA resulted in lower blastocyst rates. The negative effect of LA may be explained by the fact that LA is a precursor for arachidonic acid and thus for inflammatory mediators like prostaglandins and leukotrienes[55,56]. Interestingly, the negative effect of LA exposure seems to occur 5 days post fertilization because day 5 cleavage rates were not affected. This finding is consistent with results from a study of Tominaga et al. (2000) that reported no differences in blastocyst rates after exposing developing embryos to LA from day 1 or day 4 onwards[29]. However, in contrast to the current study, Tominaga et al. (2000) used fetal calf serum in the IVC medium[29] thus the embryos could have taken up both saturated and unsaturated fatty acids[20-22]. It is likely that the embryos took up the additional LA from the IVC media since the presence of LA positively affected the cryo-survival rates of 16-cell embryos[29].

Furthermore, it was tested if embryos could be produced *in vitro* with ≥96% essentially lipid free BSA with no additional fatty acids to check if the use of lipid free BSA alone had an impact on embryo developmental competence. Since a high intracellular lipid content is related to a lower cryo-tolerance[17,57,58], culture without fatty acids may improve the cryo-tolerance of oocytes and embryos. IVM was performed in lipid free medium and IVF and IVC were performed with ≥96% essentially lipid free BSA. Interestingly, this minimal amount of fatty acids present during *in vitro* production did not affect the day 5 cleavage rates but strongly decreased blastocyst formation rates on day 7 and 8. Future studies have to be performed to confirm if this method results in a decrease in intracellular lipid content (in this project, the required samples were collected and measured but not yet analyzed) and if as a consequence this

method results in a higher cryo-tolerance. Since a significant drop in blastocyst formation rates was observed, it is unlikely that this method will be used commercially although it may offer possibilities to freeze day 5 embryos.

Lipid analysis

For lipid analysis each collected sample was divided in two samples to enable classification of both phospholipids and neutral lipids. In this study, only data on phospholipids are presented. The phospholipid composition of embryos do play an important role in the cryoresistance properties of embryos[31,35]. Since an increase in the unsaturation level of phospholipids may improve the cryo-tolerance of embryos, the unsaturated fatty acids LA and OA were added to the IVM or IVC media. However, the main effect of the addition of LA, OA or the combination of LA and OA to both the IVM or IVC media was a decrease in the number of double bonds in phospholipids. For OA, the decrease in the number of double bonds can be explained by the higher number of double bonds found in control oocytes and embryos. Since in control oocytes and embryos, five out of seven phospholipid classes already possessed an average of more than two double bonds per fatty acid moiety, the extra incorporation of mono-unsaturated OA will not result in increased numbers of double bonds in these phospholipid classes. Since LA contains two double bonds, phospholipid molecules that have two LA tails possesses four double bonds. Although phospholipids with four or more double bonds were detected, the majority of the phospholipids have three double bonds or less which corresponds with data from literature[19,20,25,26]. Furthermore, in only one phospholipid class a 36:4 phospholipid has been detected that may correspond with a phospholipid with two LA tails although this phenomenon may also reflect alternative fatty acid moieties summing up to the same number of carbon atoms and double bonds. An example of this is a phospholipid that has a sn-1 palmitate (C16:0) and a sn-2 arachidonic acid (C20:4). Therefore, LA incorporation predominantly took place in one of the two fatty acids tails or LA was converted before incorporation by (de)saturation or by chain length elongation or truncation. Future experiments may provide a detailed insight in the process of incorporation of LA and OA in phospholipids by isolation of the phospholipid fraction and fatty acid hydrolysis. In conclusion, the addition of LA and OA did not result in an increase in the number of double bonds. Possible strategies to achieve an increase in the number of double bonds are either increasing the fatty acid concentration or using other (poly)unsaturated fatty acids. The fatty acids have to possess a higher number of double bonds than LA and OA to increase the number of double bonds normally present in the cells. Disadvantages of the use of polyunsaturated fatty acids are the susceptibility for reactive oxygen species[59] and the inflammatory mediators that can be formed of a number of polyunsaturated fatty acids[55,56].

