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Influence of the coincubation of post-thawed bull semen with elevated β-hydroxybutyrate concentrations on sperm characteristics

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Abstract

In this study, the relationships between post-thaw bull sperm characteristics and hyperketonemic conditions after coincubation with cow plasma or media were determined to investigate if such a condition could affect bull sperm characteristics. Two experiments were conducted. In experiment 1, blood samples were collected from 31 cows to prepare plasma. Cows were independently categorized into two groups according to plasma β-hydroxybutyrate (BHB) concentrations (above or below 1.2 mM). Thawed bull semen was diluted and incubated with diluted plasma; motility parameters were evaluated using Computer Assisted Semen Analysis (CASA). In experiment 2, a pooled sample of thawed semen was diluted and divided into three aliquots: without BHB (control) and treated with either 1.2 mM (1.2) or 3 mM (3) BHB. In addition to motility, flow cytometric analyses were carried out. In experiment 1, the overall motility decreased significantly in plasma containing high (≥1.2 mM) BHB compared to plasma containing low (<1.2 mM) BHB. In experiment 2, the overall motility tended to be lower in BHB (3 mM)-supplemented samples. The supplementation of 3 mM BHB increased the proportion of live superoxide-positive sperm and sperm with high mitochondrial potential, while the DNA fragmentation index decreased.

KEYWORDS
bovine, coincubation, hyperketonaemia, semen characteristics

1 | INTRODUCTION

Artificial insemination (AI) in cattle represents the most successful biotechnology in animal breeding (Kaproth & Foote, 2011), contributing to improved dairy farm efficiency. By utilizing AI, thawed sperm from bulls selected to be genetically superior for milk production traits (Parkinson & Morrell, 2019) are deposited intra-utero, prior to reaching the oocyte in the oviductal ampulla and participating in the fertilization process (Rodriguez-Martinez, 2007). During its journey from the uterine body to the oviductal ampulla, sperm are sequentially exposed to various microenvironments, which are composed of a combination of constituents derived from the blood plasma and molecules secreted from the oviduct and uterine epithelium (McKnight et al., 2014). Thus, components present in the reproductive tract may interfere with sperm motility; extended maintenance of motility and viability in

sperm after thawing is essential for achieving high conception rates (Ledesma et al., 2016).

Early lactation is a dynamic period for the dairy cow, during which the most dramatic metabolic changes are likely to occur (Roche et al., 2013). This period may last for up to 8 weeks (Heuer et al., 2001; Staples et al., 1990) and cows unable to adapt to this challenging period are more inclined to a negative energy balance (NEB; McArt et al., 2013) by mobilizing more fat reserves as non-esterified fatty acids (NEFAs; Roche et al., 2013). These NEFAs are delivered to the liver and can be completely oxidized for ATP production, exported as lipoproteins, or partially oxidized into ketone bodies and, in particular, to β-hydroxybutyrate (BHB; Allen et al., 2009). Although some elevation of this metabolite is normal as it is part of the biological adaptation process of the cow, excessive elevation of BHB may indicate poor adaptation to NEB and may result in subclinical or clinical ketosis. There is increasing evidence that the changes in blood BHB concentration which accompany the post-partum period of NEB serve as signals to the reproductive system to reduce fertility (Kalem et al., 2020). Recent reports highlight the relationships between postpartum fertility and BHB in dairy cows (Abdelli et al., 2017). Likewise, some studies showed that high BHB levels postpartum were negatively associated with subsequent conception rates (Ospina et al., 2010c; Shin et al., 2015). Others observed that high body condition losses, along with elevated BHB, affected follicular cell function (Vanholder et al., 2006), oocyte maturation (Leroy et al., 2006), corpus luteum function and early embryo development (Desmet et al., 2016), which in turn influence cow fertility. To our knowledge, no study has explicitly focused on how this metabolite in the female environment influences sperm motility. However, since it is known that BHB is present in the oviductal and uterine fluid in cows (Gómez et al., 2002), it is possible that there is an interaction between this biomarker in the plasma and sperm.

Against this background and owing to the similarities between plasma and microenvironment within the cow genital tract (Nait Mouloud et al., 2017), we hypothesized that the coincubation of thawed bull sperm with plasma collected from healthy or hyperketonemic dairy cows affects their motility. The present study was, therefore, designed as a preliminary investigation to describe how bull sperm motility is modified by coincubation with plasma from healthy or hyperketonemic dairy cows. The influence of subsequent coincubation under hyperketonemic media on sperm characteristics was also investigated.

