Oocyte developmental failure in response to elevated nonesterified fatty acid concentrations: mechanistic insights

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Abstract

Elevated plasma nonesterified fatty acid (NEFA) concentrations are associated with negative energy balance and metabolic disorders such as obesity and type II diabetes. Such increased plasma NEFA concentrations induce changes in the microenvironment of the ovarian follicle, which can compromise oocyte competence. Exposing oocytes to elevated NEFA concentrations during maturation affects the gene expression and phenotype of the subsequent embryo, notably prompting a disrupted oxidative metabolism. We hypothesized that these changes in the embryo are a consequence of modified energy metabolism in the oocyte. To investigate this, bovine cumulus oocyte complexes were matured under elevated NEFA conditions, and energy metabolism-related gene expression, mitochondrial function, and ultrastructure evaluated. It was found that expression of genes related to REDOX maintenance was modified in NEFA-exposed oocytes, cumulus cells, and resultant blastocysts. Moreover, the expression of genes related to fatty acid synthesis in embryos that developed from NEFA-exposed oocytes was upregulated. From a functional perspective, inhibition of fatty acid β -oxidation in maturing oocytes exposed to elevated NEFA concentrations restored developmental competence. There were no clear differences in mitochondrial morphology or oxygen consumption between treatments, although there was a trend for a higher mitochondrial membrane potential in zygotes derived from NEFA-exposed oocytes. These data show that the degree of mitochondrial fatty acid β -oxidation has a decisive impact on the development of NEFA-exposed oocytes. Furthermore, the gene expression data suggest that the resulting embryos adapt through altered metabolic strategies, which might explain the aberrant energy metabolism previously observed in these embryos originating from NEFA-exposed maturing oocytes.

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Introduction

Elevated plasma nonesterified fatty acid (NEFA) concentrations, arising from upregulated lipolysis, have been implicated as a key factor in the association between metabolic imbalances, cellular dysfunction, and related pathologies such as insulin resistance in mammals (Shimabukuro *et al.* 1998, Carlsson *et al.* 1999, Kruszynska *et al.* 2002, McGarry 2002). Elevated plasma NEFA concentrations are also reflected in the bovine and human ovarian follicular microenvironments (Leroy *et al.* 2004, 2005, Robker *et al.* 2009, Jungheim *et al.* 2011a, 2011b, Valckx *et al.* 2012) and, as a result, have been associated with reduced oocyte developmental competence (Jorritsma *et al.* 2004, Leroy *et al.* 2005, Aardema *et al.* 2011) and compromised human and bovine granulosa cell viability (Mu *et al.* 2001,

Vanholder *et al.* 2005). In our recent study (Van Hoeck *et al.* 2011), it was demonstrated that embryos arising from fertilized, NEFA-exposed oocytes have a significantly lower cell number, increased apoptotic cell index, aberrant transcriptional activities, altered amino acid turnover, and compromised oxidative metabolism; all indicators for a lower embryo quality and viability. Although such embryos displayed upregulated expression of the *SLC2A1* glucose transporter, they did not consume more glucose compared with control embryos, an intriguing finding, as this situation is similar to that in insulin-resistant somatic cells.

A strong metabolic role for lipid oxidation during oocyte maturation is widely acknowledged (Sturmey *et al.* 2006, Downs *et al.* 2009, Dunning *et al.* 2010). Aardema *et al.* (2011) showed that the number and size

of lipid droplets in oocytes change following exposure to NEFA during *in vitro* maturation (IVM), indicating that bovine oocytes are able to incorporate and metabolize fatty acids from the external environment. However, the mechanisms through which elevated NEFA concentrations affect oocyte development and subsequent embryo physiology are unclear.

In contrast, the mechanisms underlying NEFA cytotoxicity have been extensively investigated in somatic cells where they involve modifications to cell membrane phospholipids (Calder *et al.* 1994), increased nitric oxide production (Shimabukuro *et al.* 1997), and elevated ceramide concentrations leading to apoptosis (Shimabukuro *et al.* 1998, Maedler *et al.* 2001, Lu *et al.* 2003). Though, Mu *et al.* (2001) reported that in granulosa cells, the negative effects of NEFA were unrelated to such mechanisms.

Data on somatic cells, including pancreatic β -cells (Carlsson *et al.* 1999, Koskin *et al.* 2003), adipocytes (Furukawa *et al.* 2004), monocytes (Zhang *et al.* 2006), and skeletal muscle cells (Bonnard *et al.* 2008), suggest that mitochondrial metabolism plays a decisive role in the etiology of fatty acid-induced metabolic disruption. Fatty acids are principally metabolized by mitochondrial β -oxidation and when their supply is plentiful, there is upregulation of mitochondrial activity (Iossa *et al.* 2002), which results in elevated reactive oxygen species (ROS) production (Burton *et al.* 2003). Moreover, the mitochondrial genome possesses limited DNA repair mechanisms compared with nuclear DNA (Clayton 1991) and will potentially be more sensitive to increased ROS concentrations (Wallace 1987, Tarin 1995). In oocytes, this might be of particular relevance because mitochondria do not replicate during the early stages of preimplantation development (Cummins 2002) and each blastomere must rely on oocyte-inherited mitochondria until the blastocyst stage, when mitochondrial replication begins (Cummins 1998). Perturbations in mitochondrial function are therefore critical for oocyte development and have previously been related to embryo quality (Van Blerkom 2004).

