This item is the archived peer-reviewed author-version of:

Effect of nutritionally induced hyperlipidaemia on in vitro bovine embryo quality depends on the type of major fatty acid in the diet

Reference:

Marei Waleed, Arias Alvarez Maria, Van Hoeck Veerle, Gutierrez-Adan Alfonso, Bols Peter, Leroy Jo.- Effect of nutritionally induced hyperlipidaemia on in vitro bovine embryo quality depends on the type of major fatty acid in the diet
Reproduction, fertility and development - ISSN 1031-3613 - (2016), p. 1-33
Full text (Publishers DOI): <http://dx.doi.org/doi:10.1071/RD16297>

The effect of nutritionally-induced hyperlipidemia on *in vitro* bovine embryo quality depends on the type of the major fatty acid ~~type~~ in the diet

Waleed F.A. Marei,^{A,B,D} Maria Arias Alvarez,^A Veerle Van Hoeck,^A Alfonso Gutierrez-Adan,^C Peter E.J. Bols,^A and Jo L.M.R. Leroy,^A

^A Gamete Research Centre, Laboratory for Veterinary Physiology and Biochemistry, Department of Veterinary Sciences, University of Antwerp, Wilrijk, Belgium.

^B Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt.

^C Departamento de Reproduccion Animal, Instituto nacional de Investigacion y Tecnologia Agraria y Alimentaria (INIA), Madrid, Spain.

^D Corresponding author. Email: Waleed.Marei@uantwerpen.be

Running Title: Hyperlipidemia and embryo development

Abstract

We examined if the effects of dietary-induced hyperlipidemia on preimplantation embryo development depend on the predominant fatty acid (FA) type [in the diet](#). In a combined *in vivo- in vitro* bovine model, two groups of cows (n=6) received three consecutive diets (4-weeks each); 1) a maintenance control diet (CONT), 2) a high-starch diet rich in saturated fat (SAT), or 3) rich in omega-3 unsaturated fat (UNSAT). Two feeding sequences were used to test for carry-over effects; Group-A was fed CONT, SAT1 then UNSAT2, while Group-B received CONT, UNSAT1, then SAT2. Serum was collected after each dietary period, [analyzed and tested in bovine *in vitro* embryo culture](#). Introducing SAT and UNSAT diets induced hyperlipidemia (specifically, hypercholesterolemia and elevated free FAs) and reduced insulin sensitivity. Carry-over effects in serum metabolites and FA profile were dependent on the diet and feeding sequence. SAT1 and SAT2 serum decreased blastocyst rates, and ~~induced changes in~~ [altered blastocyst](#) mRNA expression related to apoptosis and oxidative stress. UNSAT1 and 2 serum resulted in normal embryo development and quality. ~~Some other~~ [Other](#) *in vitro* effects depended on the sequence of feeding. In conclusion, substitution of saturated fat with omega-3 fat in a high caloric diet induced [hyperlipidemia](#) ~~changes in~~ [with serum a](#) FA profile yielding similar rates and quality of blastocysts compared to normo-lipidemic controls.

Keywords: Western type diet, Hyperlipidemia, omega 3, palmitic acid, linolenic acid, embryo development,

1 Introduction

High caloric diets and obesity are associated with hyperlipidemia leading to higher risk of several metabolic disorders (Barnard *et al.* 1998; Panchal *et al.* 2011; Sanchez *et al.* 2015). This is often associated with reduced fertility as manifested by lower oocyte quality (Leary *et al.* 2015) and reduced success rates following assisted reproduction (Ferreira *et al.* 2010). Epidemiological studies indicate a 5% reduction in the chance of spontaneous conception in ovulatory women for each unit increase in body mass index (Van der Steeg *et al.* 2008). Nevertheless, obese patients have been shown to have normal implantation and pregnancy rates following oocyte donation (Styne-Gross *et al.* 2005) which may indicate that metabolic stress is imposed at earlier stages during oocyte maturation and preimplantation embryo development.

There is a fast growing scientific evidence indicating that elevated free fatty acid (FFA) concentrations in blood is a common feature and a key factor in the underlying pathogenesis of metabolic disorders associated with hyperlipidemia (Boden 2001; Zraika *et al.* 2002; Boden 2008). Hyperlipidemic conditions are reflected in the composition of the reproductive tissues and fluids, and thereby, directly impacting on the micro-environment of the maturing oocyte and growing embryo (reviewed in Leroy *et al.* 2011). In obese women, lipid composition of the follicular fluid is significantly higher in FFA concentrations; predominantly the saturated (SFA) stearic and palmitic acids (Valckx *et al.* 2014), which has been associated with poor quality cumulus oocyte complexes (COCs) and reduced cleavage rates (Jungheim *et al.* 2011). Similar effects have been described in animals, including cows, which have many similarities with human oocyte and early embryo physiology (Campbell *et al.* 2003). Deteriorated oocyte quality was reported in bovine oocytes collected during negative energy balance (NEB) (Leroy *et al.* 2004) or exposed to elevated SFAs during *in vitro* maturation (Van Hoeck *et al.* 2011) and also in mice after a maternal diet-induced obesity or a high fat diet (Igosheva *et al.* 2010; Luzzo *et al.* 2012).

The impact of maternal metabolic disorders on preimplantation embryo development is less defined and technically more difficult to study *in vivo* compared with the effects at the oocyte level. Development to the blastocyst stage was hampered when zygotes produced *in vitro* under normal conditions were transferred to the oviducts of cows under metabolic stress during NEB (Maillo *et al.* 2012). The latter indicates that the oviductal environment is also affected by the deviating maternal metabolism. In this context, recent work of Jordaens *et al.* (2015) showed that elevated SFA concentrations can alter different physiological functions of bovine oviductal epithelial cells *in vitro* and thus may lead to failure to support early embryo development.

Embryos are sensitive to the ~~FA profile~~ presence of lipids or free fatty acids in of their microenvironment (Hughes *et al.* 2011; Cagnone and Sirard 2014), and can internalize FAs, thus modifying their lipid content (Waterman and Wall 1988). This can directly affect embryo development (Haggarty *et al.* 2006). However, the biological effects of FAs are highly dependent on their chemical structure and degree of saturation (Artwohl *et al.* 2009). High SFA and omega-6 (ω -6) FA content and low intake of ω -3 FAs are typical in Western-type nutrition which contributes to the pathogenesis of the metabolic syndrome (Das 2010). The American Heart Association and the European Society of Cardiology have established targets to reduce dietary saturated fat intake (Lichtenstein *et al.* 2006; Perk *et al.* 2012). Since ω -3 polyunsaturated FAs (PUFAs) are known to have several health benefits (Ruxton *et al.* 2007), it was recommended to substitute saturated fats with unsaturated fats (particularly ω -3) in the diet (Kris-Etherton and Fleming 2015). Dietary ω -3 PUFAs reduce the risk for early preterm birth and prevent a number of pregnancy complications in women (Larque *et al.* 2012), and have been shown to improve pregnancy rates in many studies in dairy cows as reviewed by Santos *et al.* (2008). However, adverse effects of ω -3 FAs on embryo development to the blastocyst stage and embryo morphology were also reported in mice (Wakefield *et al.* 2008) and heifers (Petit *et al.* 2008).

With the complexity of interpreting results from *in vivo* experiments, it is not possible to dissociate direct FA effects on oocytes and embryos from other indirect influence of FAs on e.g. factors such as

prostaglandins (Abayasekara and Wathes 1999), progesterone (Hawkins *et al.* 1995) and growth factor concentrations (Mendoza *et al.* 2002; Fenwick *et al.* 2008). Furthermore it is difficult to speculate if the effects are exerted at the oocyte level or at the level of the embryo. While detrimental effects of elevated SFAs in the follicular fluid on oocyte quality are well characterized (Leroy *et al.* 2011), data on possible direct effect of nutritionally-induced hyperlipidemia on preimplantation embryo development are scarce. We have previously investigated the influence of hyperlipidemia on preimplantation embryo development using cows fed high-carbohydrate high-fat diet (based on palm oil), similar to a western-type diet (Leroy *et al.* 2010). Serum from these cows, which was higher in saturated and ω -6 FFAs, significantly reduced embryo development and quality when added *in vitro* during embryo culture compared to control serum (Leroy *et al.* 2010). The effect substitution of SFA with ω -3 rich fat within such high caloric diet on embryo development and quality have not been tested.

In the present study, we hypothesized that the impact of nutritionally-induced hyperlipidemia on preimplantation embryo development can differ according to the concomitant fatty acid profile. Specifically, in individuals receiving a high-fat high-carbohydrate diet, changing the type of the predominant FFA from saturated to ω -3 unsaturated can positively influence fertility by protecting or enhancing early embryo development, and *vice versa*. We also hypothesized that the feeding sequence of FFAs in the diet may affect embryo development and quality due to potential carry over effects. The aims of this study were thus: (i) to nutritionally induce hyperlipidemia on the bases of high carbohydrate diet rich in either saturated or polyunsaturated fat and in two different sequential feeding program in cows, (ii) to test the direct impact of the resulting different types of hyperlipidemic sera (collected at the end of saturated or polyunsaturated dietary period) on *in vitro* bovine early embryo development, embryo quality and embryonic gene expression patterns compared to normo-lipidemic serum collected from the same animals.

