Fatty Acid Metabolism during Maturation Affects Glucose Uptake and is Essential to Oocyte Competence

Paczkowski, M.¹, Schoolcraft, W.B.¹,², Krisher, R.L.¹

¹ National Foundation for Fertility Research, 10290 Ridgegate Circle, Lone Tree, CO 80124, (303) 781-3040;

² Colorado Center for Reproductive Medicine, 10290 Ridgegate Circle, Lone Tree, CO 80124, (303) 788-8300

Corresponding author: Rebecca Krisher; National Foundation for Fertility Research, 10290 Ridgegate Circle, Lone Tree, CO 80124; rkrisher@fertilityresearch.org

Short Title (46 char)- Oocyte fatty acid metabolism affects development
Abstract

Fatty acid oxidation (FAO) is essential for oocyte maturation in mice. The objective of this experiment was to determine the effect of etomoxir (a FAO inhibitor; 100 µM), carnitine (1 mM), and palmitic acid (1 or 100 µM) during maturation on metabolism and gene expression of the oocyte and cumulus cells, and subsequent embryo development in the mouse. Carnitine significantly increased embryo development, while there was a decrease in development following maturation with 100 µM palmitic acid or etomoxir (P<0.05). Glucose consumption per cumulus-oocyte-complex (COC) was decreased after treatment with carnitine and increased following etomoxir treatment (P<0.05). Intracellular oocyte lipid content was decreased after carnitine or etomoxir exposure (P<0.05). Abundance of Glut1 was increased after etomoxir treatment in the oocyte and cumulus cells (P<0.05), suggesting stimulation of glucose transport and potentially the glycolytic pathway for energy production when FAO is inhibited. Abundance of Cpt2 tended to increase in oocytes (P=0.1) after treatment with 100 µM palmitic acid and in cumulus cells after exposure to 1 µM palmitic acid (P=0.07). Combined with carnitine, 1 µM palmitic acid increased abundance of Acs13 (P<0.05) and Cpt2 tended to increase (P=0.07) in cumulus cells, suggesting FAO was increased during maturation in response to stimulators and fatty acids. In conclusion, fatty acid and glucose metabolism are related in the mouse COC, as inhibition of FAO increases glucose consumption. Stimulation of FAO decreases glucose consumption and lipid stores, positively affecting subsequent embryo development, while an overabundance of fatty acid or reduced FAO negatively affects oocyte quality.
Introduction


During FAO the rate limiting step is the transport of fatty acids bound to carnitine across the mitochondrial membrane, which is catalyzed by carnitine palmitoyltransferase 1 (CPT1). Carnitine is then removed from the fatty acid via CPT2, allowing the fatty acid to undergo β-oxidation within the mitochondria (McGarry and Brown 1997). Supplementation of exogenous fatty acids, such as palmitic acid, and carnitine increase the abundance of Cpt1 in pancreatic β-cell lines, thereby increasing the rate of FAO (Assimacopoulos-Jeannet, et al. 1997). Conversely, exposure to the pharmacological CPT1 inhibitor etomoxir prevents the transport of fatty acids across the mitochondrial membrane and reduces FAO (Ferguson and Leese 2006).

The importance of FAO to oocyte nuclear maturation (attainment of metaphase II and extrusion of the first polar body) is correlated to the relative concentration of intracellular lipids within the oocytes of different species (Paczkowski, et al. 2013). Porcine oocytes, which contain relatively high intracellular lipid content, are more sensitive to FAO inhibition by etomoxir than mouse (few intracellular lipids) or bovine (moderate amount of intracellular lipids) oocytes.
Higher concentrations of etomoxir are required to block nuclear maturation in the mouse than in the pig, suggesting that mouse oocytes may not rely as heavily on FAO as other species (Paczkowski, et al. 2013). However, several studies demonstrate that FAO is required to complete nuclear maturation in the mouse (Downs, et al. 2009, Dunning, et al. 2010, Hewitson, et al. 1996, Paczkowski, et al. 2013, Valsangkar and Downs 2013). Exposure to high concentrations of etomoxir (250 µM) during maturation inhibits completion of meiosis in mouse oocytes (Paczkowski, et al. 2013). After exposure to a lower concentration of etomoxir (100 µM), oocytes are able to complete nuclear maturation (Dunning, et al. 2010, Paczkowski, et al. 2013), albeit with a decrease in embryo development to the blastocyst stage post fertilization (Dunning, et al. 2010).

The objective of these experiments was to evaluate the effect of a FAO stimulator (carnitine) and inhibitor (etomoxir), as well as a fatty acid (palmitic acid), on: oocyte and cumulus cell gene expression; metabolic parameters including oocyte lipid content, glucose consumption by the COC, and ATP quantification in oocytes and cumulus cells; and subsequent embryonic development and cell allocation in a complete maturation medium that supports oocyte competence. Based on our previous studies (Paczkowski, et al. 2013), the dosage of etomoxir (100 µM) was selected to allow completion of oocyte nuclear maturation while potentially compromising cytoplasmic maturation.
Materials and Methods

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise stated.