2 | MATERIALS AND METHODS

Two experiments were conducted to determine how bull sperm characteristics are modified by coincubation with cow's plasma or media. Experiment 1 was a coincubation of thawed bull sperm with plasma from healthy or hyperketonemic dairy cows. Experiment 2 was a coincubation of thawed bull sperm with media containing 0, 1.2 or 3 mM of DL-β-hydroxybutyrate acid sodium salt, to mimic the situation of hyperketonemic conditions.

2.1 | Experiment 1

2.1.1 | Animals, blood sampling and analyses

Blood samples were collected from 31 Holstein-Friesian (14) and Montbéliarde (17) cows by one technician just before AI. The cows ranged in age from 23 months to 9 years and were within 100 days in milk (DIM).

About 10-mL of blood samples were collected from the coccygeal vein of each animal into lithium heparin tubes (FL Medical). Samples were chilled on ice packs immediately after collection and centrifuged at 733 *g* for 10 min within 4 h of collection; the supernatant was stored at −20°C until analysis.

β-Hydroxybutyrate concentrations were measured using a handheld meter (Optium Xceed; Abbott Laboratories) immediately following collection.

Briefly, Optium Xceed was used to test blood BHB concentrations, with a threshold of 1.2 mM being used to classify BHB concentrations as high (Ospina et al., 2010a). Cows were, thereafter, categorized into two groups based on their BHB concentrations: hyperketonemic, with BHB ≥1.2 mM and healthy, with BHB <1.2 mM. Thus, six cows had a BHB concentration ≥1.2 mM (hyperketonemic) and 25 cows had a BHB concentration <1.2 mM (healthy).

2.1.2 | Frozen semen and post-thawing incubation

Commercial semen straws frozen at the National Center of Artificial Insemination and Genetic Amelioration (CNIAAG, Birtouta, Algeria) were used in the current study. Bulls were housed in clean, well-ventilated and well-lit buildings or outdoors in paddocks. Their health was constantly assessed and they were fed rations that met their nutritional requirements. An ejaculate was collected from a 4-year-old Montbéliarde bull every Wednesday using an artificial vagina (initial water temperature 45°C). Semen volume was measured and sperm concentration was evaluated using a photometer (Accucell; IMV); the proportion of motile sperm was analysed by subjective visual assessment. Semen was extended with Bioxcell® (IMV Technologies) and loaded into 0.25 mL plastic straws to achieve a sperm concentration of approximately 20×10^6 sperm (spz)/mL and transferred to a refrigerator (cooled to 5°C for 20 min). After equilibration, straws were placed on a rack and frozen in liquid nitrogen (N_2) vapour (5 cm above liquid nitrogen) for 15 min, immersed in N_2 and stored in a cryobiological container.

A total of 13 straws from the same ejaculate were used in this study (one straw per replication). Each straw was thawed in a water bath at 37°C for 20 s; the content was then mixed and diluted 1:1

with physiological saline (NaCl, 0.9%). The plasma collected from cows was also diluted 1:9 (vol/vol) in physiological saline. The diluted semen (from one straw) was then divided into five aliquots; four aliquots were diluted 1:1 (vol/vol) with diluted plasma (one aliquot per cow) and one was kept as diluted semen only (without plasma supplementation; control). Samples were then incubated at 37°C in 5% CO_2: 95% air and evaluated for motility within 5, 30 and 90 min. A 5-μL sperm aliquot was placed on a pre-warmed slide and covered with a coverslip. For each sample, three microscopic fields were analysed (approximately 500 spermatozoa) using Computer Assisted Semen Analysis (SCA®CASA; Microptic SL.). The CASA system was coupled with the Nikon Eclipse E-200 microscope (Nikon Corporation) equipped with a digital camera (A312FC/C; Basler) and attached heating stage set to 37°C. The SCA settings were as follows: number of frames: 25 frames/s, optics: Ph-(negative phase contrast), analytic chamber: cover object; particle area: 10–80 μm^2; VLC cut-off values: rapid > 35 < medium > 15 < slow > 10 < and progressivity: >80% of STR.

The proportions of motile sperm (motility) and progressively motile sperm were evaluated, together with their kinematics. The kinematics recorded for each sperm were (1) curvilinear velocity (VCL; μm/s): average velocity of motile sperm over the distance travelled, including all deviations of sperm head movement; (2) average path velocity (VAP; μm/s); (3) straight line velocity (VSL; μm/s); (4) amplitude of lateral head displacement (ALH; μm); (5) beat cross-frequency (BCF; Hz); (6) wobble coefficient (WOB; calculated as VAP/VCL); (7) linearity (LIN; calculated as VSL/VCL) and (8) straightness (STR; calculated as VSL/VAP).

