Fatty acid metabolism during maturation affects glucose uptake and is essential to oocyte competence

M Paczkowski, W B Schoolcraft¹ and R L Krisher

National Foundation for Fertility Research, 10290 Ridgeway Circle, Lone Tree, Colorado 80124, USA and ¹Colorado Center for Reproductive Medicine, 10290 Ridgeway Circle, Lone Tree, Colorado 80124, USA

Correspondence should be addressed to R L Krisher; Email: rkrisher@fertilityresearch.org

Abstract

Fatty acid β -oxidation (FAO) is essential for oocyte maturation in mice. The objective of this study was to determine the effect of etomoxir (a FAO inhibitor; 100 μ M), carnitine (1 mM), and palmitic acid (1 or 100 μ M) 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 μ M palmitic acid or etomoxir ($P < 0.05$) treatment. 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 *Slc2a1* (*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 carnitine palmitoyltransferase 2 (*Cpt2*) tended to increase in oocytes ($P = 0.1$) after treatment with 100 μ M palmitic acid and in cumulus cells after exposure to 1 μ M palmitic acid ($P = 0.07$). Combined with carnitine, 1 μ M palmitic acid increased the 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 to 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.

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Introduction

Supplementation of media with energy substrates such as glucose or pyruvate is critical for the successful completion of oocyte nuclear and cytoplasmic maturation as well as development of the resulting embryo (Biggers *et al.* 1967, Downs & Mastropolo 1994, Spindler *et al.* 2000, Herrick *et al.* 2006a,b). However, recent work has shown that fatty acid β -oxidation (FAO) is another important energy source during oocyte maturation (Hewitson *et al.* 1996, Ferguson & Leese 2006, Sturmey *et al.* 2006, 2009, Downs *et al.* 2009, Dunning *et al.* 2010, Paczkowski *et al.* 2013). The oxidation of a single fatty acid can produce up to 106 ATP molecules (Berg *et al.* 2002) and thus could be an efficient source of energy production for the maturing oocyte and pre-implantation embryo.

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 & 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 (Assimakopoulos-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 & 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 inhibition of FAO 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 (Hewitson *et al.* 1996, Downs

et al. 2009, Dunning *et al.* 2010, Paczkowski *et al.* 2013, Valsangkar & Downs 2013). Exposure to high concentrations of etomoxir (250 μ M) during maturation inhibits the completion of meiosis in mouse oocytes (Paczowski *et al.* 2013). After exposure to a lower concentration of etomoxir (100 μ M), oocytes are able to complete nuclear maturation (Dunning *et al.* 2010, Paczkowski *et al.* 2013), albeit with a decrease in development of embryo 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 cumulus–oocyte complex (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 (Paczowski *et al.* 2013), the dosage of etomoxir (100 μ M) 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 unless otherwise stated.

In vitro maturation, fertilization, and culture

B6D2F1 female mice, ~3 weeks of age, were administered pregnant mare serum gonadotropin (5 IU, i.p.; EMD Biosciences, Billerica, MA, USA) 48 h before being killed. The ovaries were harvested and washed in GMOPS PLUS (Vitrolife, Englewood, CO, USA) supplemented with 5% FCS (HyClone, Thermo Scientific, Rockford, IL, USA). The 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% CO₂ and 6.3% O₂. Owing to the increased altitude of Lone Tree, Colorado, the CO₂ level was increased to maintain the pH of all media at 7.28. A completely defined oocyte maturation media was supplemented with 0.5 mM glucose, 6 mM lactate (MP Biomedicals, Santa Ana, CA, USA), 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. The COCs were matured in one of seven treatment groups: i) control; ii) 1 mM carnitine (CARN); iii) 1 μ M palmitic acid (1 μ M PALM); iv) 100 μ M palmitic acid (100 μ M PALM); v) 1 mM carnitine plus 1 μ M palmitic acid (CARN+1 μ M PALM); vi) 1 mM carnitine plus 100 μ M palmitic acid (CARN+100 μ M PALM); and vii) 1 mM carnitine plus 100 μ M 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 μ M 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 (National Research Council 2011).