Manipulation of phospholipid fatty acid chain lengths may also be a tool to improve cryoresistance of cells since fatty acids with a shorter chain length decrease the lipid phase transition temperature. However, overall fatty acids with shorter chains contain less double bonds than fatty acids with longer chains. Therefore, the drop in lipid phase temperature caused by the shorter fatty acid chains may be

counteracted by decreasing the number of double bonds[44]. Furthermore, since except for palmitic acid fatty acids that contain less than 18 carbon atoms are not abundant in oocytes[19,20,25], it was decided not to use the fatty acid chains with short chains in this study. Although the used fatty acids both consist of 18 carbon atoms, the addition of those fatty acids to IVM or IVC media affected significantly the length of the phospholipid fatty acid tails. However, a clear pattern in the changes in fatty acid chain length was observed exclusively in oocytes maturated in presence of LA, OA or a combination of LA and OA. Overall, *in vitro* maturation of oocytes in presence of LA, OA or a combination of LA and OA resulted in a shorter fatty acid chain length in phospholipids that possess more than 36 carbon atoms in fatty acid tails and in a longer fatty acid chain length in phospholipids that possess less than 36 carbon atoms. Future experiments have to provide evidence if shorter fatty acids are able to reduce the chain length of phospholipid fatty acid tails and as a consequence can increase cryo-tolerance of oocytes and embryos. Since the results of this study indicate that oocyte phospholipids are more strongly affected by addition of unsaturated fatty acids to media than blastocysts, the methods described in this study may be more suitable for improving oocyte cryopreservation than embryo cryopreservation. Especially the addition of LA to IVM media has been shown to be a potential powerful tool to improve oocyte cryoresistance by increasing the number of double bonds and shortening the chain length in fatty acids esterified to PC. PC is located mainly in the outer leaflet of the plasma membrane (for a review see Zachowski (1993)[60]) and is the most abundant phospholipid class. Therefore, the observed changes in the fatty acids esterified to PC are expected to have a stronger influence on the cryo-tolerance of cells than changes in fatty acids esterified to different phospholipid classes.

Shifts in phospholipid classes may also improve cryo-tolerance of IVP oocytes and embryos. Unpublished data from our group showed that the more cryo-tolerant *in vivo* embryos do contain more PC than the less cryo-tolerant IVP embryos. Therefore, an induced increase in the amount of PC may increase cryo-tolerance of IVP embryos. Interestingly, the addition of LA, whether or not in combination with OA, to IVM or IVC media resulted in a significant increase of the percentage of PC in respectively oocytes and blastocysts. This higher percentage of PC may explain the positive effects of LA on oocyte and embryo cryoresistance as observed in former studies[28,29,31,34].

In this study, the phospholipid composition of blastocysts from different cultures was determined to assess the variability of phospholipids of blastocysts. A study of Zeron et al. (2001) showed that the fatty acid composition of phospholipids of oocytes differs during the seasons. In summer (July – September), the oocytes contained a higher percentage of saturated fatty acids compared to in winter (December – February) where the oocytes contained higher percentages of unsaturated fatty acids.[26] In the current study, all oocytes and blastocysts used for lipid analysis were produced in December or March except for the lipid free blastocysts that were produced in June. Interestingly, control blastocysts from December and March showed a variety in fatty acid phospholipid composition in both chain length and unsaturation level. Control blastocysts from December had a lower number of double bonds in five of the seven phospholipid classes compared to control blastocysts from March. Furthermore, control blastocysts from December possess a longer chain length in six out of the seven phospholipid classes than control blastocysts from

March. This observation suggests that the phospholipid composition depends on the batches of oocytes that were collected and may be a influenced by season. Therefore, the lipid composition measured in March may not reflect the lipid composition of the blastocysts used for the cryopreservation experiments since those experiments were performed in May and June. Zeron et al. (2001) showed that for oocytes the lipid phase transition temperature was 6^0C lower in winter than in summer[26] which indicates that those oocytes are more cryoresistant in winter[36,42]. Interestingly, the cryoresistance of oocytes or blastocysts may be higher in March, since this study found an increased unsaturation level of blastocysts cultured in March compared to blastocysts cultured in December. Therefore, future studies should measure the phospholipid composition, lipid phase transition temperature and cryo-survival properties of oocytes and embryos all year round to determine the period during which oocytes and embryos have an improved cryoresistance.