2 Material and Methods

2.1 Animals, *in vivo* experimental design and diets

All experiments have been approved by the Ethical Committee of the Faculty of Biomedical, Pharmaceutical and Veterinary Sciences, Antwerp University (Approval number 2009/16, approval date April 8, 2010). Six non-pregnant, non-lactating cows were used (496 ± 36.7 kg and 2.55 ± 1.3 y). The experimental design of the *in vivo* part of this study is illustrated in figure 1. After an acclimatization period of 6 weeks, cows were randomly divided into two groups (A and B, $n=3$ each) for different dietary sequence. The experimental feeding program was divided into three successive periods (4 weeks each). During the first 4-weeks, both groups of cows received a hay-based control diet (**CONT**) with low-carbohydrate and low-fat content providing ~~enough~~ energy requirements for maintenance (National Research Council (U.S.). Subcommittee on Dairy Cattle Nutrition. 2001). During the second dietary period, Group A ($n=3$) received a high caloric diet (high-carbohydrate and high fat content) based on pure vegetable palm fat which is rich in saturated fatty acids (**SAT**), while Group B ($n=3$) received a similar high caloric diet but replacing palm oil with linseed, which is rich in polyunsaturated omega-3 fatty acids (**UNSAT**). In the third period, group A was switched to the UNSAT diet while group B was fed the SAT diet. Both SAT and UNSAT diets were designed to contain similar protein, fat and energy content. All fat supplements were rumen protected. Throughout the manuscript, the dietary sequence is indicated by numbers 1 or 2; i.e. the dietary sequence in group A is CONT > SAT1 > UNSAT2 while that in group B is CONT > UNSAT1 > SAT2. All ingredients were chemically analyzed in a specialized lab (Feed & Food Quality Lab, AVEVE, Belgium) and their composition is provided in Table 1. The total daily amount of feed was evenly divided and offered at 8:00 and 17:00. Feed refusals were negligible. During each dietary period animal weights were recorded weekly.

As detailed below, blood samples were collected at the end of each dietary period for metabolic analysis. Serum samples were also used to examine their direct effect on *in vitro* pre-implantation embryo development. To rule out major differences in reproductive hormone concentrations in serum samples,

which might influence *in vitro* embryo development (Lonergan *et al.* 2007; O'Hara *et al.* 2014), estrous cycles of all cows were synchronized prior to serum collection.

2.2 Synchronization of ovarian cycles

During each dietary period, ovarian cycles were synchronized (fig.1) using intravaginal progesterone releasing device (PRID) for 10 days with two PGF₂ α injections (Dinolytic[®], Dinoprost tromethamine, 25mg/5 ml i.m.), one day before PRID removal and 12 days later. Estrus was observed 3 days following the second PGF₂ α injection. All animals responded to the synchronization program as observed by trans-rectal ultra-sonographic monitoring of ovarian dynamics and detection of estrus signs. Blood samples were collected 3 days later ~~when~~ which corresponds to low both estrogen and progesterone levels serum concentrations are low.

2.3 Blood sampling, serum collection and analyses

Blood sampling was done at the end of each dietary period (fig. 1) before the morning feeding as previously described (Leroy *et al.* 2010). Serum and plasma were collected and stored at -80°C. A proportion of the serum samples to be used during IVC was heat-inactivated (56°C for 30 min) before storage.

Serum and plasma samples were analyzed as previously described (Leroy *et al.* 2010) using commercial photometric assays for glucose, urea, total protein, total cholesterol, HDL cholesterol, triglycerides (Roche Diagnostics, Mannheim, Germany) and NEFAs (Wako Chemicals, Neuss, Germany) at the Department of Clinical Chemistry (University Hospital, Ghent, Belgium) in a semi-automated facility. The intra- and interassay coefficients of variation (CVs) for all analyses were $\leq 5\%$. Fatty acid profile of the serum was determined using a direct transesterification method modified from Lepage and Roy (1986) using gas chromatography. Insulin concentration was measured by chemiluminescence immunoassay using Cobas Insulin Kit (Roche Diagnostics, Indianapolis, IN, USA) with a detection limits of 0.2 mU/ml.

Inter- and intra-assay CVs for insulin were within 5%. Insulin-like growth factor I (IGF-I) was determined using a commercially available radioimmunoassay (IGF-I with extraction, DSL-5600; Diagnostic Systems Laboratories, Oxon, UK) according to the manufacturer's instructions. The detection limits of IGF-I was 0.8 ng/ml, and the inter- and intra-assay CVs were <10%. Total antioxidant capacity was measured photometrically using RX Daytona (Randox Laboratories) with an interassay CV of 9%.

To estimate insulin responsiveness, the revised quantitative insulin sensitivity check index (**RQUICKI**) was computed as reported by Perseghin *et al.* (2001) using the following formula: $RQUICKI = 1 / [\log (GLUC) + \log (INS) + \log (NEFA)]$, where GLUC is blood glucose concentration (mg/dl), INS is the blood insulin concentration (μ U/mL), and NEFA is the total free fatty acid concentration (mmol/L). A decrease in RQUICKI values is indicative for reduced insulin sensitivity in human (Rabasa-Lhoret *et al.* 2003). RQUICKI has also been validated and used in cattle (Holtenius and Holtenius 2007; Schulz *et al.* 2014).

2.4 In vitro embryo production (IVP) and in vitro experimental design

Grade-I COCs were aspirated from slaughterhouse ovaries and matured in serum-free media and fertilized *in vitro* as described previously (Van Hoeck *et al.* 2011). After fertilization, presumptive zygotes were vortexed for 3 min to remove excess sperm and cumulus cells. After three washings with a modified HEPES-buffered Tyrode balanced salt solution (HEPES-TALP) and modified synthetic oviductal fluid (mSOF) medium, presumptive zygotes were cultured in groups of 25 ± 4 in 500 ml mSOF medium without a mineral oil overlay at 38.5°C, 5% CO₂, 5% O₂ and 90% N₂ until Day 8 post-fertilization as described by Leroy *et al.* (2010). mSOF medium was supplemented with 10% of the serum collected after each dietary period as described in Leroy *et al.* (2010). This *in vitro* model specifically mimics oviduct-serum protein ratios which average from 5–10% as reported earlier (Leese 1988). Serum samples used for IVC were constituted by pooling serum from the three heifers within each group at each dietary period. This

resulted in 6 pooled serum samples that were tested; CONT, SAT1 and UNSAT2 from group A, and CONT, UNSAT1 and SAT2 from group B.

We first verified that the serum samples are suitable for embryo culture compared to a commercial fetal bovine serum (FBS) used in our standard laboratory procedure. A total of 604 presumptive zygotes (in two independent repeats) were randomly assigned to ~~either~~ mSOF supplemented with either commercial fetal bovine serum (Lab control serum), or with pooled serum samples from cows of group A ~~and or~~ group B collected after feeding the control diet (Control Serum A and Control serum B). Cleavage and blastocyst rates were recorded on day 2 and 8 post fertilization, respectively. Day 8 blastocysts were fixed to determine embryo cell numbers as described below.

Further on, serum from each animal group was tested in a separate experiment. In *Experiment 1 (Group A)*, a total of 1104 presumptive zygotes (in 4 independent replicates) were randomly distributed in mSOF supplemented with CONT, SAT1, or UNSAT2 serum. In *Experiment 2 (Group B)*, a total of 1483 embryos (in 5 independent replicates) were randomly allocated to mSOF containing CONT, UNSAT1 or SAT2 serum. In both experiments, cleavage and blastocyst rates were recorded on day 2 and 8 post fertilization, respectively. Day 8 blastocysts were snap frozen in groups of 10 (3 pools for each treatment containing equal numbers of normal and expanded blastocysts) for mRNA extraction and quantification of mRNA transcript abundance of selected candidate genes. The remaining blastocysts were fixed to determine embryo quality as described below.

2.5 Assessment of embryo quality

Day 8 blastocysts were fixed in 4% formaldehyde for at least 24 h. Apoptotic cells were labelled using a fluorescein isothiocyanateconjugated *in situ* cell death detection (TUNEL) kit (Roche, Penzberg, Germany) according to the manufacturer's instructions. Embryos were permeabilized in 0.1% Triton for 5 min and incubated in the reagent (1:10 dilution of the enzyme solution [terminal deoxynucleotidyl transferase from calf thymus] in label solution [nucleotide mixture]) in a humid chamber for 45 min at

37°C. Embryos were then stained with Hoechst 33342 (10 µg/ml in phosphate-buffered saline (PBS) for 10 min), washed and mounted in small droplets of anti-fading medium (DABCO) and evaluated by fluorescence microscopy (Olympus). The total cell numbers and apoptotic cell indices were counted and calculated for each blastocyst.