Building on our previous work which showed that elevated NEFA concentrations during oocyte maturation affect oocyte developmental competence (Leroy *et al.* 2005) and the phenotype of the resulting embryos (Van Hoeck *et al.* 2011), we have sought to provide basic insights into the etiological mechanisms and have hypothesized that the negative effects of elevated NEFA exposure during oocyte IVM are linked to modifications in energy metabolism. To address this proposition, we have used our established bovine oocyte *in vitro* culture model (Van Hoeck *et al.* 2011) in which cumulus oocyte complexes (COCs) are exposed during maturation to elevated concentrations of stearic acid (SA), palmitic acid (PA), and oleic acid (OA) to examine whether

1. exposure to elevated NEFA concentrations alters expression profiles of target genes related to fatty acid metabolism in oocytes, cumulus cells, and the resulting 7-day-old embryos;
2. elevated NEFA exposure affects mitochondrial distribution in oocyte and ultrastructure;
3. reduced oocyte developmental potential arising from elevated NEFA exposure can be restored by modulating mitochondrial fatty acid oxidation; and
4. mitochondrial membrane polarity and oxygen consumption of zygotes are affected by exposing oocytes to elevated NEFA concentrations.

Results

Gene expression in oocytes matured in the presence of elevated NEFA concentrations

As shown in Fig. 1, oocytes exposed to HIGH COMBI medium displayed a significant increase in mRNA expression of lactate dehydrogenase (LDHA) and glyceraldehyde 3-phosphate dehydrogenase (GADPH), key genes involved in energy metabolism, when compared with control oocytes ($P \leq 0.02$). Elevated GADPH expression was also observed in the HIGH SA

Figure 1 Comparison of relative transcript abundance in oocytes. Oocytes were matured under control (150 μ M total, physiological, NEFA), HIGH SA (75 μ M SA), and HIGH COMBI (425 μ M total, elevated, NEFA) conditions ($n = 180$; three replicates). Bars with different superscripts are significantly different between treatments with $P \leq 0.02$.

Figure 2 Comparison of relative transcript abundance in cumulus cells. Cumulus cells from cumulus oocyte complexes ($n=180$; three replicates) matured under control (150 μM total, physiological, NEFA), HIGH SA (75 μM SA), and HIGH COMBI (425 μM total, elevated, NEFA) conditions. Bars with different superscripts are significantly different between treatments with $P \leq 0.03$.

oocytes ($P \leq 0.01$). Furthermore, the expression of glutathione peroxidase 1 (*GPX1*), a gene related to oxidative stress, was significantly upregulated in HIGH SA and HIGH COMBI oocytes ($P \leq 0.01$).

Gene expression in cumulus cells from COCs matured in the presence of elevated NEFA concentrations

Figure 2 shows that expression of the facilitated glucose transporter 1 (*SLC2A1*), *LDHA*, and glucose-6-phosphate dehydrogenase (*G6PD*), genes related to glucose metabolism, was significantly lower in HIGH SA cumulus cells compared with cumulus cells harvested from control COCs ($P \leq 0.025$). By contrast, the abundance of *GADPH* mRNA was significantly higher in HIGH SA cumulus cells. Cumulus cells collected from COCs matured under HIGH COMBI conditions displayed a significantly lower expression of *LDHA*, *GADPH*, and *G6PD* ($P \leq 0.025$). Expression of *GPX1* and DNA cytosine-5-methyltransferase 3A (*DNMT3A*) was lower in cumulus cells from COCs matured in HIGH SA and HIGH COMBI conditions

($P \leq 0.03$) compared with control COCs. Additionally, carnitine palmitoyl transferase (*CPT1A*) and adipophilin 2 (*PLIN2*) expressions were reduced in cumulus cells collected from HIGH SA COCs ($P \leq 0.03$).

Gene expression in blastocysts originating from oocytes matured in the presence of elevated NEFA concentrations

Four of the seven genes analyzed were differentially expressed among treatment groups (Fig. 3). First, the expression of a fatty acid synthetase (*ACSL1*) was dramatically upregulated in embryos originating from oocytes matured under HIGH COMBI concentrations compared with embryos arising from control and HIGH SA oocytes ($P \leq 0.05$). Secondly, acetyl CoA carboxylase (*ACCA*) expression was significantly higher in the group matured under HIGH COMBI conditions compared with the control embryos ($P \leq 0.05$), although the group matured with HIGH SA concentrations did not differ from the controls or HIGH COMBI embryos. Finally, the expression of genes related to mitochondrial

Figure 3 Expression patterns of key genes in bovine day 7 blastocysts arising from oocytes matured under control (150 μM total, physiological, NEFA), HIGH SA (75 μM SA), and HIGH COMBI (425 μM total, elevated, NEFA) conditions ($n=192$; five replicates). Bars with a and b superscripts are significantly different between treatments with $P \leq 0.05$. Bars with * and \$ superscripts tend to be significantly different between treatments with $P \leq 0.1$.

