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Lay Summary

Assisted conception is a vital technique for cattle breeding. Embryos can be produced by IVF and grow in a special substance known as embryo culture medium. Previous studies suggested that culture medium with a high level of nutrients may have a negative impact on embryo growth. Here, we developed a special culture medium with very low levels of carbohydrates, amino acids, and vitamins, which contained more lipids and a special compound, to make it easier for lipids to move into the cells. The cattle embryos in this culture medium used more lipids and less glucose, and develop much better than ones in the culture medium with high levels of nutrients. Our work provides a unique model to study embryo metabolism and to help improve culture medium.

Title: Lipid Enriched Reduced Nutrient Culture Medium Improves Bovine Blastocyst Formation

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Abstract

The refinement of embryo culture media is essential in improving embryo viability and *in vitro* production efficiency. Our previous work demonstrated that the nutrients (carbohydrates, amino acids, and vitamins) in traditional culture media far exceed the need for an embryo and producing developmentally competent embryos in a reduced nutrient environment is feasible. Here, we aim to evaluate the impact of exogenous lipid and L-carnitine supplementation on bovine blastocyst development and refine our RN condition further. Zygotes were cultured in the control medium (100% nutrients) and reduced nutrient media containing 6.25% of the standard nutrient concentrations supplemented with L-carnitine and lipid free or lipid rich BSA. Increased blastocyst development was observed in the reduced nutrient lipid rich medium compared to the other two groups. However, in both reduced nutrient conditions, blastocyst cell numbers were lower than those obtained in the control condition. We then examined the expression level of 18 transcripts correlated with lipid metabolism, glucose metabolism, redox balance, and embryo quality, along with mitochondrial DNA copy numbers, ATP productions, and lipid profile. The results indicated lipid metabolism, embryo quality, and redox enzyme related genes were upregulated while glucose related gene was downregulated in embryos derived from reduced nutrient lipid rich condition Finally, we identified that the lipid rich BSA has enriched linoleic, stearic, oleic, palmitic, and alpha-linoleic fatty acids, a lipid profile that may contribute to the increased lipid metabolism and improved blastocyst development of the bovine embryos under the reduced nutrient condition.

Lay Summary

Assisted conception is a vital technique for cattle breeding. Embryos can be produced by IVF and grow in a special substance known as embryo culture medium. Previous studies suggested that culture medium with a high level of nutrients may have a negative impact on embryo growth. Here, we developed a special culture medium with very low levels of carbohydrates, amino acids, and vitamins, which contained more lipids and a special compound, to make it easier for lipids to move into the cells. The cattle embryos in this culture medium used more lipids and less glucose, and develop much better than ones in the culture medium with high levels of nutrients. Our work provides a unique model to study embryo metabolism and to help improve culture medium.

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Introduction

In vitro embryo production (IVP) is a reliable and cost-effective technique increasingly used in cattle breeding. In the last decade, the number of bovine blastocysts produced *in vitro* transferred to a recipient has significantly increased worldwide (Viana 2021). However, improvement of pregnancy rates upon transfer of IVP embryos is still a major concern in cattle farming, with significant financial implications (Ferre, et al. 2020, Hansen 2020). This might be correlated with reduced oocyte quality after in vitro maturation, , suboptimal in vitro culture (IVC) media that resulted in poor embryo quality, cryopreservation of the embryos, pregnancy losses due to poor uterine environment or transfer techniques, etc. (Ferre, et al. 2020, Hansen 2020). Technological advances have allowed the study of metabolite use in individual embryos *in vitro*, indicating mouse, bovine, human, and feline embryos utilize less than 20% of the metabolites supplied in culture medium (Herrick, et al. 2020, Krisher, et al. 2015). In mouse, it has been possible to produce embryos *in vitro* using a reduced nutrient medium (Ermisch, et al. 2020). However, elevated concentrations of pyruvate and lactate in the first step medium and essential amino acids (EAA) and glutamine in the second step medium were needed to support embryo implantation and development after transferring these embryos to a recipient (Ermisch, et al. 2020). We recently reported that the percentage of bovine blastocyst formation was not affected until nutrient provision was decreased to 6.25% of standard medium concentrations (Herrick, et al. 2020). These results open a new frontier in medium formulation for in vitro embryo culture in cattle, suggesting embryos might not need such a nutrient rich environment for successful development.

Fatty acids are essential molecules that support cellular membrane structure and promote inter- and intra- cellular signaling (Albanese and Dainiak 2003), as well as being a potent source for the production of ATP via fatty acid beta-oxidation (Schulz 2008). Embryos metabolize fatty acids during pre-implantation development in several species, including humans (Haggarty, et al. 2006), mouse (Hillman and Flynn 1980) and cattle (Sutton-McDowall, et al. 2012). In cattle, fatty acid metabolism has been correlated with supplementation of L-carnitine to the culture medium. Particularly, addition of L- carnitine has been beneficial for lipid metabolism and mitochondrial function (Takahashi, et al. 2013), allowing improved embryo development (Sutton-McDowall, et al. 2012) and increased lipid metabolism (Held-Hoelker, et al. 2017, Takahashi, et al. 2013). L-carnitine is needed for the transport of fatty acids into mitochondria where beta-oxidation occurs (Chankitisakul, et al. 2013). Another function of L-carnitine for embryo development is through its antioxidant action to the mitochondria that was described previously (Jiang, et al. 2019, Truong, et al. 2016, Wu, et al. 2011). However, compared to carbohydrates and amino acids, little is known about the effect of fatty acid supplementation during IVC on bovine embryo development. Previous work in our laboratory has demonstrated that bovine embryos become increasingly dependent on fatty acid oxidation when cultured with reduced concentrations of carbohydrates, amino acids, and vitamins (Herrick, et al. 2020). Importantly, the culture medium used in that study contained a preparation of bovine serum albumin (BSA) with very low levels of fatty acids (essentially fatty acid-free), so the primary source of fatty acids available to the embryos would have been

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intracellular lipids. The medium also did not include L-carnitine, an essential co-factor for entry of fatty acids into the mitochondria that has been shown to stimulate lipid metabolism and embryo development (Sutton-McDowall, et al. 2012, Takahashi, et al. 2013).