2.2 | Experiment 2

2.2.1 | Frozen semen

Frozen semen samples from 10 Swedish Red AI bulls of proven fertility from Viking Genetics were used. The collection and freezing of the semen were performed using standard procedures (Lima-Verde et al., 2018). Semen was collected twice weekly from the bulls using an artificial vagina. The semen was extended in Andromed® (Minitube International) to achieve a sperm concentration of 69×10^6/mL and was used to fill plastic straws (0.25 mL), which were then sealed. The semen was equilibrated for 4 h at 4°C before freezing in liquid nitrogen vapour using a programmable freezing machine, according to the standard practice at this commercial semen station.

2.2.2 | Sperm preparation and post-thawing incubation

A total of 10 frozen semen straws were thawed in a water bath at 37°C for 20 s, pooled and mixed. Sperm concentration was measured using a Nucleocounter® SP-100™ (ChemoMetec) as described by Morrell et al. (2018). After evaluating the sperm cell count, each sample was aliquoted into three samples, which were diluted to 2 million sperm either with basic TALP extender without BHB (control) or with TALP supplemented with different concentrations (1.2 mM or 3 mM) of DL-β-hydroxybutyrate acid sodium salt (Sigma-Aldrich; H6501). These BHB concentrations were typically associated with subclinical and clinical ketosis, respectively (McArt et al., 2013).

The sperm samples were incubated at 37°C in 5% CO_2 in air. The TALP medium composition was 100 mM NaCl, 21.6 mM Na lactate, 1 mg/mL Na pyruvate, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 10 mM Hepes and 10 μg/mL penicillin/streptomycin. The incubation period used for all treatments was 180 min. Assays were performed immediately after thawing except for the Sperm Chromatin Structure Assay where the sperm samples were added to TNE buffer 1:1 (vol/vol) at 2 million sperm/mL and stored at −80°C for later analysis. All chemicals used in this experiment were obtained from Sigma-Aldrich.

Three replicates were performed. For each replicate, sperm characteristics were evaluated at predetermined incubation times (30, 90 and 180 min). In addition to these time points, sperm motility and cell kinematics were evaluated immediately after coincubation (within 5 min). We were not able to evaluate sperm cell characteristics using flow cytometry immediately after coincubation because the flow cytometry assays required at least 30 min of preparation.

2.2.3 | Sperm motility variables

The assessment of sperm motility and cell kinetics was performed using SpermVision software version 3.5 (Minitüb GmbH) connected to an Olympus BX51 microscope (Olympus). Sperm motility characteristics (mentioned above) were determined with a 10 objective and ×10 ocular at 38°C. For each sample, eight fields were randomly selected, and at least 200 sperm per field were evaluated.

2.2.4 | Measurement of sperm cell characteristics using flow cytometry

Flow cytometry (FC) was performed using a FACSVerse (BD Biosciences) cytometer.

Sperm viability assessment

Sperm viability was estimated using SYBR-14 and propidium iodide (PI) stain (Live-Dead® Sperm Viability Kit L-7011; Invitrogen) according to the procedure described by Kumaresan et al. (2017) evaluating sperm cell characteristics of post-thawed bull semen. The stock solution of SYBR-14 (1 mM) was diluted 50 times (0.5 μL of SYBR-14 to 24.5 μL of PBS); the working solution was prepared each day. We added 1.2 μL of diluted SYBR-14 and 3 μL PI (2.4 mM) to 300 μL of sperm suspension containing 2 million sperm and incubated at 37°C for 10 min before analysis using flow cytometry. In total, 30,000 sperm-specific events were evaluated, identified and classified

according to the degree of intactness of the plasma membrane, as membrane intact (live: SYBR14-positive/PI-negative), membrane damaged (dead: SYBR14-negative/PI-positive).

Sperm reactive oxygen species

Sperm reactive oxygen species [ROS; superoxide (SO), hydrogen peroxide (H_2O_2)] were determined using the procedure given by Kumaresan et al. (2017). Sperm (2 million in 300 μL of PBS) were stained with 9 μL of 40 μM Hoechst 33258 (Sigma), 9 μL of 40 μM hydroethidine (HE; Invitrogen) and 9 μL of 2 mM 2′,7′ dichlorodihydrofluorescein diacetate (Invitrogen) for 30 min at 37°C before flow cytometric analysis. After gating to identify sperm, the sperm detected were classified as the proportions of: live, SO-negative; live, SO-positive; dead, SO-positive; live, H_2O_2-negative; live, H_2O_2-positive; dead, H_2O_2-negative and dead, H_2O_2-positive by region statistics.