For interpretation of the lipid analysis data in this study, it is essential to focus critically on the limitations of the used lipid analysis method. In other words, multiple limitations have been notified that concern the lipid analysis method. Firstly, not all measured phospholipids were able to be annotated and as a consequence the presented data presented are derived from the part of the data analyzed. This limitation is illustrated in an example concerning the PG molecules in the derived blastocysts of experiment 1 and 2 that were analyzed during two independent runs. In the blastocysts from experiment 1, PG 34:1, PG 36:1 and PG 36:2 were detected (individual data not shown). However, in the blastocysts from experiment 2, only PG 34:1 and PG 36:1 were detected: the number of double bonds per PG for each sample and each treatment group was exactly equal to 1.0. This example illustrates that the annotated phospholipids may not provide a fair and solid reflection of the total phospholipid class. This limitation is especially important in PC since a relatively large portion of PC could not be annotated (data not shown) due to the detection method on the mass spectrometer that was based only on detection of negative charged ions which is indirect for PC. Detection of positive charged PC ions is more straight forward and has a higher efficiency but has to be performed in a special high performance liquid chromatograph (HPLC) mass spectrometry run that was not performed in this study. Secondly, the results from a pilot experiment indicated that 10 pmol PC 14:0/14:0 per sample could be detected by mass spectrometry (data not shown) but that 5 pmol PC 14:0/14:0 would be better suitable for these data. Based on these findings, it was decided to use 5 pmol PC 14:0/14:0 for the next experiments. However, no PC 28:0 (likely corresponding with PC 14:0/14:0) was annotated in the samples worked up with 5 pmol PC 14:0/14:0. Unfortunately, given this experimental short coming it was not possible to produce an absolute quantitative analysis of the phospholipid data. Instead, a relative comparison was calculated between the different phospholipid classes. A limitation of this method is that percentages of phospholipids classes can strongly be influenced by an increase in the amount of a single phospholipids class.

Cryopreservation

At the beginning of this study, no operational embryo cryopreservation method for bovine embryos was available at our laboratory. Therefore, this study first focused on applying and testing a proven

cryopreservation protocol for bovine embryos. The used cryopreservation protocol resulted in re-expanded and hatched blastocysts after thawing. However, the re-expansion and hatching rates for control embryos were tremendously lower than rates reported in literature. Unfortunately, the development of thawed embryos had to be assessed *in vitro* because recipient cows could not be used due to the short time span of this project. Nowadays, the use of ethylene glycol has allowed direct transfer of cryopreserved embryos to recipient cows, thereby eliminating the need for multiple washing steps for thawing[7]. In the current study, it was decided to incorporate washing steps in the *in vitro* thawing protocol since the thawed embryos were placed in a small volume (500 µl) in which ethylene glycol was not diluted to the level that exists when the embryos are transferred directly in the uterus. Note that as a consequence by using these thawing steps the embryos were exposed to (decreasing concentrations of) ethylene glycol for approximately 20 minutes at room temperature. Although the levels of ethylene glycol were relatively low, the metabolism of the embryos is active at room temperature and therefore sensitive for the toxic effects of ethylene glycol during the whole thawing process[14]. The suboptimal thawing process may have lowered the cryo-survival rates significantly. Furthermore, the cryo-survival rates may have been influenced by the total length of culture in SOF medium. The cryopreserved day 7 and 8 blastocysts have been cultured in SOF medium for 6 or 7 days before cryopreservation and for an additional 3 days after cryopreservation. Although the used cryopreservation and thawing methods are not optimal for bovine embryo cryopreservation, a comparison between the different groups could still be analyzed. Blastocysts cultured in presence of both LA and OA tended to have lower re-expansion rates than control blastocysts or blastocysts cultured only in presence of OA. In a future study, the numbers of blastocysts have to be increased to confirm if re-expansion after thawing of cryopreserved IVP blastocysts is negatively affected by the presence of both LA and OA in the IVC media. In contrast, former studies reported a positive effect of the presence of LA in IVM or IVC media on cryo-survival rates[28,29,31,34]. The positive effect of LA is speculated to be related to induced changes in membrane permeability for water and cryo-protectants[34] and to induced changes in lipid composition[20,21,36]. In this study, LA in combination with OA did not result in an increase in the number of double bonds in fatty acid tails of phospholipids and did not improve blastocyst cryoresistance. Lipid analysis of embryos that are more cryotolerant due to culture in presence of LA[28,29,31,34] may clarify if the lipid composition can explain the differences in cryo-tolerance.