In Vitro Maturation, Fertilization, and Culture

B6D2F1 female mice, approximately three weeks of age, were administered pregnant mare serum gonadotropin (PMSG; 5 IU, i.p.; EMD Biosciences, Billerica, MA) 48 h prior to euthanasia. Ovaries were harvested and washed in GMOPS PLUS (Vitrolife; Englewood, CO) supplemented with 5% fetal calf serum (FCS; HyClone, Thermo Scientific, Rockford, IL). Oocytes surrounded by a complete layer of cumulus cells were recovered by ovarian dissection and matured in vitro for 18 h at 37 °C, 8.3% CO$_2$ and 6.3% O$_2$. Due to the increased altitude of Lone Tree, CO, the CO$_2$ was increased to maintain the pH of all media at 7.28. A completely defined oocyte maturation media (OMM) was supplemented with 0.5 mM glucose, 6 mM lactate (MP Biomedicals, Santa Ana, CA), 0.2 mM pyruvate, 10 ng/mL epidermal growth factor (EGF), 0.5 mg/mL G-MM™ Recombumin (Vitrolife), and 2 mg/mL fetuin. This medium is our standard mouse maturation medium, and successfully supports oocyte nuclear and cytoplasmic maturation. Cumulus-oocyte-complexes were matured in one of seven treatment groups: 1) control; 2) 1 mM carnitine (CARN); 3) 1 μM palmitic acid (1 μM PALM); 4) 100 μM palmitic acid (100 μM PALM); 5) 1 mM carnitine plus 1 μM palmitic acid (CARN + 1 μM PALM); 6) 1 mM carnitine plus 100 μM palmitic acid (CARN + 100 μM PALM); and 7) 1 mM carnitine plus 100 μM etomoxir (CARN + ETO). As EGF is present in the basal media, FAO is expected to be an active pathway in the COC; therefore, supplementation of carnitine to the basal medium may
further augment FAO. The concentrations of 1 and 100 µM palmitic acid used are similar to those reported in bovine follicular fluid during both a normal physiological state and negative energy balance with elevated lipolysis (Leroy, et al. 2005, Van Hoeck, et al. 2011). All mouse protocols were approved by the National Foundation for Fertility Research (NFFR) Ethics in Research Committee and followed animal care and use guidelines, as described by the *Guide for the Care and Use of Laboratory Animals* (Committee for the Update of the Guide for the Care and Use of Laboratory Animals and National Research Council 2010).

Cumulus-oocyte-complexes were transferred to Human Tubal Fluid (HTF; Hogan 1994) supplemented with 0.4 g bovine serum albumin (BSA; MP Biomedicals) post maturation. Spermatozoa were collected from the vas deferens and cauda epididymis from an adult B6D2F1 male (greater than 10 weeks of age) and incubated at 37 °C, 8.3% CO$_2$ and 6.3% O$_2$ in HTF for 1 h to allow for capacitation. Spermatozoa were further diluted in HTF and added to the fertilization drops for a final concentration of 1 x 10$^6$ sperm/mL. Gametes were then co-incubated for 6 h.

Presumptive zygotes were cultured in G1 PLUS (Vitrolife) at 37 °C, 8.3% CO$_2$ and 6.3% O$_2$ for 48 h. Cleavage was assessed 22 h post-insemination (hpi). After 48 h of culture, embryos were transferred to G2 PLUS (Vitrolife) for an additional 64 h and blastocyst development was assessed on day four (96 hpi). Blastocysts were stained for inner cell mass (ICM), trophectoderm (TE), and total cell number (Thouas, et al. 2001) on day five (112 hpi). Briefly, blastocysts were incubated in 300 µg/mL propidium iodide and 1.25% Triton X-100 in phosphate buffered saline for 5 sec and then incubated in 0.25 µg/mL bizbenzimide (Hoescht 33258) in 100% ethanol overnight at 4 °C. Blastocysts were mounted on glass slides in a drop of glycerol under a cover slip. Cell numbers were counted using fluorescent microscopy.
Quantitative PCR

Cumulus-oocyte-complexes were matured in vitro for 18 h as described above. Oocytes were mechanically denuded following IVM using a pulled glass pipette and cumulus cells were isolated from maturation media via centrifugation at 16,000 g for 5 min. Oocytes that had reached metaphase II, indicated by the extrusion of the first polar body, were frozen at -80 °C in groups of ten oocytes per treatment in PicoPure lysis buffer (Applied Biosystems; Carlsbad, CA). Four biological replicates of ten oocytes each were collected for each treatment. RNA was extracted using the PicoPure RNA Isolation Kit (Applied Biosystems) with on-column DNase treatment (Qiagen; Valencia, CA). Total RNA extracted from oocytes and cumulus cells were loaded into the cDNA reaction; therefore, resulting quantitative PCR (qPCR) data were analyzed by relative quantification as described below. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacture’s protocol. Primer design and qPCR were performed as previously described (Paczkowski, et al. 2011, Paczkowski, et al. 2013). Primers for genes related to FAO (acyl-Coenzyme A dehydrogenase long chain, Acadl; acyl-CoA synthetase long chain family member 3, Acsl3; carnitine palmitoyltransferase 2, Cpt2), glucose transport (glucose transporter 1, Glut1), and oxidative stress (glutaredoxin 2, Glrx2; thioredoxin reductase 1, Txnrd1) were designed for qPCR using Primer3 (Rozen and Skaletsky 2000). Peptidylprolylisomerase A (Ppia) was used as the reference gene as transcript abundance remained stable (not statistically different) between treatments. Accession number, primer sequence, and product length of target and reference genes are presented in Table 1. Primer specificity was determined by melt curve analysis and gel electrophoresis, and resulting PCR products were cloned into pCR 2.1 TOPO vectors and
transformed into One Shot TOP10 chemically competent *E. coli* (Invitrogen Life Technologies; Grand Island, NY). Plasmids were sequenced by the DNA sequencing facility at Colorado State University (Fort Collins, CO) to confirm the identity of the transcript and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen Life Technologies). Quantitative PCR was performed on three-fold diluted sample cDNA run in duplicate. Target genes were analyzed using iQ SYBR Green Supermix (Bio-Rad; Hercules, CA) and an Applied Biosystems 7300 Real Time PCR system. A standard curve was generated from serial dilutions of EcoRI digested plasmids ($10^7$ to $10^1$ molecules) and the efficiency of the primers was calculated.