Figure 4 Electron microscopic images of mitochondria in oocytes matured under control (150 μ M total, physiological, NEFA; A and B) conditions and HIGH COMBI (425 μ M total, elevated, NEFA; C and D) conditions at magnifications 6000 $\times$ (A and C) and 25 000 $\times$ (B and D). Mitochondria (M), vesicles (V), and lipid droplets (LD) were distributed randomly in the cytoplasm.

biogenesis and oxidative stress (transcription factor A mitochondria, *TFAM*, and manganese superoxide dismutase, *MNSOD* respectively) tended to be increased in HIGH COMBI embryos ($P \leq 0.1$).

Mitochondrial ultrastructure and distribution in oocytes matured in the presence of elevated NEFA concentrations

Transmission electron microscopic analysis of matured oocytes did not reveal any obvious differences in spatial distribution of mitochondria (Fig. 4, 1A and 2A). As expected (Bracket *et al.* 1980, Mohr & Trounson 1981), the mitochondria of the oocyte appeared small and electron dense with few cristae, and there was little obvious difference between oocytes from the different treatments. In all the treatments, the cytoplasm was divided into organelle-rich and organelle-free zones. The organelle-rich zones displayed a slightly more peripheral localization in the ooplasm and were characterized by an apparently random distribution of mitochondria. Moreover, mitochondrial ultrastructure did not appear to change in response to HIGH NEFA exposure (Fig. 4, 1B and 2B).

Development of oocytes matured in the presence of elevated NEFA concentrations after inhibiting β -oxidation

As shown in Table 1, the presence of β -mercaptoacetate (β -MA) had no significant effect on cleavage and blastocyst rates obtained from the fertilization of oocytes matured in control maturation medium. However, blastocyst production from oocytes matured in the presence of β -MA and HIGH COMBI NEFA was higher than when β -MA was absent (26.5 vs 18.8%; $P = 0.05$).

Oxygen consumption in zygotes originating from oocytes matured in the presence of elevated NEFA concentrations

No significant difference was observed in the average oxygen consumption of zygotes originating from oocytes matured under control (1.25 ± 0.07 nl/embryo per h) and HIGH COMBI (1.11 ± 0.11 nl/embryo per h) conditions.

Ratio of high vs low-polarized mitochondrial membranes in zygotes originating from oocytes matured in the presence of elevated NEFA concentrations

Confocal images (Fig. 5) showed that the proportion of red pixel intensity (highly polarized membrane) out of the total red and green pixel intensity (total mitochondrial mass) was not significantly different between treatments. However, the latter percentage tended to be

Table 1 Developmental competence of NEFA-exposed oocytes in the presence and absence of β -mercaptoacetate (β -MA), an inhibitor of the mitochondrial β -oxidation.

<i>n</i> (%)	Control	Control + β -MA	HIGH COMBI	HIGH COMBI + β -MA
Oocytes	208	204	208	200
Cleaved	172 (82.7)	160 (78.4)	162 (77.9)	154 (77.0)
Blastocysts from oocytes matured	54 (26.0)	49 (24.0)	39 (18.8)*	53 (26.5) [†]
Blastocysts from cleaved zygotes	54 (31.4)	49 (30.6)	39 (24.1)	53 (34.4)

Cleavage rate at day 2 p.i., number of formed blastocysts at day 7 p.i. relative to the number of matured oocytes or to the number of cleaved zygotes. Oocytes ($n=820$; five replicates) were matured under the following conditions: i) control (150 μ M total, physiological, NEFA), ii) HIGH COMBI (425 μ M total, elevated, NEFA), iii) control (150 μ M total, physiological, NEFA) + 0.1 mM β -MA, and iv) HIGH COMBI (425 μ M total, elevated, NEFA) + 0.1 mM β -MA. Data marked with different superscripts are significantly different ($P < 0.05$).

Figure 5 Confocal microscopic images (400× magnification) of JC1-stained zygotes (day 1 p.i.) originating from oocytes matured under control (150 μ M total, physiological, NEFA; A and B) and HIGH COMBI (425 μ M total, elevated, NEFA; C and D) conditions with view at mid-equatorial plane (A and C) and view at top plane (B and D). Green fluorescence indicates low-polarized mitochondria. Red fluorescence indicates high-polarized mitochondria.

higher in HIGH COMBI zygotes compared with the controls for the top plane ($55.0\% \pm 1.2$ vs $51.2\% \pm 2.6$ respectively; $P=0.09$) and for the average of both the top and the mid-equatorial planes ($53.8\% \pm 1.2$ vs $50.9\% \pm 2.4$ respectively; $P=0.09$). No difference in mitochondrial membrane polarity ratios could be detected between treatments for the mid-equatorial plane ($52.9\% \pm 1.2$ vs $50.7\% \pm 2.5$ respectively; $P=0.17$).