2.6 RNA extraction, reverse transcription and quantification of mRNA transcript abundance in blastocysts

Poly-A RNA of day 8 blastocysts was extracted using the Dynabeads mRNA Direct Extraction KIT (Dynal Biotech, Madrid, Spain), and immediately followed by reverse transcription (RT) using BioTag enzyme (Bioline, Ecogen, Spain) according to manufacturer's instructions and following MIQE guidelines (Bustin *et al.* 2009). The cDNA samples were quantified using real-time quantitative RT-PCR (qPCR) for a housekeeping gene, *H2AFZ*, and genes of interest associated with metabolism (*GAPDH*, *SLC2A1*, *SLC2A4*, *G6PD*); fatty acid transport and synthesis (*SREBF*, *ACCA*, *PLIN2*, *PTGS2*); apoptosis (*BCL2*); oxidative stress (*SHC1SHC*, *GPX1*, *SOD2*); and *de novo* methylation (*DNMT3A*). Gene full name, primer sequences and product sizes are shown in Supplementary Table 1. Quantification was performed as described previously (Bermejo-Alvarez *et al.* 2010). Cycle threshold (CT) values were normalized against that of the endogenous control (*H2AFZ*) as previously validated for bovine blastocysts (Gomez *et al.* 2009). Fold changes in the relative gene expression of the target genes were determined using the formula $2^{-\Delta\Delta CT}$ as described by Livak and Schmittgen (2001). Data are presented as Log 2 of fold change as described by Cagnone and Sirard (2014).

2.7 Statistical analyses

All statistical procedures were carried out using SPSS 21.0 for Windows (Chicago, IL, USA). Data from each experimental animal group were analyzed separately. In the *in vivo* experiment, numerical data from serum parameters after each dietary period were equally distributed, and analyzed by repeated measure ANOVA. *P*-values were corrected by Bonferroni test for multiple dependent comparisons. In the *in vitro*

experiment, cleavage and blastocyst rates were compared using a binary logistic regression models; the effect of treatments, replicates and their interaction were assessed. The interaction terms were not significant and were excluded from the final models. Gene expression data were analyzed by one-sample T-test for difference of the group mean from “0” which is the log 2 fold change value of controls as described in Cagnone and Sirard (2014). Numerical data are expressed as mean $\pm$ SD unless otherwise stated. Values of $P < 0.05$ were considered statistically significant, while P values > 0.05 and < 0.1 were indicated as tendencies.

3 Results

3.1 Effect of diets on animals and serum composition

Feeding high caloric diets resulted in increased body weight at the end of the experiment compared with body weights after feeding the control diet (weight gain for Group A: 33.5 ± 14.37 Kg ($P = 0.03$) and for Group B: 43.7 ± 27.57 ($P = 0.056$)). This was associated with altered serum concentrations of some metabolites and hormones as shown in Table 2. All diets resulted in similar concentrations of total serum protein and urea in both experimental groups and along the experimental period, except in group A, where feeding UNSAT2 significantly decreased blood urea levels compared to SAT1 ($P < 0.05$), both of which were not different from control ($P > 0.05$).

Importantly, switching to high caloric diets (either SAT or UNSAT) significantly increased serum NEFA and cholesterol (mainly HDL) concentrations, and reduced RQUICKI values ($P < 0.05$) compared to the controls. Triglyceride concentrations were not affected. In addition to these common effects, the dietary sequence resulted in specific differential effects in serum metabolites. Changing from SAT1 to UNSAT2 diet significantly ($P < 0.05$) reduced NEFA concentrations, and increased total anti-oxidant capacity, RQUICKI value, and IGF-I concentrations ($P < 0.05$). In contrast, switching from UNSAT1 to SAT2 decreased serum HDL cholesterol to control levels, while high NEFAs and total cholesterol concentrations were persistent.

3.2 *Effect of diets on serum fatty acid profile*

Elevated dietary fat supplementation (regardless of their type) resulted in a significantly increased total serum free FA content ($P < 0.05$). In the serum of animals fed SAT diet, this was primarily due to a 2-fold increase in Palmitic acid (PA, $P < 0.05$) compared to control, together with higher omega-6 linoleic acid (Table 3). This resulted in higher ω -6: ω -3 ratio compared to control, and higher saturated: ω -3 ratio in SAT1 compared with control serum, and in SAT2 compared with UNSAT1 serum. In animals fed UNSAT-diet, the increase in total FAs was primarily due to an increase in ω -3 unsaturated alpha linolenic acid (ALA; $P < 0.05$) together with elevated ω -6 (LA) and stearic acid. This resulted in ω -6: ω -3 and saturated: ω -3 ratios similar to control ($P > 0.05$) (Table 3).

3.3 *Effect of SAT and UNSAT serum on embryo development in vitro*

Zygotes cultured in media supplemented with CONT serum samples from group A and B had similar or higher embryo cleavage (day 2) and blastocyst (day 8) rates compared with standard laboratory control (Table 4), indicating suitability for embryo culture. Blastocysts in all groups had similar average total cell numbers ($P > 0.05$).

~~Thereafter~~Subsequently, serum from control, SAT and UNSAT feeding periods from each animal group was supplemented (10%) to culture media. Cleavage rates on day 2 were similar among different serum samples ($P > 0.05$). In experiment 1 (group A), supplementation of SAT1 serum significantly reduced blastocyst rate on day 8 compared with CONT serum ($P < 0.05$). However, the UNSAT2 serum resulted in a less marked decrease in blastocyst yield which was statistically similar to the CONT serum ($P > 0.05$), and resulted in blastocyst Total cell numbers of the blastocyst produced in the presence of the UNSAT2 serum total cell numbers that were also similar to the control ($P > 0.05$) and significantly higher than SAT1 ($P < 0.05$) (Table 5). In experiment 2 (group B), blastocyst yield tended to be higher in UNSAT1 and lower in SAT2 compared to CONT serum ($P < 0.1$). SAT2 blastocyst rate was significantly lower than UNSAT1 ($P < 0.05$). Changes in total cell numbers were not statistically significant but followed the

same pattern as in Experiment 1. In addition, blastocysts developed in the presence of SAT-serum showed a higher apoptotic cell index compared with control in Exp. 1, and compared with UNSAT in both experiments ($P < 0.02$).

3.4 Effect of SAT and UNSAT serum on transcription in blastocysts

Blastocysts cultured in mSOF supplemented with SAT1 serum showed a significant decrease ($P < 0.05$) in *BCL2* transcription compared to its control (figure 2). Similarly, SAT2 serum resulted in a lower *BCL2* expression, as well as a higher *SOD2* and a tendency to increase *G6PD* mRNA expression compared to its control (figure 2). Embryo cultured in the presence of UNSAT1 serum resulted in a higher *DNMT3A* compared to the control, while in UNSAT2 serum there was a tendency to decrease *SHC1-SHC* gene expression. ~~There was no treatment~~ Treatments had no effect on *GPX1* and transcripts involved in FA transport and synthesis (*SREBF*, *ACCA*, *PLIN2*, *COX2*), and glucose transport and metabolism (*SLC2A1*, *SLC2A4*, *GAPDH*).

4 Discussion

It has been shown earlier that hyperlipidemic serum from cows fed a high starch, high-saturated-fat diet hampers embryo development and quality when added *in vitro* during embryo culture (Leroy *et al.* 2010). We now expanded on this concept using the same combined *in vivo* - *in vitro* model to examine whether changing the FA type to a more polyunsaturated profile ~~would~~ can counteract these negative effects. As expected, serum metabolites and FA profile were dependent on the type of fat supplement and sequence of feeding, which differentially influenced embryo development and quality *in vitro*.

Introducing the high-starch high-fat diet, regardless of the predominant FAs, significantly increased cholesterol (by >26%) and FFA concentrations (by >33%). Elevated total blood cholesterol, mainly in the form of HDL, is a common consequence of high fat diets in cows (Wehrman *et al.* 1991; Childs *et al.* 2008). These changes are also similar to those associated with western-type diets and obesity in humans

(Boden 2001; Gallagher *et al.* 2010). Although total cholesterol in SAT1 serum was not statistically different from CONT, it reached hypercholesterolemic levels (>130 mg/ml) similar to those reported in high lactating dairy cows (Guretzky *et al.* 2006). In contrast to Wehrman *et al.* (1991) and unlike humans where it is common to detect an increase in serum triglycerides associated with nutritionally induced hyperlipidemia (Fredrickson and Lees 1965), here the triglyceride concentrations in plasma of the cows were not affected by the high-fat diet. This is however in line with other previous findings where dietary-induced hyperlipidemia was not associated with elevated serum triglycerides in cows (Childs *et al.* 2008; Leroy *et al.* 2010). ~~A rapid turnover of very low density lipoproteins, where the majority of triglycerides reside, might explain the unaffected triglyceride levels among different dietary treatments (Palmquist 1976; Newman *et al.* 2016).~~ The majority of triglycerides reside in very low density lipoproteins (VLDL), which have a rapid turnover either for milk production in lactating dairy cows (Palmquist 1976; Newman *et al.* 2016), or by increased uptake into the adipose tissue for storage (Doreau and Chilliard 1997). Fat storage substantiated by the weight gain observed in the cows used in this study after the dietary supplementation period may possibly explain the unaffected circulating triglyceride concentrations.

