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The effect of elevated non-esterified fatty acid concentrations on bovine spermatozoa and on oocyte *in vitro* fertilization.

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Abstract

Elevated non-esterified fatty acids (NEFA) concentrations, present in follicular and oviductal fluid, have been postulated as a causative link between metabolic disorders and subfertility. High NEFA conditions can directly disrupt oocyte maturation and developmental capacity after fertilization. However, their influence on sperm function and the fertilization process is not known. This study investigated the fertilization process under high NEFA conditions. To differentiate between effects on both sperm and oocytes or only sperm, different experiments were conducted. In the first experiment, both gametes were simultaneously incubated during *in vitro* fertilization (IVF) under different conditions: 1) NEFA-free, solvent-free control conditions, 2) solvent control, 3) physiological concentrations of oleic (OA), palmitic (PA) and stearic (SA) acid or 4) pathophysiological concentrations of OA, PA and SA. In the second experiment, sperm was incubated (4 h) under the same treatment conditions before routine IVF. Gamete co-incubation resulted in reduced fertilization and cleavage rates and increased prevalence of polyspermy. In the second experiment, embryo developmental capacity and quality were not affected although sperm motility and plasma membrane integrity were decreased. In conclusion, lipolytic conditions affected the fertilization process mainly through an effect on the oocyte. Spermatozoa were still able to fertilize even though these conditions reduced sperm function.

Additional keywords: fertility, maternal metabolism, free fatty acids, embryo

Introduction

It is generally accepted that maternal metabolic disorders, associated with upregulated lipolysis, are linked with decreased fertility (Leroy *et al.* 2015). Reduced insulin sensitivity, obesity or catabolic disorders (as seen in e.g. negative energy balance (NEB) or ketosis in dairy cows) are characterised by elevated concentrations of non-esterified fatty acids (NEFAs) in the blood. They are also reflected in the oocyte's micro-environment, which has significant carry over effects on *in vitro* embryo quality and viability up to seven days after fertilization (Aardema *et al.* 2011; Van Hoeck *et al.* 2011). Underlying mechanisms are related to increased reactive oxygen species (ROS) production and altered mitochondrial activity within the oocytes, increased lipid accumulation in the oocytes and resultant morulae, and reduced oxidative phosphorylation and altered metabolism in resultant blastocysts (Aardema *et al.* 2011; Van Hoeck *et al.* 2011; Van Hoeck *et al.* 2013; Van Hoeck *et al.* 2015; Marei *et al.* 2017). Likewise, early bovine embryos are highly sensitive to changes in their micro-environment, the oviduct (Rizos *et al.* 2003; Leese *et al.* 2008). As the maternal metabolic state is reflected in blood and follicular fluid, it may furthermore influence oviductal fluid composition; a recent study showed that NEFAs are present in the oviductal fluid and mirror the concentrations seen in plasma of healthy cows (Jordaens *et al.* 2017a). Although the oviduct has been suggested to modulate its micro-environment by alleviating potential lipotoxic effects (Jordaens *et al.* 2017b), it remains debatable whether this gatekeeper function is sufficient to actually protect the embryo from an adverse climate. *In vivo* studies have indicated that the reproductive tract of lactating dairy cows, which suffer NEB, is compromised in supporting the development of *in vitro* produced zygotes to the blastocyst stage compared to heifers (Rizos *et al.* 2010) and dry cows (Maillo *et al.* 2012). NEFAs might be key molecules in the integration of these metabolic and reproductive malfunctions. More in-depth *in vitro* research has indeed shown that elevated NEFA concentrations during embryo culture are detrimental for early embryo development. Integration of gene expression and DNA methylation patterns of these embryos suggested that cellular pathways, particularly oxidative metabolism, apoptosis, endoplasmic reticulum stress and lipid metabolism, were affected due to elevated NEFA exposure during *in vitro* embryo culture (Desmet *et al.* 2016).

The oviduct not only plays a vital role in early embryo development, it is also a complex mediator in the selection of spermatozoa, storage in the sperm reservoir, regulation of sperm motility, guidance of spermatozoa towards the egg and finally, fertilization (Holt and Fazeli

2010; Coy *et al.* 2012; Ghersevich *et al.* 2015). At ovulation, sperm detaches from the oviductal epithelium through hyperactivation and capacitation and, reaching the oocyte, they undergo the acrosome reaction and penetrate the cumulus cells and zona pellucida (Kolle 2015). Knowledge on the influence of changes in the oviductal environment, and more specifically in free fatty acid (FFA) concentrations due to an altered maternal metabolic state, on sperm functionality and the fertilization process is limited. Modifications in concentration and type of FFAs present in the micro-environment, in which fertilization takes place, may affect, for example, the sperm mitochondrial function (Paillamanque *et al.* 2016). It has been proven that mitochondrial β -oxidation of FFAs is one of the metabolic pathways used by sperm to produce adenosine triphosphate (ATP) as a supply of energy in order to undergo activation (Ferramosca and Zara 2014). Furthermore, an altered FFA environment may influence sperm membrane fluidity due to incorporation of these FFAs into spermatozoal membranes (Neill and Masters 1972; Alvarez and Storey 1995; Rooke *et al.* 2001), subsequently affecting both sperm motility and viability (Aksoy *et al.* 2006; Am-in *et al.* 2011). For example, Hossain *et al.* (2007) observed an improvement of motility, viability and acrosome reaction of boar sperm after 4 h of incubation with unsaturated FFAs. Addition of saturated FFAs, on the contrary, inhibited the acrosome reaction in hamster spermatozoa (Meizel and Turner 1983), probably as a result of reduced membrane fluidity and, subsequently, reduced membrane fusion required for the acrosome reaction. Furthermore, Quinn and Whitfingham (1982) observed that IVF of mouse oocytes in the presence of PA and OA was inhibited thus providing an insight in the harmful effect of FFAs on the fertilization process. But the question arises as to which extent these FFAs reduce fertilization by reducing sperm function or by a direct effect on the oocyte.

Therefore, we hypothesized that elevated NEFA concentrations, associated with maternal lipolytic disorders, may directly affect sperm physiology and the fertilization process itself leading to reduced embryo development and quality. To investigate this hypothesis, we aimed to assess the influence of elevated NEFA concentrations on the process of fertilization. In order to differentiate between effects on the oocyte and/or on spermatozoa, i) both oocytes and spermatozoa were incubated in the presence of different NEFA conditions during IVF or ii) spermatozoa were exposed to different NEFA conditions before IVF. Effects on fertilization rate, embryo developmental capacity and quality and sperm viability and functionality were evaluated.