The COCs were transferred to human tubal fluid (HTF; Nagy *et al.* 2003) supplemented with 0.4 g BSA (MP Biomedicals) post maturation. The spermatozoa were collected from the vas deferens and cauda epididymis of an adult B6D2F1 male (>10 weeks of age) and incubated at 37 °C, 8.3% CO₂ and 6.3% O₂ in HTF for 1 h to allow for capacitation. They were further diluted in HTF and added to the fertilization drops for a final concentration of 1×10^6 sperm/ml. The gametes were then co-incubated for 6 h.

Presumptive zygotes were cultured in G1 PLUS (Vitrolife) at 37 °C, 8.3% CO₂ and 6.3% O₂ 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 4 (96 hpi). The blastocysts were stained for inner cell mass (ICM), trophectoderm (TE), and total cell number (Thouas *et al.* 2001) on day 5 (112 hpi). Briefly, blastocysts were incubated in 300 μ g/ml propidium iodide and 1.25% Triton X-100 in PBS for 5 s and then incubated in 0.25 μ g/ml bisbenzimidazole (Hoescht 33258) in 100% ethanol overnight at 4 °C. The blastocysts were mounted on glass slides in a drop of glycerol under a cover slip. The cell numbers were counted by fluorescent microscopy.

Quantitative PCR

The COCs were matured *in vitro* for 18 h as described earlier. The oocytes were mechanically denuded following *in vitro* maturation (IVM) using a pulled glass pipette and the cumulus cells were isolated from maturation media via centrifugation at 16 000 *g* for 5 min. The 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). 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). Total RNA extracted from oocytes and cumulus cells were loaded into the cDNA reaction; therefore, the resulting quantitative PCR (qPCR) data were analyzed by relative quantification as described below. cDNA was synthesized using the High Capacity cDNA RT Kit (Applied Biosystems) following the manufacture's protocol.

Primer design and qPCR were performed as previously described (Paczowski *et al.* 2011, 2013). Primers for genes related to FAO (acyl-coenzyme A dehydrogenase long-chain (*Acadl*), acyl-CoA synthetase long-chain family member 3 (*Acs3l3*), and *Cpt2*), glucose transport (glucose transporter 1 (*Slc2a1* (*Glut1*))), and oxidative stress (glutaredoxin 2 (*Glr2*) and thioredoxin reductase 1 (*Txnrd1*)) were designed for qPCR using Primer3 (Rozen & 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 *Escherichia coli* (Invitrogen Life Technologies). The plasmids were sequenced in the DNA-sequencing facility at Colorado State University (Fort Collins, CO, USA) to confirm the identity of the transcript and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen Life Technologies). QPCR was carried out on threefold diluted sample cDNA run in duplicate. The target genes were analyzed using iQ SYBR Green Supermix (Bio-Rad) and an Applied Biosystems 7300 Real-Time PCR system. A standard curve was generated from serial dilutions of EcoRI-digested plasmids (10^7 – 10^1 molecules) and the efficiency of the primers was calculated.

Metabolic assays

Glucose colorimetric assay

At 18 h post maturation, 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, USA) 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, USA). 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 per h) during oocyte maturation, the concentration obtained from the spent media was subtracted from the average concentration of unspent media and converted to picomole glucose using the volume of the drop used for maturation (50 μl), the number of COC per drop (ten 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

COCs were matured *in vitro* and oocytes and cumulus cells were collected separately as described earlier. Denuded oocytes were pooled into three biological replicates of ten oocytes per treatment and frozen at -80°C in 10 μl PBS. 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 IVM, the oocytes were denuded and fixed for a minimum of 24 h in 500 μl of a 2% glutaraldehyde and 2% formaldehyde solution. The oocytes were then stained overnight in 10 $\mu\text{g}/\text{ml}$ Nile red. Images were taken at $200\times$ on a fluorescent microscope and analyzed using the freely available ImageJ software (version 1.46, National Institutes of Health, Bethesda, MD, USA; 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 analyses

Percent oocyte maturation, embryonic cleavage, and 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.* 2014). 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 ANOVA using Number Cruncher Statistical System (NCSS, Kaysville, UT, USA). When ANOVA detected

Table 1 Summary of quantitative PCR primers of target and reference genes in the mouse.

