Nonesterified Fatty Acid-Induced Endoplasmic Reticulum Stress in Cattle Cumulus Oocyte Complexes Alters Cell Metabolism and Developmental Competence

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ABSTRACT

Reduced oocyte quality has been associated with poor fertility of high-performance dairy cows during peak lactation, due to negative energy balance. We examined the role of nonesterified fatty acids (NEFAs), known to accumulate within follicular fluid during under- and overnutrition scenarios, in causing endoplasmic reticulum (ER) stress of in vitro maturated cattle cumulus-oocyte complexes (COCs). NEFA concentrations were: palmitic acid (150 μM), oleic acid (200 μM), and steric acid (75 μM). Abattoir-derived COCs were randomly matured for 24 h in the presence of NEFAs and/or an ER stress inhibitor, salubrinal. Total and hatched blastocyst yields were negatively impacted by NEFA treatment compared with controls, but this was reversed by salubrinal. ER stress markers, activating transcription factor 4 (Atf4) and heat shock protein 5 (Hspa5), but not Atf6, were significantly up-regulated by NEFA treatment within whole COCs but reversed by coincubation with salubrinal. Likewise, glucose uptake and lactate production, measured in spent medium samples, showed a similar pattern, suggesting that cumulus cell metabolism is sensitive to NEFAs via an ER stress-mediated process. In contrast, while mitochondrial DNA copy number was recovered in NEFA-treated oocytes, oocyte autofluorescence of the respiratory chain cofactor, FAD, was lower following NEFA treatment of COCs, and this was not reversed by salubrinal, suggesting the negative impact was via reduced mitochondrial function. These results reveal the significance of NEFA-induced ER stress on bovine COC development, revealing a potential therapeutic target for improving oocyte quality during peak lactation.

cattle, cumulus oocyte complex, endoplasmic reticulum stress, lipids, nonesterified fatty acids

INTRODUCTION

The maternal environment during the periconception period (encompassing the final stages of oocyte maturation through to implantation) can have profound effects on the developmental competence of oocytes. In particular, the follicular environment mirrors the maternal metabolic state, hence over- and undernutrition influence oocyte developmental competence and subsequent embryo health. Fatty acid metabolism within the cumulus-oocyte complex (COC) is essential for oocyte competence, as evidenced by the fact that inhibition with etomoxir, an agent that inhibits β-oxidation, during in vitro maturation (IVM) results in poor embryo yields [1]. Furthermore, enhanced fatty acid metabolism occurs by improving β-oxidation capacity when IVM medium is supplemented with L-carnitine to IVM, reducing lipid content and improving subsequent developmental outcomes [2].

High-performance dairy cows, especially those with milk yields above 10,000–20,000 L/cow/yr, have reduced fertility during peak lactation as a consequence of negative energy balance. Negative energy balance mobilizes nonesterified fatty acids (NEFAs) as an alternative to carbohydrate oxidation, sparing glucose for milk production. This can result in up to a 3-fold increase in circulating NEFA levels in the early postpartum period compared with prepartum serum levels [3, 4]. More significantly, even though the rise in serum NEFA levels is not necessarily consistent between lactating cows and heifers [5], follicular fluid levels of specific NEFAs—palmitic, stearic, and oleic acids [5]—are significantly higher in lactating cows. It has been proposed that these increased follicular NEFA concentrations damage the oocyte [6], explaining the reduced fertility within high-performance dairy cows. Experimental demonstration of the impact of NEFAs in dairy cows has been conducted largely using IVM of abattoir-derived COCs in the presence of elevated levels of NEFAs, replicating levels within ovarian follicular fluid during the early postpartum period. This has convincingly demonstrated that NEFAs—palmitic, oleic, and steric acids—reduce oocyte developmental competence [7–9]. Similarly in mouse, high NEFA levels, specifically palmitic acid, during IVM reduces oocyte mitochondrial activity and developmental competence [10]. This response to high lipid levels in mouse COCs was demonstrated to involve endoplasmic reticulum (ER) stress pathways because treatment with the well-characterized ER...
stress inhibitor, salubrinal, recovered both oocyte mitochondrial activity and oocyte developmental competence [10]. These studies provide insight into cellular alterations that might mechanistically contribute to lactation-associated impairments in cattle oocyte maturation and developmental competence.