Materials and Methods

Experimental design

In order to evaluate the effect of elevated NEFA exposure during sperm capacitation (4 h) and fertilization (22 h), fertilization medium was supplemented with the following NEFA treatments:

- i) STANDARD: FA-free and solvent-free medium,
- ii) SOLVENT: FA-free medium with 0.45 % absolute ethanol,
- iii) BASAL: physiological NEFA concentrations (72 μ M total NEFA containing 28 μ M SA, 23 μ M PA, and 21 μ M OA) in 0.45 % absolute ethanol,
- iv) HIGH COMBI: a combination of elevated NEFA concentrations equivalent to those measured in the serum during high lipolytic conditions (720 μ M total NEFA, containing 280 μ M SA, 230 μ M PA, and 210 μ M OA) in 0.45 % absolute ethanol.

Since no data are available regarding the concentrations of FFAs in oviductal fluid in cows under metabolic stress conditions, the concentrations of FFAs used here are based on serum concentrations in cows during NEB (Leroy *et al.* 2005) which may correlate with the oviductal fluid concentrations (Jordaens *et al.* 2017a). A basal NEFA treatment group was implemented as a control in this study in order to improve the physiological relevance of our *in vitro* model as these NEFAs are present at basal concentrations in the environment in which oocytes mature and embryos develop *in vivo* (Leroy *et al.* 2005).

121 *Experiment 1. NEFAs during in vitro fertilization*

122 In the first experiment, cumulus oocyte complexes (COCs) and spermatozoa were
123 exposed to the different treatment conditions during *in vitro* fertilization (22 h). In order to
124 evaluate developmental competence and other functional parameters, the following
125 experiments were performed:

- 126 i) Developmental competence was assessed by determining embryo cleavage (day 2 post
127 insemination (p.i.) and development to the blastocyst stage (day 8 p.i.) using 770 COCs
128 in 3 replicates. To determine if the effect of NEFAs on cleavage rate was due to
129 differences in fertilization rate, 400 COCs were fertilized in the presence of NEFAs.
130 Presumptive zygotes (22 h p.i.) were fixed and stained with aceto-orcein to evaluate
131 pronuclear formation (n = 237).
- 132 ii) In order to evaluate embryo quality, intracellular lipid content of 42 presumptive
133 zygotes (22 h p.i.) and 45 morulae (day 5 p.i.) (derived from 317 COCs, 2 replicates)
134 were measured using BODIPY 493/503. In addition, metabolic activity of day 7
135 blastocysts was determined using micro-fluorometric assays on spent medium (from
136 day 7 to 8 p.i.) (n = 96 blastocysts derived from 419 COCs, 2 replicates). Cell
137 differentiation and apoptosis of resultant day 8 blastocysts were evaluated by an
138 immunostaining with Hoechst 33342, CDX2 and cleaved caspase-3 in the same
139 experiment (n = 64 blastocysts). Post-hatching outgrowth of day 8 blastocysts was
140 assessed until day 14 p.i. (n = 161 blastocysts derived from 770 COCs, 3 replicates).

142 *Experiment 2. NEFAs during sperm capacitation*

143 In the second experiment, spermatozoa were exposed during capacitation (up to 4 h) to
144 the different treatment conditions before IVF in FA-free fertilization medium. In order to
145 evaluate the effect on sperm functionality, fertilizing capacity and subsequent embryo
146 development, the following experiments were performed:

- 147 i) Integrity of plasma and acrosomal membranes of spermatozoa after 1, 2, 3 and 4 h
148 exposure were evaluated using a combined fluorescence staining with propidium
149 iodide and PSA conjugated to fluorescein isothiocyanate (n = 9762 spermatozoa, 5
150 replicates). Furthermore, sperm motility after 4 h exposure was evaluated using
151 computer-assisted sperm analysis (CASA) (n = 8610 spermatozoa, 3 replicates).

ii) To determine if NEFA treatment influences the fertilizing capacity of the exposed spermatozoa, developmental competence of resultant embryos was assessed. Embryo cleavage (day 2 p.i.) and development to the blastocyst stage (day 8 p.i.) were recorded using 791 COCs which were fertilized using the pre-exposed spermatozoa in FA-free fertilization medium (3 replicates). In addition, quality of day 7 blastocysts was assessed by determining their metabolic activity (from day 7 to 8 p.i.) (n = 82 blastocysts derived from 651 COCs, 3 replicates), cell differentiation and apoptosis (day 8 p.i.) (n = 46 blastocysts).

Preparation of NEFA treatments

Media containing NEFAs were prepared as previously described by Van Hoeck *et al.* (2011). All chemicals were purchased from Sigma-Aldrich (Bornem, Belgium), unless otherwise stated. Stearic acid (SA, C18:0), palmitic acid (PA, C16:0), and oleic acid (OA, C18:1) were dissolved in stock solutions of absolute ethanol at different concentrations according to the experiment and treatment; 28, 23 and 21 mM for the BASAL treatment and 112, 230 and 210 mM for the HIGH COMBI treatment, respectively. These stock solutions were vortexed for 3 min and added to fertilization media containing FA-free bovine serum albumin (BSA) as a carrier to obtain the desired final concentration. All media, except the FA-free and solvent-free medium, were sonicated for 4 h and filter-sterilized under aseptic conditions.

In vitro embryo production

In vitro embryo production procedures were performed as previously described (Van Hoeck *et al.* 2011), using immature COCs retrieved from bovine ovaries collected from a local abattoir within 2 h of slaughter. Briefly, unexpanded COCs surrounded by five or more cumulus cell layers (quality grade I) were used. Selected COCs were matured in groups of 50-60 in 500 µl serum-free maturation medium composed of TCM199 supplemented with 0.75 % w/v FA-free BSA, 0.4 mM glutamine, 0.2 mM sodium pyruvate, 0.1 mM cysteamine, 50 mg/ml gentamycin and 20 ng/ml murine epidermal growth factor in 4-well plates for 24 h in humidified air with 5 % CO₂ at 38.5°C. After *in vitro* maturation (IVM), COCs were co-incubated for 22 h in groups of 50-100 with spermatozoa at a final concentration of 10⁶/ml in 500 µl fertilization medium (containing 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 sodium pyruvate, 36 mM sodium lactate, 2 µl/ml phenol red, 6 mg/ml FA-free BSA, 50 µg/ml gentamycin and 0.72 U/ml heparin) in a humidified 5 % CO₂ incubator at 38.5°C.