Gene symbol	Accession number	Forward primer sequence	Reverse primer sequence	Product length (bp)	Sequence identity (%) ^a
<i>Acadl</i>	NM_007381	5'-ctgcttgccatcaacatc-3'	5'-ttcgaatataggcatga-3'	150	100
<i>Acs13</i>	NM_001033606	5'-tgctgagctgtgtgtcttt-3'	5'-gatccgatccatgattcc-3'	160	100
<i>Cpt2</i>	NM_009949	5'-tgctcgctcaggataaacag-3'	5'-cgattgaatgccatgaa-3'	122	100
<i>Glrx2</i>	NM_001038592	5'-gttccatggccaagaagatt-3'	5'-gcctccaataaatcgtccat-3'	161	99
<i>Slc2a1</i>	NM_011400	5'-ggcctgactactggctttgt-3'	5'-agaggccacaagtctgcatt-3'	164	100
<i>Txnrd1</i>	NM_001042513	5'-acaatgaatggctccaaga-3'	5'-aaatttgctgctccttag-3'	105	100
<i>Ppia</i>	NM_008907	5'-ggcaaatgctggacaaa-3'	5'-tcagtctggcagtcagata-3'	60	100

^aSequence identity refers to the similarity in transcript sequence between PCR products and accession number, and confirms that the gene of interest has been amplified.

significant differences ($P < 0.05$), treatment differences were determined using a Fisher's least significant difference (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). The expression ratios were generated from equation (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 pairwise fixed reallocation randomization tests with 50 000 iterations and significance was determined with a P value < 0.05 .

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CT_{\text{target}} (\text{control-sample})}}{(E_{\text{ref}})^{\Delta CT_{\text{ref}} (\text{control-sample})}}. \quad (1)$$

Results

Oocyte maturation and embryo development

We hypothesized that embryonic development would be decreased after FAO inhibition during 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 with 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 with control oocytes ($P < 0.05$; Table 2). Cleavage of oocytes matured in CARN post fertilization was not different compared with control oocytes ($P > 0.05$; Fig. 1); however, there was a significant increase in day 4 blastocysts of both cleaved embryos and of total presumptive zygotes ($P < 0.05$) after oocyte maturation in CARN. Cleavage and day 4 blastocyst of total presumptive zygotes were significantly reduced in the 100 μM PALM treatment compared with control oocytes ($P < 0.05$); however, the addition of carnitine

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).

Treatment	n	Percent (%)
Control	102	98.04 $\pm$ 1.38 ^{*,†}
CARN	102	99.02 $\pm$ 9.80 [*]
100 μM PALM	104	91.35 $\pm$ 2.77 [†]
CARN+100 μM PALM	100	90.00 $\pm$ 3.02 [†]
CARN+ETO	106	94.34 $\pm$ 2.26 ^{†,‡}

^{*,†,‡}Columns across treatments with different symbols are significantly different, $P < 0.05$.

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,d}Columns across treatments with different letters are significantly different, $P < 0.05$.

(CARN+100 μM PALM) was able to rescue embryo development on day 4 such that it was not different than control ($P > 0.05$; Fig. 1). Supplementation of CARN+ETO significantly decreased day 4 blastocyst development (per zygote and per cleaved embryo) compared with controls ($P < 0.05$; Fig. 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 day 4 blastocysts of total presumptive zygotes compared with control oocytes respectively ($P > 0.05$). However, the proportion of day 4 blastocysts of cleaved embryos was reduced (78.90 ± 3.93 vs $88.99 \pm 3.01\%$; $P < 0.05$) in the lower concentration of palmitic acid (1 μM) compared with 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 with control embryos ($P > 0.05$; Table 3). However, the number of cells within the ICM was significantly decreased in the embryos derived from oocytes matured in 1 μM PALM or CARN+1 μM PALM compared with 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 in the percent of 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

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 μ M palmitic acid (1 μ M PALM), carnitine plus 1 μ M palmitic acid (CARN + 1 μ M PALM), or carnitine plus 100 μ M etomoxir (CARN + ETO).