We hypothesized that elevated levels of specific NEFAs, mimicking those in the follicular fluid of lactating dairy cattle, induce COC ER stress and oocyte mitochondrial dysfunction, leading to compromised oocyte developmental competence. Hence, the aim of this study was to investigate the influence of elevated NEFA levels during IVM on COC metabolism and ER stress markers, and determine whether NEFA-induced ER stress could be reversed using salubrinal, an ER stress inhibitor.

MATERIALS AND METHODS

Unless specified, all chemicals were purchased from Sigma-Aldrich.

Preparation of Nonesterified Fatty Acid Media Supplement

NEFA aliquots were prepared as previously described [11]. Briefly, 44.85 mM stocks of oleic acid (C18:1), stearic acid (C18:0), and palmitic acid (C16:0) were made with absolute ethanol. One ml of this stock was added to 0.5 g fatty acid-free (FAP) bovine serum albumin (BSA) (MP Biomedicals) in 30 ml Milli-Q water followed by 5 ml of 0.1 M NaOH and was gently stirred until the solution was clear. Aliquots were frozen, freeze-dried, and reconstituted in IVM medium prior to culture.

Collection and Culture of COCs

In vitro oocyte maturation was performed using VitroMat (IVF Vet Solutions) + 6 mg/ml FAF BSA + 0.1 IU/ml FSH (Purogen; Organon). Cattle ovaries were transported from a local abattoir (Thomas Foods) in warm saline (30°C–35°C). Follicles were aspirated using an 18-gauge needle attached to a 10 ml syringe, and intact, unexpanded COCs surrounded by four or more cumulus layer cells and ungranulated ooplasm were selected in undiluted follicular fluid, quickly washed twice in IVM medium, and then transferred into pre-equilibrated IVM culture drops overlaid with paraffin oil (Merck). Treatment groups were 1) control (IVM medium), 2) NEFA (IVM medium + 150 mM palmitic acid + 200 mM oleic acid + 75 mM steric acid) [9], 3) salubrinal (IVM medium + 400 mM salubrinal; Merck), and 4) NEFA + salubrinal. Groups of 10 COCs were cultured in 100 μl of media for 23 h at 38.5°C with 6% CO2 in humidified air.

Preliminary experiments were conducted to assess the impact of supplementing IVM cultures (NEFA) with three pharmacological inhibitors of ER stress (tauroursodeoxycholic acid, phenylbutyric acid, and salubrinal) on on-time development post-IVF. After two experimental replicates, blastocyst development rates on Day 8 (D8) were tauroursodeoxycholic acid = 53%, phenylbutyric acid = 58%, and salubrinal = 71%. Hence, in standard IVM conditions, salubrinal supplementation improved developmental competence over other ER stress inhibitors. The salubrinal concentration used in this and subsequent experiments were based on levels previously described in the literature [10, 12] and our own dose-testing experiments using 200 nM or 400 nM salubrinal (Supplemental Table S1; Supplemental Data are available online at www.biolreprod.org).

To determine the effect of NEFA supplementation on oocyte lipid content, a cohort of COCs (n = 20 per treatment group) were mechanically demembrated by repeat pipetting (denuded oocyte, DO), cultured in 0.1% saponin for 23 h at 38.5°C, and incubated overnight with 1 μM bafilomycin A1, 1 μM ATP5, 0.1 M NaOH and was gently stirred until the solution was clear. Aliquots were frozen, freeze-dried, and reconstituted in IVM medium prior to culture.

In Vitro Embryo Culture

At the completion of IVM, COCs were washed once in wash medium—VitroWash (IVF Vet Solutions) + 4 mg/ml FAF BSA—transferred into 500 μl of IVF medium—VitroFert (IVF Vet Solutions) + 4 mg/ml FAF BSA + 10 IU/ml heparin + 25 μM penicillin/g + 12.5 μM hypotaurine + 1.25 μM epinephrine—and overlaid with paraffin oil. Two straws of bull sperm (Semex Australia Pty Ltd.) of proven fertility were thawed, prepared using a discontinuous Percoll gradient (45%:90%; GE Healthcare), and added to IVF wells at a final concentration of 1 × 10^5 sperm/ml. Following 23 h of co-incubation with sperm (D1), presumptive zygotes were mechanically demembrated by repeated pipetting, washed once in wash medium, and groups of five embryos were transferred into 20 μl of pre-equilibrated VitroCleave (IVF Vet Solutions) supplemented with 4 mg/ml FAF BSA overlaid with paraffin oil, and cultured at 38.5°C in 6% CO2, 7% O2, and 87% N2. On D5, five embryos were transferred into 20 μl of VitroBlast (IVF Vet Solutions) + 4 mg/ml FAF BSA and cultured at 38.5°C in 6% CO2, 7% O2, and 87% N2. Fertilization rates and on-time embryo development were assessed on D8. Five experimental replicates were performed, with 50 COCs used per treatment group within replicates.