In experiment 1, motile spermatozoa were isolated from frozen bull semen of proven *in vitro* fertility by centrifugation on a discontinuous Percoll gradient (90 % and 45 %, Biosciences, Amersham, UK) for subsequent fertilization. The fertilization medium was supplemented with ethanol or different NEFA concentrations according to the above mentioned treatment groups. In experiment 2, spermatozoa were isolated by swim-up technique as described by Mortimer (2000) with minor modifications to avoid repeated centrifugation of spermatozoa during NEFA treatments. Semen from the same bull used in experiment 1 was washed to remove diluent and cryoprotectant. Aliquots of 30 µl sperm suspension were added to the bottom of 15 ml tubes containing 2 ml of the four different fertilization media without heparin (a capacitation inducer for bovine sperm (Parrish 2014)) supplemented with NEFAs or solvent as described above. The tubes were incubated under an angle of 45° for up to 4 h in humidified air with 5 % CO₂ at 38.5°C. Afterwards, the supernatant was collected and centrifuged at 155 g for 10 min. The supernatant was discarded and the resulting sperm pellet was washed and used for fertilization in NEFA-free fertilization medium.

In both experiments, presumptive zygotes were vortexed for 3.5 min to remove excess sperm and cumulus cells after 22 h of fertilization. The presumptive zygotes were cultured in groups of 25 ± 4 in 75 µl serum-free, FA-free modified synthetic oviductal fluid (mSOF) medium supplemented with 2 % w/v FA-free BSA and with 10 µg/ml insulin, 5.5 µg/ml transferrin and 6.7 ng/ml selenium (Thermo Fisher Scientific, Waltham, USA) in a reduced surface 96-well dish (modular incubator: 5 % CO₂, 5 % O₂ and 90 % N₂ at 38.5°C).

Embryo developmental competence

Total cleavage rate, 2-cell block, number of embryos with ≥ 4-cells and fragmentation were recorded at day 2 h p.i.. Zygotes were categorised as fragmented if ≥ 20 % of their cellular mass was fragmented (Stringfellow *et al.* 2010). Blastocyst yield was assessed at day 7 and 8 p.i. using an inverted Olympus CKX41 microscope (Olympus, Aartselaar, Belgium).

Evaluation of fertilization status

The fertilization rate of the oocytes was assessed by aceto-orcein staining as described by Prentice-Biensch *et al.* (2012). Following 22 h of IVF, presumptive zygotes were transferred to HEPES-buffered TALP and vortexed for 3.5 min to remove excess sperm and cumulus cells. Subsequently, they were mounted on glass slides (max. 10 presumptive zygotes per slide) under coverslip supported with silicon drops and fixed in methanol:acetic acid (3:1) for at least 24 h. Presumptive zygotes were stained in 2 % w/v orcein in 45 % acetic acid for at least 3 min and rinsed in glycerol: acetic acid: water (1:1:3). The presence and numbers of pronuclei, and extrusion of the 2 polar bodies, were then investigated using a light Olympus microscope IX71 at 40x magnification. Oocytes were considered not fertilized if still in metaphase-I or metaphase-II. Normal fertilization was defined by the presence of two pronuclei and the extrusion of both polar bodies, or fusion of the male and female pronucleus. Polyspermic fertilization was characterized by the presence of more than two pronuclei.

Zygote and morula lipid droplet content

Zygotes and morulae were fixed at day 2 and 5 p.i., respectively, in 4 % paraformaldehyde, stored in 1 mg/ml PVP-PBS at 4°C and stained as previously described by Van Hoeck *et al.* (2015) with minor modifications. Briefly, zygotes and morulae were washed twice in PBS with 0.3 % (w/v) polyvinylpyrrolidone (PBS-PVP), permeabilized for 30 min in PBS containing 0.1 % (w/v) saponin (Fiers, Kuurne, Belgium) and 0.1 M glycine and washed in PBS-PVP. Nuclei were counterstained with 50 µg/ml Hoechst 33342 (Thermo Fisher Scientific) for 10 min and zygotes and morulae were subsequently washed twice in PBS-PVP. Finally, lipid droplets were stained with the specific neutral lipid stain; BODIPY 493/503 (Thermo Fisher Scientific) in PBS (10 µg/ml) for 1.5 h, washed twice in PBS-PVP and transferred to droplets of PBS-PVP under oil in glass-bottom dishes for confocal imaging.

High resolution images were obtained using a Nikon Eclipse Ti-E inverted microscope attached to a microlens-enhanced dual spinning disk confocal system (UltraVIEW VoX; PerkinElmer, Zaventem, Belgium) equipped with 405 and 488 nm diode lasers for excitation of blue and green fluorophores, respectively. For each zygote or morula, a z-stack of 20 µm (1 µm interval) was consistently taken at the lower quarter of the zygote or morula. Images were further analyzed using Volocity 6.0.1 software (PerkinElmer) to evaluate lipid droplet content. Droplets were considered as relevant from a size of 0.5 µm³ onwards to eliminate false

positive counting of background pixels. The number of lipid droplets, their mean volume (in μm^3) and total volume of lipid droplet content (in μm^3) were calculated.

Blastocyst's glucose, lactate and pyruvate consumption

Glucose, lactate and pyruvate utilization by day 7 blastocysts was determined using micro-fluorometric assays of spent medium as described by Guerif *et al.* (2013) with minor modifications following Marei *et al.* (2017). Briefly, day 7 blastocysts were individually transferred from their routine culture medium to 8 μl droplets of mSOF medium (under mineral oil) supplemented with 0.5 mM glucose and 0.4 mM sodium pyruvate, but lactate- and phenol red-free. After 24 h of incubation, blastocysts were removed, fixed in 4 % paraformaldehyde and stored in 1 mg/ml PVP-PBS at 4°C. Morphological stage (young, normal, expanded, hatching, hatched) of all blastocysts was recorded at day 7 and day 8 p.i. Culture plates containing spent media droplets and empty droplets (blanks) with the oil overlay were stored at -80°C until analysis.

Initial fluorescence of the glucose, pyruvate and lactate reaction cocktails (10 μl) containing all substrates (for composition see Guerif *et al.* (2013)) was measured using a Tecan Infinite® M200 spectrophotometer (Tecan Group Ltd., Männedorf, Switzerland) in a V-shaped black 96-well plate. The assay mixtures were excited at 340 nm with emitted fluorescence measured at 460 nm. Subsequently, 1 μl of spent medium, blanks or standards (six points from 0 to 0.5 mM) (lactate and glucose; Analox Instruments, Stourbridge, UK) was added. These mixtures were incubated at 37°C for 30 min before final fluorescence measurement. Spent media samples and blanks were analyzed in duplicates, while standards were analyzed in triplicates. Consumption of glucose and pyruvate and production of lactate over the 24 h period were calculated as the difference between concentrations in blanks and spent media samples from each blastocyst and expressed as pmol/embryo/h $\pm$ SEM.