We hypothesized that the development of bovine embryos cultured in a reduced nutrient medium would be improved if the embryos are provided with L-carnitine and additional extracellular fatty acids. The objective of this study was to compare the development of embryos cultured in our standard medium with a full complement of carbohydrates, amino acids, and vitamins and minimal extracellular lipids (lipid free BSA) with that of embryos cultured in a reduced nutrient medium with L-carnitine and the absence or presence of extracellular fatty acids. To better understand the relationship of metabolic regulation and embryo development, we also examined expression of metabolic genes, along with relative mitochondrial DNA (mtDNA) copy number, ATP production, and lipid content of individual blastocysts. Understanding the molecular mechanisms related to blastocyst development together with improved formulation of IVC medium will help improve IVP technologies for agricultural and biomedical purposes.

Materials and Methods

Unless specified otherwise, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The gas concentrations used for in vitro maturation (IVM) and in vitro fertilization were 7.5% CO_2 and atmospheric O_2, and 7.5% CO_2 and 6.5% O_2 for IVC. These gas concentrations are increased to compensate for

the elevation of our laboratory (~1830 m above sea level) and are approximately equal to 6.0% CO_2 and 5% O_2 at sea level (media pH = 7.2 to 7.3) (Herrick, et al. 2016a).

Quantitative analysis of fatty acid content of albumins

Samples of lipid free (FAF-BSA) and lipid rich BSA (FrV–BSA) were dissolved in MilliQ water at 100 mg/mL and analyzed in triplicate over two runs using gas chromatography – mass spectrometry (GC-MS) to determine differences in fatty acid content. To this end, fatty acids bound to albumin were extracted using organic solvent. Liquid samples (350 µL) were first acidified with 20 µL of concentrated HCl, then added with 2 mL of methanol containing 10 µg of C19:0 free acid as internal standard, and 4 mL of chloroform. After a brief vortexing, 1.15 mL of water was added, followed by 10-min vigorous mixing. Then the mixture was centrifuged at 3750 g for 10 min. Chloroform extract in the lower phase was recovered and the solvent was removed under nitrogen. To the dried sample, 200 µL of 3 M methanolic HCl was added and the sample was incubated at 60°C for 1 h. After derivatization, the sample was cooled to ambient temperature, added with 300 µL of hexane and 900 µL of water, vortexed for 1 min, and centrifuged at 2500 rpm for 1 min. The hexane layer on the top was recovered and concentrated to various volumes to be injected to GC-MS. Samples (1 µL) were injected onto a Thermo Trace 1310 GC coupled to an ISQ-LT MS. The injector was held at 250 ℃ and a 30:1 split ratio. MS transfer line and source were both held at 250 ℃. FAME separation was achieved on a 30m DB-WAXUI column (J&W, 0.25 mm ID, 0.25 µm film thickness). The oven temperature was held at 200°C for 1 min, ramped at

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10°C/min to 250°C and held for 1 min. Mass detector was operated under full scan mode (50-650 m/z, 5 scans per sec) and electron impact ionization. Calibration curves were prepared by a series of dilutions of authentic standards of fatty acids which were derivatized as described above.

Oocyte In vitro maturation

Ovaries were collected at an abattoir by a commercial supplier (BPO Parts LLC, CO, USA) and transported to the laboratory in warmed 0.9% saline. Bovine cumulus-oocyte complexes (COC) were isolated from antral follicles (2-6 mm) within 2.5 h of ovary collection and washed 2-3 times in 3-(N-morpholino)-propanesulfonic acid (MOPS) - buffered medium. In vitro maturation was carried out for 22-24 h in groups of 5-7 COC in 50 µL drops of a defined maturation medium containing 50 ng/ml recombinant murine EGF, 0.1 IU/ml recombinant human FSH (Follistim, Merck & Co., Inc), 0.125 mg/ml recombinant human hyaluronan (Novozymes, Bagsvaerd, Denmark), and 2.5 mg/ml recombinant human albumin (AlbIX, Novozymes) under oil.

In vitro fertilization and embryo culture

After IVM, COC were washed using a commercial, serum-free fertilization medium (BO-IVF; IVF Bioscience, Falmouth, UK) and transferred to 45 µl drops of BO-IVF (10-12 COC/drop) under oil. Cryopreserved spermatozoa from a single bull were thawed and processed by density gradient centrifugation (45%:90%, PureSperm, Nidacon, Mölndal, Sweden), followed by two washes in a MOPS-buffered medium. Spermatozoa were diluted with BO-IVF medium

194 and added to drops containing COCs for a final concentration of 1 x 10^6
195 spermatozoa/mL. Gametes were co-incubated 18–20 h.
196 Presumptive zygotes were removed from fertilization drops and denuded of
197 remaining cumulus cells and loosely bound spermatozoa by shaking on a
198 vortex mixer for 2.5 min. After three washes in MOPS-buffered medium, groups
199 of 10 zygotes were randomly allocated to 20 µL drops of serum-free, bovine
200 Optimized Embryo Culture Medium 1(bOEC1) (Herrick, et al. 2020). On day 3
201 (72 h in bOEC1, 96 h post-insemination), cleavage to at least the 2-cell stage
202 was evaluated and embryos with more than 4-cells were washed and
203 transferred to 20 µL drops of fresh, bovine Optimized Embryo Culture Medium
204 2(bOEC2) designed for compaction and blastocyst formation (Herrick, et al.
205 2020). For the final 96 h of culture, embryos were cultured in groups of 5 in 20
206 µl drops under oil.
207 Concentrations of salts (NaCl, KCl, KH_2PO_4, $CaCl_2$-$2H_2O$, $MgSO_4$-$7H_2O$, and
208 $NaHCO_3$), antibiotics (gentamicin, 25 µg/ml), macromolecules (hyaluronan,
209 0.125 mg/mL), and growth factors (insulin, transferrin, and selenium, ITS) were
210 kept the same in all treatments to maintain consistent osmolarity and pH.
211 Nutrients (glucose/fructose, citrate, lactate, pyruvate, amino acids, vitamins,
212 and EDTA) were diluted to 6.25% of control (reduced nutrient, RN). The control
213 medium was supplemented with 8 mg/mL lipid free BSA (Herrick, et al. 2016b).
214 The RN media were supplemented with 5 mM L-carnitine supplementation
215 (Sutton-McDowall, et al. 2012) and 8 mg/ml of lipid-free or lipid-rich BSA
216