Treatment	n	Total	TE	ICM	% ICM of total
Control	67	99.18 $\pm$ 4.30*	93.96 $\pm$ 3.85*	16.25 $\pm$ 0.96*	14.74 $\pm$ 0.61*
CARN	60	93.28 $\pm$ 3.95*	87.38 $\pm$ 3.62*	15.41 $\pm$ 1.22* [‡]	15.17 $\pm$ 1.08*
1 μ M PALM	58	88.97 $\pm$ 4.58*	84.42 $\pm$ 6.34*	12.31 $\pm$ 0.91 [†]	13.55 $\pm$ 0.93*
CARN + 1 μ M PALM	46	93.13 $\pm$ 5.56*	87.20 $\pm$ 7.41*	12.95 $\pm$ 0.73* [‡]	14.35 $\pm$ 1.35*
CARN + ETO	38	65.97 $\pm$ 4.20 [†]	56.93 $\pm$ 3.59 [†]	10.00 $\pm$ 0.88 [†]	15.25 $\pm$ 1.31*

*[†],[‡]Columns across treatments with different symbols are significantly different, $P < 0.05$.

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 μ M PALM, or CARN + 100 μ M PALM, respectively, compared with control oocytes (Fig. 2). However, there were no significant differences in the expression of oocyte gene when the concentration of palmitic acid was lowered to 1 μ M, in the presence or absence of carnitine (1 μ M PALM and CARN + 1 μ M PALM), compared with control oocytes ($P > 0.05$; Fig. 2). Abundance of *Cpt2* tended to be increased in cumulus cells after COC treatment with 1 μ M PALM ($P = 0.07$) or CARN + 1 μ M PALM ($P = 0.07$) compared with cumulus cells from untreated COC (Fig. 3). There was a significant increase in abundance of *Acs13* in cumulus cells after COC treatment with CARN + 1 μ M PALM compared with control cumulus cells ($P < 0.05$). Interestingly, there tended to be a decrease ($P = 0.08$) in *Acs13* in cumulus cells after treatment with CARN + 100 μ M PALM; however, *Acs13* was not different when 100 μ M PALM was added without carnitine. There was a significant increase in transcript abundance of *Slc2a1* in oocytes and *Cpt2* and *Slc2a1* in cumulus cells in response to FAO inhibition (CARN + ETO) compared with untreated oocytes and cumulus cells ($P < 0.05$; Figs 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 with 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 with the control ($P < 0.05$; Fig. 4A). There was no difference in glucose consumption per COC after maturation in 1 or 100 μ M PALM alone or in combination with carnitine compared with controls ($P > 0.05$; Fig. 4A and B). There was also no significant difference in ATP concentration per oocyte after any experimental treatment compared with control oocytes, except that treatment with 1 μ M PALM (0.19 ± 0.04 pmol ATP/oocyte) significantly increased ATP concentration per oocyte compared with control oocytes (0.06 ± 0.01 pmol ATP/oocyte; $P < 0.05$).

Oocyte maturation in CARN or CARN + 1 μ M PALM significantly reduced intracellular lipid content compared with control oocytes ($P < 0.05$; Fig. 5A). However, there was no significant difference in lipid content in oocytes matured in 1 μ M PALM ($P > 0.05$). When concentrations of palmitic acid were increased

Figure 2 Transcript abundance in oocytes after maturation with 1 mM carnitine (CARN), 1 μ M palmitic acid (1 μ M PALM), 100 μ M PALM, carnitine plus 1 μ M palmitic acid (CARN + 1 μ M PALM), CARN + 100 μ M PALM, or carnitine plus 100 μ M etomoxir (CARN + ETO). Genes related to FAO (acyl-coenzyme A dehydrogenase long chain (*Acadl*), acyl-CoA synthetase long-chain family member 3 (*Acs13*), and carnitine palmitoyltransferase 2 (*Cpt2*)), glucose transport (glucose transporter 1 (*Slc2a1*)), and oxidative stress (glutaredoxin 2 (*Glrx2*) and thioredoxin reductase 1 (*Txnrd1*)) were analyzed. * $P < 0.05$ and [†] $P < 0.1$.