Blastocyst differential and apoptosis staining

The inner cell mass (ICM) / trophectoderm (TE) cell ratio as well as the apoptotic cell index of day 8 blastocysts were evaluated by an immunostaining adapted from Marei *et al.* (2017). Blastocysts fixed after metabolic assay were used for immunostaining. After an overnight permeabilization in 0.1 % Triton X-100 and 0.05 % Tween-20 in PBS at 4°C, blastocysts were

incubated in blocking solution (containing 10 % normal goat serum and 0.05 % Tween-20 in PBS) for 2 h at 4°C. Afterwards, they were transferred to a primary antibody mixture containing 1:1 mouse-anti-caudal type homeobox 2 (CDX2) (Biogenex, San Ramon, USA) and rabbit-anti-cleaved caspase-3 (Cell Signaling Technology, 1:250 in blocking solution) and incubated overnight at 4°C. Negative control embryos were transferred to a mixture of normal rabbit and mouse antibodies at the same concentrations. Blastocysts were subsequently washed twice and incubated overnight at 4°C in blocking solution containing a mixture of FITC-labelled goat-anti-rabbit (1:200) and Texas Red-labelled goat-anti-mouse (1:100) secondary antibodies (Thermo Fisher Scientific). Finally, nuclei of all blastocysts were stained in 50 µg/ml Hoechst 33342 (Thermo Fisher Scientific) for 10 min at room temperature. Blastocysts were evaluated under a fluorescence Olympus microscope IX71 with a blue DAPI filter (excitation 360-370 nm, emission 420-460 nm; for Hoechst stained total cells), a green FITC filter (excitation 460-490 nm, emission 520-540 nm; for cleaved caspase-3 positive apoptotic cells) and a red TRITC filter (excitation 510-550 nm, emission > 570 nm; for CDX2 positive TE cells). The apoptotic cell index was calculated as the proportion of apoptotic cells from total cell count and expressed as percentages. Data were expressed as mean ± SEM.

Post-hatching blastocyst outgrowth culture

Extended culture of blastocysts was performed as described by Rodina *et al.* (2009) with minor modifications. On day 9 p.i., blastocysts were washed in HEPES-TALP. All blastocysts were cultured individually in 75 µl medium composed of TCM199 supplemented with 5 % foetal bovine serum and 11 µM gentamycin in a reduced surface 96-well dish (modular incubator: 38.5°C, 5% CO₂, 5% O₂ and 90% N₂) until day 14 p.i. Blastocyst stage was recorded and an image of the blastocysts was taken on a daily basis with an inverted Olympus CKX41 microscope. The “average diameter” was calculated based on the measurement of two diameters, orthogonal of each other, per blastocyst based on the images with the Cell* software (Olympus Soft Imaging Solution, Münster, Germany) and expressed in µm.

Sperm plasma membrane and acrosome integrity

Plasma membrane and acrosome integrity were evaluated using a combined fluorescent staining technique as described by Celeghini *et al.* (2007) with minor modifications. Thawed

semen was washed and transferred to the different fertilization media without heparin supplemented with NEFAs or solvent as described above for 1 to 4 h during swim-up. After incubation, the supernatant was centrifuged for 10 min at 1000 rpm and the supernatant was removed. A 30 μ l aliquot of the remaining sperm pellet was transferred to a warmed tube containing 0.5 mg/ml propidium iodide (PI) in PBS and 100 μ g/ml PSA conjugated to fluorescein isothiocyanate (FITC-PSA) in 10 % sodium azide. The samples were incubated at 37°C for 8 min in the dark. Afterwards, 40 μ g/ml Hoechst 33342 (Thermo Fisher Scientific) in PBS was added and the samples were incubated at 37°C for 10 min in the dark. Sperm suspension (8 μ l) was placed on a slide, coverslipped and immediately analyzed under a fluorescence Olympus microscope IX71 with green FITC filter (excitation 460-490 nm, emission 520-540 nm) and blue DAPI filter (excitation 360-370 nm, emission 420-460 nm). Sperm cells with a damaged plasma membrane (PI positive) showed a red-stained sperm head and sperm cells with an intact plasma membrane (PI negative) showed a blue-stained sperm head. Sperm cells with a reacted acrosome (FITC-PSA positive) had a green acrosome region. Results are expressed as average percentage of spermatozoa with intact plasma membrane or reacted acrosome $\pm$ SEM.

Sperm motility

Computer-assisted sperm analysis (CASA) was performed using a Hamilton Thorne motility analyser (CEROS version 12.3d; Hamilton-Thorne Research, Beverly, MA, USA). The parameter settings were as previously described Rahman *et al.* (2012): frame rate 60 Hz; number of frames 30; minimum contrast 20; minimum cell size 10 pixels; non-motile head size 5 pixels; non-motile head intensity 20; medium pathway velocity (VAP) cut-off 50 μ m/s; straightness (STR) cut-off 70%; low VAP cut-off 30 μ m/s; and low straight line velocity (VSL) cut-off 15 μ m/s. Three microliters of diluted semen was placed on a prewarmed (37°C) Makler chamber and sperm motility parameters were analyzed in different focus fields (varying between 6 and 11 fields per treatment per replicate) using a 10 $\times$ negative phase contrast objective. The following variables were assessed: total motility (TM, %), progressive motility (PM, %), rapid progressive motility (VAP > 50 μ m/s, %), medium progressive motility (30 μ m/s < VAP < 50 μ m/s, %), VAP (μ m/s), VSL (μ m/s), curvilinear velocity (VCL, μ m/s), STR (%), linearity (LIN, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, μ m) and beat cross-frequency (BCF, Hz) (Goovaerts *et al.* 2006).

339

340 *Statistical analysis*

341 All statistical analyses were carried out with SPSS 24 (for Windows, Chicago, IL, USA).
342 fertilization, cleavage and blastocyst rate were compared among the three treatment groups
343 using a binary logistic regression model. Outgrowth, cell counts, apoptotic cell indexes,
344 glucose and pyruvate consumption, lactate production, lipid droplet content and sperm
345 quality parameters were compared among all treatments using a linear mixed-model ANOVA.
346 A Bonferroni post hoc test was performed to correct for multiple comparisons. Replicate
347 (random factor), treatment (fixed factor) and the interaction between them were taken into
348 account. All interactions were not significant and were omitted from the final model. Data
349 were first tested for normal distribution and homogeneity of variances by performing the
350 Kolmogorov–Smirnov and Levene’s test, respectively, and log transformed if necessary.
351 Differences were considered significant at $P < 0.05$.

Results

Validation of solvent control

Addition of 0.45 % absolute ethanol did not affect developmental competence and quality of the resultant embryos (experiment 1 and 2) and all parameters of sperm functionality (experiment 2) compared to the solvent-free, NEFA-free lab control ($P > 0.1$). Thus, the solvent-free, NEFA-free lab control was excluded from further analyses.