Conclusions

This study aimed to increase the unsaturation level of phospholipids of IVP embryos by the addition of unsaturated fatty acids LA and OA to IVM or IVC media with the final goal to improve membrane properties and hence cryo-tolerance of IVP embryos. To summarize, the presence of LA, OA or a combination of LA and OA during IVM did not affect blastocysts rates. The presence of OA during IVC did not affect blastocyst rates as well. In contrast, the presence of LA during IVC, whether or not in combination with OA, significantly reduced blastocyst rates. However, the largest drop in blastocyst rates was observed when IVC was performed with lipid free BSA. Lipid analysis of oocytes and blastocysts respectively maturated or cultured in presence of LA, OA or a combination of LA and OA showed that the

unsaturation level of phospholipids was lower than in control oocytes or blastocysts. The fatty acid chain length was also affected by the presence of LA, OA or a combination of LA and OA, although no clear pattern in the changes was discovered in blastocysts. The results from the lipid analysis indicate that presence of LA, OA or a combination of LA and OA probably did not lower the lipid phase transition temperature and did not increase cryo-tolerance of blastocysts. Indeed, the cryo-survival rates after slow freezing of blastocysts exposed to OA or a combination of LA and OA during IVC were not affected. The results from this study indicate that the cryo-tolerance may be improved by IVM or IVC in presence of fatty acids that possess more double bonds or have shorter chains than LA and OA. Finally, the results of this study indicate that oocytes maturated in presence of LA may have a higher cryoresistance due to a higher number of double bonds and a shorter chain length of fatty acids esterified to PC.

Future perspectives

In this study, all collected lipid samples were divided in two samples to enable both phospholipid and neutral lipid analysis. The results of the phospholipid analysis are presented in this study and the neutral lipid analysis will be performed in the upcoming months to determine the effects of unsaturated fatty acids during *in vitro* maturation or culture on intracellular lipid content. The phospholipid analysis should be repeated with a higher amount of internal referent to enable quantitative analysis of the phospholipids. Furthermore, the results of this study illustrate the importance of lipid analysis of oocytes and embryos all year round since the phospholipid composition of blastocysts varies during the seasons. Future studies have to determine the lipid composition of oocytes and embryos during the different seasons. Furthermore, the results of the phospholipid analysis in this study indicate that future studies should focus on fatty acids that possess more double bonds or have shorter fatty acid chain lengths than LA and OA to increase the cryo-tolerance of embryos. Finally, the cryopreservation properties of the blastocysts derived from *in vitro* maturation or culture in presence of unsaturated fatty acids should be determined in a laboratory that has established a working slow freezing or vitrification method. Since this study indicated that culture with ≥96% essentially lipid free BSA may offer possibilities to freeze day 5 embryos, the cryopreservation properties of day 5 embryos cultured with ≥96% essentially lipid free BSA should be compared to cryopreservation properties of control day 5 embryos.