On D8, the allocation of cells to the inner cell mass (ICM) and trophectoderm (TE) in a cohort of blastocysts was determined using a preembedding differential staining—20% Coomassie Blue (CBB) in PBS and 20% Neutral Red (NR) BODIPY in PBS. Two straws of bull sperm (Semex) were incubated in 100 μg/ml propidium iodine in 1% Triton X-100 (VitroWash (no protein)) for 30 sec followed by 25 μg/ml Hoechst 33342 in 100% ethanol overnight. Embryos were mounted on microscope slides with glycerol and visualized using an epifluorescence microscope (excitation: 340–380 nm and emission: 440–480 nm), with TE cells appearing pink and ICM appearing blue. A total of 20 embryos per treatment over five replicates were analyzed.

Expression of ER Stress Marker Genes

Groups of 40 COCs per treatment group collected over four experimental replicates were snap frozen and stored at −80°C. RNA was extracted using Trizol (Invitrogen, Life Technologies) as per the manufacturer’s instructions with the inclusion of 7.5 μg of glycoblue (Ambion, Life Technologies) during RNA purification. RNA concentration was determined using a Nanodrop ND1000 Spectrophotometer (Biolab) before reverse transcribing using random primers (Roche) and Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Quantitative RT-PCR was performed in an ABI 7700 (AB Applied Biosystems, Life Technologies) thermocycler. A level of CDNA was determined with TaqMan Gene Expression Master Mix (AB Applied Biosystems) using Taqman gene expression assays (ACTB FAM B03372917s-g1; ATF4 FAM B03221058-g1; ATF6 FAM B03287802-s1; HSP5 FAM B03244880-m1) purchased from Applied Biosystems, Life Technologies. Reactions were run in triplicate on an AB7900HT PCR system (Applied Biosystems) using the manufacturer’s specified amplification settings. Each reaction used 0.5 μl of Taqman assay, 5 μl of Taqman master mix, 5 μg cDNA, and 120 μl to a final volume of 10 μl. Real-time PCR data was analyzed using the 2^-ΔΔCt method; activating transcription factor 4 (ATF4), ATF6, and heat shock protein 70 kDa 5 (HSP5; also known as GRP78) were normalized to ACTB and expressed as the fold change relative to a calibrator CDNA sample, which was included in each run.

Glucose and Lactate Metabolism

Spent media (100 μl) from 23 h IVM of 10 COCs from all four treatment groups were collected, transferred into eppendorf tubes, and snap frozen. Glucose and lactate concentrations were determined using a COBAS Integra400 chemical analyzer (Roche) and were normalized to concentrations within blank media drops (culture drops without COCs) to determine changes in glucose and lactate levels. Levels of glucose consumed and lactate produced were expressed as pmol/COC/h. Three experimental replicates were performed, with each replicate containing four separate groups of COCs per treatment.

Quantification of Mitochondrial DNA Copy Number

The mitochondrial DNA (mtDNA) copy number in individual oocytes or blastocysts was quantified as previously described [16]. Briefly, following 23 h IVM in the presence of NEFAs and salubrinal, COCs were mechanically demembrated during their cumulus removal using 5 IU/ml hyaluronidase in wash medium and repeat pipetting. Denuded oocytes or D8 blastocysts were washed with 1 mg/ml PVP in PBS, collected individually into 1.5 ml siliconized low-retention microcentrifuge tubes (Fisher Scientific) with 5 μl of PVP in PBS, snap frozen in liquid nitrogen, and stored at −80°C until use. Genomic DNA was isolated from each sample using a QIAamp DNA Micro Kit (Qiagen), according to the manufacturer’s protocol. Carrier DNA (1 μg) (Qiagen) was added to each sample during DNA extraction. Genomic DNA was eluted twice with 50 μl of water and diluted 10 times for quantitative PCR. The quantification standards were prepared as described earlier [12]. Real-time
quantitative PCR using the primer pair 5′-CGT GAT GTC AAG GTG TAG CC-3′ and 5′-CCA GAC ACA CTT CTT AGT ATG-3′ was performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems) and a Rotor-Gene 6000 (Corbett) real-time rotary analyzer. Standard curves were created separately for each run and sample copy number was generated from the equation of Ct value against copy number for the corresponding standard curve. Data was normalized to the control (− salubrinal − NEFA) group. Five DOs and five expanded blastocysts were collected per treatment group.