217 **Determination of blastocyst cell number and allocation**

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Expanded, hatching and fully hatched blastocysts were fixed for 20 min in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) and then stored in PBS with 0.5% BSA (MP Biomedicals) until staining. Blastocysts were washed three times in PBS with 0.1% polyvinylpyrrolidone (PVP) and 0.1% Triton X-100 (TX100) and then permeabilized in PBS with 1.0% TX100 (30 min). After blocking (2 h) in PBS with 0.1% TX100, 0.1 M glycine, 0.5% BSA, and 10% (v/v) horse serum, blastocysts were incubated with primary antibodies (18 to 24 h, 4°C) for SRY-box 2 (SOX2, Biogenex, Fremont, CA, rabbit monoclonal, anti-human; AN579) and caudal type homeobox 2 (CDX2, Biogenex, mouse monoclonal, anti- human; MU392A) (Bakhtari and Ross 2014, Herrick, et al. 2016b) to detect inner cell mass (ICM) and trophectoderm (TE) cells, respectively. Following three washes in PBS with 0.1% PVP and 0.1% TX100, blastocysts were incubated (1 h) with secondary antibodies (Alexa Fluor 488 donkey anti-rabbit IgG (A-2126, SOX2) and Alexa Fluor 555 goat anti-mouse IgG (A-21424, CDX2; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Blastocysts were washed three times and mounted on a glass slide in ProLong Gold Antifade reagent containing DAPI (Life Technologies, Thermo Fisher Scientific). Cells were visualized using a fluorescent microscope (Olympus BX52) and counted using the manual count function of MetaMorph software. Cells positive for SOX2 were considered ICM cells and cells positive for CDX2 were considered TE cells (Fig 1B). The total number of cells in the blastocyst was calculated as the sum of SOX2 and CDX2 positive cells.

Gene expression analysis

Gene expression analysis was carried out by real-time quantitative PCR (RT-qPCR) for the following genes (Table 1): fatty acid metabolism: *ACADL*, *ACSL3, ECHS1*, *MT-CO2*, *CPT1B*, *PPARGC1A*; glucose metabolism: *HK1*, *LDHA*, *PDHA1*, *PDK1, TIGAR*, *TALDO1*; embryo quality: *PLAC8*, *POU5F1*, *PTGS2;* redox balance: *GLRX2*, *TXNRD1*, *SOD1*. A total of four biological replicates (pools of 8 hatching and fully hatched blastocysts) for each treatment (control, lipid free and lipid rich RN media) were used for after snap freezing in liquid nitrogen in 10 µL PBS + 0.01 % PVP. RNA extraction was performed using the PicoPure RNA Isolation Kit (Thermofisher Scientific, Waltham, MA) with on-column DNase treatment (Qiagen, Germantown, MD). Complementary DNA (cDNA) was synthesized using iScript™ cDNA Synthesis Kit (Bio-rad, Hercules, CA) following the manufacture's protocol. The cDNA samples were diluted 1:3 using RNase free water and stored at −20 °C until qPCR was run. For each biological sample, PCR reaction was performed in duplicate using 12.5 µL Power SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, CA), 2.5 µL 10 µM primer mix and 5 µL 1:3 diluted cDNA sample. The qPCR program was: 50° C for 2 min for first cycle, 95 °C for 2 min for second cycle followed by 40 cycles of amplification step at 95 °C for 10 s and 59 °C for 1 min. A melting curve was analyzed for each experiment to assess the specificity of primer amplification. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). Normalization of Ct values was obtained using expression of *GAPDH*.

Mitochondrial DNA copy number of individual blastocysts

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The quantification of relative mtDNA copy number per cell was carried out using individual blastocysts collected from control, RN lipid free and RN lipid rich treatments as previously described (Wu, et al. 2015). Briefly, blastocysts were washed in 1X PBS without calcium and magnesium with 0.01% PVP, collected individually and stored in 10 µL PBS + 0.01% PVP at −80°C. DNA extraction was carried out using the QIAamp DNA micro kit (Qiagen, Germantown, MD) according to the manufacturer's protocol with RNA carrier (1 µg/µL; supplied with the kit) added to each sample. The DNA samples were analyzed using RT-qPCR to calculate mtDNA copy number relative to the amount of nuclear DNA. Primer sequences for amplification of mtDNA and nuclear DNA (*i.e. GAPDH*) are detailed in Table 2. RT-qPCR of mitochondrial DNA and nuclear DNA was performed simultaneously for each sample in triplicate using SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and Quantstudio 5 real time machine (Applied Biosystems). The program of amplification was as follows: 50° C for 2 min for first cycle; 95° C for 2 min for second cycle; 95° C per 15 sec, 60° C for 30 sec and 72°C per 1 min for 40 cycles. The Ct value for *GAPDH* was subtracted from that for bovine mtDNA region to give the ΔCt value. mtDNA copy number per nuclear genome (two *GAPDH* gene copies) was calculated as $2\times2^{\wedge\Delta Ct}$. DNA samples of each category were compared on the same RT-qPCR plate in order to produce comparable results.