Experiment 1. NEFAs during in vitro fertilization

Embryo developmental competence

Addition of elevated NEFA concentrations during IVF reduced cleavage and the number of oocytes reaching the four-cell stage compared to the SOLVENT ($P < 0.05$) and BASAL group ($P < 0.05$ for ≥ 4 -cell embryos and $P < 0.1$ for cleavage). Concomitantly, fertilization in the presence of elevated NEFA concentrations increased the number of embryos arrested at the two-cell stage compared to the BASAL and SOLVENT groups ($P < 0.05$). No significant difference in fragmentation at day 2 p.i. was observed among treatment groups ($P > 0.1$). Overall blastocyst formation at day 8 p.i. was not significantly different among treatment groups ($P > 0.1$). However, less oocytes were able to develop to the expanded, hatching or hatched blastocyst stage at day 8 p.i. ($P < 0.05$), and the proportion of blastocysts reaching the hatching or hatched stage was lower under elevated NEFA conditions compared to solvent conditions ($P < 0.05$) (table 1).

Fertilization status

Normal fertilization rate (two pronuclei or syngamy) was lower in the presence of elevated NEFA concentrations compared to basal NEFA concentrations and the solvent control ($P < 0.05$). Both basal and elevated NEFA concentrations increased the incidence of polyspermy compared to the solvent control ($P < 0.05$). Concomitantly, more oocytes remained unfertilized after high NEFA exposure compared to basal NEFA exposure ($P < 0.05$). The proportion of unfertilized oocytes also tended to be more after high NEFA exposure compared to the solvent control ($P < 0.1$) (table 2).

Lipid droplet content in zygotes and morulae

Supplementation of fertilization medium with basal or elevated NEFA concentrations did not affect total lipid content, number of lipid droplets and average size of lipid droplets in zygotes and morulae compared to the solvent control ($P > 0.1$).

Blastocyst metabolism

Glucose consumption, pyruvate consumption and lactate production by blastocysts were similar among all treatment groups ($P > 0.1$) (figure 1).

Blastocyst cell differentiation and apoptosis

Total cell count, ICM count and apoptotic cell index were similar among the treatment groups ($P > 0.1$). Elevated NEFA exposure during IVF increased TE cell count compared to the solvent control ($P < 0.05$). Additionally, blastocysts from the HIGH COMBI group had a higher TE/ICM ratio compared to blastocysts from the SOLVENT and BASAL group ($P < 0.05$) (table 3).

Post-hatching blastocyst outgrowth

Supplementation of fertilization medium with basal or elevated NEFA concentrations did not affect the number of blastocysts that continued to grow starting from day 9 p.i. ($P > 0.1$). The percentage of blastocysts that continued to grow was 73.0% (SOLVENT group), 78.3% (BASAL group) and 76.9% (HIGH COMBI group).

The average diameter of the hatched blastocysts from day 9 until 14 p.i. was similar among the treatment groups ($P > 0.1$) (figure 2). The average daily growth (calculated as the net growth divided by the number of days) was also not significantly different among treatment groups ($P > 0.1$).

Experiment 2. NEFAs during sperm capacitation

Sperm plasma membrane and acrosome integrity

Significantly less spermatozoa had an intact plasma membrane after 1 or 4 h exposure to elevated NEFA concentrations compared to exposure to basal NEFA concentrations or the solvent control ($P < 0.05$). In addition, the proportion of spermatozoa with intact plasma membrane tended to be lower in the BASAL group compared to the SOLVENT group after 4 h

exposure ($P < 0.1$). No significant differences in plasma membrane integrity were found after 2 or 3 h exposure among the treatment groups ($P > 0.1$) (figure 3).

The percentage of spermatozoa with a reacted acrosome was not significantly affected after 1, 2, 3 or 4 h exposure to the different treatment conditions ($P > 0.1$).

Sperm motility

While total and progressive motility percentages were significantly reduced by both HIGH COMBI and BASAL treatment compared to the SOLVENT treatment, HIGH COMBI-treated spermatozoa were the lowest in rapid progressive motility compared to the SOLVENT treatment ($P < 0.05$). Contrarily, the percentage of medium progressive spermatozoa was higher in the presence of elevated NEFA concentrations compared to basal NEFA concentrations ($P < 0.05$) and tended to be higher compared to the solvent control ($P < 0.1$). Exposure to basal NEFA concentrations also significantly reduced total, progressive and rapid progressive motility percentages compared to the solvent control ($P < 0.05$). The velocity parameters; VAP, VSL and VCL, were significantly reduced in the HIGH COMBI group compared to the other treatment groups ($P < 0.05$). This was also reflected in the values of amplitude of lateral head displacement ($P < 0.05$) and beat cross frequency ($P < 0.05$ compared to the SOLVENT group, $P < 0.1$ compared to the BASAL group). No significant differences were observed among the treatment groups for LIN, STR and WOB values ($P > 0.1$) (table 4).

Embryo developmental competence

Developmental competence of embryos after IVF with pre-exposed spermatozoa was not different among the treatment groups. Cleavage rate at day 2 p.i. and blastocyst yield at day 8 were similar among the treatment groups ($P > 0.1$).

Blastocyst metabolism

Glucose and pyruvate consumption as well as lactate production by blastocysts were similar among all treatment groups ($P > 0.1$) (figure 4).

Blastocyst cell differentiation and apoptosis

Total cell count, ICM count and TE/ICM ratio were similar in day 8 blastocysts among treatment groups ($P > 0.1$). The number of TE cells tended to be lower under BASAL conditions

compared to SOLVENT conditions ($P < 0.1$). The apoptotic cell index was not significantly different among treatment groups ($P > 0.1$) (table 5).

Discussion

Both oocyte and embryo are very sensitive to high NEFA conditions in their environment (Van Hoeck *et al.* 2011; Van Hoeck *et al.* 2013; Van Hoeck *et al.* 2015; Desmet *et al.* 2016). However, little is known about the impact of adverse metabolic conditions on the fertilization process. In the present study, we hypothesized that elevated NEFA concentrations negatively affect the process of IVF by directly hampering sperm and/or oocyte quality. We observed that IVF in the presence of elevated NEFA concentrations affected fertilization itself. When spermatozoa alone were exposed to elevated NEFA concentrations, their functionality seemed to be decreased, but, in contrast, oocytes exhibited normal development after IVF using spermatozoa, which were exposed to elevated NEFA concentrations. These findings provide further understanding of the functionality of and interaction between the gametes in a metabolically compromised micro-environment.