Determination of FAD and NAD(P)H in Oocytes

At the completion of IVM, COCs were mechanically denuded by repeated pipetting in wash medium and transferred into 5 μl drops of media in glass bottom confocal dishes (Cell E&G) and then overlaid with paraffin oil. The fluorescence intensity of autofluorescent redox cofactors—oxidized flavin adenine dinucleotide (FAD) and reduced nicotinamide adenine dinucleotide (NAD(P)H)—were determined using a Fluoview FV10i confocal microscope using green (excitation = 473 nm, emission = 490–590 nm) and blue (excitation = 405 nm, emission = 420–520 nm) filters, respectively. Images were captured at 40× magnification and laser, sensitivity, and imaging parameters were kept constant between replicates. Fluorescent standard beads (InSpeck; Molecular Probes) were prepared as per the manufacturer’s instructions and imaged simultaneously with experimental samples to account for any variations in laser intensity between experimental replicates. The raw data was normalized to the standards as previously described [17]. Fluorescence intensity from the maximal cross-sectioned image of the DO was measured using Image J software (National Institute of Health). Three independent experiments were performed; with fluorescence intensity measurements derived from images of 10 DO per treatment per replicate.

Reactive Oxygen Species, Anti-Oxidants, and Active Mitochondria Within Mature Oocytes

After 23 h of culture, COCs were denuded by repeat pipetting and transferred in the dark into VitroWash + 4 mg/ml FAF BSA + 20 μM peroxynitro/L-phenylalanine (PFI: 3′,6′-bis[4,5,5-tetramethyl-1,3,2-dioxaborolane-2-yl]s-propylbenzofuran-3,9-xanthene-1-one) for 1 h, 12.5 μM monochlorobimane (MCB) 30 min, and 200 nM Mitotracker Red CMXROS (MTR) (Molecular Probes) for 15 min at 38.5°C. Oocytes were washed once in VitroWash + 4 mg/ml FAF BSA and transferred into 2 ml smears of wash medium in glass bottom confocal dishes. PFI is an aryl boronate probe that fluoresces upon reaction with H$_2$O$_2$ [17, 18] and was synthesized as described earlier [17]. It has higher specificity for H$_2$O$_2$ and peroxynitrite over other reactive oxygen species (ROS) compared to commonly used nonspecific ROS probes such as 2′,7-dichlorofluorescein diacetate, MCB fluoresces when bound to low molecular weight thiols compound. However, 99% of intracellular fluorescence intensity is attributed to binding with reduced GSH [19]. MTR accumulation within mitochondria is dependent on membrane potential; hence positive staining represents active mitochondria.

Intra-oocyte fluorescence was visualized using a Fluoview FV10i confocal microscope (MCB: excitation = 405 nm and emission = 519; MTR: excitation = 578 nm and emission = 598) with laser, magnification, and image settings remaining constant across replicates and raw data was normalized to fluorescence intensity of fluorescent standard beads (InSpeck). The images were processed and analyzed using Image J software and macros [17]. Briefly, individual channels were separated and converted to 8-bit gray scale. The oocyte was selected as the region of interest using a constant threshold, and the background of the image was excluded. Mean intensity and texture analyses (grey-level co-occurrence matrix, GLCM) were applied using batch macro plugins (available on the Image J website: http://rsb.info.nih.gov/ij/plugins). GLCM represents the texture patterns (uniformity/smoothness/roughness) of positive staining by determining the probability of pixel intensity patterns, hence indicating patterns of localization, and has been extensively utilized in ultrasound imaging, dermatology, and cancer research [20–22]. Within GLCM, three levels of texture analyses were determined; entropy represents orderliness/uniformity of the fluorescence (overall texture); contrast represents the finer homogeneity/regularity and predictability of fluorescence as seen in wrinkles and organelles (fine texture); and homogeneity represents density differences between neighboring pixels (pixel texture). Increased entropy and contrast values indicate increased heterogeneity of fluorescence within the oocyte, and increasing correlation values represent decreased heterogeneity, hence increased homogeneity staining patterns.