Lipid profile of individual blastocysts

Individual blastocysts obtained from control and RN lipid rich treatments were washed several times using PBS + 0.01% PVP before collection using 1 µL of PBS + 0.01% PVP, snap freezing and storage at -80°C. Lipids were extracted

292 from D7 blastocysts using 100% methanol, and the extracts were profiled using
293 a chromatographically coupled time of flight mass spectrometer (LC-MS).
294 Briefly, a lipid extract of each sample was injected onto a Waters Acquity UPLC
295 system in discrete, randomized blocks with a pooled QC injection after every 8
296 sample injections. The sample injections were then separated using a Waters
297 Acquity UPLC CSH Phenyl Hexyl column (1.7 µM, 1.0 x 100 mm), using a
298 gradient made of 2mM ammonium hydrioxide + 0.1% formic acid and
299 acetonitrile + 0.1% formic acid. The column and samples were held at 65 °C
300 and 6 °C, respectively. The column eluent was infused into a Waters Xevo G2
301 TOF-MS with an electrospray source in positive mode. The scanning was run
302 using 50-2000 m/z at 0.2 seconds module, alternating MS (6 V collision energy)
303 and MSE mode (15-30 V ramp). Calibration was performed using sodium iodide
304 with 1 ppm mass accuracy. The capillary voltage was held at 2200 V, source
305 temp at 150 °C, and nitrogen desolvation temperature at 350 °C with a flow rate
306 of 800 L/hr. Data analysis was run using a non-targeted data acquisition (GC-
307 MS and UPLC-MS). For each sample, raw data files were converted to .cdf
308 format, and matrix of molecular features as defined by retention time and mass
309 (m/z) was generated using XCMS software in R (Smith, et al. 2006) for feature
310 detection and alignment. Raw peak areas were quantile normalized. While
311 outlier injections were detected based on total signal and PC1 of principle
312 component analysis. The mean area of the chromatographic peak was
313 calculated among replicate injections for QC samples. Features were grouped
314 using RAMClustR (Broeckling, et al. 2014), which groups features into spectra
315 based on co-elution and covariance across the full dataset. Spectra are used
316 to determine the identity of observed compounds in the experiment.

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Compounds were annotated based on spectral matching to in-house, NISTv14, 1- SToP spectral databases (Broeckling, et al. 2016).

ATP quantification

Individual blastocysts obtained from control, lipid free and lipid rich RN treatments were collected in 10 µL of PBS + 0.01% PVP and frozen at -80°C. ATP concentrations were determined by the ATP bioluminescent somatic cell assay kit (Sigma Chemical Co., St. Louis, MO) as previously described with minor modifications (Cukurcam, et al. 2004, Silva, et al. 2015, Simsek-Duran, et al. 2013). Briefly, 1:5 diluted ATP assay mix was added to individual wells in an opaque 96-well plate. In a separate tube, somatic cell ATP-releasing reagent was mixed with each sample or standards, and added to the assay mix. The amount of light emitted was immediately measured using a Synergy 2 plate reader for luminescence (BioTek, Winooski, VT). ATP concentration was calculated by comparison to a standard curve ranging from 60 fmol to 2 pmol/100 µL and normalized (per cell) using the average total cell number for blastocysts from each treatment.

Statistical analysis

Statistical analyses were completed using IBM SPSS (IBM, Armonk, NY). Student's T-test (two treatments) or one-way ANOVA (multiple comparisons) were conducted depending on the number of experimental groups. For the analysis of lipid content, ANOVA was conducted on each compound using the analysis of variance function of R package. For this analysis, P-values were adjusted for false positives using the Bonferroni-Hochberg method using

342 p.adjust function of R package. Principal Component Analysis (PCA) was
343 performed on mean-centered and pareto variance-scaled data using the R
344 package pcaMethods (Stacklies, et al. 2007). Results were considered
345 statistically significant when $P < 0.05$. Unless otherwise stated, results are
346 presented as mean ± S.E.M.
347
348 **Results**
349 **Determination of fatty acid abundance in lipid free and lipid rich BSA**
350 The lipid rich BSA had a total concentration of 14.3 µg of fatty acids per mg of
351 albumin, whereas the lipid free BSA had 0.13 µg of fatty acids per mg of albumin
352 (Table 3). There was a higher concentration (µg per mg albumin) of several
353 fatty acids in lipid rich BSA with respect to lipid free BSA including linoleic
354 (C18:2, 3.21), stearic (C18:0, 3.07), oleic (C18:1, 2.87), palmitic (C16:0; 2.71),
355 and alpha-linoleic fatty acids (C18:3, 1.83) (Table 3). However, because the
356 overall amount of fatty acid was considerably lower, the total concentration of
357 even the most abundant fatty acid, palmitic acid, in lipid free BSA, was only
358 0.04 µg per mg of albumin compared to 2.7 µg palmitic acid per mg of albumin
359 in lipid rich BSA.
360
361 **Effect of culture environment on embryo development**
362 Our objective in this experiment was to investigate the effect of exogenous lipid
363 availability on embryo development in a RN culture environment. Embryo
364 cleavage (≥ 79.9%) was not different between treatments (Figure 1A).
365 However, blastocyst development (per cleaved embryos) was higher in the RN
366 lipid rich medium compared to the control and RN lipid free treatments (Figure

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1A). There was no difference in blastocyst hatching between embryos cultured in RN lipid rich and control media, while fewer blastocysts hatched in the RN lipid free treatment group (Figure 1A). Blastocysts produced in RN lipid free and RN lipid rich media had fewer TE, ICM, and total cells than those produced in control medium (TE: control, 160 ± 9.0; RN lipid free, 99.0 ± 5.9; RN lipid rich, 99.0 ± 9.1; ICM: control, 43.0 ± 4.0; lipid free, 19.0 ± 2.9; RN lipid rich, 25.0 ± 6.1; total: control 204.0 ± 10.2, RN lipid free, 124.0 ± 8.7; RN lipid rich, 118.0 ± 7.3, Figure 1B-C)

Effect of culture environment on blastocyst gene expression

Amongst the fatty acid metabolism genes, *MT-CO2* had higher expression ($P<0.05$) in both RN lipid free and lipid rich media, and *ECHS1* was also upregulated in the RN lipid free ($P<0.05$) and RN lipid rich ($P=0.08$) media when compared to control medium. *PPARGC1A* followed the same trend, and were upregulated ($P<0.05$) in both RN lipid free and lipid rich media (Figure 2A). The glucose metabolism genes *HK1* had a lower expressions in both RN lipid free ($P<0.05$) and RN lipid rich ($P=0.05$) media, and *LDHA* was also downregulated ($P<0.05$) in both RN conditions when compared to the control medium (Figure 2B). The embryo quality related gene, *PLAC8*, was upregulated ($P<0.05$) in both RN lipid free and RN lipid rich media, and the redox enzyme, *TXNRD1,* was also more abundant in RN lipid free ($P<0.05$) and RN lipid rich ($P=0.09$) media when compared to the control medium (Figure 2C).