Impact of NEFAs during in vitro fertilization

In the first part of the study, oocytes and spermatozoa were simultaneously exposed to different NEFA concentrations during IVF (22h). Elevated NEFA concentrations reduced embryo cleavage, thus implying a reduced fertilization rate. Indeed, less oocytes were fertilized under high NEFA conditions as shown by the aceto-orcein staining. Interestingly, the incidence of polyspermy differed significantly between treatments, thereby suggesting that the oocyte's defence mechanisms against multiple sperm penetration were affected under high NEFA conditions. Following fertilization, the zona pellucida is normally modified by cortical-granule contents in a process called "hardening", which prevents the binding and penetration of additional sperm (Okabe 2013). Damage to the oocyte by different pathways can induce polyspermy. For example, oxidative stress has been shown to decrease Ca^{2+} reserves and affect cortical granule migration by damaging microfilaments leading to polyspermy in mouse oocytes (Jiao *et al.* 2013). Similar mechanisms could be affected by increased NEFA concentrations in the present study, however, more in-depth research is

necessary to confirm this. After high NEFA exposure, more zygotes remained at the two-cell stage and less good quality embryos (containing four cells or more) were formed at day 2 p.i. It has previously been shown that lipolytic conditions during oocyte maturation or early embryo development compromise the viability of the subsequent embryo (Desmet *et al.* 2016; Marei *et al.* 2017). We could not substantiate an overall decrease in blastocyst formation by elevated NEFA concentrations in the present study. A lower proportion of blastocysts attained the hatching/hatched stage at day 8 p.i after high NEFA exposure during IVF. This may suggest that the embryos that were not fertilized/cleaved are those with the lowest quality that would have been arrested at later stages of development, not reaching the blastocyst stage. Furthermore, the embryos that did form were less able to reach the more advanced blastocysts stages, possibly leading to a delay in further development.

Further study demonstrated that the presence of elevated NEFA concentrations during IVF only affected the quality of resultant blastocysts to a limited extend. Fertilization is associated with an increased ATP demand (Chappel 2013), mainly derived from lactate and pyruvate (Dumollard *et al.* 2004), but also lipids may be used as an energy source during fertilization (Fleming and Saacke 1972; Ferguson and Leese 1999). An excess of energy substrates, such as elevated NEFA concentrations, can alter embryo metabolism as substantiated by Van Hoeck *et al.* (2011) and Desmet *et al.* (2016). No significant differences in blastocyst metabolism could be detected in this study. Similarly, De Bie *et al.* (2017) and Marei *et al.* (2017) demonstrated that metabolism of both COCs and blastocysts was not affected after high NEFA exposure during IVM. A possible explanation of the unaltered blastocyst metabolism in the present study could be related to the embryo plasticity, with acceptable development and functionality reported for a wide variety of environmental conditions (Gardner 1998; Sturmey and Leese 2008; Gardner and Harvey 2015). Another reason could be that the excess of available energy substrates is not taken up by the COCs and metabolized during IVF. We showed that lipid accumulation in zygotes and morulae was not affected under high NEFA conditions. Contrarily, Aardema *et al.* (2011) and Van Hoeck *et al.* (2015) observed a significant increase in lipid droplet content in oocytes and morulae, respectively, originating from immature oocytes exposed to elevated NEFA concentrations during IVM. The authors suggested that storage of FAs in lipid droplets may have a protective function against lipotoxicity. Moreover, Lolicato *et al.* (2015) demonstrated that cumulus cells store the circulating NEFAs in neutral lipids to prevent accumulation in the oocyte itself.

However, Ferguson and Leese (1999) demonstrated a decrease in lipid content in the period between the end of oocyte maturation and formation of a two-cell embryo, thereby suggesting lipid consumption rather than accumulation during fertilization.

Our data showed that blastocysts contained more TE cells and had a higher TE/ICM ratio after high NEFA exposure compared with those in the control group. Embryos may compensate for the metabolic insult earlier in development by relatively enlarging their TE to enhance implantation (Eckert *et al.* 2012). Additionally, the ability of the embryo to grow after hatching is a prerequisite for implantation (Gonda and Hsu 1980). However, our data suggested rather a reduction in outgrowth potential, although this finding was not statistically significant. It has been postulated that reduced outgrowth is related to slow cleavage of the embryo (Lee *et al.* 2015) as we observed in the present study. Overall, embryo development is not only disturbed when high NEFA concentrations are supplemented during oocyte maturation or embryo culture, but here we show evidence that the fertilization window is also sensitive to a lipotoxic environment.

Impact of NEFAs during sperm capacitation

In the second part of the study, only spermatozoa were exposed to elevated NEFA concentrations to specifically investigate the effect of FFAs, as present in the micro-environment in which fertilization takes place, on sperm quality and to distinguish between effects on oocytes and/or sperm cells. Sperm plasma membrane integrity was reduced under elevated NEFA conditions after different exposure periods. These conditions did not perturb the acrosome reaction. In addition, several motility and velocity parameters were negatively affected in the presence of elevated NEFA concentrations while others were not. It appears that contradictory results are present in literature regarding the impact of FAs on sperm quality. Moreover, the effect on motility and viability are strongly dependent on the type of FAs present in the sperm's environment. For example, Pellicer-Rubio and Combarrous (1998) and Hossain *et al.* (2007) noted that the unsaturated OA may exert an adverse effect on acrosome intactness of fresh goat and boar spermatozoa, respectively, through induction of cell membrane damage. In contrast, Hossain *et al.* (2007) observed an improvement of sperm motility and viability in the presence of OA assumingly due to its supportive role in ATP production and in increasing Ca^{2+} and cyclic adenosine monophosphate (cAMP) concentrations. It was demonstrated in the latter study that the saturated PA and SA slightly

increased sperm motility but failed to induce the acrosome reaction probably as a result of tight packaging in the sperm membrane. In the present study, spermatozoa were exposed to a mixture of the three predominant FFAs present in the follicular fluid and serum of cows during an episode of NEB, with the highest fraction of FFAs being saturated. It is possible that the effect on sperm quality of one of these FFAs was alleviated or aggravated by another FFA. It is also important to notice that spermatozoa were treated during swim-up. This technique selects a population of viable and motile spermatozoa (Shamsuddin and Larsson 1993) and thus resembles the physiological selection in the female reproductive tract by accomplishing both cleansing and selection of spermatozoa. Nonetheless, differences in motility and membrane integrity due to the treatment were still observed in the present study which implies that the sperm selection by swim-up did not alleviate these effects.