**Metabolic Assays**

**Glucose Colorimetric Assay**

At 18 h post maturation (hpm), maturation media from the seven treatment groups was snap frozen and stored at -80 °C. For each treatment, spent (media that had been exposed to ten COC) and unspent media (media that had not been exposed to COC) were collected from each dish. Glucose concentrations were determined using the Glucose Assay Kit (BioVision; Mountain View, CA) following the manufacturer’s instructions. Briefly, 25 μL of media were diluted 1:2 and incubated with 2 μL glucose probe, 2 μL glucose enzyme mix, and 46 μL assay buffer for 30 min at 37 °C, protected from light. After incubation, samples were read immediately on an EPOCH microplate reader (BioTek; Winooski, VT). To determine glucose concentration (mM), the average amount of glucose (nmol) in each well was divided by the volume of sample analyzed. To determine glucose consumption (pmol/COC/h) during oocyte maturation, the concentration obtained from the spent media was subtracted from the average concentration of unspent media and converted to pmol glucose using the volume of the drop used.
for maturation (50 µL), the number of COC per drop (10 COC), and the length of maturation (18 h). It is important to note that glucose consumption values were not corrected for the number of cumulus cells per drop, but are based on the number of COC. A limit of this methodology is that if differences exist in cumulus cell number, resulting either from the initial cumulus investment or treatment effects on cumulus cell survival and/or viability, they are not reflected.

**ATP Concentration**

Cumulus-oocyte-complexes were matured in vitro and oocytes and cumulus cells were collected separately as described above. Denuded oocytes were pooled into three biological replicates of ten oocytes per treatment and frozen at -80 ºC in 10 µL phosphate buffered saline. ATP concentrations, in pmol ATP/oocyte, were calculated using the ApoSENSOR Assay Kit and standard curve (BioVision), following the manufacturer’s instructions.

**Lipid Content**

Lipid content was analyzed by Nile Red staining as previously described (Genicot, et al. 2005), with slight modifications. Briefly, after in vitro maturation, oocytes were denuded and fixed for a minimum of 24 h in 500 µL of a 2% glutaraldehyde and 2% formaldehyde solution. Oocytes were then stained overnight in 10 µg/mL Nile Red. Images were taken at 200x on a fluorescent microscope and analyzed using ImageJ (version 1.46, National Institutes of Health; Marei, et al. 2012). The mean signal intensity for each oocyte and background was determined three times and used to calculate the final signal intensity of each oocyte.

**Statistical Analysis**
Percent oocyte maturation, embryonic cleavage, blastocyst development (of total oocytes and of cleaved embryos) were analyzed using the generalized linear mixed model (GLIMMIX) procedure in SAS (SAS Institute Inc., Cary, NC, USA) with a binomial error distribution and a probit link function (Littell, et al. 1996), as each embryo was scored as a 1 or 0 depending on whether or not it achieved the desired stage of development (MII oocyte, cleaved embryo, or blastocyst; Herrick, et al. 2013). In all analyses, treatment was considered to be a fixed factor and replicate was included as a random factor. Cell number, glucose concentration, ATP concentration, and lipid content were normally distributed and were analyzed by a one-way analysis of variance (ANOVA) using NCSS (Number Cruncher Statistical System; Kaysville, UT). When ANOVA detected significant differences (P < 0.05), treatment differences were determined using a Fisher’s LSD multiple comparison test. For relative quantification, data were analyzed using the relative expression software tool, REST 2005 version 1.9.12 (Pfaffl, et al. 2002). Expression ratios were generated from Eq. 1 using PCR efficiencies (E) of the target and reference genes and the ΔCT values of the control and sample (treatment) oocytes or cumulus cells. The threshold cycle (CT) values represent the PCR cycle when the SYBR Green fluorescence rises above the background fluorescence, or threshold. The levels of significance were calculated by pair-wise fixed reallocation randomization tests with 50,000 iterations and significance was determined with a P-value less than 0.05.

Eq. 1: \[
\text{ratio} = \frac{(E_{\text{target}})^{\Delta CT \text{ target (control-sample)}}}{(E_{\text{ref}})^{\Delta CT \text{ ref (control-sample)}}}
\]
Results

Oocyte Maturation and Embryo Development

We hypothesized that embryonic development would be decreased after FAO inhibition during in vitro maturation (IVM), whereas a FAO stimulator, carnitine, would increase embryonic development. Furthermore, exogenous palmitic acid would increase embryonic development when supplemented alone or in conjunction with carnitine. There were no significant differences in the percentage of oocytes completing nuclear maturation in oocytes matured in CARN or CARN + ETO compared to control oocytes (P > 0.05; Table 2). Nuclear maturation of oocytes matured in 100 µM PALM and CARN + 100 µM PALM were significantly decreased compared to control oocytes (P < 0.05; Table 2). Cleavage of oocytes matured in CARN post fertilization was not different compared to control oocytes (P > 0.05; Figure 1); however, there was a significant increase in d4 blastocysts of both cleaved embryos and of total presumptive zygotes (P < 0.05) after oocyte maturation in CARN. Cleavage and d4 blastocyst of total presumptive zygotes was significantly reduced in the 100 µM PALM treatment compared to control oocytes (P < 0.05); however, the addition of carnitine (CARN + 100 µM PALM) was able to rescue embryo development on d4 such that it was not different than control (P > 0.05; Figure 1). Supplementation of CARN + ETO significantly decreased d4 blastocyst development (per zygote and per cleaved embryo) compared to controls (P < 0.05; Figure 1).

Because supplementation of 100 µM PALM was inhibitory to maturation and embryonic development, we subsequently analyzed these parameters after supplementation with a lower concentration of palmitic acid (1 µM PALM). Reducing the concentration of palmitic acid alone (1 µM) or in conjunction with carnitine did not significantly alter nuclear maturation, embryo
cleavage or the proportion of d4 blastocysts of total presumptive zygotes compared to control oocytes, respectively (P > 0.05). However, the proportion of d4 blastocysts of cleaved embryos was reduced (78.90% ± 3.93% vs. 88.99% ± 3.01%; P < 0.05) in the lower concentration of palmitic acid (1 µM) compared to control oocytes, respectively.

Supplementation of carnitine (CARN) or palmitic acid (1 µM PALM), or the combination of the two (CARN + 1 µM PALM), did not affect total cell number or the number of TE cells compared to control embryos (P > 0.05; Table 3). However, the number of cells within the ICM was significantly decreased in embryos derived from oocytes matured in 1 µM PALM or CARN + 1 µM PALM compared to controls (P < 0.05). Addition of etomoxir (CARN + ETO) during maturation significantly decreased the number of total, TE, and ICM cells (P < 0.05; Table 3). There were no significant differences in the ICM:TE ratio (data not shown) or the percent ICM cells of total blastocyst cells between any of the treatments and control (P > 0.05; Table 3).