Effect of culture environment on blastocyst mtDNA copy number and ATP content

To determine whether the provision of fatty acids in a reducing nutrient environment had an effect on mitochondrial number, the relative mtDNA copy number was assessed. Interestingly, blastocysts produced in RN lipid free medium had lower relative mtDNA copy number than those produced in control and RN lipid rich media (Figure 3A). Blastocyst ATP content normalized per cell was not significantly different between embryos produced in control, RN lipid free, and RN lipid rich media (Figure 3B).

Effect of culture environment on individual blastocyst lipid profile

The principal component analysis using blastocyst lipid content identified 2 different clusters that separated the blastocysts obtained from the control and those produced with RN lipid rich medium (Figure 4A). A total of 43 lipids were annotated, 12 of which were significantly reduced in blastocysts cultured in RN lipid rich compared to control (Figure 4B).

Discussion

Our previous work demonstrated that bovine embryos only used a small amount of the nutrients, and the blastocyst formation was not affected until nutrient concentrations were reduced to 6.25% of the nutrients present in control condition (Herrick, et al. 2020). Such remarkable resilience to reduced concentrations of nutrients may be explained by the ability of bovine embryos to utilize their internal stored lipids as the energy source when other nutrients are scarce. Therefore, We hypothesized that promoting fatty acid oxidation may restore the declined blastocyst formation in the 6.25% RN condition seen in our previous study (Herrick, et al. 2020). When L-carnitine is included in the 6.25%

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RN condition, we observed the blastocyst development was restored when compared with the 100% control, and surprisingly, a significantly improved blastocyst development was observed when growing bovine embryos in the 6.25% RN condition with supplementation of lipid rich BSA (i.e. FrV BSA) and L-Carnitine. It is well documented that lipid metabolism, especially the utilization of endogenous lipid, is indispensable for energy production and proper development of bovine embryos (Sutton-McDowall, et al. 2012, Takahashi, et al. 2013). The importance of lipid metabolism on bovine embryo development was also demonstrated in our previous work, in which the use of a fatty acid oxidation inhibitor, etoxomir, blocked blastocyst formation and hatching (Herrick, et al. 2020). However, whether exogenous lipid supplementation would benefit bovine embryo development is debated. Replacing lipid rich fetal bovine serum (FBS) with low lipid BSA resulted in improved bovine embryo cryotolerance and quality (Del Collado, et al. 2015, Rizos, et al. 2003). It is suspected that the high lipid content in FBS may result in excessive lipid accumulation, therefore, resulted in comprised embryo quality (Del Collado, et al. 2015). In a recent mouse study, we also observed compromised blastocyst development when lipid rich albumins was supplemented in the culture media and compared with the lipid free albumins (Logsdon, et al. 2021). However, the variability observed in different albumin products may be not due to the amount of lipid, instead, the different lipid profiles and variations of other contaminants in these albumins may have played bigger roles in embryo development (Logsdon, et al. 2021). Another important note from our mouse study was that addition of L-Carnitine mitigated the blastocyst developmental differences between the lipid rich and lipid free albumin (Logsdon, et al. 2021). L-Carnitine

442 can enhance lipid metabolism and mitochondrial activity during embryo
443 development by facilitating fatty acids transportation into mitochondria, where
444 ATP is generated by fatty acid beta-oxidation (Held-Hoelker, et al. 2017). In this
445 study, we confirmed the positive effect of exogenous lipid supplementation and
446 the importance of L-Carnitine supplementation to facilitate lipid metabolism
447 during embryo development. As shown in earlier studies, when access to other
448 nutrients are readily available, excessive lipid supplementation may be
449 detrimental to bovine embryo development (Del Collado, et al. 2015, Rizos, et
450 al. 2003). Therefore, it is important to note that the positive effect we seen here
451 may depend on the availability of other nutrients.
452
453 In order to elucidate what specific fatty acid profiles are associated with
454 improved embryo development in the lipid rich RN medium, we performed lipid
455 content analysis on both lipid free BSA and lipid rich BSA. These two BSAs
456 have different concentrations of linoleic, stearic, oleic, palmitic, and alpha-
457 linoleic fatty acids (table 3), which are classified as non-esterified fatty acids
458 (NEFA). Oleic acid was reported as an important energy source and cellular
459 structure in both oocyte and embryo development (Fayezi, et al. 2018), and
460 high concentration of linoleic and oleic acid in the embryo culture medium
461 resulted in improved bovine embryo recovery from thawing (Karasahin 2019).
462 It is also important to note that the concentrations of the fatty acids used in our
463 study were not as high as those used in other work. Therefore, the beneficial
464 effect of exogenous lipid supplementation on embryo development could be
465 related to the specific fatty acid profiles instead of the quantity, and the fatty

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466 acids we identified here may be important candidates to constitute an optimal
467 fatty acid profile for embryo development.
468
469 Analysis of genes involved in this study gives us a few hints on how RN
470 environment controls the embryo metabolism and development. *HK1* controls
471 phosphorylation of glucose to glucose-6-phosphaste (G6P) and is the gateway
472 enzyme of glucose metabolism that plays a central role in modulating multiple
473 signaling pathways. The activity of hexokinase is closely related to glucose
474 consumption, and progressively increased in embryos at morula stage (Banliat,
475 et al. 2022, Houghton, et al. 1996). *LDHA* is the gene that is highly expressed
476 in bovine blastocysts (Harvey, et al. 2007) and controls the conversion of
477 pyruvate to lactate and participate in maintaining redox balance by converting
478 NADH to NAD+. Both *HK1* and *LDHA* were downregulated and genes involved
479 in fatty acid metabolism and mitochondrial oxidation (*ECHS1*, *MT-CO2,*
480 *PPARGC1A*) were upregulated in blastocysts collected from the RN media.
481 These results indicate that pyruvate was not preferentially converted to lactate
482 in the RN media. Instead, it may be routed to the mitochondria and, along with
483 fatty acids, used for ATP production via oxidative phosphorylation. These
484 results also suggested that embryos from RN environment preferentially relied
485 on fatty acids as the energy source. The shift from glycolysis to fatty acid
486 oxidation could affect the proliferation rate, which might explain the reduced cell
487 number in RN derived embryos (De Oliveira and Liesa 2020). In addition, such
488 shift in energy source may result in less regeneration of NAD+ and more
489 production of ROS through oxidative phosphorylation, challenging the ability of
490 the embryos in maintaining proper redox balance. This speculation is supported