In addition, both dietary-induced hyperlipidemic conditions (SAT1 and UNSAT1) were associated with reduced RQUICKI values indicating reduced insulin sensitivity. This is a key component of several metabolic diseases including obesity and type II diabetes (Rabasa-Lhoret *et al.* 2003). In contrast to QUICKI calculations, RQUICKI takes into consideration FFA concentrations which improves its association with insulin sensitivity in non-obese individuals (Perseghin *et al.* 2001). It was noticed, however, that switching from SAT-rich to UNSAT-rich diet significantly increased RQUICKI values back to control levels. Unsaturated ω -3 rich diets in human have been shown to improve insulin sensitivity particularly linked to type-II diabetes (Popp-Snijders *et al.* 1987; Farsi *et al.* 2014). This effect was explained by the lower NEFA concentrations associated with ω -3 diets (Farsi *et al.* 2014). Here we also noticed that blood NEFAs were significantly reduced in cows when unsaturated-fat diet was introduced after a period of saturated-fat rich diet. Moreover, blood urea nitrogen (BUN) was

significantly higher in the SAT1 group (5.99 mM vs. 4.33 mM in UNSAT2). Exposure of bovine oocytes during *in vitro* maturation to high urea levels (6 mM) reduced cleavage rates and subsequent development to the blastocyst stage (De Wit *et al.* 2001), however direct effects on embryos have not been demonstrated. It was reported that increased BUN levels are associated with decreased conception rates in cattle, however, only when concentrations exceed 20 mg/dl (7.14 mM) (Ferguson *et al.* 1993). Nevertheless, the overall metabolic changes induced by switching from SAT to UNSAT FA profile suggest a lower risk of infertility.

As mentioned in the introduction, systemic metabolic changes are reflected in the reproductive fluid composition. Embryos are sensitive to the lipid composition of their immediate microenvironment and can accumulate and metabolize cholesterol and fatty acids from the media (Ferguson and Leese 1999; Kim *et al.* 2001). In a previous report in sheep, inclusion of ω -3 enriched serum in embryo culture medium increased the total FA and ω -3 content (ng/blastocyst) of Day 7 blastocysts when compared with embryos cultured with FA-free media containing BSA (Hughes *et al.* 2011). Studies from our group have shown that Day 7 embryos recovered from high lactating dairy cows during NEB displayed a dark appearance of the cytoplasm and were categorized as poor quality compared to embryos collected from non-lactating heifers or beef cows (Leroy *et al.* 2005). Whereas feeding cows more PUFAs significantly increased the proportion of excellent and good quality embryos compared to those fed Ca salt of palm oil which is rich in palmitic acid (Cerri *et al.* 2009). These effects may be partially mediated by changes in the oviductal environment during early embryo development.

While the studies listed above are very informative, investigating specific dietary effects on early embryo development *in vivo* is complicated. Therefore, in the present study, we opted to combine an *in vivo* feeding model with an *in vitro* embryo exposure model. We found that SAT1 hyperlipidemic serum supplementation during *in vitro* culture significantly reduced *in vitro* embryo development to the blastocyst stage (by ~8%) and reduced blastocyst quality compared to the preceding CONT serum. UNSAT2 serum collected ~~later~~ afterwards displayed carry-over effects in its fatty acid profile ~~and but~~ led

to a normal blastocyst rate and quality. With the opposite feeding sequence, switching from CONT to UNSAT1 also resulted in hyperlipidemic serum (hypercholesterolemia and elevated NEFAs) and tended to increase blastocyst rates with normal cell numbers and apoptotic cell index compared to CONT. Subsequently, SAT2-serum resulted in a substantial drop (by about 13%) in blastocyst rates compared to UNSAT1 with a significantly upregulated apoptosis. To explain the differences caused by dietary sequence, a closer look to the serum fatty acid profile is informative. The composition of the pooled serum samples used in the present study can be described by the average concentrations showed in Table 2 and 3, while the variation between heifers within each group was omitted by pooling of serum samples for *in vitro* supplementation. For each diet, comparing this one pooled serum sample of group A to that of group B (obviously numerically and not statistically), it is noticeable that feeding high caloric diet rich in ω -3 (UNSAT1) after a control dietary period produced serum with lower PA, ω -6 linoleic acid and ω -6: ω -3 ratio if compared to UNSAT2. UNSAT2 (which was fed after saturated dietary period) apparently contained relatively higher saturated and ω -6 fatty acids following recovery from SAT1 period. Both saturated PA and ω -6 LA are known to inhibit embryo development *in vitro* when added during maturation (Marei *et al.* 2010; Van Hoeck *et al.* 2011). LA was also detrimental when added during culture (Hochi *et al.* 1999). These extended carry over effects may be related to altered cellular fatty acid composition which slowly change in response to dietary fat alteration (Field *et al.* 1985; Gibney and Hunter 1993). Fat mobilization from saturated FA-rich depots ~~may~~ can occur after stopping SAT-supplementation and may explain the relatively higher serum saturated fatty acids in UNSAT2 compared to UNSAT1.

Other changes in the serum profile such as elevated cholesterol and glucose concentrations are less likely to contribute to the effects on embryo development; It has been previously shown that treatment of embryos with cholesterol and HDL cholesterol had no effect on embryo development under standard culture conditions (Moss *et al.* 2012). Similarly, cleavage stage bovine embryos have very limited capacity to utilize glucose until the blastocyst stage (Gardner *et al.* 2015) and the differences in serum

glucose concentrations observed among SAT and UNSAT samples are unlikely to cause any differential effect on developmental competence especially considering that the serum was ~~used~~ added to the embryo culture medium at 10% v/v and therefore providing low glucose concentrations in the medium (~0.4mM). Human embryos have been reported to consume higher amounts of glucose (Gardner *et al.* 2011) compared to values described for bovine embryos (Guerif *et al.* 2013) which may suggest that human embryos may be more sensitive to perturbations in glucose concentrations in their micro-environment.

Looking at the gene expression patterns of the blastocysts produced under different serum conditions, a distinct inhibitory effect of SAT serum on *BCL2* expression was noticed in both SAT1 and SAT2. *BCL2* is known to have anti-apoptotic role and its balance with the pro-apoptotic Bax is essential in regulating cell death (Chao and Korsmeyer 1998). Down-regulation of *BCL2* results in downstream activation of caspases and induces chromatin condensation and fragmentation (Marsden *et al.* 2002). Palmitic acid, which is significantly higher in SAT1 and SAT2 sera have been previously shown to downregulate *BCL2* and induce lipo-apoptotic effects in other cell types like pancreatic cells (Landau *et al.* 2006). This might explain the significant increase in apoptotic cell ratio reported here in blastocysts produced in SAT1 and SAT2 culture conditions. It can also explain the reduced cell proliferation in SAT1 as indicated by lower total cell numbers in the blastocysts. In addition, SAT2 serum supplementation which resulted in the lowest developmental potential, increased *SOD2* expression in blastocysts. Mitochondrial SOD is required for scavenging superoxide anions and balancing ROS generation (Borgstahl *et al.* 1992). This may indicate that these embryos are under oxidative stress. Compensatory upregulation of *SOD2* expression and antioxidant activity in the face of increased ROS have been reported in other cell types (Borgstahl *et al.* 1992; Lei *et al.* 2012). Furthermore, this is also supported by the tendency to increase *G6PD* expression in SAT2 group. *G6PD* catalyses the first step of pentose phosphate pathway that supplies NADPH for antioxidant defense mechanisms (Kletzien *et al.* 1994). The other candidate genes analyzed in this study, which are involved in fatty acid transport, synthesis or accumulation and in metabolism, were not significantly affected by any of the serum conditions. UNSAT1 blastocysts had

significantly higher DNMT3A suggesting a regulatory role in the *de novo* DNA methylation and epigenetic regulation. This was not evident in UNSAT2 group which still had higher saturated fatty acid content and high ω -6: ω -3 compared to UNSAT1 as discussed above.

Considerable caution is required when extrapolating data acquired from animal models to human fertility. Distinct differences in fat digestion and subsequent metabolism between ruminants and non-ruminants are known described (Doreau and Chilliard 1997). However, similarities in the pathophysiological changes in serum FA profile associated with metabolic stress in human and cattle, as described in the introduction, together with the ethical and practical considerations, augment the value of the bovine model in studying the impact of metabolic stress on reproductive performance.

In conclusion, substitution of saturated fat with an ω -3 fat source within a typical Western type high-fat and high-carbohydrate diet resulted in ω -3 rich hyperlipidemic serum conditions, among other changes in serum metabolites ~~suggesting~~ suggesting that we may expect a differential impact on fertility. By using a combined *in vivo* - *in vitro* model we learned that such effects can be exerted at the level of the pre-implantation embryo. Changes in the serum FA profile composition to a more ω -3 rich profile alleviates the detrimental effects of SAT-rich hyperlipidemia on embryo development, quality, and gene expression patterns related to apoptosis. However, carry-over effects from previous saturated FA-rich diets are evident and should therefore be considered as a limiting factor and may ~~require~~ indicate longer ω -3 supplementation. While considering species differences, ~~T~~these results suggest that, for infertile patients suffering metabolic disorders associated with hyperlipidemia, shifting the major FA type in the diet from saturated to omega-3 FA may improve embryo quality and fertility outcomes by directly affecting the embryo's micro-environment.

Funding

This study was funded by Antwerp University BOF project number 24281 and FWO research project number G019214N. Gene expression analysis was supported by Grant number AGL2012-39652-C02-01 from the Spanish Ministry of Science and Innovation.

Disclosure Summary

The authors have no conflicts of interest to disclose.