Appendix

Additional experiment lipid raft distribution in oocytes as a potential marker for cryoresistance

Another approach to increase cryo-survival of cells is to stabilize the fluid lipid phase and limit chilling injuries by the incorporation of cholesterol into membranes[18]. Cholesterol is enriched in specific membrane micro domains that are called lipid rafts[61]. Lipid rafts are defined as small (10-200 nm), heterogenous sterol- and sphingolipid-enriched domains that compartmentalize cellular processes at the cell surface or within one organelle[62]. This study determined the lipid raft distribution in bovine oocytes which is the first step in establishing a method in which lipid rafts can be used as cryoresistance markers. The lipid raft distribution in bovine oocytes was studied with confocal images of immunofluorescence staining of flottilin-1 and caveolin-1. However, immunofluorescence images of flottilin-1 revealed a aspecific binding of flottilin-1 to the cumulus and oocytes (data not shown). Caveolin-1 seems to bind specific according to the immunofluorescence images and the specific binding was confirmed with a Western Blot (data not shown). Caveolin-1 organizes around the extruding polar body (Figure 6A and 6B) even before condensation of the polar body (Figure 6C) as is also described for Caenorhabditis elegans[63]. This phenomenon may play a key role in polar body extrusion by preventing fusion of the polar body with the oolemma. Since the polar body membrane contains many lipid rafts and is thus enriched in cholesterol, the fusion of the polar body membrane with another membrane is limited[64,65]. The organization of lipid rafts around the polar body may also play a role in preventing fusion of sperm cells with the polar body during fertilization. Furthermore, lipid rafts may also play a role in preventing cryodamage. Not only the meiotic spindle is very sensitive to cryodamage[66] but also the formation of meiotic spindles and polar bodies is negatively influenced by cooling[67]. Interestingly, cooling bovine oocytes to 0ºC and 4ºC causes division of MII spindles and formation of two identical spindles without a polar body[67]. Since the results of this study indicate that lipid rafts play a role in a polar body extrusion, the role of lipid rafts in (preventing) cryodamage should be studied.

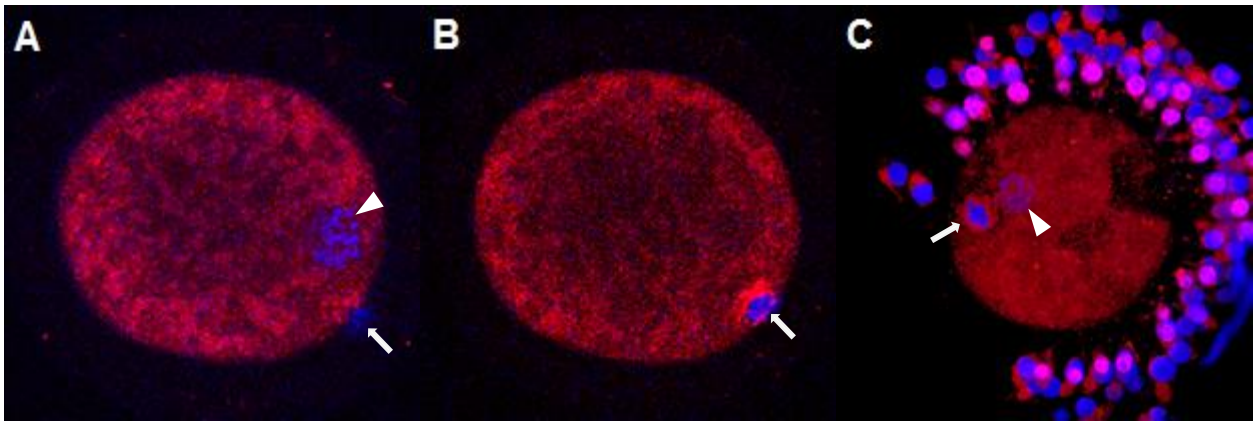

Figure 6 Lipid raft distribution in oocytes. Confocal images of an oocyte at metaphase II (A and B, two z-stacks of the same oocyte) and of an oocyte short before polar body condensation (C). Metaphase II plates are marked with arrow heads and polar bodies with arrows. Merges show lipid rafts in red and DNA in blue. Note the organization of caveolin-1 around the extruding polar body.