by fact that higher level of redox enzyme *TXNRD1* was observed in embryos from RN environment, therefore, providing a higher antioxidant capacity to counterbalance the elevated ROS level in the embryos. The upregulation of *PLAC8* in the blastocysts from RN conditions is also interesting. It's upregulation in more competent bovine blastocysts have been shown in at least three different studies (Salilew-Wondim, et al. 2021). PLAC8 promotes autophagy and improves the proliferation of human trophoblast cells (Feng, et al. 2021), suggesting its potential role in improving bovine embryo implantation via the mechanism of PLAC8-induced autophagy in trophoblast cells.

Blastocysts cultured in RN lipid rich medium had a higher mtDNA copy number than those from RN lipid free medium. This is the sign that embryos in RN lipid rich medium tend to increase the number of mitochondria and utilize more fatty acids as the energy source and shift their metabolism to favor mitochondrial oxidative phosphorylation. Our lipid profile analysis of individual blastocyst supported this notion, as 12 out of 43 annotated lipids containing fatty acids were significantly reduced in blastocysts cultured in RN lipid rich medium compared to the control. However, the ATP production of individual cells remain similar among all three conditions. The similar ATP production suggested that embryos under different culture conditions have similar cellular energy demands to support their development. However, the embryos in the RN lipid free condition may experience environmental stress due to nutrient availability. Therefore, mitochondrial replication in these embryos is compromised. As a result, each individual mitochondrial may have to have elevated oxidative phosphorylation activities to meet the ATP demand. It is important to note that

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the regulation of mitochondrial function, energy production, and metabolism is a highly complex process involving multiple factors and signaling pathways (reviewed in (Harvey 2019)). Additional characterizations, such as mitochondrial activities, redox status, embryo transcriptional and translational activities, etc., may help us better understand the underlying mechanisms.

In summary, blastocyst development was significantly improved after supplementing fatty acids and L-carnitine to a medium with RN concentrations. The mechanism underlying this phenomenon may be related to increased lipid metabolism in the mitochondria in the RN environment. It is important to note that the benefit of supplementing fatty acid may depend on the availability of other nutrients. The reduced blastocyst cell numbers and increased expression of embryo quality related genes provided conflicting interpretations of the quality of the resulting embryos. Future in-depth analyses of transcriptome, proteome, and post-transfer embryo viability will be needed to fully evaluate the metabolic mechanisms and developmental competence of the embryos produced from the RN environment. The novel RN media may also provide a unique model to study embryo metabolism and facilitate the optimization of culture media. Ultimately, the ability of bovine embryos to develop in an environment with such low concentrations of carbohydrates and amino acids demonstrated the remarkable plasticity of preimplantation embryo development and urged us to re-examine what should be considered as optimal IVC environment.

Figure Legends

Figure 1. Effects of culture environment on embryo development and blastocyst cell numbers. (A) Embryo development in control medium and reduced nutrient (RN) media supplemented lipid free and lipid rich BSA (control, n = 587; RN lipid free, n = 573; RN lipid rich, n = 585, the experiments were replicated seven times). The percentage of cleaved embryos was calculated of the total number of cumulus-complexes oocytes matured and fertilized (cleaved/COC); the percentage of blastocysts (blastocyst/cleaved) and of hatching/fully hatched blastocysts (hatching/cleaved) of cleaved embryos were calculated. (B) A representative image of a bovine blastocyst stained by CDX2 (red) and SOX2 (green) to assess blastocyst cell numbers. Scale bar, 20 μm. (C) The number of cells in the trophectoderm (TE) and inner cell mass (ICM), and the total number of cells were determined in bovine blastocysts from control (n = 39), RN lipid free (n = 6) and RN lipid rich (n = 20) media. Data are reported as mean ± SEM. Different superscripts indicate a significant difference between treatments ($P<0.05$).

Figure 2. Effect of culture environment on blastocyst gene expression related to fatty acid metabolism (A), glucose metabolism (B) and embryo quality and redox balance (C). The gene expression value was arbitrarily set to 1 for samples from the control medium. Comparisons were made between samples from the control medium with the ones from RN lipid free and RN lipid rich media. The experiments were replicated four times. Data are reported as

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565 mean ± SEM. Different superscripts indicate a significant difference between
566 treatments (P<0.05).
567
568 **Figure 3.** Mitochondrial copy number and ATP quantification of individual
569 bovine blastocysts produced in different culture environments. (A) The
570 quantification of relative mtDNA copy number per cell of individual blastocysts
571 were compared between control (n=19), RN lipid free(n=18) and RN lipid rich
572 media (n=19). The experiments were replicated three times. (B) ATP
573 quantification of individual bovine blastocysts were compared between control
574 (n=15), RN lipid free (n=15), and RN lipid rich media (n=15). Data are shown
575 as pmoL/cell obtained by dividing the ATP content of individual blastocysts by
576 the average total cell number for that treatment. The experiments were
577 replicated three times. Data are reported as mean ± SEM. Different
578 superscripts indicate a significant difference between treatments (P<0.05).
579
580 **Figure 4.** Effect of culture environment on individual blastocyst lipid contents.
581 (A) Principal component analysis obtained using lipid content of the
582 blastocysts produced with control (n = 14) and RN lipid rich media (n = 15). (B
583 and C) A total of 12 out of 40 tested lipids were different between blastocysts
584 from the control and RN lipid rich media. Compounds were annotated based
585 on spectral matching to in-house, NISTv14, 1 - SToP spectral databases
586 (Broeckling, et al. 2016).
587
588 **Declaration of Interest**
589 There is no conflict of interest that could be perceived as prejudicing the
590 impartiality of the research reported.

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Contributions

RP, RLK conceived the study; RP, JRH, AFE, JB, YY performed experiments; WBS, JPB, RLK contributed project administration; RP, MZ, YY analyzed data; RP, MZ, YY, RLK wrote the paper.