Gene Expression

We hypothesized that transcript abundance of genes involved in FAO would be increased in the oocyte and cumulus cells after treatment with carnitine and/or palmitic acid, as stimulation of this metabolic pathway or provision of extracellular substrates may increase FAO pathway activity. However, in the presence of a FAO inhibitor (etomoxir), transcript abundance of FAO genes would decrease and abundance of glucose transport genes would increase in response to the metabolic needs of the oocyte and cumulus cells. Our results demonstrate that there tended to be an increase in abundance of Txnrd1 (P = 0.08), Cpt2 (P = 0.1), and Glrx2 (P = 0.09) transcripts in oocytes treated with CARN, 100 µM PALM, or CARN + 100 µM PALM,
respectively, compared to control oocytes (Figure 2). However, there were no significant differences in oocyte gene expression when the concentration of palmitic acid was lowered to 1 µM, in the presence or absence of carnitine (1 µM PALM, CARN + 1 µM PALM), compared to control oocytes (P > 0.05; Figure 2). Abundance of Cpt2 tended to be increased in cumulus cells after COC treatment with 1 µM PALM (P = 0.07) or CARN + 1 µM PALM (P = 0.07) compared to cumulus cells from untreated COC (Figure 3). There was a significant increase in abundance of Acsl3 in cumulus cells after COC treatment with CARN + 1 µM PALM compared to control cumulus cells (P < 0.05). Interestingly, there tended to be a decrease (P = 0.08) in Acsl3 in cumulus cells after treatment with CARN + 100 µM PALM; however, Acsl3 was not different when 100 µM PALM was added without carnitine. There was a significant increase in transcript abundance of Glut1 in oocytes and Cpt2 and Glut1 in cumulus cells in response to FAO inhibition (CARN + ETO) compared to untreated oocytes and cumulus cells (P < 0.05; Figures 2 and 3).

Metabolic Assays

We hypothesized that glucose consumption in the COC and intracellular lipid content of the oocyte would be increased while ATP concentration in the oocyte would be decreased after treatment with etomoxir compared to control oocytes, as the oocyte might increase glucose consumption to compensate for decreased energy production via FAO in the COC. Furthermore, supplementation of exogenous FAO stimulators and fatty acids would decrease glucose consumption and lipid content, as the oocyte increased its level of FAO. Our results demonstrated that there was a significant decrease in glucose consumption per COC after treatment with CARN compared to the control (P < 0.05; Figure 4A). There was no difference in
glucose consumption per COC after maturation in 1 or 100 \( \mu M \) PALM alone or in combination with carnitine compared to controls (\( P > 0.05 \); Figures 4A and 4B). There was also no significant difference in ATP concentration per oocyte after any experimental treatment compared to control oocytes, except that treatment with 1 \( \mu M \) PALM (0.19 \( \pm \) 0.04 pmol ATP/oocyte) significantly increased ATP concentration per oocyte compared to control oocytes (0.06 \( \pm \) 0.01 pmol ATP/oocyte; \( P < 0.05 \)).

Oocyte maturation in CARN or CARN + 1 \( \mu M \) PALM significantly reduced intracellular lipid content compared to control oocytes (\( P < 0.05 \); Figure 5A). However, there was no significant difference in lipid content in oocytes matured in 1 \( \mu M \) PALM (\( P > 0.05 \)). When concentrations of palmitic acid were increased (100 \( \mu M \) PALM) there was a significant increase in intracellular lipid content compared to control oocytes (Figure 5B). Carnitine in conjunction with high concentrations of palmitic acid (CARN + 100 \( \mu M \) PALM) was able to rescue intracellular lipid content to levels comparable to the control oocytes (\( P > 0.05 \); Figure 5B). Oocytes derived from COC treated with etomoxir during maturation had significantly less lipid content than all other treatments (\( P < 0.05 \); Figure 5A).
Discussion

The findings of this study have further clarified the role of lipids during mouse oocyte maturation. Although FAO was not directly measured, the use of agents known to inhibit FAO during maturation of the mouse COC, even at concentrations that do not affect nuclear maturation or embryo cleavage, negatively impacted oocyte quality resulting in impaired blastocyst development and reduced embryo quality. Similarly, use of a reagent that stimulates FAO, carnitine, during oocyte maturation increased blastocyst development but not embryo quality. In addition, elevated levels of exogenous palmitic acid during in vitro oocyte maturation negatively impacted subsequent embryo cleavage and blastocyst development. The addition of agents that stimulate or inhibit FAO during in vitro maturation of mouse COC resulted in changes in expression of genes related to redox balance, fatty acid metabolism, and glucose transport in both cumulus cells and the oocyte itself, and directly affected intracellular lipid content of the oocyte. Interestingly, glucose consumption and fatty acid metabolism appear to be related in the mouse COC. This has also been proposed in bovine embryos following treatment of oocytes with elevated fatty acids (Van Hoeck, et al. 2011). Thus, it appears essential for optimal oocyte quality that FAO and intracellular lipid content are controlled within a tolerated range during in vitro oocyte maturation in the mouse.