Statistical Analyses

The study design was a 2 x 2 factorial one; hence, a general linear model was used (SPSS statistical software, version 22; IBM) and two main effects (NEFA and salubrinal) were built into the model (two-way ANOVA). With this kind of statistical model, main effects or interactions between the two treatments are reported, and unlike a one-way ANOVA, no post hoc analyses were performed. Embryo development data was arcsine transformed prior to analyses. $P < 0.05$ was considered statistically significant. Data was presented in graphs as means ± SEM and significant main effects and interaction terms presented.

RESULTS

In Vitro Embryo Culture

Following IVM in the presence or absence of NEFAs and salubrinal, COCs were fertilized and cultured, and on-time embryo development was assessed 8 days later. Regardless of treatment, there were no significant differences in cleavage rates (Fig. 1A). However, the presence of NEFAs significantly decreased total blastocyst development compared to the control group (main effect: $- \text{NEFA} = 50.2\% ± 1.5\% \text{ vs. } + \text{NEFA} = 43.4\% ± 3.7\%$; $P = 0.020$; Fig. 1B), whereas salubrinal supplementation during IVM recovered total blastocyst rates (main effect: $- \text{salubrinal} = 43.3\% ± 1.5\% \text{ vs. } + \text{salubrinal} = 50.4\% ± 3.7\%$; $P = 0.025$; Fig. 1B). Furthermore, regardless of NEFA supplementation, salubrinal treatment significantly improved hatched blastocyst developmental rates (main effect: $- \text{salubrinal} = 9.6\% ± 1.8\% \text{ vs. } + \text{salubrinal} = 12.8\% ± 2.0\%$; $P = 0.048$; Fig. 1D).

The D8 blastocysts were stained to determine cell allocation, namely, ICM versus TE cells. There were no significant differences in the number of ICM, TE, and total cells within blastocysts and the ICM:total cell ratio (Table 1).

Expression of ER Stress Marker Genes

The gene expression of three ER stress marker genes, that is, ATF4, ATF6, and HSPA5, were assessed in COCs matured for 23 h in the presence or absence of NEFAs and salubrinal. There were no significant differences in the expression of ATF6 between the treatment groups (Fig. 2B). There was a significant interaction between salubrinal and NEFA treatments regarding the expression of ATF4 and HSPA5 ($P < 0.05$) with the presence of NEFA significantly increasing the expression of both genes but the mRNA levels were maintained at control levels in the NEFA + salubrinal group (Fig. 2, A and C). This indicates that the presence of NEFAs during IVM-induced ER stress response act via the ATF4 pathway (including HSPA5), but not the ATF6 pathway and that salubrinal treatment, as expected, prevents the increase in mRNA expression.

Glucose and Lactate Metabolism

Following 23h of IVM culture, spent media were analyzed to determine glucose consumption, lactate production, and the lactate:glucose ratio to indicate glycolytic activity. While there were no main effects of NEFA or salubrinal supplementation, there were significant interactions between salubrinal and NEFAs treatment for glucose uptake ($P = 0.001$; Fig. 3A), lactate production ($P = 0.006$; Fig. 3B), and lactate:glucose ratio ($P = 0.024$; Fig. 3C), with the presence of both (+ NEFA + salubrinal) resulting in similar levels of glucose, lactate, and ratios relative to the control group (− NEFA − salubrinal). Hence, the presence of NEFAs or salubrinal alone reduced glucose consumption and lactate production compared to the control and the + NEFA + salubrinal groups. Furthermore, the COCs within the control and + NEFA + salubrinal groups had
lower lactate:glucose ratios compared to the + NEFA and + salubrinal treatment groups (Fig. 3C), suggesting that less glucose was being utilized via glycolysis and was diverted to other glucose-metabolizing pathways.

Mitochondrial DNA Copy Number

As an indicator of mitochondrial replication, mtDNA copy number in oocytes and blastocysts derived from COCs matured in the presence of NEFAs and salubrinal were determined. In both oocytes and blastocysts, there was a main effect of salubrinal supplementation significantly increasing mtDNA copy number, regardless of the presence or absence of NEFA (main effect: \( P < 0.05 \); Fig. 4, A and B). Furthermore, there was a significant interaction term between salubrinal and NEFA supplementation, resulting in a significant increase in mtDNA copy number in both oocytes and D8 blastocysts (\( P < 0.05 \)) compared to oocytes exposed to NEFA alone. Indeed, within oocytes, mtDNA copy number was 1.9-fold higher in the + NEFA + salubrinal group compared to the control (– NEFA – salubrinal).