Additional observation: black appearance of embryos cultured in presence of unsaturated fatty acids

On day 7 and 8 of the fourth replicate of experiment 2, a small minority of the embryos of the experimental groups showed a black appearance (Figure 7A). Interestingly, also the black embryos could hatch (Figure 7B) and resulted in morphologically normal hatched blastocysts (Figure 7C) while empty zona pellucidas were black (Figure 7D). This indicates that the black apperance of the embryos was caused by something that had sticked on the outside of the embryo. The blastocyst rates in the experimental groups were lower than in the control group (data not shown), however the stadium of most black embryos in the experimental groups could not be determined and therefore blastocysts could have been missed in those groups. A possible explanation of the black appearance of the embryos is the presence of mold in the culture, as structures that could be hyphae were observed in the embryos (Figure 7A).

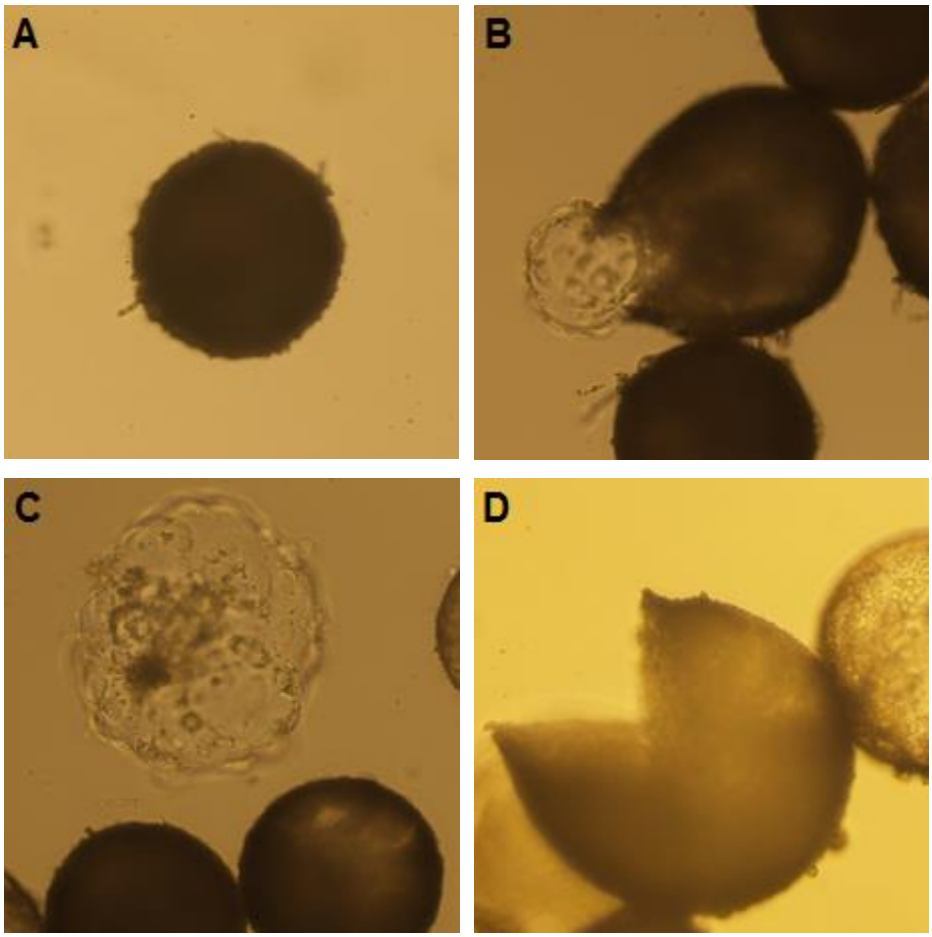

Figure 7 Black appearance of embryos cultured in presence of unsaturated fatty acids. Embryo (A), hatching blastocyst (B), hatched blastocyst (C) and empty zona pellucida (D) cultured in presence of unsaturated fatty acids. Note the black appearance of the embryos, which was observed in one out of eight cultures and only in the groups that were exposed to unsaturated fatty acids.