In these experiments, supplementation of exogenous carnitine to stimulate FAO in the COC during maturation increased embryonic development post fertilization but did not alter embryo quality, as reflected by total cell number and cell allocation in the resulting blastocysts. Stimulation of FAO with carnitine reduced intracellular lipid content in the oocyte, demonstrating that carnitine increased utilization of intracellular fatty acid stores. Interestingly, provision of extracellular palmitic acid at a low concentration (1 µM) could not maintain
intracellular lipid content in the presence of carnitine. Conflicting data exists in the literature regarding the benefit of carnitine, and thus an increase in FAO, during in vitro oocyte maturation in a number of species. It is important to note that these studies were performed in different species with varying amounts of intracellular lipids, which could have a dramatic effect on how carnitine influences oocyte competence. Supplementation of maturation medium with 0.6 mg/mL (approximately 3 mM) carnitine had no effect on nuclear maturation in bovine oocytes (Chankitisakul, et al. 2013) or on blastocyst development in hybrid mice (Moawad, et al. 2013). Addition of carnitine at concentrations ranging from 0.3 mg/mL to 5 mg/mL (1.5 to 25 mM) during maturation of porcine oocytes had no effect on subsequent blastocyst development post fertilization (Somfai, et al. 2011). Supplementation of carnitine during maturation of bovine oocytes tended to increase ATP content (Chankitisakul, et al. 2013), in contrast to our results in mice; however, a higher concentration of carnitine (approximately 3 mM) was used in the bovine study. In humans, carnitine is present in follicular fluid but concentration is not correlated with the success of assisted reproduction (Montjean, et al. 2012). In another study with hybrid mice, the addition of carnitine to stimulate FAO during oocyte maturation did not affect blastocyst development, although embryos were more advanced with increased inner cell mass cells reflecting increased embryo quality (Dunning, et al. 2010), which we did not observe. Although similar concentrations of carnitine (1 mM) were used by Dunning et al. (2010) and in the current experiment, differences observed regarding the effect of carnitine on embryo development and quality could be due in part to differences in maturation media. For example, the increased concentration of glucose (8.6 mM glucose compared to 0.5 mM glucose in our maturation media) used by Dunning et al. (2010) could have provided an alternative energy substrate to the oocyte. If carnitine were to stimulate FAO in mouse oocytes to a point that depleted intracellular
fatty acid stores, the COC may be able to switch to other energy substrates to sustain oocyte quality. That glucose might act in this manner is suggested by the increase we observed in Glut1 transcripts, a glucose transporter, in both oocytes and cumulus cells when FAO was inhibited using etomoxir. That glucose and fatty acid metabolism may be conversely related is also supported by the decrease in glucose consumption by the COC in the presence of carnitine that we observed.

Data obtained from the current experiment and previous reports suggest that oocyte intracellular lipid content and developmental competence are correlated, and that there is an optimal range of intracellular lipids that is compatible with high oocyte quality. Good quality bovine oocytes have high levels of intracellular lipids which appears optimal for this species, as oocytes with low lipid content have reduced developmental potential (Jeong, et al. 2009). Bovine oocytes that were denuded prior to maturation had decreased developmental competence and had significantly less lipid content than cumulus enclosed oocytes (Auclair, et al. 2013), suggesting that cumulus cells help to regulate the accumulation and/or metabolism of intracellular lipids during oocyte maturation. On the other hand, lipotoxicity in mouse oocytes, resulting from elevated intracellular lipid storage due to a high fat diet, is associated with decreased mitochondrial membrane potential and fertilization, suggesting decreased oocyte competence (Wu, et al. 2010). In our experiment, inhibition of FAO with etomoxir significantly decreased lipid content within the oocyte, which was associated with decreased embryonic development post fertilization. Decreased oocyte quality was also associated with elevated intracellular lipids when excess palmitic acid was present in the media. However, a moderate decrease in intracellular lipids induced by carnitine improved subsequent embryo development. Developmental competence may be directly related to levels of intracellular lipid, but more
likely lipid content indirectly reflects a balance in the activity of multiple metabolic pathways necessary to successfully support future embryo development.

Supplementation of fatty acids has been demonstrated to affect oocyte maturation and embryonic development in other species. In bovine oocytes, supplementation of α-linolenic acid during oocyte maturation increased the percentage of oocytes resuming meiosis and progressing to metaphase II (Marei, et al. 2012). However, supplementation of a different fatty acid, linoleic acid, decreased nuclear maturation in bovine oocytes (Marei, et al. 2012). High concentrations of stearic and palmitic acid present during oocyte maturation in cattle decreases subsequent blastocyst development and cell number, and increases the number of apoptotic cells within the blastocyst (Van Hoeck, et al. 2011). Supplementation of palmitic acid alone during bovine oocyte maturation, at concentrations used in the current experiment (100 µM), significantly decreased cleavage and development to the 8-cell stage, and higher concentrations negatively affected blastocyst development (Aardema, et al. 2011). Elevated levels of free fatty acids in follicular fluid are also associated with poor COC morphology in infertility patients undergoing assisted reproduction (Jungheim, et al. 2011). In the current experiment we observed a similar negative effect of elevated fatty acids during oocyte maturation. Maturation medium supplemented with a high concentration of palmitic acid (100 µM) inhibited nuclear maturation, with or without carnitine. Elevated levels of palmitic acid alone were also inhibitory to embryo cleavage and preimplantation embryo development. Even low levels of palmitic acid (1 µM) decreased development to the blastocyst stage (of cleaved embryos), and 1 µM palmitic acid, with or without carnitine, decreased ICM cell numbers, potentially reflecting reduced embryo quality.
We also demonstrated a significant increase in oocyte intracellular lipids after treatment with elevated palmitic acid during maturation. This suggests that lipids can be taken up by mouse COC from their environment, and that in an elevated fatty acid environment the accumulation of lipids in the oocyte cytoplasm reaches levels that have negative consequences for oocyte quality. This decrease in embryo cleavage and development after exposure to and uptake of exogenous palmitic acid from the environment is similar to that observed in bovine oocytes after exposure to 100 and 250 µM palmitic acid during oocyte maturation (Aardema, et al. 2011). However, bovine oocytes appear to metabolize these fatty acids, as intracellular oocyte lipid droplet size was decreased after exposure to higher concentrations (250 µM) of palmitic acid, although the number of lipid droplets per oocyte was unchanged until very elevated (500 µM) concentrations were used, when lipid droplets per oocyte also decreased (Aardema, et al. 2011). This is contrary to what was observed in the current experiment, where exogenous fatty acids resulted in increased intracellular oocyte lipid content, although the concentrations of palmitic acid used in the current study were lower than those used to elicit a response by Aardema, et al. (2011). In addition, there may be species specific differences in oocyte fatty acid metabolism between the cow and mouse resulting in differences in storage versus utilization. Similar to what we observed in mouse oocytes, Ferguson and Leese (1999) demonstrated that bovine embryos cultured in vitro take up and store triglycerides from the culture medium if serum is included. It is likely that species differences in the accumulation and utilization of intracellular lipid stores exist, given the differences in amount of intracellular lipids observed in the mouse and bovine oocyte (Genicot, et al. 2005). The mouse oocyte appears to have a finite ability to metabolize these fatty acids, although supplementation of maturation media with carnitine can increase this capacity, reducing intracellular lipids and increasing
subsequent embryo development to levels equal to control oocytes. From our results we are unable to determine whether FAO was increased in the presence of carnitine and elevated palmitic acid and if the intracellular lipid stores were maintained by the incorporation of the palmitic acid supplied in the medium, or if higher concentrations of palmitic acid inhibited FAO resulting in no change in intracellular lipid levels. However, the increase in Cpt2 expression in oocytes in the presence of elevated palmitic acid suggests that FAO may be increased. Low levels of palmitic acid did increase oocyte ATP content, again suggesting that oocytes can take up fatty acids from the environment and metabolize them via FAO. However, development was negatively affected in this treatment and embryo quality may have been reduced. Based on our work and that of others, elevated levels of at least some fatty acids are detrimental to oocyte quality, resulting in compromised embryo development. Although palmitic, stearic, and linoleic acids are present in follicular fluid of cattle (Renaville, et al. 2010), and potentially also in mouse follicular fluid, the benefit of fatty acids during oocyte maturation in vitro may be dependent on the specific fatty acid(s) and the concentration at which it is supplemented, as well as species.