Discussion

Elevated NEFA concentrations represent a common feature of a distorted maternal metabolism, typically present in obese and type II diabetes patients (Reaven *et al.* 1988, Mooradian *et al.* 2008). Epidemiological studies suggest that such metabolic disorders are associated with decreased fertility (Pasquali *et al.* 2003, Metwally *et al.* 2007), and there is growing evidence that elevated NEFA concentrations play a key role in this process as they may alter the biochemical composition of the follicular microenvironment (Leroy *et al.* 2005, Robker *et al.* 2009, Jungheim *et al.* 2011a, 2011b, Valckx *et al.* 2012). Such adverse follicular conditions during oocyte maturation can interfere with the oocyte's developmental capacity (Jorritsma *et al.* 2004, Leroy *et al.* 2005, 2012, Aardema *et al.* 2011). Furthermore, embryos originating from oocytes exposed to elevated NEFA concentrations exhibit an altered oxidative metabolism, compromised glucose metabolism, and an altered gene expression pattern as they develop up to day 7 (Van Hoeck *et al.* 2011).

In this study, we investigated the etiological mechanisms by which these events occur. We have used cows in negative energy balance (NEB) as a model system to investigate the lipotoxic effects of elevated NEFA on the oocyte. Although there are obvious differences between ruminant NEB and human obese of type II diabetes conditions, both ultimately lead to upregulated lipolysis and to elevated serum NEFA concentrations. The types and the concentrations of the NEFA used in the present model are based on the studies on follicular fluid *in situ* under conditions of upregulated lipolysis and thus are physiologically relevant (Leroy *et al.* 2005). We know from human follicular fluid analyses that our bovine observations accurately replicate the situation in the human. Furthermore, human serum NEFA concentrations under obese conditions (Hall & Saunders 1979, Reaven *et al.* 1988, Stolba *et al.* 1993) are very similar to the bovine serum NEFA concentrations during NEB (Leroy *et al.* 2005). We therefore hypothesized that the compromised oocyte developmental competence, which results from NEFA exposure during the final stages of maturation, is caused by modifications in oocyte energy metabolism, a concept based on the fundamental research in myocardial cells exposed to elevated NEFA concentrations, in which high fatty acid β -oxidation rates contribute to the development of myocardiopathies (for an overview: Lopaschuk *et al.* (2010)). Interestingly, Igosheva *et al.* (2010) also reported that excessive nutrient exposure before and during conception in obese mice might be associated with a compromised oocyte and embryo mitochondrial metabolism.

At the molecular level, oocytes matured in medium with elevated concentrations of palmitic, stearic, and OA showed a significant increase in mRNA encoding *GADPH* and *LDHA*. *GADPH* catalyzes the sixth step of

glycolysis, converting NAD^+ to NADH and thus generating reducing power. LDHA is also recognized as a strong cytosolic reductant (Lane & Gardner 2000) as it preferentially converts lactate into pyruvate (Dumollard *et al.* 2007, Wilding *et al.* 2009), with the conversion of NAD^+ to NADH. It is also noteworthy that the products of these two genes regulate transcription by virtue of their ability to interact with the transcriptional cofactor Oct1 in a REDOX-dependent manner (Zheng *et al.* 2003). Furthermore, the HIGH COMBI oocytes showed significant upregulation of *GPX1* expression, a ROS-related gene, which supports our proposition that the REDOX status of the oocyte is affected by exposure to high NEFA. *GPX1* is of fundamental importance since it acts in the detoxification of hydrogen peroxide. Interestingly, increased expression of *GADPH* will spare NAD(P)H, which is essential for the recycling of glutathione. In other words, the differential expression of these specific genes in response to NEFA exposure may provide a mechanism for the imbalance in intracellular REDOX potential, as reported in the previous studies in leucocytes (Kakinuma & Minakami 1978, Listenberger *et al.* 2001), a state characterized by an imbalance between pro-oxidant molecules including ROS and antioxidant defenses. A moderate increase in ROS levels can stimulate cell growth and proliferation and is physiologically important. Conversely, excessive ROS will cause cellular injury (e.g. damage to DNA, lipid membranes, and proteins). Mitochondria are major sites for ROS production and excessive ROS can affect their function in oocytes and embryos (for review: Agarwal *et al.* (2012)).

In contrast to the observations in the oocyte, cumulus cells from HIGH NEFA-exposed COCs exhibited down-regulated expression of genes encoding enzymes involved in REDOX regulation: *GADPH*, *GPX1*, *G6PD*, and *LDHA*. Furthermore, expression of the *DNMT3A* gene was significantly downregulated in HIGH COMBI and HIGH SA cumulus cells, pointing to the possibility of altered methylation status. Cumulus cells are vital for the oocyte to complete successful maturation and sustain further development (Tanghe *et al.* 2002). Moreover, cumulus cells likely play a role in regulating REDOX homeostasis by providing glutathione to the oocyte (Geshi *et al.* 2000). If the cumulus cells do not succeed in safeguarding this system and fail to protect the oocytes, there could be a compensating mechanism in oocytes to upregulate their own defense against ROS. Thus, impaired viability and anti-ROS defense of the cells surrounding the oocyte most likely reflects jeopardized oocyte capacity to defend against ROS, consistent with the significant upregulated *GPX1* and *LDHA* expression in the oocyte.

