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

V Van Hoeck, J L M R Leroy, M Arias Alvarez¹, D Rizos², A Gutierrez-Adan², K Schnorbusch, P E J Bols, H J Leese³ and R G Sturmey³

Veterinary Physiology and Biochemistry, Department of Veterinary Sciences, Faculty of Biomedical, Pharmaceutical and Veterinary Sciences, Gamete Research Center, University of Antwerp, Universiteitsplein 1 - Gebouw U, B-2610 Wilrijk, Belgium, ¹Departamento Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Ciudad Universitaria, s/n, 28040 Madrid, Spain, ²Departamento de Reproducción Animal, Instituto Nacional de Investigacion y Tecnologia Agraria y Alimentaria, Ctra. de la Coruña Km. 5,9, 28040 Madrid, Spain and ³Hull-York Medical School, University of Hull, Hertford Building, Cottingham Road, HU6 7RX Hull, UK

Correspondence should be addressed to V Van Hoeck; Email: veerle.vanhoeck@ua.ac.be

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.

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 follicle microenvironments (Leroy et al. 2004, 2005, Robker et al. 2009, Junghem 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. 2003), 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 (lossa 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 ≤ 0.02). Elevated GADPH expression was also observed in the HIGH SA

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![Figure 1](https://via.placeholder.com/150)  
**Figure 1** Comparison of relative transcript abundance in oocytes. Oocytes were matured under control (150 μM total, physiological, NEFA), HIGH SA (75 μM SA), and HIGH COMBI (425 μM total, elevated, NEFA) conditions (n=180; three replicates). Bars with different superscripts are significantly different between treatments with P ≤ 0.02.
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
biogenesis and oxidative stress (transcription factor A mitochondria, TFAM, and manganese superoxide dismutase, MNSOD respectively) tended to be increased in HIGH COMBI embryos ($P < 0.1$).

**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 $\beta$-oxidation**

As shown in Table 1, the presence of $\beta$-mercaptoacetate ($\beta$-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 $\beta$-MA and HIGH COMBI NEFA was higher than when $\beta$-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 ($1.25 \pm 0.07 \text{ nl/embryo per h}$) and HIGH COMBI ($1.11 \pm 0.11 \text{ nl/embryo per h}$) 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

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<th>Table 1 Developmental competence of NEFA-exposed oocytes in the presence and absence of $\beta$-mercaptoacetate ($\beta$-MA), an inhibitor of the mitochondrial $\beta$-oxidation.</th>
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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 $\mu$M total, physiological, NEFA), ii) HIGH COMBI (425 $\mu$M total, elevated, NEFA), iii) control (150 $\mu$M total, physiological, NEFA) +0.1 mM $\beta$-MA, and iv) HIGH COMBI (425 $\mu$M total, elevated, NEFA) +0.1 mM $\beta$-MA. Data marked with different superscripts are significantly different ($P < 0.05$).
higher in HIGH COMBI zygotes compared with the controls for the top plane (55.0% ± 1.2 vs 51.2% ± 2.6 respectively; P=0.09) and for the average of both the top and the midequatorial planes (53.8% ± 1.2 vs 50.9% ± 2.4 respectively; P=0.09). No difference in mitochondrial membrane polarity ratios could be detected between treatments for the midequatorial plane (52.9% ± 1.2 vs 50.7% ± 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, Junghem 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 cardiomyopathies (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 transcripational 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 \(\beta\)-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 mito-
chondrial distribution in oocytes matured in the
presence and absence of elevated NEFA. Although
mitochondrial organization differs between morpho-
logically good and poor oocytes and may be associated
with different developmental capacity after IVF
(Stojkovic et al. 2001), transmission electron micro-
scopic 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 (Sturmey 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 Sturmey 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 μg/ml gentamicin, and murine epididymal 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 25–41 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 myo-inositol, 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 (Dynal Biotech, 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 (ACSF1), 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 among treatments).
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 (Sturmey 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 μg/ml JC1 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 Velocity 6.0.1 software and data on pixel intensity in each channel were determined with ImageJ, a Java image-processing program, with sample origin

Table 2 Details of primers used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’–3’)</th>
<th>Fragment size (bp)</th>
<th>GenBank accession no.</th>
</tr>
</thead>
</table>
| H2AFZ  | AGGACGACTGACGTTGGGATGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGTGAGTGGGGHG

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Etiology of NEFA toxicity in oocytes
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 ± 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–Kleus 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. 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.

Funding

This work was supported by the Flemish Research Fund (FWO grant 1106910N: V Van Hoeck), GEMINI COST ACTION FA0702 (short mission scientific stay in RGS’s lab), the Spanish Ministry of Science and Innovation (A Gutierrez-Adan by Grant AGL2009-11358; D Rizos by Grant AGL2009-11810), and the Special Research Fund (BOF-UA: J L M R Leroy). M Arias Alvarez is postdoctoral researcher funded by the Spanish Ministry for Science and Education. The PerkinElmer Ultraview Vox was purchased with support of the Hercules Foundation (Hercules Type 1: AUHA-09-001).

Acknowledgements

The authors acknowledge Els Merckx and Silke Andries, of the University of Antwerp Gamete Research Center, for their outstanding technical assistance with IVP and also Meg Stark, of the University of York Technology Facility, for excellent guidance in electron microscopy and the Ministry for Education and Science of Spain.
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