Concurrent with the increase in FAO via carnitine supplementation, we observed a decrease in glucose consumption, suggesting an inverse relationship between these two metabolic pathways in the COC. The increased ability of the COC to utilize fatty acids as an energy substrate may decrease the need for the COC to metabolize glucose, or allow glucose to be stored or directed to other essential pathways. The ‘glucose-fatty acid cycle’ is an important concept which describes the inter-regulation of glucose and fatty acid metabolism to maintain homeostasis (Holness and Sugden 2003). In a previous study we showed that abundance of Pfk1, which is involved in the glycolytic pathway, was increased in bovine oocytes after inhibition of FAO, corresponding to an increase in glucose uptake by the COC (Paczkowski, et
Inhibition of fatty acid metabolism in mouse and pig embryos also results in changes in glucose metabolism (Hewitson, et al. 1996, Sturmey, et al. 2009). Similarly, obese mice had decreased blastocyst expression of Glut1 compared to non-obese mice, suggesting a down regulation of glucose uptake when fatty acid levels are elevated (Bermejo-Alvarez, et al. 2012). Elevated serum glucose levels in pregnant women correspond to reduced levels of CPT1 activity in placental extracts, thereby decreasing FAO (Visiedo, et al. 2013). Furthermore, overexpression of glucose transporter Glut1 in mouse cardiac tissue increases glucose uptake and oxidation while decreasing FAO, despite a high supply of fatty acids (Yan, et al. 2009). A relationship between FAO and glucose consumption and oxidation has also been reported in rat hepatocytes (Berry, et al. 1993), rat myocytes (Abdel-aleem, et al. 1994), rat skeletal muscle (Kaushik, et al. 2001), and rat (Lavrentyev, et al. 2004, Saddik, et al. 1993) and mouse cardiac tissue (Campbell, et al. 2002).

Genes associated with oxidative stress tended to increase in abundance in oocytes after maturation with a stimulator of FAO (carnitine), or when carnitine and palmitic acid were supplemented together. The data suggests that oxidative stress is increased in oocytes when FAO increases, resulting in a concurrent increase in abundance of genes to maintain redox balance. These findings are reminiscent of our previous study where we demonstrated that inhibition of FAO with etomoxir decreased expression of oxidative stress genes (Paczkowski, et al. 2013). However, sustained inhibition of FAO may also increase oxidative stress, as excessive accumulation of fatty acids within the cytoplasm can also result in reactive oxygen species formation (Furukawa, et al. 2004).

In oocytes, stimulation or inhibition of FAO caused few changes in expression of genes related to fatty acid metabolism (Acad1, Acs13, and Cpt2). Previous reports indicate that
stimulation of FAO with carnitine can alter oocyte and embryo gene expression. Carnitine supplementation during maturation increases the expression of both anti- and proapoptotic genes, as well as transcription factors in bovine oocytes (You, et al. 2012). Bovine blastocysts, after culture with carnitine, had elevated expression of genes related to ATP synthesis and oxidative phosphorylation and decreased expression of Bax, a proapoptotic gene (Takahashi, et al. 2013). High concentrations of palmitic acid, resulting in increased oocyte lipid content and decreased development post fertilization, tended to increase expression of Cpt2, suggesting that oocytes may have increased the movement of fatty acids into the mitochondria. Whether this resulted in increased mitochondrial FAO is unknown. When carnitine is added to excess palmitic acid conditions, returning oocyte lipid content and subsequent embryo development to levels equal to control, transcription of the genes involved in fatty acid metabolism were also no longer different than in control oocytes, although Glrx2 was elevated, suggesting a possible cellular response to increased reactive oxygen species production with elevated FAO. When bovine oocytes are exposed to elevated concentrations of stearic, palmitic and oleic acids (425 µM total), the resulting blastocysts have increased expression of genes related to de novo methylation, cellular and oxidative stress, and glucose metabolism, leading to disrupted oocyte and embryo metabolism (Van Hoeck, et al. 2011, Van Hoeck, et al. 2013). Abundance of ER stress markers are also elevated in COC from mice fed a high-fat diet and in granulosa cells from obese women (Wu, et al. 2010). Our gene expression results would also suggest that increased fatty acids in maturation medium leads to aberrant gene expression and metabolism.