Although sperm functionality declined under elevated NEFA conditions, their fertilizing capacity was not affected as cleavage rate and blastocyst yield were not altered compared to the control groups. An increase in saturated FA content would be expected to disrupt sperm functions by limiting the fluidity of the plasma membrane, which is, in turn, crucial for such biological events as fusion with the oolemma (Meizel 1984). Quinn and Whitfingham (1982), for example, demonstrated that exposure of mouse sperm to PA and/or OA inhibited fertilization, although neither PA nor OA had any marked effect on sperm motility. We have to take into account that our study was performed under *in vitro* conditions where sperm is present in excess (Ward *et al.* 2002). In contrast, the number of spermatozoa reaching the site of fertilization *in vivo* is much lower (Hawk 1987). It could be possible that the effect of elevated NEFA concentrations can have a more detrimental effect on these low numbers than observed *in vitro*.

Sperm transmits not only its nuclear DNA but also several components that actively participate in early embryo development (Jenkins and Carrell 2011; Kumar *et al.* 2013). Environmental insults, such as paternal diet, have been shown to exhibit profound effects on early embryo development and phenotype (Mitchell *et al.* 2011; Binder *et al.* 2012; Soubry 2015). Likewise, it could be possible that an environmental insult, e.g. high NEFA conditions as present in a metabolically compromised oviductal environment, induces modifications in the resultant blastocysts apart from its impact on sperm functionality. However, our study revealed no difference in blastocyst quality and metabolism in relation to sperm NEFA-

exposure treatments. Thus, we suggest that the exposure window of only 4 h is not sufficient to alter embryo quality through changes in the sperm cells.

Are oocytes, sperm or both affected?

Finally, it appears that exposing both oocytes and sperm cells simultaneously during IVF affected the fertilization process and further embryo development. Sperm functionality decreased in a metabolically compromised environment but sufficient sperm cells retained their fertilizing capacity and led to normal embryo development. When comparing both experiments, we suggest that elevated NEFA concentrations in the micro-environment surrounding the fertilization process could reduce this process mainly through alterations in the oocyte rather than in the spermatozoa. A possible explanation could be that the cumulus cells, surrounding the oocyte, may be affected by elevated NEFA concentrations. Cumulus cells attract (Eisenbach 1999), trap (Bedford and Kim 1993), and select spermatozoa (Carrell *et al.* 1993), and prevent the premature hardening of the zona pellucida (Tanghe *et al.* 2002). Moreover, the communication between cumulus cells and oocytes through gap junctions is necessary to support normal fertilization (Tanghe *et al.* 2003). It was recently shown that IVM under high NEFA conditions alter cumulus cell mitochondrial activity, increase their cellular and endoplasmic reticulum stress levels and induce apoptosis (Marei *et al.* 2017). More research is necessary to investigate possible effects of high NEFA exposure on the cumulus cells and their interaction with the oocyte and sperm during fertilization. These experiments were performed using the sperm of the same bull in order to differentiate between possible effects of high NEFA concentrations on the fertilization process and sperm functionality. Although this study provides the first insight in the effect of NEFAs on IVF, similar experiments should be performed using sperm originating from different bulls as their sperm could react differently (Palma and Sinowatz 2004). Furthermore, addition of ethanol significantly increased the osmolarity in the media used for the solvent, basal NEFA and high NEFA treatment up to the higher range above which sperm motility is reduced (Guthrie *et al.* 2002). Little information is available on the effect of osmolarity on IVF in bovine. In rodents, it is known that this increase in osmolarity does not affect IVF (Miyamoto and Chang 1973). The fact that the basal and high NEFA treatment were compared to a solvent control still endorses our conclusions as differences in sperm functionality were observed after addition of NEFAs compared to the solvent control.

Conclusions

We show evidence that elevated NEFA concentrations, as observed in females suffering metabolic disorders associated with upregulated lipolysis, impair the fertilization process. Although sperm functionality was affected by the presence of these lipolytic conditions, adequate numbers of viable sperm cells can retain their ability to fertilize the oocytes and support further embryo development. In contrast, simultaneous incubation of the gametes in the presence of elevated NEFA concentrations reduced the fertilization rate and increased the incidence of polyspermy. Subsequent embryo development seemed to be affected at early cleavage and blastocyst stages. In addition, resultant blastocysts exhibited differences in cell differentiation. These findings imply that the fertilization process is disturbed due to alterations in the oocyte rather than the sperm cells. Further research is required to unravel underlying mechanisms leading to the decline in fertilization. Overall, these data provide the first insights into the impact of the oviductal environment, influenced by maternal metabolic health, on both oocytes and sperm and their interaction.

List of abbreviations

ALH: amplitude of lateral head displacement; ATP: adenosine triphosphate; BCF: beat cross-frequency; BSA: bovine serum albumin; cAMP: cyclic adenosine monophosphate; CASA: computer-assisted sperm analysis; COC: cumulus oocyte complex; FFA: free fatty acid; ICM: inner cell mass; IVC: *in vitro* culture; IVF: *in vitro* fertilization; IVM: *in vitro* maturation; LIN: linearity; MP: medium progressive motility; NEB: negative energy balance; NEFA: non-esterified fatty acid; OA: oleic acid; PA: palmitic acid; PM: progressive motility; ROS: reactive oxygen species; RP: rapid progressive motility; SA: stearic acid; STR: straightness; TE: trophectoderm; TM: total motility; VAP: average pathway velocity; VCL: curvilinear velocity; VSL: straight-line velocity; WOB: wobble.

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637 **Conflicts of Interest**

638 The authors declare no conflicts of interest.

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Fig. 1. Glucose consumption, pyruvate consumption and lactate production of Day-7 blastocysts after NEFA exposure during *in vitro* fertilisation. Bars are presented as mean $\pm$ s.e.m. ($n = 67$ blastocysts). \$Bars with a dollar sign tend to be different from each other ($P < 0.1$).

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901

902 **Fig. 2.** Average diameter of hatched blastocysts originating from oocytes exposed to the different conditions
 903 during *in vitro* fertilisation, from Day 9 until Day 14 p.i. Bars are presented as mean $\pm$ s.e.m. ($n = 85$
 904 blastocysts). No significant differences could be detected between the treatment groups.

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Fig. 3. The percentage of spermatozoa with intact plasma membrane after 1, 2, 3 and 4 h NEFA exposure. Bars are presented as mean $\pm$ s.e.m. ($n = 5$ replicates). ^{a,b}Bars with different letters are significantly different ($P < 0.05$). ^{\$}Bars with a dollar sign tend to be different from each other ($P < 0.1$).

Fig. 4. Glucose consumption, pyruvate consumption and lactate production of Day-7 blastocysts originating from routinely matured oocytes and NEFA-exposed spermatozoa. Data are presented as mean $\pm$ s.e.m. ($n = 66$ blastocysts). No significant differences could be detected between the treatment groups.