As a result of our own work (Van Hoeck *et al.* 2011) and that of others (Rizos *et al.* 2002, Lonergan *et al.* 2003, Sirard *et al.* 2006), it can be concluded that the nutritional environment during maturation can affect

the resulting embryo. We therefore examined whether blastocysts originating from NEFA-exposed oocytes displayed differences in transcript abundance of key genes. The HIGH COMBI blastocysts tended to display upregulated expression of *MNSOD*, an enzyme that plays a protectant role against oxidative stress (Harvey *et al.* 1995), which might be the result of a compensatory mechanism in response to a shifted REDOX potential at earlier stages of development and in turn affect the activity of REDOX-sensitive transcription factors, a suggestion supported by our previous demonstration of *SLC2A1* mRNA upregulation in HIGH COMBI embryos (Van Hoeck *et al.* 2011). Moreover, the HIGH COMBI embryos showed higher expression of *TFAM*, which is essential in stabilizing mitochondrial DNA and for which overexpression is reported to be related to oxidative stress (Suarez *et al.* 2008). Interestingly, a significant increase was also observed in the mRNA encoding enzymes involved in the fatty acid synthesis pathways; namely *ACSL1* and *ACCA* in HIGH COMBI blastocysts. *ACSL1* is involved in the activation of long-chain fatty acid for β -oxidation; however, when malonyl Co A, the product of *ACCA*, is also abundant, there is a shift toward synthesis of triglycerides. The parallel increase in the mRNA abundance of *ACSL1* and *ACCA* suggests increased lipogenesis in such embryos as *ACSL1* has been closely linked to triglyceride synthesis, and overexpression suggests a mechanism for sequestering fatty acids as triacylglycerols in lipid droplets (Li *et al.* 2010). The ability to store NEFA in lipid droplets might have a protective function in channeling fatty acids away from other lipotoxic pathways that may have been triggered at oocyte level (Cnop *et al.* 2001, Li *et al.* 2010). However, previous studies in somatic cell lines have demonstrated that excessive accumulation of lipids in peripheral tissues is closely associated with the pathological state of insulin resistance in type II diabetes (Zhang & Zhang 2012). Therefore, the question arises whether such adaptations toward lipid storage provide protective/compensatory mechanisms or rather activate other toxicity-related pathways. Both options may have an important impact during further embryo development, implantation, and fetal development, problems that might not become apparent until post-implantation, fetal, or even neonatal development.

As the organization, positioning, and morphology of mitochondria within the oocyte are indicative of the energy requirements of key events during oocyte maturation (Sun *et al.* 2001), we evaluated mitochondrial distribution in oocytes matured in the presence and absence of elevated NEFA. Although mitochondrial organization differs between morphologically good and poor oocytes and may be associated with different developmental capacity after IVF (Stojkovic *et al.* 2001), transmission electron microscopic analyses did not reveal obvious differences in

oocyte mitochondrial distribution or morphology between HIGH COMBI oocytes and controls.

We next sought to investigate the functional effects of exposure to high NEFA on mitochondrial activity, by testing the effect of β -oxidation inhibition during oocyte maturation. A strong metabolic role for lipid oxidation during oocyte maturation has been substantiated previously in mouse (Downs *et al.* 2009, Dunning *et al.* 2010) and bovine oocytes (Sturme *et al.* 2006). Using β -MA, an enzyme that inhibits the β -oxidation, we found that the developmental potential of HIGH COMBI oocytes could be rescued if β -oxidation was inhibited during oocyte maturation ($P=0.05$). This suggests that mitochondrial fatty acid β -oxidation is involved in developmental failure in bovine oocytes exposed to elevated NEFA concentrations. By contrast, control oocytes exposed to basal NEFA concentrations did not display significant differences in postfertilization development after inhibition of β -oxidation, which is in line with the findings from Sturme *et al.* (2006). We have previously shown that the oxygen consumption of embryos originating from NEFA-exposed oocytes is significantly reduced (Van Hoeck *et al.* 2011) and therefore wished to discover whether such a reduction in oxygen consumption was already evident directly after maturation. As it was not possible to measure the respiration of the oocyte alone, due to the presence of surrounding cumulus cells (Wilding *et al.* 2009), oxygen consumption was determined in zygotes originating from HIGH NEFA-exposed oocytes. We also measured the ratio of high vs low-polarized mitochondrial membranes in these zygotes to provide a global picture of mitochondrial activity as this feature has been positively correlated with the level of respiration (Miwa & Brand 2003, Van Blerkom *et al.* 2006). The oxygen consumption of zygotes was not significantly different between treatments. Likewise, confocal analyses did not reveal major differences in the activity of mitochondria in zygotes arising from the different treatments, although there was a tendency for an increased ratio of high vs low-polarized mitochondrial membranes in HIGH NEFA zygotes. One possible explanation for this observation might relate to the metabolic plasticity of the early embryo. Measuring oxygen consumption and the ratio of high vs low-polarized mitochondrial membranes provides information on overall oxidative process in terms of ATP synthesis from oxidative phosphorylation. However, the identity of the major substrate oxidized to generate the ATP cannot be determined from such experiments. The observation that inhibition of β -oxidation during maturation restores developmental capacity in NEFA-exposed oocytes does, however, strongly suggest that the pathways adopted by the early embryo to generate substrates for oxidation are crucial in determining ongoing viability and might be influenced by the conditions under which the oocyte matures. If the oocyte is unable to oxidize fatty acids for the generation

of ATP, one can speculate that it alters its metabolism to oxidize alternative exogenous substrates such as pyruvate, lactate, and glucose (Sutton-McDowall *et al.* 2010).