The REDOX State—FAD and NAD(P)H—of Oocytes

The REDOX state of the oocyte following COC exposure to NEFAs and salubrinal was determined by measuring the autofluorescence of NAD(P)H (cytoplasmic and mitochondria) and FAD (mitochondria). There were no differences in NAD(P)H intensity within the oocyte between treatment groups. However, the presence of NEFA significantly reduced FAD intensity compared to the control group, resulting in a significantly lower REDOX ratio (main effects: \( P < 0.001 \) and \( P = 0.007 \); Fig. 5, B and C). In addition, there was a significant interaction between NEFA and salubrinal treatment, with salubrinal supplementation unable to recover FAD autofluorescence in oocytes exposed to NEFAs (interaction: \( P = 0.005 \); Fig. 5B), but this did not translate to an altered REDOX ratio.

**TABLE 1.** Cell allocation of Day 8 blastocysts derived from cumulus-oocyte complexes cultured in the presence of nonesterified fatty acids (NEFAs) and salubrinal.

| Salubrinal | NEFA | ICM \(^a\) | TE \(^b\) | Total \(^b\) | ICM:Total \(^a\) |
|-------------|------|------------|--------|----------|-------------|
| –           | –    | 65.2 ± 5.2 | 105.7 ± 8.8 | 170.9 ± 11.9 | 0.38 ± 0.02 |
| +           | –    | 61.9 ± 4.4 | 103.8 ± 7.4 | 165.6 ± 10.0 | 0.38 ± 0.02 |
| –           | +    | 51.1 ± 4.9 | 112.9 ± 8.4 | 164.0 ± 11.3 | 0.32 ± 0.02 |
| +           | +    | 56.8 ± 3.6 | 108.8 ± 6.1 | 165.6 ± 8.2  | 0.34 ± 0.02 |

\(^a\) Data are presented as means ± SEM.

\(^b\) Total = inner cell mass (ICM) + trophoderm (TE).
Reactive Oxygen Species (ROS), Anti-Oxidants, and Active Mitochondria Within Mature Oocytes

Given that FAD is a cofactor of oxidative phosphorylation, hence exclusively localized to the mitochondria, the influence of NEFA and salubrinal supplementation during oocyte maturation on total mitochondrial activity and downstream outcomes such as intracellular anti-oxidant and ROS levels was determined. Denuded oocytes were exposed to probes for active mitochondria (MTR), GSH (MCB), and H₂O₂ (PF1) (Fig. 6). There were main effects for both NEFA and salubrinal supplementation to result in significantly lower mean intensity levels of GSH (MCB main effects: NEFA, \( P < 0.05 \), and salubrinal, \( P = 0.031 \); Table 2) and H₂O₂ (PF1 main effects: NEFA, \( P < 0.05 \), and salubrinal, \( P = 0.005 \); Table 2) within oocytes. However, there were no significant differences in the mean intensity of MTR (Table 2), indicating that although oocytes treated with NEFA had lower mtDNA copy numbers, this did not affect total mitochondrial membrane potential.

Given that mitochondrial potential is also dependent on localization, that is, even distribution of mitochondria throughout the oocyte is related to improved development competence [23, 24], texture analyses (GLCM) were applied to determine if NEFA and salubrinal supplementation altered the patterns of positive staining of MCB (GSH), PF1 (H₂O₂), and MTR (mitochondrial membrane potential). Entropy is a measure of heterogeneity and predictability of positive fluorescence patterns; contrast is a measure of the degree of roughness of fluorescence, such as the presence of wrinkles or organelles; and correlation predicts the relationship between the intensity of fluorescence between neighboring pixels, hence fine texture and fluorescence pattern variations. High units of entropy and contrast are related to increased heterogeneity, while increased correlation values are related to more homogeneous fluorescence patterning.