Table 1. Embryo developmental competence after NEFA exposure during *in vitro* fertilisation

Data are presented as total numbers (%). ^{a,b}Values with different superscripts in the same row differ significantly between treatment groups ($P < 0.05$). ^{\$}Values marked with a dollar sign in the same row tend to be different from each other ($P < 0.1$)

Parameter	Treatment		
	Solvent	Basal	High Combi
Oocytes	190	196	195
Total cleaved	157 (82.6) ^a	160 (81.6) ^{ab\$}	141 (72.3) ^{b\$}
2-Cell block / cleaved embryos	18 (11.5) ^a	21 (13.1) ^a	35 (24.8) ^b
≥4-Cell embryos / total oocytes	90 (47.4) ^a	85 (43.4) ^a	68 (34.9) ^b
Fragmented embryos / total oocytes	44 (23.2)	39 (19.9)	31 (15.9)
Day-8 blastocysts / total oocytes	69 (36.3)	55 (28.1)	54 (27.7)
Day-8 expanded, hatching and hatched blastocysts / total oocytes	62 (32.6) ^a	49 (25.0) ^{ab}	41 (21.0) ^b
Day-8 hatching and hatched blastocysts / total blastocysts	41 (59.4) ^a	35 (63.6) ^{ab}	24 (44.4) ^b

Table 2. Fertilisation status of oocytes after NEFA exposure during *in vitro* fertilisation

Data are presented as total numbers (%). ^{a,b}Values with different superscripts in the same row differ significantly between treatment groups ($P < 0.05$). ^{\$}Values marked with a dollar sign in the same row tend to be different from each other ($P < 0.1$)

Parameter	Treatment		
	Solvent	Basal	High Combi
Oocytes	63	52	70
Normal fertilisation	47 (74.6) ^a	38 (73.1) ^a	38 (54.3) ^b
Polyspermy	0 (0.0) ^a	4 (7.7) ^b	4 (5.7) ^b
No fertilisation	16 (25.4) ^{ab\$}	10 (19.2) ^a	28 (40.0) ^{b\$}

Table 3. Cell differentiation and apoptosis in Day-8 blastocysts after NEFA exposure during *in vitro* fertilisation

Data are presented as mean $\pm$ s.e.m. ($n = 41$ blastocysts). ^{a,b}Values with different superscripts in the same row differ significantly between treatment groups ($P < 0.05$)

Parameter	Treatment		
	Solvent	Basal	High Combi
Total cell count	92 $\pm$ 10	122 $\pm$ 8	124 $\pm$ 10
Trophectoderm (TE) cell count	55 $\pm$ 9 ^a	73 $\pm$ 6 ^{ab}	90 $\pm$ 7 ^b
Inner cell mass (ICM) cell count	37 $\pm$ 5	50 $\pm$ 5	35 $\pm$ 5
TE/ICM ratio	1.8 $\pm$ 0.39 ^a	1.9 $\pm$ 0.29 ^a	4.2 $\pm$ 1.04 ^b
Apoptotic cell index (%)	3.1 $\pm$ 1.03	3.1 $\pm$ 0.55	3.0 $\pm$ 0.78

Table 4. Computer-assisted sperm analysis (CASA) of spermatozoa exposed to elevated NEFA concentrations during 4 h

Data are presented as mean $\pm$ s.e.m. ($n = 3$ replicates). ^{a,b}Values with different superscripts in the same row differ significantly between treatment groups ($P < 0.05$). ^{§,§}Values marked with a dollar or section sign in the same row tend to be different from each other ($P < 0.1$)

Parameter	Treatment		
	Solvent	Basal	High Combi
Total motility (TM, %)	53.4 $\pm$ 2.45 ^a	41.1 $\pm$ 2.09 ^b	44.9 $\pm$ 2.75 ^b
Progressive motility (PM, %)	34.6 $\pm$ 2.33 ^a	24.0 $\pm$ 1.41 ^b	22.6 $\pm$ 1.60 ^b
Rapid progressive motility (RP, %)	30.7 $\pm$ 2.37 ^a	20.7 $\pm$ 1.45 ^b	17.1 $\pm$ 1.36 ^b
Medium progressive motility (MP, %)	3.9 $\pm$ 0.52 ^{ab§}	3.4 $\pm$ 0.49 ^a	5.5 $\pm$ 0.52 ^{b§}
Average path velocity (VAP, $\mu\text{m s}^{-1}$)	51.8 $\pm$ 2.45 ^a	47.0 $\pm$ 2.04 ^a	37.9 $\pm$ 1.86 ^b
Straight-line velocity (VSL, $\mu\text{m s}^{-1}$)	47.8 $\pm$ 2.58 ^a	42.9 $\pm$ 1.98 ^a	33.5 $\pm$ 1.99 ^b
Curvilinear velocity (VCL, $\mu\text{m s}^{-1}$)	82.0 $\pm$ 3.08 ^a	75.1 $\pm$ 3.18 ^a	62.4 $\pm$ 2.24 ^b
Straightness (STR, %)	82.4 $\pm$ 1.28	80.6 $\pm$ 1.28	79.6 $\pm$ 1.32
Linearity (LIN, %)	54.0 $\pm$ 1.55	53.0 $\pm$ 1.32	52.0 $\pm$ 1.20
Wobble (WOB, %)	61.6 $\pm$ 1.16	61.4 $\pm$ 1.01	61.1 $\pm$ 0.89
Amplitude of lateral head displacement (ALH, μm)	3.0 $\pm$ 0.08 ^a	2.9 $\pm$ 0.11 ^a	2.5 $\pm$ 0.07 ^b
Beat cross frequency (BCF, Hz)	8.5 $\pm$ 0.33 ^{a§}	7.6 $\pm$ 0.32 ^{ab§§}	6.5 $\pm$ 0.25 ^{b§§}

Table 5. Cell differentiation and apoptosis in Day-8 blastocysts after pre-exposure of spermatozoa to NEFA followed by routine *in vitro* fertilisation

Data are presented as mean $\pm$ s.e.m. ($n = 39$ blastocysts). [§]Values marked with a dollar sign in the same row tend to be different from each other ($P < 0.1$)

Parameter	Treatment		
	Solvent	Basal	High Combi
Total cell count	108 $\pm$ 10	85 $\pm$ 6	99 $\pm$ 10
Trophectoderm (TE) cell count	78 $\pm$ 7 [§]	58 $\pm$ 5 [§]	75 $\pm$ 8
Inner cell mass (ICM) cell count	30 $\pm$ 4	27 $\pm$ 4	24 $\pm$ 4
TE/ICM ratio	3.1 $\pm$ 0.46	4.0 $\pm$ 1.15	3.8 $\pm$ 0.47
Apoptotic cell index (%)	6.3 $\pm$ 1.92	6.9 $\pm$ 0.58	7.8 $\pm$ 1.36