The follicular microenvironment before conception is crucial for maturing oocytes (for an overview: Leroy *et al.* (2012)). As such, using a bovine *in vitro* production model, we were able to demonstrate that elevated NEFA concentrations during the final phase of oocyte maturation compromise further development and even alter the gene expression pattern of the resultant embryo, which substantiates our previous work (Leroy *et al.* 2005, Van Hoeck *et al.* 2011). However, Britt (1994) hypothesized that the developmental competence of oocytes, in high-yielding dairy cows, is determined by their biochemical environment during a long period (up to 80 days) of follicular growth before ovulation. So far, studies determining the effects of a long-term NEFA exposure during folliculogenesis and oogenesis have not been performed, though might be crucial in determining the final fate of oocytes dwelling in NEFA-exposed follicles.

Conclusions

Our data indicate that the follicular conditions under which the oocyte completes final maturation have a significant effect on the subsequent embryo in terms of gene and molecular/cellular phenotypic expression, particularly with regard to metabolically critical REDOX- and FAS-related gene function, as well as influencing the metabolic strategy of the early embryo. We can speculate that such profound alterations apparent at the oocyte stage may persist in fetal development and in the offspring.

Materials and Methods

Preparation of NEFA treatments

All chemicals were purchased from Sigma unless otherwise stated. SA, PA, and OA were dissolved in a stock solution of pure ethanol at concentrations of 25, 150, and 200 mM respectively. These ethanol stock solutions were vortex mixed for 4 min and diluted in working solutions to obtain the desired final concentration in maturation medium. The serum-free maturation medium consisted of TCM199 supplemented with 0.75% BSA free of fatty acids, 0.4 mM glutamine, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 μ g/ml gentamicin, and murine epidermal growth factor (20 ng/ml). All solutions were shaken for 45 min and filter sterilized under aseptic conditions.

In vitro embryo production

Bovine ovaries were collected at local abattoirs as soon as possible after killing and transported immediately to the laboratory. They were washed three times in warm saline

solution (38 °C) supplemented with 0.5% kanamycin sulfate. Follicles with a diameter of 2–6 mm were aspirated. Unexpanded COCs surrounded by five or more cumulus cell layers (quality grade I) were matured *in vitro* as described by Leroy *et al.* (2010). The COCs were washed in 500 µl maturation medium and matured in groups of 50–60 COCs in 500 µl maturation medium in four-well plates (Nunc, Langenselbold, Germany) for 22–24 h in humidified air with 5% CO₂ at 38.5 °C. After IVM, all COCs were coincubated in groups of 100–120 with spermatozoa at a final concentration of 10⁶ sperm cells/ml for 20 h at 38.5 °C in 500 µl fertilization medium in a humidified 5% CO₂ incubator. For all experiments, frozen semen from a bull of proven IVF (Van Hoeck *et al.* 2011) was thawed and live spermatozoa were selected by centrifugation on a discontinuous Percoll gradient (90 and 45%, Amersham Biosciences). The final sperm–egg ratio was adjusted to 5000:1. Fertilization medium consisted of 114 mM NaCl, 3.1 mM KCl, 0.3 mM Na₂HPO₄, 2.1 mM CaCl₂·2H₂O, 0.4 mM MgCl₂·6H₂O, 25 mM bicarbonate, 1 mM pyruvate, 36 mM lactate, 2 µl/ml phenol red, 6 mg/ml BSA, 50 µg/ml gentamycin, and 10 µl/ml heparin. After coincubation with spermatozoa, the presumptive zygotes were vortexed for 4 min to remove excess sperm and cumulus cells. After three wash steps in HEPES-TALP and modified SOF medium, presumptive zygotes were cultured in groups of 25±4 in 50 µl modified SOF medium with mineral oil overlay (modular incubator: 38.5 °C, 5% CO₂, 5% O₂, and 90% N₂) until day 7 p.i. The SOF medium comprised 108 mM NaCl, 7.2 mM KCl, 1.2 mM KH₂PO₄, 0.8 mM MgSO₄·7H₂O, 0.6 mM sodium lactate, 25 mM NaHCO₃, 0.0266 mM phenol red, 0.73 mM sodium pyruvate, 1.78 mM CaCl₂·2H₂O, 0.34 mM trisodium citrate, 2.755 mM myoinositol, 3% v/v BME 50x, 1% v/v MEM 100x, 0.4 mM glutamine, 5% fetal bovine serum, and 50 µg/ml gentamycin.

Composition of the oocyte maturation treatments

The types and concentrations of free fatty acids used in this study are based on bovine *in vivo* studies in serum and in follicular fluid (Leroy *et al.* 2005) and are physiologically appropriate, as circulating NEFA concentrations in women suffering lipolysis-linked metabolic disorders, including obesity (Reaven *et al.* 1988, Stolba *et al.* 1993), are very similar to NEFA concentrations detected in bovine during an episode of upregulated lipolysis (Leroy *et al.* 2005).