REFERENCES

- Abayasekara, D.R., and Wathes, D.C. (1999) Effects of altering dietary fatty acid composition on prostaglandin synthesis and fertility. *Prostaglandins Leukot Essent Fatty Acids* **61**(5), 275-87
- Artwohl, M., Lindenmair, A., Roden, M., Waldhausl, W.K., Freudenthaler, A., Klosner, G., Ilhan, A., Luger, A., and Baumgartner-Parzer, S.M. (2009) Fatty acids induce apoptosis in human smooth muscle cells depending on chain length, saturation, and duration of exposure. *Atherosclerosis* **202**(2), 351-62
- Barnard, R.J., Roberts, C.K., Varon, S.M., and Berger, J.J. (1998) Diet-induced insulin resistance precedes other aspects of the metabolic syndrome. *J Appl Physiol* (1985) **84**(4), 1311-5
- Bermejo-Alvarez, P., Lonergan, P., Rath, D., Gutierrez-Adan, A., and Rizos, D. (2010) Developmental kinetics and gene expression in male and female bovine embryos produced in vitro with sex-sorted spermatozoa. *Reprod Fertil Dev* **22**(2), 426-36
- Boden, G. (2001) Free fatty acids-the link between obesity and insulin resistance. *Endocr Pract* **7**(1), 44-51
- Boden, G. (2008) Obesity and free fatty acids. *Endocrinol Metab Clin North Am* **37**(3), 635-46, viii-ix
- Borgstahl, G.E., Parge, H.E., Hickey, M.J., Beyer, W.F., Jr., Hallewell, R.A., and Tainer, J.A. (1992) The structure of human mitochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles. *Cell* **71**(1), 107-18
- Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., and Wittwer, C.T. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* **55**(4), 611-22

Cagnone, G., and Sirard, M.A. (2014) The impact of exposure to serum lipids during in vitro culture on the transcriptome of bovine blastocysts. *Theriogenology* **81**(5), 712-22 e1-3

Campbell, B.K., Souza, C., Gong, J., Webb, R., Kendall, N., Marsters, P., Robinson, G., Mitchell, A., Telfer, E.E., and Baird, D.T. (2003) Domestic ruminants as models for the elucidation of the mechanisms controlling ovarian follicle development in humans. *Reprod Suppl* **61**, 429-43

Cerri, R.L., Juchem, S.O., Chebel, R.C., Rutigliano, H.M., Bruno, R.G., Galvao, K.N., Thatcher, W.W., and Santos, J.E. (2009) Effect of fat source differing in fatty acid profile on metabolic parameters, fertilization, and embryo quality in high-producing dairy cows. *J Dairy Sci* **92**(4), 1520-31

Chao, D.T., and Korsmeyer, S.J. (1998) BCL-2 family: regulators of cell death. *Annu Rev Immunol* **16**, 395-419

Childs, S., Hennessy, A.A., Sreenan, J.M., Wathes, D.C., Cheng, Z., Stanton, C., Diskin, M.G., and Kenny, D.A. (2008) Effect of level of dietary n-3 polyunsaturated fatty acid supplementation on systemic and tissue fatty acid concentrations and on selected reproductive variables in cattle. *Theriogenology* **70**(4), 595-611

Das, U.N. (2010) Essential Fatty Acids: Biochemistry and Physiology. In 'Metabolic Syndrome Pathophysiology.' pp. 181-200. (Wiley-Blackwell)

De Wit, A.A., Cesar, M.L., and Kruip, T.A. (2001) Effect of urea during in vitro maturation on nuclear maturation and embryo development of bovine cumulus-oocyte-complexes. *J Dairy Sci* **84**(8), 1800-4

Doreau, M., and Chilliard, Y. (1997) Digestion and metabolism of dietary fat in farm animals. *Br J Nutr* **78 Suppl 1**, S15-35

Farsi, P.F., Djazayery, A., Eshraghian, M.R., Koohdani, F., Saboor-Yaraghi, A.A., Derakhshanian, H., Zarei, M., Javanbakht, M.H., and Djalali, M. (2014) Effects of supplementation with omega-3 on insulin sensitivity and non-esterified free fatty acid (NEFA) in type 2 diabetic patients. *Arq Bras Endocrinol Metabol* **58**(4), 335-40

Fenwick, M.A., Fitzpatrick, R., Kenny, D.A., Diskin, M.G., Patton, J., Murphy, J.J., and Wathes, D.C. (2008) Interrelationships between negative energy balance (NEB) and IGF regulation in liver of lactating dairy cows. *Domest Anim Endocrinol* **34**(1), 31-44

Ferguson, E.M., and Leese, H.J. (1999) Triglyceride content of bovine oocytes and early embryos. *J Reprod Fertil* **116**(2), 373-8

Ferguson, J.D., Galligan, D.T., Blanchard, T., and Reeves, M. (1993) Serum urea nitrogen and conception rate: the usefulness of test information. *J Dairy Sci* **76**(12), 3742-6

Ferreira, R.C., Halpern, G., Figueira Rde, C., Braga, D.P., Iaconelli, A., Jr., and Borges, E., Jr. (2010) Physical activity, obesity and eating habits can influence assisted reproduction outcomes. *Womens Health (Lond Engl)* **6**(4), 517-24

Field, C.J., Angel, A., and Clandinin, M.T. (1985) Relationship of diet to the fatty acid composition of human adipose tissue structural and stored lipids. *The American Journal of Clinical Nutrition* **42**(6), 1206-20

Fredrickson, D.S., and Lees, R.S. (1965) A System for Phenotyping Hyperlipoproteinemia. *Circulation* **31**, 321-7

Gallagher, E.J., Leroith, D., and Karnieli, E. (2010) Insulin resistance in obesity as the underlying cause for the metabolic syndrome. *Mt Sinai J Med* **77**(5), 511-23

Gardner, D.K., Gardner, D.K., Harvey, A.J., and Harvey, A.J. (2015) Blastocyst metabolism. *Reprod Fertil Dev* **27**, 638-654

Gardner, D.K., Wale, P.L., Collins, R., and Lane, M. (2011) Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Hum Reprod* **26**(8), 1981-6

Gibney, M.J., and Hunter, B. (1993) The effects of short- and long-term supplementation with fish oil on the incorporation of n-3 polyunsaturated fatty acids into cells of the immune system in healthy volunteers. *European journal of clinical nutrition* **47**(4), 255-259

Gomez, E., Gutierrez-Adan, A., Diez, C., Bermejo-Alvarez, P., Munoz, M., Rodriguez, A., Otero, J., Alvarez-Viejo, M., Martin, D., Carrocera, S., and Caamano, J.N. (2009) Biological differences between in vitro produced bovine embryos and parthenotes. *Reproduction* **137**(2), 285-95

Guerif, F., McKeegan, P., Leese, H.J., and Sturmey, R.G. (2013) A simple approach for CONsumption and RElease (CORE) analysis of metabolic activity in single mammalian embryos. *PLoS One* **8**(8), e67834

Guretzky, N.A., Carlson, D.B., Garrett, J.E., and Drackley, J.K. (2006) Lipid metabolite profiles and milk production for Holstein and Jersey cows fed rumen-protected choline during the periparturient period. *J Dairy Sci* **89**(1), 188-200

526 Haggarty, P., Wood, M., Ferguson, E., Hoad, G., Srikantharajah, A., Milne, E., Hamilton, M., and
 527 Bhattacharya, S. (2006) Fatty acid metabolism in human preimplantation embryos. *Hum Reprod* **21**(3),
 528 766-73

 529
 530 Hawkins, D.E., Niswender, K.D., Oss, G.M., Moeller, C.L., Odde, K.G., Sawyer, H.R., and Niswender, G.D.
 531 (1995) An increase in serum lipids increases luteal lipid content and alters the disappearance rate of
 532 progesterone in cows. *J Anim Sci* **73**(2), 541-5

 533
 534 Hochi, S., Kimura, K., and Hanada, A. (1999) Effect of linoleic acid-albumin in the culture medium on
 535 freezing sensitivity of in vitro-produced bovine morulae. *Theriogenology* **52**(3), 497-504

 536
 537 Holtenius, P., and Holtenius, K. (2007) A model to estimate insulin sensitivity in dairy cows. *Acta Vet*
 538 *Scand* **49**, 29

 539
 540 Hughes, J., Kwong, W.Y., Li, D., Salter, A.M., Lea, R.G., and Sinclair, K.D. (2011) Effects of omega-3 and -6
 541 polyunsaturated fatty acids on ovine follicular cell steroidogenesis, embryo development and molecular
 542 markers of fatty acid metabolism. *Reproduction* **141**(1), 105-18

 543
 544 Igosheva, N., Abramov, A.Y., Poston, L., Eckert, J.J., Fleming, T.P., Duchon, M.R., and McConnell, J. (2010)
 545 Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and
 546 zygotes. *PLoS One* **5**(4), e10074

 547
 548 Jordaens, L., Arias-Alvarez, M., Pintelon, I., Thys, S., Valckx, S., Dezhkam, Y., Bols, P.E., and Leroy, J.L.
 549 (2015) Elevated non-esterified fatty acid concentrations hamper bovine oviductal epithelial cell
 550 physiology in three different in vitro culture systems. *Theriogenology* **84**(6), 899-910

 551
 552 Jungheim, E.S., Macones, G.A., Odem, R.R., Patterson, B.W., Lanzendorf, S.E., Ratts, V.S., and Moley, K.H.
 553 (2011) Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function
 554 during in vitro fertilization. *Fertil Steril* **95**(6), 1970-4