Figure 3 Transcript abundance in cumulus cells after oocyte maturation with 1 mM carnitine (CARN), 1 μ M palmitic acid (1 μ M PALM), 100 μ M PALM, carnitine plus 1 μ M palmitic acid (CARN + 1 μ M PALM), CARN + 100 μ M PALM, or carnitine plus 100 μ M etomoxir (CARN + ETO). Genes related to FAO (acyl-coenzyme A dehydrogenase long chain (*Acadl*), acyl-CoA synthetase long-chain family member 3 (*Acs13*), and carnitine palmitoyltransferase 2 (*Cpt2*)), glucose transport (glucose transporter 1 (*Slc2a1*)), and oxidative stress (glutaredoxin 2 (*Glr2*) and thioredoxin reductase 1 (*Txnrd1*)) were analyzed. * $P < 0.05$ and + $P < 0.1$.

(100 μ M PALM), there was a significant increase in intracellular lipid content compared with control oocytes (Fig. 5B). Carnitine in conjunction with high concentrations of palmitic acid (CARN + 100 μ M PALM) was able to rescue intracellular lipid content to levels comparable with the control oocytes ($P > 0.05$; Fig. 5B). Oocytes derived from COC treated with etomoxir during maturation had significantly less lipid content than all other treatments ($P < 0.05$; Fig. 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, such as 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 IVM 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, for optimal oocyte quality, it appears essential 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 μ M) 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

Figure 4 (A) Glucose consumption per COC after maturation with 1 mM carnitine (CARN), 100 μ M palmitic acid (100 μ M PALM), carnitine plus 100 μ M palmitic acid (CARN + 100 μ M PALM), or carnitine plus 100 μ M etomoxir (CARN + ETO) and (B) glucose consumption per COC after maturation with 1 μ M palmitic acid (1 μ M PALM) or carnitine plus 1 μ M palmitic acid (CARN + 1 μ M 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) and (B) lipid content in oocytes after maturation with 100 μM PALM or CARN+100 μM PALM. ^{a,b,c,d}Columns across treatments with different letters are significantly different, $P < 0.05$.

maturation medium with 0.6 mg/ml (~ 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 to 5 mg/ml (1.5–25 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 (~ 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 ICM with 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 with 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 *Slc2a1* 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 indicate 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 before 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 μ M), significantly decreased cleavage and development to the eight-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 μ M) 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 μ M) decreased development to the blastocyst stage (of cleaved embryos), and 1 μ M 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 μ M 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 μ M) of palmitic acid, although the number of lipid droplets per oocyte was unchanged until very elevated (500 μ M) 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 vs utilization. Similar to what we observed in mouse oocytes, Ferguson & 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 whether the intracellular lipid stores were maintained by the incorporation of the palmitic acid supplied in the medium, or whether 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 follicular fluid of mouse, 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 & Sugden 2003). In a previous study, we showed that abundance of *Pfkfb1* (*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 al.* 2013). Inhibition of fatty acid metabolism in mouse and pig embryos also results in changes in glucose metabolism (Hewitson *et al.* 1996, Sturme *et al.* 2009). Similarly, obese mice had decreased blastocyst expression of *Slc2a1* compared with non-obese mice, suggesting a downregulation 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 *Slc2a1* 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 (Saddik *et al.* 1993, Lavrentyev *et al.* 2004) and mouse cardiac tissues (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 indicate 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 whereby 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 formation of reactive oxygen species (Furukawa *et al.* 2004).

In oocytes, stimulation or inhibition of FAO caused few changes in the expression of genes related to fatty acid metabolism (*Acadl*, *Acsl3*, 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 the 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 *Glx2* 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 μ M total), the resulting blastocysts have increased the 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, 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 indicate 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 before 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 is also increased in COC after treatment with rosiglitazone, an agent known to inhibit FAO (Dunning *et al.* 2014). Similar to the observed increase in *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 (Assimakopoulos-Jeannot *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 the expression of these genes in oocytes (a nonsignificant increase of 2.5- to threefold was detected), a decrease in lipid content in oocytes in the presence of low palmitic acid was observed, suggesting increased lipid metabolism. This result indicates 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 indicate that lipid metabolism, as well as related

intracellular lipid stores, is 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 IVM 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.

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