Standard serum-free maturation systems are devoid of fatty acids, although the physiological environment, in which the oocyte matures *in vivo*, contains physiological, basal concentrations of NEFA (Leroy *et al.* 2005). In order to improve the relevance of our *in vitro* model, we therefore used a maturation medium supplemented with basal NEFA concentrations as control medium. We showed that inclusion of physiological concentrations of the key NEFA in maturation media formulations (three replicates; 595 oocytes) does not affect developmental competence compared with standard serum-free maturation media ($P>0.1$, details in Van Hoeck *et al.* (2011)). Moreover, in previous studies, we identified SA as the most toxic NEFA for oocyte developmental competence

(Leroy *et al.* 2005, Van Hoeck *et al.* 2011). We therefore focused on the following NEFA treatments in this study:

1. control=physiological NEFA concentrations (150 µM total NEFA comprising 25 µM SA, 50 µM PA, and 75 µM OA)
2. HIGH SA=elevated SA concentrations (75 µM SA)
3. HIGH COMBI=combination of elevated NEFA concentrations (425 µM total NEFA, comprising 75 µM SA, 150 µM PA, and 200 µM OA).

RNA extraction, RT, and quantification of mRNA transcript abundance

For gene expression analysis, cumulus cells were separated from the mature oocytes by repeated aspiration with a small-bore glass pipette. Poly(A) RNA of cumulus cells, oocytes, and day 7 blastocysts was extracted using a Dynabead mRNA Direct Extraction KIT (Dyna Bead, Madrid, Spain) according to the manufacturer's instructions, with minor modifications. Immediately after extraction, the RT reaction was carried out following the manufacturer's instructions (Bioline, London, UK), using poly(T) primer, random primers, and the MMLV reverse transcriptase enzyme, in a total volume of 40 µl, in order to prime the RT reaction and to synthesize cDNA. Tubes were heated to 70 °C for 5 min to denature the secondary RNA structure and the RT reaction was completed with the addition of 100 units of reverse transcriptase. The mixture was incubated at 42 °C for 60 min to allow the RT of RNA, followed by 70 °C for 10 min to denature the RT enzyme. The quantification of all mRNA transcripts was carried out by real-time quantitative RT-PCR (qPCR). For qPCR, four groups of cDNA per experimental group were used with two replicates for all genes of interest. Experiments were conducted to compare relative levels of each transcript and histone *H2AFZ* in every sample. The PCR was performed by adding a 2 µl aliquot of each sample to the PCR mix, containing the specific primers, to amplify the genes of interest. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 2. For quantification, qPCR was performed as described previously (Bermejo-Alvarez *et al.* 2010). PCR conditions were optimized to achieve efficiencies close to 1 and the comparative cycle threshold (CT) method used to quantify expression levels as described by Schmittgen & Livak (2008). Quantification was normalized to the endogenous control *H2AFZ*. The genes chosen have been associated with aspects of embryo energy metabolism: *LDHA*, *GADPH*, facilitated glucose transporter 1 (*SLC2A1*), and *G6PD*; oxidative stress: *MNSOD* and *GPX1*; *de novo* methylation: *DNMT3A*; mitochondrial biogenesis: *TFAM*; and fatty acid metabolism: *CPT1A*, adipophilin 2 (*PLIN2*), *ACCA*, mitochondrial uncoupling protein 2 (*UCP2*), and transcription factor A, mitochondrial (*ACSL1*), all of which have been reported to play a key role in NEFA toxicity pathways in several cell types. A total of 180 COCs were used to analyze gene expression in cumulus cells and oocytes in three independent repeats and 192 blastocysts were used for gene expression analysis in five independent repeats (samples equally distributed along treatments).

Table 2 Details of primers used for qRT-PCR.

Gene	Primer sequence (5'–3')	Fragment size (bp)	GenBank accession no.
H2AFZ	AGGACGACTAGCCATGGACGTGTG CCACCACCAGCAATTGTAGCCTTG	212	NM_016750
PLIN2	ACAACACACCCCTCAACTGG CTGCCTGCCTACTTCAGACC	211	BT029909.1
MNSOD	GCTTACAGATTGCTGCTTGT AAGGTAATAAGCATGCTCCC	101	S67818.1
CPT1A	CTGCCCGCCTGGGAAATGCTGT CAGTCTCTCCTCCCCGGGCTGG	328	NM_001034349.2
ACSL1	TGACTGTTGCTGGAGACTGG TGTGCTTCTCCTGTGCGATG	250	NM_001076085.1
LDHA	TTCTTAAGGAAGAACATGTC TTCACGTTACGCTGGACCAA	310	NM_174099.2
GPX1	GCAACCAGTTTGGGCATCA CTCGCACTTTTCGAAGAGCATA	116	NM_174076.3
ACCA	AAGCAATGGATGAACCTTCTTC GATGCCCAAGTCAGAGAGC	196	FN185963.1
GADPH	ACCCAGAAGACTGTGGATGG AYGCCTGCTTACCACCTTC	247	BC102589
SLC2A1	CTGATCCTGGGTCGCTTCAT ACGTACATGGGCACAAAACCA	68	NM_174602.2
G6PD	CGCTGGGACGGGGTGCCCTTCATC CGCCAGGCCTCCCGCAGTTCATCA	347	XM_583628.4
DNMT3A	CTGGTGCTGAAGGACTTGGGC CAGAAGAAGGGGCGGTCATC	318	AY271299