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Table 1. Primer sequences used for gene expression analysis.

Name ID	Accession Number ID	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
ACADL	NM_001076936.1	CCCGTGTCCAGACAATCTATG	GATGTGGGCAGATGTCTACTG
ACSL3	NM_001205468.2	TTGGCTTTCCTACGAAGATG	CCCTGGTCTCACAGAAGATG
CPT1B	NM_001034349.2	CCAAGAACATCTCCGGAAACA	ATTCCCTCCAGCCCTACTT
ECHS1	NM_001025206.2	TCAGAGTGAAGGCCTTGTTG	CAAGGCCTAATGTGACCTGAA
HK1	NM_001012668.2	CTGCTTGACAAAGCCATCAA	CGTCGTAGCCACAGGTCAT
GAPDH	NM_001034034.2	TCATCATCTCTGCACCTTCTG	ATGCCAAAGTGGTCATGGA
GLRX2	NM_001040523.2	CCCGCACTAAGACCATGTA	CTGCATTTCCCAAAGATGA
LDHA	NM_174099.2	CAGATTGCAACCACTTCCA	GCAAGTTGCTTGTTGTTTCC
MT-CO2	NC_006853.1	CCAAGATGCAACATCACCAATC	TGGGTCAGCTTTGTCGTTAG
PLAC8	NM_001025325.2	TGAACGAATGCTGCCTATGG	CAGGCAATCCTTGCAAATGG
POU5F1	NM_174580.3	GCCAAGCTCCTAAAGCAGAA	TTGAAACTGAGCTGCAAAGC
TXNRD1	NM_174625.5	AGGCAGCCAAATATGACAAG	CGTAGGGCTTGACCTAACAA
PPARGC1A	NM_177945.3	TTGCCCAGATCTTCCTGAAC	CACTTGAGTCCACCCAGAAA
PDK1	NM_001205957.1	GTCACCAGCCAGAATGTTCA	TCCGATGAGATAGGCTTCCT
PTGS2	NM_174445.2	AAGATCTCCTTCCTGCGAAA	ATCAGGCACAGGAGGAAGAG
PDHA1	NM_001101046.2	AGAGTGCTGGTGGCATCTC	GAACGGTCTGCATCATCCT
SOD1	NM_174615.2	CTTCGAGGCAAAGGGAGATAC	CTTGTGTATTGTCTCCAAACTGATG
TIGAR	NM_001076370.1	GAGTGCCCAGCATTCACA	CATTCTGACCGGCTTCTTTC
TALDO	NM_001035283.2	GCGCCTCATTGAGCTGTA	CTGGGCGAAGGAGAAGAG

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Table 2. Primer sequences used for relative mitochondrial DNA copy number assay of individual bovine blastocysts.

Name ID	Accession Number ID	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
mtDNA region	AY526085.1	GGGCTACATTCTCTACACCAAG	GTGCTTCATGGCCTAATTCAAC
GAPDH	AB098985.1	ATATAGCTGCCTGACCTTTCTG	GGATTGGGAGCAACAGGTATTA

Table 3. Fatty acid content (µg per mg albumin) of lipid free and lipid rich BSA used in these experiments.

	Fatty Acid								
	Total	**Palmitic (C16:0)**	**Stearic (C18:0)**	**Oleic (C18:1)**	**Linoleic (C18:2)**	**Alpha-linolenic (C18:3)**	**Palmitoleic (C17:0)**	**Myristic (C16:1)**	**Magaric (C14:0)**
Lipid free BSA	0.13	0.04	0.03	0.03	0.00	0.00	0.00	0.01	0.01
Lipid rich BSA	14.33	2.71	3.07	2.87	3.21	1.83	0.31	0.16	0.17

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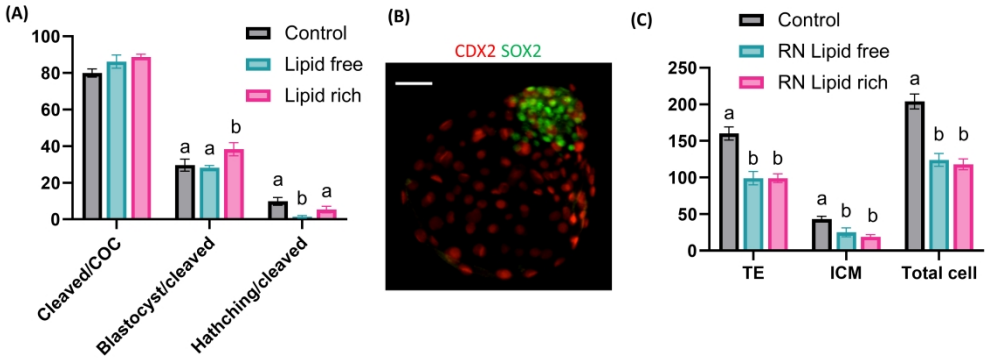

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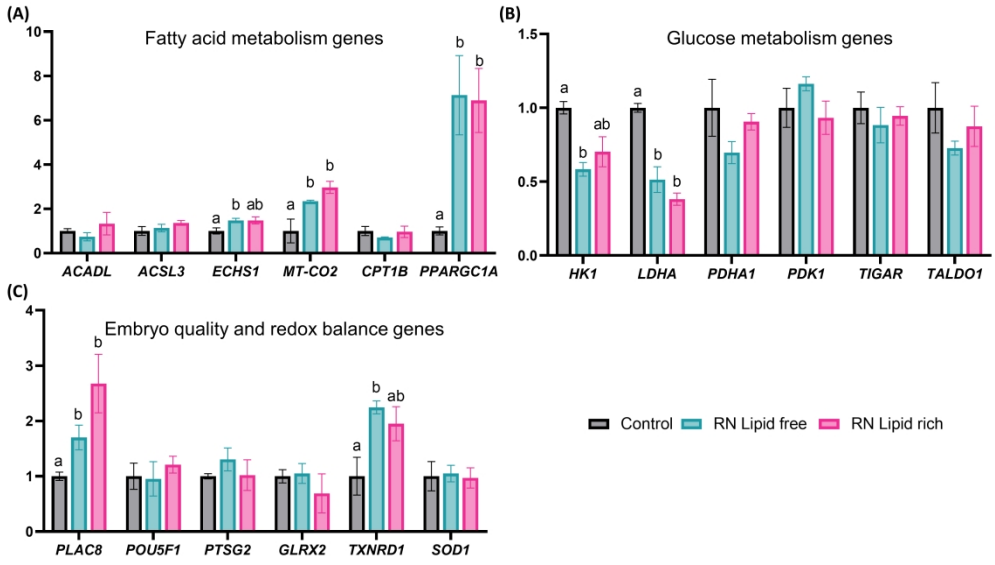