Entropy, contrast, and correlation values of MCB- and PF1-positive fluorescence were all significantly affected by NEFA and salubrinal supplementation (Table 2), with NEFA and salubrinal treatment significantly decreasing entropy and contrast and increasing correlation, resulting in smoother and predictable positive fluorescence compared to oocytes cultured in the absence of NEFA and salubrinal. Furthermore, there was a significant interaction between salubrinal and NEFA treatments in regards to contrast measurements of both MCB (interaction, \( P = 0.025 \), Table 2) and PF1 (interaction, \( P <
0.001, Table 2), resulting in significantly lower levels of contrast, hence even smoother positive-staining patterns and the absence of defined wrinkles or organelle-specific fluorescence localization.

In regards to MTR fluorescence, entropy and correlation were not significantly altered by NEFA and salubrinal treatment. However, the presence of NEFAs resulted in significantly higher correlation values compared to the absence of NEFA (main effect: $P = 0.022$; Table 2), hence fine details and positive-staining patterns of MTR were significantly increased in the presence of NEFA.

Overall, NEFA and salubrinal supplementation resulted in more predictable (entropy) and smoother (contrast and correlation) fluorescence localization of MCB and PF1 and finer patterns of MTR localization compared to oocytes cultured in the absence of NEFAs or salubrinal. Combined, these results indicate that NEFA and salubrinal supplementation during IVM reduces intra-oocyte ROS and anti-oxidants levels, and the homogeneous localization suggests more even dispersion, as opposed to concentration around specific organelles.

**DISCUSSION**

The reduction of fertility during the postpartum period in high-performance dairy cows is a major cost to dairy farmers, especially those requiring 365-day calving patterns to accommodate pasture-based nutrition. Poor oocyte quality has been at least partly responsible for poor postpartum fertility, with the damaging influence of high NEFA levels in follicular fluid affecting oocyte quality as a suggested mechanism [5]. While lipids play an important role in pre-implantation embryo development (reviewed by [25]), the lipotoxic effects of elevated NEFAs include increased intra-oocyte accumulation [7] and reduced postfertilization embryo development [3, 9, 26].

We hypothesized that high NEFA levels during maturation induce ER stress in cattle COCs and assessed their effect on developmental competence, COC ER stress-gene responses, and metabolism as well as the impact of an ER stress inhibitor. Lipotoxicity induces cellular damage and is particularly damaging to organelles such as mitochondria and ER [27]. The ER stress pathway involves $HSPA5$, inducing three main pathways: IRE1$\alpha$ (inositol requiring 1), PERK (PKR-like ER kinase), and ATF6 (activating transcription factor 6) and includes increased gene expression followed by phosphorylation of downstream targets and activation of stress responses [28]. Here, we confirmed that when incubated with NEFAs during IVM, significantly higher levels of lipid accumulate within the oocyte and there was a significant increase in COC expression of two of the three ER stress marker genes: $ATF4$ (downstream from PERK) and $HSPA5$, but not $ATF6$ (Fig. 2). This pattern of increased expression of both $ATF4$ and $HSPA5$ were also seen in mouse COCs following in vivo exposure to a high fat diet or in vitro culture in the presence of elevated lipid concentrations [29, 30]. Importantly, $ATF4$ and $HSPA5$ were also up-regulated in mouse COCs exposed to lipid-rich follicular fluid [31], suggesting that similar effects might occur in bovine COCs developing in the presence of high follicular levels of NEFA as occurs during lactation.
Salubrinal is a selective inhibitor of elongation initiation factor 2 (eIF2, upstream from ATF4), which acts by inhibiting the dephosphorylation of the alpha subunit and is a recognized pharmacological inhibitor of ER stress [28]. Salubrinal supplementation recovered blastocyst development and ER stress gene response in NEFA-treated COCs, further confirming that high-level lipid environments during oocyte maturation are detrimental to developmental competence. Hence, this study is the first to demonstrate that the ER stress pathway is induced in cattle COCs in response to high lipid content. That ATF4, a chaperone-inducing transcription factor, and ER-localized chaperone HSPA5 were up-regulated by NEFA suggests that protein-folding pathways are disrupted by high NEFA levels and at least partly normalized by salubrinal. Similarly, a lack of effect of NEFA treatment on ATF6 gene expression suggests that protein translation is less affected. However, whether high NEFA levels directly affects these specific signaling pathways and whether they impinge upon the apoptosis machinery remains unclear.