Conversely, more changes were observed in expression of these genes in cumulus cells. Surprisingly, Cpt2 expression was elevated in cumulus cells when FAO was inhibited. This is in agreement with our previously reported findings in mouse and bovine oocytes, that etomoxir
treatment, at a concentration that did not affect completion of nuclear maturation, reduced oocyte lipid content (Paczkowski, et al. 2013). Etomoxir treatment also decreased oocyte lipid content in this study. We hypothesize that in the COC, FAO may undergo an initial increase in activity as a response to etomoxir, increasing transcription of essential FAO genes and briefly metabolizing lipids prior to inhibition. This hypothesis is supported by recent reports in mouse COC where expression of genes involved in FAO were altered after FAO inhibition (Brisard, et al. 2014). Abundance of genes involved in FAO are also increased in COC after treatment with rosiglitazone, an agent known to inhibit FAO (Dunning, et al. 2014). Similar to the observed increase of Cpt2 transcripts in cumulus cells after maturation with low levels of palmitic acid, with or without carnitine, an increase in Cpt1 has been documented in pancreatic cell lines when palmitate, oleate, and linoleate are supplied (Assimacopoulos-Jeannet, et al. 1997). After maturation with low palmitic acid concentrations and carnitine, Cpt2 and AcsL3 gene transcripts were elevated in cumulus cells, suggesting that FAO may have been increased in this treatment. Although we did not observe similar changes in expression of these genes in oocytes (a non-significant increase of 2.5 to 3-fold was detected), a decrease in lipid content in oocytes in the presence of low palmitic acid was observed, suggesting increased lipid metabolism. This result suggests that expression of genes related to fatty acid metabolism in the cumulus cells reflect the status of lipids in the oocyte itself, even when significant changes in transcription of these genes in the oocyte are not detected. This is further supported by recent work in cattle suggesting that cumulus cells have an essential role in regulating lipid metabolism of the oocyte (Auclair, et al. 2013).

In conclusion, oocyte quality, as reflected by subsequent embryo development, is negatively impacted when FAO is inhibited or when oocytes are exposed to an elevated
concentration of fatty acid, demonstrating the importance of fatty acid metabolism to optimal oocyte maturation. Increasing fatty acid metabolism with carnitine partially negates the negative effects of elevated fatty acids during maturation, and carnitine alone can be beneficial to subsequent embryo development. These results suggest that lipid metabolism, as well as related intracellular lipid stores, are important to oocyte quality, but must be finely balanced within a specific range to support developmental competence. However, we found no benefit from the supplementation of low concentrations of palmitic acid during in vitro maturation on oocyte quality in the mouse. Of particular interest, there appears to be a carefully orchestrated balance between glucose and fatty acid metabolism in mouse COC, in which increased FAO results in reduced lipid content and lowered glucose consumption, while increasing oocyte competence.
Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial, or not-for-profit sector.
References


Herrick JR, Lane M, Gardner DK, Behboodi E, Memili E, Blash S, Echelard Y &
Krisher RL 2006b Metabolism, protein content, and in vitro embryonic
development of goat cumulus-oocyte complexes matured with physiological
concentrations of glucose and L-lactate. *Molecular Reproduction and
Development* 73 256-266.

Herrick JR, Strauss KJ, Schneiderman A, Rawlins M, Stevens J, Schoolcraft WB &
Krisher RL 2013 The beneficial effects of reduced magnesium during the
oocyte-to-embryo transition are conserved in mice, domestic cats and humans.

Hewitson LC, Martin KL & Leese HJ 1996 Effects of metabolic inhibitors on mouse
preimplantation embryo development and the energy metabolism of isolated inner


Holness MJ & Sugden MC 2003 Regulation of pyruvate dehydrogenase complex
activity by reversible phosphorylation. *Biochemical Society Transactions* 31
1143-1151.

Jeong WJ, Cho SJ, Lee HS, Deb GK, Lee YS, Kwon TH & Kong IK 2009 Effect of
cytoplasmic lipid content on in vitro developmental efficiency of bovine IVP

Jungheim ES, Macones GA, Odem RR, Patterson BW, Lanzendorf SE, Ratts VS &
Moley KH 2011 Associations between free fatty acids, cumulus oocyte complex


Pfaffl MW, Horgan GW & Dempfle L 2002 Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30** e36, 1-10.


Figure Legends

Figure 1. Embryonic development after oocyte maturation with 1 mM carnitine (CARN), 100 µM palmitic acid (100 µM PALM), carnitine plus 100 µM palmitic acid (CARN + 100 µM PALM), or carnitine plus 100 µM etomoxir (CARN + ETO). \(^{a,b,c}\) Columns across treatments with different letters are significantly different, \(P < 0.05\).

Figure 2. Transcript abundance in oocytes after maturation with 1 mM carnitine (CARN), 1 µM palmitic acid (1 µM PALM), 100 µM palmitic acid (100 µM PALM), carnitine plus 1 µM palmitic acid (CARN + 1 µM PALM), carnitine plus 100 µM palmitic acid (CARN + 100 µM PALM), or carnitine plus 100 µM etomoxir (CARN + ETO). Genes related to FAO (acyl-Coenzyme A dehydrogenase long chain, \(Acadl\); acyl-CoA synthetase long chain family member 3, \(Acsl3\); carnitine palmitoyltransferase 2, \(Cpt2\)), glucose transport (glucose transporter 1, \(Glut1\)), and oxidative stress (glutaredoxin 2, \(Glrx2\); thioredoxin reductase 1, \(Txnrd1\)) were analyzed. *, \(P < 0.05\); +, \(P < 0.1\).