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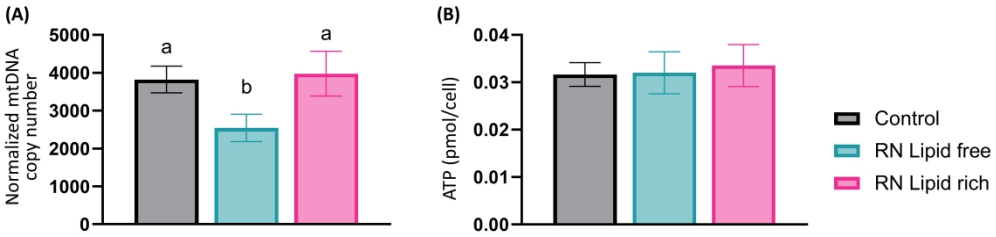
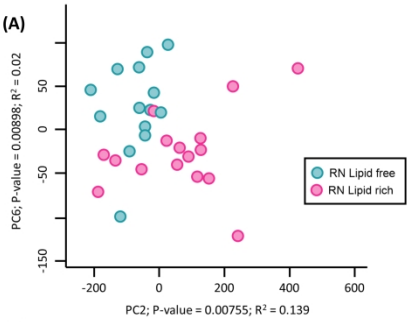


315x80mm (300 x 300 DPI)

(A)



(B)

Compound ID	P-value	Name of the lipid*
PC(36:2)	<0.05	phosphatidylcholine 36:2 (CHEBI:64433)
TG(56:3)	<0.05	triacylglycerol 56:3 (CHEBI:85843)
PC(32:1)	<0.05	phosphatidylcholine 32:1 (CHEBI:66849)
TG(53:2)	<0.05	triacylglycerol 53:2 (CHEBI:85739)
TG(56:2)	<0.05	triacylglycerol 56:2 (CHEBI: 132034)
PC(36:1)	<0.05	phosphatidylcholine 36:1 (CHEBI: 66857)
GlcCer(d18:1/18:0)	<0.05	β-D-glucosyl-N-octadecanoylsphingosine (CHEBI: 84719)
TG(54:2)	<0.05	triacylglycerol 54:2 (CHEBI: 85743)
TG(59:3)	<0.05	triacylglycerol 59:3 (CHEBI: 85738)
TG(53:3)	<0.05	triacylglycerol 53:3 (CHEBI: 85738)
TG(56:5)	<0.05	triacylglycerol 56:5 (CHEBI: 85751)
PC(36:3)	<0.05	phosphatidylcholine 36:3 (CHEBI: 64523)

(C)

Compound ID	P-value	Name of the lipid*
GlcCer(d18:1/24:0)	0.05	β-D-glucosyl-N-(tetracosanoyl)sphingosine (CHEBI: 84744)
TG(54:1)	0.09	triacylglycerol 54:1 (CHEBI: 90305)
TG(50:2)	0.09	triacylglycerol 50:2 (CHEBI: 84662)
PC(36:4)	0.09	phosphatidylcholine 36:4 (CHEBI: 64520)
TG(52:3)	0.10	triacylglycerol 52:3 (CHEBI: 84661)
TG(53:4)	0.10	triacylglycerol 53:4 (CHEBI: 85737)
DG(36:1)	0.14	diacylglycerol 36:1 (CHEBI: 86980)
Cer(d18:1/24:1)	0.14	N-(15Z)-tetracosenoylsphingosine (CHEBI: 74450)
SM(d18:0/16:1)	0.17	Sphingomyelin (d18:0/16:1(9Z)) (CHEBI: 90008)
TG(52:2)	0.18	triacylglycerol 52:2 (CHEBI: 85736)
PC(34:1)	0.19	phosphatidylcholine 34:1 (CHEBI: 64517)
GlcCer(d18:1/22:0)	0.24	β-D-glucosyl-N-(docosanoyl)sphingosine (CHEBI: 62107)
TG(54:3)	0.25	triacylglycerol 54:3 (CHEBI: 84659)
TG(52:1)	0.29	triacylglycerol 52:1 (CHEBI: 90302)
TG(53:1)	0.29	triacylglycerol 53:1 (CHEBI: 138351)
TG(52:4)	0.36	triacylglycerol 52:4 (CHEBI: 84660)
SM(d18:1/26:0)	0.36	N-hexacosanoylsphingosine-1-phosphocholine (CHEBI: 83361)
TG(54:4)	0.37	triacylglycerol 54:4 (CHEBI: 85742)
TG(50:1)	0.41	triacylglycerol 50:1 (CHEBI: 84665)
DG(38:5)	0.41	diacylglycerol 38:5 (CHEBI: 85704)
TG(58:1)	0.43	triacylglycerol 58:1 (CHEBI: 140903)
TG(51:3)	0.43	triacylglycerol 51:3 (CHEBI: 85731)
PC(34:2)	0.50	phosphatidylcholine 34:2 (CHEBI: 64516)
TG(56:7)	0.56	triacylglycerol 56:7 (CHEBI: 85749)
Cholesterol	0.6	cholesterol (CHEBI: 16113)
TG(56:6)	0.6	triacylglycerol 56:6 (CHEBI: 85750)
TG(51:1)	0.78	triacylglycerol 51:1 (CHEBI: 85733)
TG(49:1)	0.92	triacylglycerol 49:1 (CHEBI: 85728)
TG(54:5)	0.92	triacylglycerol 54:5 (CHEBI: 85741)

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