 555
 556 Kim, J.Y., Kinoshita, M., Ohnishi, M., and Fukui, Y. (2001) Lipid and fatty acid analysis of fresh and frozen-
 557 thawed immature and in vitro matured bovine oocytes. *Reproduction* **122**(1), 131-8

 558
 559 Kletzien, R.F., Harris, P.K., and Foellmi, L.A. (1994) Glucose-6-phosphate dehydrogenase: a
 560 "housekeeping" enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress.
 561 *FASEB J* **8**(2), 174-81

 562
 563 Kris-Etherton, P.M., and Fleming, J.A. (2015) Emerging nutrition science on fatty acids and cardiovascular
 564 disease: nutritionists' perspectives. *Adv Nutr* **6**(3), 326S-37S

 565

Landau, Z., Forti, E., Alcala, M., and Birk, R.Z. (2006) Palmitate induced lipoapoptosis of exocrine pancreas AR42J cells. *Apoptosis* **11**(5), 717-24

Larque, E., Gil-Sanchez, A., Prieto-Sanchez, M.T., and Koletzko, B. (2012) Omega 3 fatty acids, gestation and pregnancy outcomes. *Br J Nutr* **107** Suppl 2, S77-84

Leary, C., Leese, H.J., and Sturmey, R.G. (2015) Human embryos from overweight and obese women display phenotypic and metabolic abnormalities. *Hum Reprod* **30**(1), 122-32

Leese, H.J. (1988) The formation and function of oviduct fluid. *J Reprod Fertil* **82**(2), 843-56

Lei, S., Liu, Y., Liu, H., Yu, H., Wang, H., and Xia, Z. (2012) Effects of N-acetylcysteine on nicotinamide dinucleotide phosphate oxidase activation and antioxidant status in heart, lung, liver and kidney in streptozotocin-induced diabetic rats. *Yonsei Med J* **53**(2), 294-303

Leroy, J.L., Opsomer, G., De Vlieghe, S., Vanholder, T., Goossens, L., Geldhof, A., Bols, P.E., de Kruif, A., and Van Soom, A. (2005) Comparison of embryo quality in high-yielding dairy cows, in dairy heifers and in beef cows. *Theriogenology* **64**(9), 2022-36

Leroy, J.L., Rizos, D., Sturmey, R., Bossaert, P., Gutierrez-Adan, A., Van Hoeck, V., Valckx, S., and Bols, P.E. (2011) Intrafollicular conditions as a major link between maternal metabolism and oocyte quality: a focus on dairy cow fertility. *Reprod Fertil Dev* **24**(1), 1-12

Leroy, J.L., Van Hoeck, V., Clemente, M., Rizos, D., Gutierrez-Adan, A., Van Soom, A., Uytterhoeven, M., and Bols, P.E. (2010) The effect of nutritionally induced hyperlipidaemia on in vitro bovine embryo quality. *Hum Reprod* **25**(3), 768-78

Leroy, J.L., Vanholder, T., Delanghe, J.R., Opsomer, G., Van Soom, A., Bols, P.E., Dewulf, J., and de Kruif, A. (2004) Metabolic changes in follicular fluid of the dominant follicle in high-yielding dairy cows early post partum. *Theriogenology* **62**(6), 1131-43

Lichtenstein, A.H., Appel, L.J., Brands, M., Carnethon, M., Daniels, S., Franch, H.A., Franklin, B., Kris-Etherton, P., Harris, W.S., Howard, B., Karanja, N., Lefevre, M., Rudel, L., Sacks, F., Van Horn, L., Winston, M., and Wylie-Rosett, J. (2006) Diet and Lifestyle Recommendations Revision 2006: A Scientific Statement From the American Heart Association Nutrition Committee. *Circulation* **114**(1), 82-96

Livak, K.J., and Schmittgen, T.D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods* **25**(4), 402-408

605 Lonergan, P., Woods, A., Fair, T., Carter, F., Rizos, D., Ward, F., Quinn, K., and Evans, A. (2007) Effect of
 606 embryo source and recipient progesterone environment on embryo development in cattle. *Reprod Fertil*
 607 *Dev* **19**(7), 861-8
 608
 609 Luzzo, K.M., Wang, Q., Purcell, S.H., Chi, M., Jimenez, P.T., Grindler, N., Schedl, T., and Moley, K.H.
 610 (2012) High fat diet induced developmental defects in the mouse: oocyte meiotic aneuploidy and fetal
 611 growth retardation/brain defects. *PLoS One* **7**(11), e49217
 612
 613 Maillo, V., Rizos, D., Besenfelder, U., Havlicek, V., Kelly, A.K., Garrett, M., and Lonergan, P. (2012)
 614 Influence of lactation on metabolic characteristics and embryo development in postpartum Holstein
 615 dairy cows. *J Dairy Sci* **95**(7), 3865-76
 616
 617 Marei, W.F., Wathes, D.C., and Fouladi-Nashta, A.A. (2010) Impact of linoleic acid on bovine oocyte
 618 maturation and embryo development. *Reproduction* **139**(6), 979-88
 619
 620 Marsden, V.S., O'Connor, L., O'Reilly, L.A., Silke, J., Metcalf, D., Ekert, P.G., Huang, D.C., Cecconi, F.,
 621 Kuida, K., Tomaselli, K.J., Roy, S., Nicholson, D.W., Vaux, D.L., Bouillet, P., Adams, J.M., and Strasser, A.
 622 (2002) Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome
 623 c/Apaf-1/caspase-9 apoptosome. *Nature* **419**(6907), 634-7
 624
 625 Mendoza, C., Ruiz-Requena, E., Ortega, E., Cremades, N., Martinez, F., Bernabeu, R., Greco, E., and
 626 Tesarik, J. (2002) Follicular fluid markers of oocyte developmental potential. *Hum Reprod* **17**(4), 1017-22
 627
 628 Moss, J.I., Garrett, T.J., and Hansen, P.J. (2012) Involvement of free cholesterol and high-density
 629 lipoprotein in development and resistance of the preimplantation bovine embryo to heat shock. *J Anim*
 630 *Sci* **90**(11), 3762-9
 631
 632 National Research Council (U.S.). Subcommittee on Dairy Cattle Nutrition. (2001) 'Nutrient requirements
 633 of dairy cattle.' 7th rev. edn. (National Academy Press: Washington, D.C.) xxi, 381 p.
 634
 635 Newman, A., Mann, S., Nydam, D.V., Overton, T.R., and Behling-Kelly, E. (2016) Impact of dietary plane
 636 of energy during the dry period on lipoprotein parameters in the transition period in dairy cattle. *J Anim*
 637 *Physiol Anim Nutr (Berl)* **100**(1), 118-26
 638
 639 O'Hara, L., Forde, N., Kelly, A.K., and Lonergan, P. (2014) Effect of bovine blastocyst size at embryo
 640 transfer on day 7 on conceptus length on day 14: can supplementary progesterone rescue small
 641 embryos? *Theriogenology* **81**(8), 1123-8
 642
 643 Palmquist, D.L. (1976) A kinetic concept of lipid transport in ruminants. *J Dairy Sci* **59**(3), 355-63
 644

645 Panchal, S.K., Poudyal, H., Iyer, A., Nazer, R., Alam, A., Diwan, V., Kauter, K., Sernia, C., Campbell, F.,
646 Ward, L., Gobe, G., Fenning, A., and Brown, L. (2011) High-carbohydrate high-fat diet-induced metabolic
647 syndrome and cardiovascular remodeling in rats. *J Cardiovasc Pharmacol* **57**(1), 51-64

648

649 Perk, J., De Backer, G., Gohlke, H., Graham, I., Reiner, Z., Verschuren, M., Albus, C., Benlian, P., Boysen,
650 G., Cifkova, R., Deaton, C., Ebrahim, S., Fisher, M., Germano, G., Hobbs, R., Hoes, A., Karadeniz, S.,
651 Mezzani, A., Prescott, E., Ryden, L., Scherer, M., Syvanne, M., Scholte op Reimer, W.J., Vrints, C., Wood,
652 D., Zamorano, J.L., Zannad, F., European Association for Cardiovascular, P., Rehabilitation, and
653 Guidelines, E.S.C.C.f.P. (2012) European Guidelines on cardiovascular disease prevention in clinical
654 practice (version 2012). The Fifth Joint Task Force of the European Society of Cardiology and Other
655 Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of
656 nine societies and by invited experts). *Eur Heart J* **33**(13), 1635-701

657

658 Perseghin, G., Caumo, A., Caloni, M., Testolin, G., and Luzi, L. (2001) Incorporation of the fasting plasma
659 FFA concentration into QUICKI improves its association with insulin sensitivity in nonobese individuals. *J*
660 *Clin Endocrinol Metab* **86**(10), 4776-81

661

662 Petit, H.V., Cavalieri, F.B., Santos, G.T., Morgan, J., and Sharpe, P. (2008) Quality of embryos produced
663 from dairy cows fed whole flaxseed and the success of embryo transfer. *J Dairy Sci* **91**(5), 1786-90

664

665 Popp-Snijders, C., Schouten, J.A., Heine, R.J., van der Meer, J., and van der Veen, E.A. (1987) Dietary
666 supplementation of omega-3 polyunsaturated fatty acids improves insulin sensitivity in non-insulin-
667 dependent diabetes. *Diabetes Res* **4**(3), 141-7