Mitochondrial morphological evaluation, ultrastructure, and distribution

Immediately following maturation, COCs were fixed in 4% formaldehyde for 1–3 h and prepared for transmission electron microscopy according to Abe *et al.* (1999). COCs were washed in PBS and postfixed for 1 h with 1% osmium tetroxide in PBS at 0–4 °C. Subsequently, the COCs were individually embedded in 1% agar before the epoxy resin embedding process. All samples were dehydrated in ascending concentrations of ethanol solutions (50–100%), substituted in propylene oxide, and embedded in epoxy resin. Ultra-thin sections were prepared using an ultramicrotome. These sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope operated at 60 kV. In total, 15 COCs, equally distributed along treatments, were visualized.

Evaluation of the effect of β -oxidation inhibition

β -Oxidation was inhibited by incubation with β -MA, a competitive inhibitor of 3-hydroxyl CoA dehydrogenase, a component of the β -oxidation pathway (Sturme *et al.* 2006). During a serum-free maturation period of 24 h, bovine COCs were exposed to the following treatments: i) control, ii) HIGH COMBI, iii) control + 0.1 mM β -MA, and iv) HIGH COMBI + 0.1 mM β -MA. Following IVF, zygotes were cultured in SOF (+5% of FCS) medium as described earlier. Cleavage (2 days p.i.) and blastocyst rates (7 days p.i.) were defined as the number of cleaved zygotes or blastocysts formed per oocyte matured respectively. The number of blastocysts from cleaved zygotes was also recorded. A total of 820 COCs, equally allocated over treatments, was used in five independent replicates.

Oxygen consumption analysis

As the maturing oocyte is surrounded by cumulus cells, it is not relevant to measure respiration of the oocyte alone (Wilding *et al.* 2009). Oxygen consumption and mitochondrial membrane potential were therefore measured in zygotes after they had been denuded of cumulus oophorus. Individual zygotes day 1 p.i. were loaded into a PCR Glass micropipette (Drummond, Broomal, PA, USA) and allowed to respire for 30 min to form an oxygen gradient, which was measured in real time using a nanorespirometer (Unisense, Aarhus, Denmark) and converted to oxygen consumption rate using SensorTrace Pro (Unisense) according to the previous studies (Lopes *et al.* 2005, Van Hoeck *et al.* 2011). In total, 54 zygotes, equally distributed along treatments, were analyzed on three independent occasions.

Analysis of the ratio of high vs low-polarized mitochondrial membranes

Bovine zygotes were stained with 5,5',6,6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazoly-carbocyanine iodide (JC1) following the method of Van Blerkom *et al.* (2002). Briefly, zygotes at day 1 p.i. were washed three times in PBS and incubated in SOF culture medium containing 2 μ g/ml JC-1 in a modular incubator (38.5 °C, 5% CO₂, 5% O₂, and 90% N₂) for 25 min. Subsequently, the zygotes were washed three times and transferred into imaging dishes, protected from light, and immediately evaluated on a PerkinElmer Ultraview Vox confocal microscope (Waltham, MA, USA) in the FITC (bandpass 500–550 nm), rhodamine isothiocyanate (bandpass 580–650 nm) channels with narrow band filter sets as shown in Fig. 5. Images were acquired using Volocity 6.0.1 software and data on pixel intensity in each channel were determined with ImageJ, a Java image-processing program, with sample origin

blinded to the scorer. In each zygote, the ratio of high vs low-polarized mitochondrial membranes was estimated as the percentage of the red pixel intensity (highly polarized membrane) out of the total red and green pixel intensity (total mitochondrial mass). A total of 62 zygotes, equally distributed along treatments, were analyzed, at both the top and the midequatorial plane (Fig. 5), on three independent occasions.

Statistical analyses

Data are expressed as mean $\pm$ S.E.M. except for the β -MA experiment. Relative transcript abundance was analyzed using the SigmaStat (Jandel Scientific) software package using one-way ANOVA with multiple pairwise comparisons using the Student–Newman–Keuls method *post hoc*. Other statistical procedures were carried out with PASW Data Collection 5.6 (for Windows, Chicago, IL, USA). For the β -MA experiment, cleavage and blastocyst rates were compared between the three treatments using a binary logistic regression model taking replicate, treatment, and the interaction term into account. Where no significant interaction was present, the term was omitted from the final model. For oxygen consumption and ratio of high vs low-polarized mitochondrial membranes, a mixed model ANOVA, taking treatment as fixed factor and replicate as random factor, was used to compare differences between the three groups. Replicate, treatment, and the interaction term were taken into account. If no significant interaction was present, the term was omitted from the final model. Data transformations were not required for inequality of variance between groups nor for achieving normality for any data.

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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