Figure 3. Transcript abundance in cumulus cells after oocyte maturation with 1 mM carnitine (CARN), 1 µM palmitic acid (1 µM PALM), 100 µM palmitic acid (100 µM PALM), carnitine plus 1 µM palmitic acid (CARN + 1 µM PALM), carnitine plus 100 µM palmitic acid (CARN + 100 µM PALM), or carnitine plus 100 µM etomoxir (CARN + ETO). Genes related to FAO (acyl-Coenzyme A dehydrogenase long chain, \(Acadl\); acyl-CoA synthetase long chain family member 3, \(Acsl3\); carnitine palmitoyltransferase 2, \(Cpt2\)), glucose transport (glucose transporter 1, \(Glut1\)), and oxidative stress
(glutaredoxin 2, Glrx2; thioredoxin reductase 1, Txnrd1) were analyzed. *, P < 0.05; +, P < 0.1.

Figure 4. (A) Glucose consumption per COC after maturation with 1 mM carnitine (CARN), 100 µM palmitic acid (100 µM PALM), carnitine plus 100 µM palmitic acid (CARN + 100 µM PALM), or carnitine plus 100 µM etomoxir (CARN + ETO); (B) Glucose consumption per COC after maturation with 1 µM palmitic acid (1 µM PALM) or carnitine plus 1 µM palmitic acid (CARN + 1 µM PALM). a,b,c Columns across treatments with different letters are significantly different, P < 0.05.

Figure 5. (A) Lipid content in oocytes after maturation with 1 mM carnitine (CARN), 1 µM palmitic acid (1 µM PALM), carnitine plus 1 µM palmitic acid (CARN + 1 µM PALM), or carnitine plus 100 µM etomoxir (CARN + ETO); (B) Lipid content in oocytes after maturation with 100 µM palmitic acid (100 µM PALM) or carnitine plus 100 µM palmitic acid (CARN + 100 µM PALM). a,b,c Columns across treatments with different letters are significantly different, P < 0.05.
Table 1. Summary of quantitative PCR primers of target and reference genes in the mouse.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Accession Number</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
<th>Product Length</th>
<th>Sequence Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acadl</td>
<td>NM_007381</td>
<td>5'-cttgcttggcatcaacatc- 3’</td>
<td>5'-ttcgcaatatagggcatga- 3’</td>
<td>150 bp</td>
<td>100%</td>
</tr>
<tr>
<td>Acsl3</td>
<td>NM_001033606</td>
<td>5'-tgctgagcttgtgtgtcttt- 3’</td>
<td>5'-gatccgatccatgatttcc- 3’</td>
<td>160 bp</td>
<td>100%</td>
</tr>
<tr>
<td>Cpt2</td>
<td>NM_009949</td>
<td>5'-tgctcgetcaggataacag- 3’</td>
<td>5'-eggattgaatgcctgga- 3’</td>
<td>122 bp</td>
<td>100%</td>
</tr>
<tr>
<td>Glrx2</td>
<td>NM_001038592</td>
<td>5'-gttccatggecaagaagatt- 3’</td>
<td>5'-gctccttctaaatctgctgcatg- 3’</td>
<td>161 bp</td>
<td>99%</td>
</tr>
<tr>
<td>Glut1</td>
<td>NM_011400</td>
<td>5'-ggcctgactactgctttg- 3’</td>
<td>5'-agaggccacaaggtgcctt- 3’</td>
<td>164 bp</td>
<td>100%</td>
</tr>
<tr>
<td>Txnrd1</td>
<td>NM_001042513</td>
<td>5'-acaatgaatggcctcagaag- 3’</td>
<td>5'-aataatggctgcctgctt- 3’</td>
<td>105 bp</td>
<td>100%</td>
</tr>
<tr>
<td>Ppia</td>
<td>NM_008907</td>
<td>5'-ggccttcttgctggac- 3’</td>
<td>5'-tcagttttgctgctgca- 3’</td>
<td>60 bp</td>
<td>100%</td>
</tr>
</tbody>
</table>

Sequence identity refers to the similarity in transcript sequence between PCR products and accession number, and confirms that the gene of interest has been amplified.
Table 2. Nuclear maturation of mouse oocytes after treatment with 1 mM carnitine (CARN), 100 µM palmitic acid (100 µM PALM), carnitine plus 100 µM palmitic acid (CARN + 100 µM PALM), or carnitine plus 100 µM etomoxir (CARN + ETO).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>102</td>
<td>98.04 ± 1.38&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>CARN</td>
<td>102</td>
<td>99.02 ± 9.80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>100 µM PALM</td>
<td>104</td>
<td>91.35 ± 2.77&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CARN + 100 µM PALM</td>
<td>100</td>
<td>90.00 ± 3.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CARN + ETO</td>
<td>106</td>
<td>94.34 ± 2.26&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Columns across treatments with different letters are significantly different, P < 0.05.
Table 3. Total, trophectoderm (TE), and inner cell mass (ICM) cell numbers following in vitro maturation and fertilization after treatment with 1 mM carnitine (CARN), 1 µM palmitic acid (1 µM PALM), carnitine plus 1 µM palmitic acid (CARN + 1 µM PALM), or carnitine plus 100 µM etomoxir (CARN + ETO).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Total</th>
<th>TE</th>
<th>ICM</th>
<th>% ICM of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>67</td>
<td>99.18 ± 4.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93.96 ± 3.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.25 ± 0.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.74 ± 0.61&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CARN</td>
<td>60</td>
<td>93.28 ± 3.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>87.38 ± 3.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.41 ± 1.22&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>15.17 ± 1.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 µM PALM</td>
<td>58</td>
<td>88.97 ± 4.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>84.42 ± 6.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.31 ± 0.91&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.55 ± 0.93&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CARN + 1 µM PALM</td>
<td>46</td>
<td>93.13 ± 5.56&lt;sup&gt;a&lt;/sup&gt;</td>
<td>87.20 ± 7.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.95 ± 0.73&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>14.35 ± 1.35&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CARN + ETO</td>
<td>38</td>
<td>65.97 ± 4.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.93 ± 3.59&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.00 ± 0.88&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.25 ± 1.31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> Columns across treatments with different letters are significantly different, P < 0.05.
Figure 1.

Embryonic Development

- **Cleavage**
- d4 Blastocyst of Cleaved
- d4 Blastocyst of Total
Figure 2.
Figure 3.
Figure 4.
Figure 5.