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Courses

Writing for academic publication

Provided by PhD Course Centre Graduate School of Life Sciences

07/03/2016 – 29/06/2016

Course description

This is a 10-session course during which you will write an article based on your own research, for submission to a peer-reviewed journal in your field. A grant proposal could also be the focus. This is a course on writing, not on 'English', and is as relevant for native English speakers as for those with English as a second or further language.

Aim of the course is:

• Planning and drafting your research article or conference paper;

• Writing and editing more purposefully, applying practical insights from genre research, psychology, reading (and writing) research, academic publishing and other areas;

• Finishing the paper, and submitting it to a conference or a peer-reviewed journal or (for proposals) a funding agency;

• Publishing (or receiving funding).

Personal experience

The course described above was adapted to fit the needs of the HP students but sometimes it was difficult to apply the structures discussed in the course in the HP report since the structures were designed for papers. I found the session in which we read each other's introduction very useful since this session provided hands-on advice on what I wrote. I think the session in which the editing method was introduced was also useful because it provided me insights on how to start editing. I wish I would have learned as much in the other sessions as in the two mentioned sessions but unfortunately I did not since the other sessions were less hands-on and did not provide much new information.

Giving effective oral presentations

Provided by PhD Course Centre Graduate School of Life Sciences

06/04/2016, 13/04/2016 and 20/04/2016

Course description

There is more to a successful presentation than having good slides and knowing your stuff. Effective presentations are also about communication, energy, confidence, knowing your audience. This intensive and hands-on course offers you the opportunity to get a fresh perspective on presenting, try out new techniques and experiment with ideas you may not have considered before.

Personal experience

I did not have experience in presenting in English and therefore I really appreciated the opportunity to gain some experience under the guidance of a professional. I liked that this course was really practical, we

practiced presenting a lot. I found it really interesting to try out new techniques for presentations and although not all techniques are applicable for scientific presentations, it gave me new insights in how to boost my presentations.

Introductory Statistics Course

Provided by the faculty of Veterinary Medicine

15/12/2015 and 17/12/2015

Course description

This course is designed as an introductory course for Modern Methods in Data Analysis (see later). During this two-day course, students are introduced to the use of R and R studio for statistical computing. In addition, statistical methods that are taught during the BSc Veterinary Medicine are reviewed.

Personal experience

The introduction to the use of R was very useful for me because I had never used R or R studio before and the course Modern Methods in Data Analysis required basic knowledge of R and R studio.

Modern Methods in Data Analysis

Provided by MSc Epidemiology, University of Utrecht, Utrecht, the Netherlands

04/01/2016 – 22/01/2016

Course description

This course provides statistical methods to study the association between (multiple) determinants and the occurrence of an outcome event. The course starts with an introduction to likelihood theory, using simple examples and a minimum of mathematics. Next, the most important regression models used in medical research are introduced. Topics are: maximum-likelihood methods, logistic regression, model validation and regression diagnostics, Poisson regression, and analysis of `event-history´ data, including an extensive discussion of the Cox proportional hazards regression model. Also, the basic principles of longitudinal data analysis are taught.

Personal experience

I found this course very useful because my knowledge about statistics was very limited. The only statistic courses I followed were courses provided in the regular program of the bachelor in Veterinary Medicine. Those courses did not focus on the use of statistical programs and therefore this course helped me a lot in gaining skills with statistical programs. Furthermore, new statistical methods were introduced and my understanding of several statistical methods was increased. I still found it difficult to decide which statistical method I should use for my own data but one of the teachers was willing to help me choose a statistical method. With the statistical skills I gained during this course I could do the statistics on my own data.

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