668

669 Rabasa-Lhoret, R., Bastard, J.P., Jan, V., Ducluzeau, P.H., Andreelli, F., Guebre, F., Bruzeau, J., Louche-
670 Pellissier, C., Maltrepierre, C., Peyrat, J., Chagne, J., Vidal, H., and Laville, M. (2003) Modified
671 quantitative insulin sensitivity check index is better correlated to hyperinsulinemic glucose clamp than
672 other fasting-based index of insulin sensitivity in different insulin-resistant states. *J Clin Endocrinol*
673 *Metab* **88**(10), 4917-23

674

675 Ruxton, C., Reed, S., Simpson, M., and Millington, K. (2007) The health benefits of omega-3
676 polyunsaturated fatty acids: a review of the evidence. *J Hum Nutr Diet* **20**(3), 275-85

677

678 Sanchez, I., Reynoso-Camacho, R., and Salgado, L.M. (2015) The diet-induced metabolic syndrome is
679 accompanied by whole-genome epigenetic changes. *Genes Nutr* **10**(4), 471

680

681 Santos, J.E., Bilby, T.R., Thatcher, W.W., Staples, C.R., and Silvestre, F.T. (2008) Long chain fatty acids of
682 diet as factors influencing reproduction in cattle. *Reprod Domest Anim* **43 Suppl 2**, 23-30

683

684 Schulz, K., Frahm, J., Meyer, U., Kersten, S., Reiche, D., Rehage, J., and Danicke, S. (2014) Effects of
685 prepartal body condition score and peripartal energy supply of dairy cows on postpartal lipolysis, energy
686 balance and ketogenesis: an animal model to investigate subclinical ketosis. *J Dairy Res* **81**(3), 257-66

Styne-Gross, A., Elkind-Hirsch, K., and Scott, R.T., Jr. (2005) Obesity does not impact implantation rates or pregnancy outcome in women attempting conception through oocyte donation. *Fertil Steril* **83**(6), 1629-34

Valckx, S.D., Arias-Alvarez, M., De Pauw, I., Fievez, V., Vlaeminck, B., Fransen, E., Bols, P.E., and Leroy, J.L. (2014) Fatty acid composition of the follicular fluid of normal weight, overweight and obese women undergoing assisted reproductive treatment: a descriptive cross-sectional study. *Reprod Biol Endocrinol* **12**(1), 13

Van der Steeg, J.W., Steures, P., Eijkemans, M.J., Habbema, J.D., Hompes, P.G., Burggraaff, J.M., Oosterhuis, G.J., Bossuyt, P.M., van der Veen, F., and Mol, B.W. (2008) Obesity affects spontaneous pregnancy chances in subfertile, ovulatory women. *Hum Reprod* **23**(2), 324-8

Van Hoeck, V., Sturmey, R.G., Bermejo-Alvarez, P., Rizados, D., Gutierrez-Adan, A., Leese, H.J., Bols, P.E., and Leroy, J.L. (2011) Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *PLoS One* **6**(8), e23183

Wakefield, S.L., Lane, M., Schulz, S.J., Hebart, M.L., Thompson, J.G., and Mitchell, M. (2008) Maternal supply of omega-3 polyunsaturated fatty acids alter mechanisms involved in oocyte and early embryo development in the mouse. *Am J Physiol Endocrinol Metab* **294**(2), E425-34

Waterman, R.A., and Wall, R.J. (1988) Lipid interactions with in vitro development of mammalian zygotes. *Gamete Res* **21**(3), 243-54

Wehrman, M.E., Welsh, T.H., Jr., and Williams, G.L. (1991) Diet-induced hyperlipidemia in cattle modifies the intrafollicular cholesterol environment, modulates ovarian follicular dynamics, and hastens the onset of postpartum luteal activity. *Biol Reprod* **45**(3), 514-22

Zraika, S., Dunlop, M., Proietto, J., and Andrikopoulos, S. (2002) Effects of free fatty acids on insulin secretion in obesity. *Obes Rev* **3**(2), 103-12

Table 1. Ingredient composition and chemical analysis of the CONT, SAT and UNSAT diets.

Ingredients	Dry matter (kg)		
	CONT diet	SAT diet	UNSAT diet
Hay	6.64	6.64	6.64
Wheat straw	0.84	0.84	0.84
Ground corn	0.26	3.48	3.48
Concentrate mixture ^a	0.54	2.7	2.7
Linseed extract	0.59	0.72	0
Butterboost ^b	0	0.43	0
Linseed oil	0	0	0.12
Protected Exfoliated Linseed ^c	0	0	0.93
Dry matter intake (DMI; kg per day)	8.9	14.8	14.7
Starch g/kg DM	39.2	190.4	190.0
Sugars g/kg DM	79.5	57.6	55.9
Total digestible protein g/kg DM	77.5	86.3	88.9
Crude Fat g/kg DM	32.3	59.4	60.6
Crude Fiber g/kg DM	232.7	164.2	160.7
Energy content Kcal/kg DM	1324.1	1561.9	1566.9

^a Superbeef F5TM (Quartes, BE). ^b Commercial rumen-protected pure vegetable palm fat containing concentrated high levels of saturated (SAT) palmitic acid (Trouw Nutrition International, BE). ^c Commercial rumen-protected linseeds as a source of unsaturated (UNSAT) omega-3 alpha-linolenic fatty acid (Trouw Nutrition International, BE). DM; Dry matter. SAT and UNSAT diets were iso-caloric.

Table 2. Biochemical composition of bovine serum collected on the last day of each dietary period (Day 28).

	Group A			Group B		
	Control <u>serum</u>	SAT1 <u>serum</u>	UNSAT2 <u>serum</u>	Control <u>serum</u>	UNSAT1 <u>serum</u>	SAT2 <u>serum</u>
Total protein (g/dl)	7.37±0.86	7.23±1.17	7.25±0.21	6.57±0.51	7.07±0.75	7.27±0.55
Urea (mmol/l)	5.27±0.54 ^{ab}	5.99±1.26 ^a	4.33±0.47 ^b	5.11±0.95	5.11±1.21	4.22±0.77
NEFA (mmol/l)	0.12±0.04 ^a	0.16±0.04 ^b	0.12±0.01 ^a	0.11±0.01 ^x	0.22±0.06 ^y	0.23±0.19 ^{xy}
Total cholesterol (mg/dl)	125.6±17.6 ^a	158.7±27.2 ^a	206.5±28.7 ^b	118.0±21.3 ^x	160.7±40.0 ^y	162.0±43.7 ^y
HDL cholesterol (mg/dl)	68.0±6.24 ^a	91.3±12.86 ^b	103.0±4.24 ^b	68.3±2.08 ^x	97.0±7.55 ^y	75.7±15.2 ^{xy}
Triglycerides (mg/dl)	28.0±4.00	22.3±11.93	28.0±7.07	34.0±6.56	32.0±12.49	25.7±6.81
Total anti-oxidant capacity (mmol/l)	1.10±0.01 ^{ab}	1.06±0.03 ^a	1.12±0.02 ^b	1.02±0.08 ^x	1.11±0.08 ^{xy}	1.17±0.04 ^y
Glucose (mmol/l)	3.61±0.25 ^a	3.83±0.33 ^{ab}	4.16±0.08 ^b	3.44±0.22 ^x	3.83±0.22 ^y	3.90±0.09 ^y
RQUICKI	0.75±0.10 ^a	0.53±0.18 ^b	0.78±0.02 ^a	0.83±0.06 ^x	0.61±0.06 ^y	0.67±0.12 ^y
IGF-I (ng/ml)	127±23.7 ^a	113±18.8 ^a	179±53.2 ^b	88±21.3	100±24.8	102±34.9

Data is shown as mean ± SD from three cows in each group. Cows in group A were fed control, saturated fat-rich (SAT1) and unsaturated-fat rich (UNSAT2) diets subsequently for 28 days each. Cows in groups B were fed control, UNSAT1 and SAT2 diets subsequently for 28 days each. Different superscripts within each group indicate significance difference at $P < 0.05$.

Table 3. Average fatty acid profiles of the total fat extract from bovine serum collected on the last day of each dietary period (Day 28).