Mitochondria replication is largely suppressed between metaphase II (mature oocyte) and the blastocyst stage [32]. Mitochondrial activity during oocyte maturation is critical for developmental competence; mitochondrial health and function rely on numerous factors, including the number of mitochondria present (biosynthesis), localization/distribution, maturity, and activity (reviewed by [33]). In this current study, a broad approach was undertaken to investigate the influence of NEFA supplementation during maturation: mtDNA copy was determined; autofluorescence—FAD and NAD(P)H—was measured to indicate REDOX state; active mitochondria were

FIG. 6. Representative images of COC-derived oocytes following culture in the presence of NEFA and salubrinal and stained with monochlorobimane (MCB) to indicate reduced glutathione (GSH) levels; peroxylfluor 1 (PF1) to assess H2O2, and Mitotracker Red CMXRos (MTR) to measure mitochondrial membrane potential. Bar = 50 μm.
measured using a fluorescent probe against membrane potential; and levels of ROS and the anti-oxidant (reduced GSH) were determined to ascertain the influence of NEFA supplementation on the oocyte’s ability to cope with stress.

NEFA supplementation during oocyte maturation resulted in significantly lower mtDNA copy number in both mature oocytes and blastocyst-staged embryos. This agrees with metabolism shifting away from oxidative phosphorylation (lower FAD), reducing the REDOX state of the oocyte and decreased production of H₂O₂, a by-product of normal oxidative phosphorylation. Furthermore, while there were no significant differences in mitochondrial membrane potential (as measured using Mitotracker Red CMXRos), fluorescence intensity was more variable in oocytes treated with NEFAs, regardless of salubrinal treatment. While salubrinal supplementation recovered mtDNA copy number in NEFA-treated oocytes, oocyte oxidative metabolism (FAD and H₂O₂) was still significantly decreased when compared to the whole COC and oocyte was also investigated. The presence of elevated NEFAs during IVM reduced glucose uptake and lactate production by COCs, but this was recovered by inclusion of salubrinal. Because glucose uptake and lactate production is almost entirely restricted to the cumulus cells of the COC [35], this may infer that the cumulus cells in particular are sensitive to NEFA-induced ER stress. It has recently been reported that the cumulus layer of bovine COCs is protective of potential damage to the oocyte caused by NEFAs [13, 14]; our data reveals that the cumulus cells themselves are sensitive to lipotoxicity and respond accordingly.

In addition, NEFA supplementation resulted in significantly lower levels of ROS production (indicated by PF1, an H₂O₂ probe), accompanied by reduced levels of anti-oxidants (GSH). Furthermore, texture analyses suggest less variation and more homogeneous localization of intra-oocyte ROS and GSH (entropy, contrast, and correlation) following NEFA and salubrinal treatment, possibly due to more disperse and nonorganelle-specific positive-staining profiles. Interestingly, Van Hoeck and colleagues [26] reported NEFA supplementation reduced the gene expression of glutathione peroxidase 1 (GPX1) within the oocyte, an enzyme that converts reduced glutathione to oxidized glutathione. However, no other genes involved in glutathione reduction, oxidation, or synthesis were significantly affected.

In addition to studying mitochondrial kinetics using numerous different approaches, this study also utilized fluorophores to study anti-oxidant, ROS, and mitochondrial membrane potential in single, live oocytes, as opposed to traditional methods such as fluorometric assay kits requiring pools of oocytes and measurement of a single compound per sample. However, one limitation of this method is optical shadowing because refractive index mismatches create an artificial optical shadowing effect toward the center of the oocyte. Optical clearing can be performed; however, samples are required to be fixed and permeabilized with glycerol and benzyl alcohol [36]. Apart from Mitotracker Red CMXRos, the fluorescent intensities and localization of positive staining of the fluorophores used in the current study are compromised during the fixation process; hence, optical clearing is not a viable option in this case. However, we have previously demonstrated that the optical shadowing is minimal and differences in positive staining due to treatment effects can be readily detected [17].
In summary, this study demonstrated that NEFA supplementation during IVM of cattle COCs impairs oocyte health and metabolism and that ER stress, especially within cumulus cells, is a contributing mechanism through which lipotoxicity is acting. Inhibitors of ER stress, such as salubrinal, are promising therapeutic options in recovering the negative impacts of elevated NEFAs, as seen in obese women and during the peak lactation period in dairy cattle. While treatment of a herd with therapeutics may be unrealistic, given that we saw significant improvements in blastocyst development following treatment only during the 24 h IVM period, a single application may prove useful within in vitro embryo production, an emerging reproductive technology within both the dairy and the beef industries.

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