Mean ($\mu\text{mol/l}$) $\pm$ SD	Group A			Group B		
	Control <u>serum</u>	SAT1* <u>serum</u>	UNSAT2* <u>serum</u>	Control <u>serum</u>	UNSAT1* <u>serum</u>	SAT2* <u>serum</u>
Palmitic acid (C16:0)	757 $\pm$ 124 ^a	1387 $\pm$ 409 ^b	947 $\pm$ 186 ^a	760 $\pm$ 31 ^x	799 $\pm$ 37 ^x	1328 $\pm$ 329 ^y
Stearic acid (C18:0)	988 $\pm$ 208 ^a	1063 $\pm$ 327 ^{a,b}	1620 $\pm$ 360 ^b	1035 $\pm$ 21 ^x	1501 $\pm$ 151 ^y	948 $\pm$ 230 ^x
Oleic acid (C18:1, ω-9)	639 $\pm$ 119.3	731 $\pm$ 105.52	661 $\pm$ 182.7	669 $\pm$ 49.1	587 $\pm$ 152.5	643 $\pm$ 98.8
Linoleic acid (C18:2, ω-6)	1274 $\pm$ 163 ^a	2239 $\pm$ 585 ^b	2850 $\pm$ 857 ^b	1137 $\pm$ 242 ^x	2219 $\pm$ 341 ^y	2183 $\pm$ 970 ^y
Linolenic acid (C18:3, ω-3)	679 $\pm$ 143.1 ^a	514 $\pm$ 79.6 ^a	1215 $\pm$ 190.3 ^b	638 $\pm$ 193.5 ^x	1031 $\pm$ 170.4 ^y	444 $\pm$ 182.4 ^x
Omega 3 fatty acids	869 $\pm$ 25.3 ^a	724 $\pm$ 108.6 ^a	1428 $\pm$ 239.1 ^b	816 $\pm$ 191.7 ^x	1247 $\pm$ 171.0 ^y	645 $\pm$ 162.6 ^x
Omega 6 fatty acids	1606 $\pm$ 220 ^a	2694 $\pm$ 724 ^b	3295 $\pm$ 938 ^b	1438 $\pm$ 237 ^x	2549 $\pm$ 357 ^y	2615 $\pm$ 975 ^{xy}
Omega 9 fatty acids	662 $\pm$ 121.9	752 $\pm$ 108.4	675 $\pm$ 184.6	689 $\pm$ 45.8	610 $\pm$ 147.7	661 $\pm$ 91.3
Saturated fatty acids	1855 $\pm$ 340	2552 $\pm$ 753	2669 $\pm$ 563	1900 $\pm$ 50 ^x	2389 $\pm$ 178 ^y	2371 $\pm$ 553 ^{xy}
Odd chain fatty acids	130 $\pm$ 24.2 ^a	76 $\pm$ 19.6 ^b	100 $\pm$ 11.0 ^{a,b}	137 $\pm$ 7.9 ^x	94 $\pm$ 21.0 ^y	68 $\pm$ 9.3 ^z
Omega 5 and 7 fatty acids	134 $\pm$ 21.0 ^a	207 $\pm$ 24.3 ^b	141 $\pm$ 49.7 ^{a,b}	132 $\pm$ 8.6 ^x	113 $\pm$ 23.1 ^x	207 $\pm$ 26.0 ^y
Total fatty acids	5256 $\pm$ 925 ^a	7005 $\pm$ 1698 ^b	8307 $\pm$ 1230 ^b	5112 $\pm$ 347 ^x	7000 $\pm$ 738 ^y	6567 $\pm$ 1635 ^{xy}
Omega 6: Omega 3 ratio	1.88 $\pm$ 0.18 ^a	3.68 $\pm$ 0.44 ^b	2.33 $\pm$ 0.13 ^a	1.79 $\pm$ 0.16 ^x	2.05 $\pm$ 0.12 ^x	3.99 $\pm$ 0.54 ^y
Saturated: Omega 3 ratio	2.15 $\pm$ 0.15 ^a	3.48 $\pm$ 0.50 ^b	1.91 $\pm$ 0.26 ^a	2.44 $\pm$ 0.70	1.94 $\pm$ 0.26 [§]	3.76 $\pm$ 0.96 [§]

Data are shown as mean $\pm$ SD from 3 cows in each group. SAT, saturated fat-rich diet; UNSAT, unsaturated fat-rich diet; *1 and 2 indicate the sequence of feeding. Different superscripts within each group indicate significance difference at $P < 0.05$. § indicates $P = 0.057$ between UNSAT 1 and SAT2

Table 4. Bovine embryo development in CONT serum samples from heifers in both experimental groups *versus* commercial serum control (FBS).

	Lab. control serum	Group A	Group B
	Commercial FBS	CONT serum	CONT serum
Total number of oocytes (n)	201	214	189
Cleaved zygotes n(%)	156 (77.61)	155 (72.43)	154 (81.48)
Blastocyst from oocytes matured n (%)	49 (24.38)	71 (33.18)	59 (31.22)
Blastocysts from cleaved zygotes n (%)	49 (31.41) ^a	71 (45.81) ^b	59 (38.31) ^{ab}
Blastocyst total cell number (mean±SD)	155.94±30.19	157.75±44.27	154.81±41.16

^{a,b} Data marked with different superscripts per row significantly differ between treatments ($P < 0.05$).

Table 5. Developmental potential of bovine embryos cultured in different serum conditions.

	Group A serum			Group B serum		
	Control	SAT 1	UNSAT2	Control	UNSAT1	SAT2
Total fertilized (n)	371	372	361	412	532	539
Cleavage (%)	275 (74.1)	278 (74.7)	250 (69.3)	289 (70.2)	385 (72.4)	393 (72.9)
Blastocysts/cleaved (%)	123 (44.7) ^a	102 (36.7) ^b	96 (38.4) ^{ab}	99 (34.3) ^{xy}	154 (40.0) ^{x§}	105 (26.7) ^{y§}
Total cell number (mean±SD)	120±24.3 ^{ab}	108±15.5 ^a	126±25.7 ^b	111±24.4	123±27.5	109±18.3
Apoptosis index (mean±SD)	0.06±0.04 ^a	0.10±0.05 ^b	0.06±0.03 ^a	0.08±0.04 ^{xy}	0.06±0.04 ^x	0.12±0.05 ^y

Data marked with different superscripts per row within each group significantly differ between treatments

($P < 0.05$). While § indicates tendency to be different from control ($P < 0.1$).

750 **Supplementary Table 1.** Details of primers used for qRT-PCT

Gene Symbol	Gene Name	Forward and reverse primer sequences (5'-3')	Product Size (bp)	Gene Bank Accession No.
H2AFZ	H2A histone family member Z	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	212	NM_016750
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	ACCCAGAAGACTGTGGATGG AYGCCTGCTTCACCACCTTC	247	BC102589
SOD2	Mitochondrial superoxide dismutase 2	GCTTACAGATTGCTGCTTGT AAGGTAATAAGCATGCTCCC	101	S67818.1
DNMT3A	DNA cytosine-5- methyltransferase 3 alpha	CTGGTGCTGAAGGACTTGGGC CAGAAGAAGGGGCGGTCATC	318	AY271299
SCL2A1	Solute carrier family 2 (facilitated glucose transporter) member 1	CTGATCCTGGGTGCGTTTCAT ACGTACATGGGCACAAAACCA	68	NM_174602.2
GPX1	Glutathione peroxidase 1	GCAACCAGTTTGGGCATCA CTCGCACTTTTCGAAGAGCATA	116	NM_174076.3
ACCA	Acetyl-coa carboxylase, carboxytransferase, alpha subunit	AAGCAATGGATGAACCTTCTTC GATGCCCAAGTCAGAGAGC	196	FN185963.1
G6PD	Glucose-6-phosphate dehydrogenase	CGCTGGGACGGGGTGCCCTTCATC CGCCAGGCCTCCCGCAGTTCATCA	264	NM_001244135.1
SLC2A4	Solute carrier family 2 (facilitated glucose transporter) member 4	TGCGCCATCTTGATGACTGT GGCCAGGGCCAATTTCAAAG	115	NM_174604.1
SREBF	Sterol regulatory element-binding protein	GACGGCCAGGTGAATCCAGA CAGGACCATCTCTGCCCTCA	224	NM_001113302.1
PLIN2	Lipid droplet-associated protein	CGTCCCTGTCAGCAATGCAGAG TGCGAGTTCTGGACAGGCTCTG	235	NM_173980
PTGS2	Prostaglandin-endoperoxide synthase-2	ATCTACCCGCCTCATGTTTCCT GGATTAGCCTGCTTGTCTGGA	187	AF031698
BCL2	B- cell lymphoma 2	GTCCCGCCTCTTCACCTTTTCAG GATTCTGGTGTTTCCCCGTTGG	147	NM_009741.4
SHC1SHC	Src homology 2 domain-containing transforming protein 1	GTGAGGTCTGGGGAGAAGC GGTTCGGACAAAGGATCACC	334	NM_001075305

751

752

Figure Legends

Fig. 1. Experimental design and timetable. After 4 weeks of feeding hay-based Control diet (CONT), heifers (n=6) were switched to carbohydrate-rich diets with high Saturated (SAT) or Unsaturated (UNSAT) fat content in 2 different sequences indicated by the number 1 and 2 after SAT and UNSAT. During each dietary period, Ovarian cycles were synchronized during each dietary period using intravaginal progesterone releasing device (PRID) and prostaglandin F₂ α injections (PG). Blood samples were collected at the end of each dietary period (Day 28) and serum was used later in bovine *in vitro* embryo culture system to test effects of SAT- and UNSAT- serum composition (including carry-over effects) on embryonic development and quality~~IVC~~.

Fig. 2. Gene expression of candidate genes in blastocysts produced in the presence of control, SAT and UNSAT serum supplementation from animal groups **A** and **B**. Candidate genes analysed are involved in regulation of fatty acid transport and synthesis (*SREBF*, *ACCA*, and *PLIN2*), Apoptosis (*BCL2*), oxidative stress (*SOD2*, *GPX1*, *SHC1-SHC*), metabolism (*G6PD*, *GADPH*, *SLC2A1* and *SLACA4*), PG synthesis (*COX2*) and methylation (*DNMT3A*). Data are shown as mean $\pm$ SEM of log 2 of fold change relative to *H2AFZ* gene expression. Bars with asterisks in SAT and UNSAT groups indicate significant difference at $P < 0.05$ compared to control ($\equiv 0$) whereas § indicates tendency ($P > 0.05$ and < 0.1).