Mitochondrial activity assessment

Mitochondrial membrane potential (MMP) of sperm was measured using the lipophilic cationic probe JC-1. A total of 2 μL of 3 mM 55,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1; Invitrogen) were added to 300 μL of sperm-TALP extender containing 2 million sperm and incubated for 30 min at 37°C in air, shielded from light. The JC-1 fluorescence was measured in the FL1 (527/32 nm) and FL2 (586/42 nm) channels of the flow cytometer. In total, 50,000 cells were evaluated and classified into two categories: sperm with reduced function (sperm with green fluorescent mitochondria) and sperm with highly active mitochondria (red fluorescent mitochondria).

Acrosome reaction assay

The acrosome reaction status of sperm was assessed using fluorescein isothiocyanate-peanut agglutinin (Sigma-Aldrich). We added 3 μL of fluorescein isothiocyanate-peanut agglutinin and 3 μL of PI (2.4 mM) to 300 μL of PBS containing 2 million sperm and 5 μL of Hoechst 33342 (50 μg/mL; Invitrogen) and the mixture was incubated at 37°C for 10 min under dark conditions. The samples were analysed using a flow cytometer with the same parameters as for viability, with the addition that Hoechst 33342 fluorescence (FL5) was excited with a violet 405-nm laser and collected using a 528/45-nm band-pass filter. In total, 30,000 events were evaluated for each sample. After gating to identify sperm, four populations were discerned: live acrosome intact (LAI), live acrosome reacted (LAR), dead acrosome intact (DAI) and dead acrosome reacted (DAR).

Sperm chromatin structure assay

The procedure followed was designed by Evenson and Jost (2000), with modifications. Briefly, sperm samples frozen and stored in TNE buffer were thawed on ice, 100 μL of each sperm suspension was subjected to partial DNA denaturation by mixing with 200 μL of Triton X-100 (Sigma-Aldrich) detergent solution (0.08 N HCl, 0.1% Triton X-100; pH 1.2). About 30 s later, the sperm were stained with 600 μL of acridine orange (AO; Sigma-Aldrich) staining solution (200 mM of Na_2HPO_4; 0.1 mol/L of citric acid buffer, pH 6.0; 1 mM of EDTA; 150 mM of NaCl and 6 μg/mL of AO). Within 3–5 min after the addition of AO the degree of chromatin stability was quantified by flow cytometric (FACSVerse; BD Biosciences) measurement of the metachromatic shift from green (stable, dsDNA) to red (denatured, ssDNA) AO fluorescence. For each sample, 10,000 events were analysed and the proportion of sperm with single-stranded DNA, i.e., the DNA fragmentation index (%DFI), was calculated.

2.3 | Statistical analysis

Statistical analyses were done using R (version 3.5.1; R Foundation for Statistical Computing, Vienna, Austria) via RStudio (version 1.1.383; RStudio Inc.). Repeated-measures analysis of variance (ANOVA) in R was performed for all measured variables. Diagnostic plots for assessing the normality of residuals and effects in models fit were obtained using the qqnorm function of R. Post-hoc comparisons were computed using the lsmeans function from the lsmeans package in R. The results were represented by the least square means (LSM) ± standard error of mean (SEM). Stacked line plots of the variables in the study were generated using Prism 6.07 (GraphPad Software, Inc.). p-Values < .05 were considered to be significant.

3 | RESULTS

3.1 | Experiment 1

In this experiment, adding cow plasma significantly affected total and progressive sperm motility, as well as all kinematic parameters recorded by Computer Assisted Semen Analysis (CASA) except linearity (LIN), straightness (STR) and wobble (WOB) (Table 1). As can be observed, the total motility was lower by eight percentage points ($p < .05$) when sperm were coincubated with plasma of hyperketonemic cows (BHB concentrations ≥1.2 mM) compared to those incubated with plasma of healthy cows (BHB concentrations <1.2 mM) at 30 min of incubation.

The progressive motility was higher at 30 min when sperm were coincubated with plasma of hyperketonemic cows compared to the control; but it was similar to those incubated with plasma of healthy cows.

Similarly to the results for total motility, curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), lateral head displacement (ALH) and beat cross frequency (BCF) were significantly influenced by adding cow plasma (Table 1). Higher values of VAP, VCL, VSL and ALH were observed when sperm were coincubated with plasma (from hyperketonemic or healthy cows) compared to the control at the first 5 min. At 30 min of incubation, VAP, VCL, VSL, ALH and BCF had significantly lower values ($p < .05$) when sperm were coincubated with plasma of hyperketonemic cows compared to coincubation with plasma from healthy cows. However,

TABLE 1 Least square means ± SEM of sperm motility descriptors in the different plasma media according to high or low BHB levels and the control group within 5 min, at 30 and 90 min.

		Time				*p*-Value[3]		
Sperm motility descriptors	Value (*N*[1])	Within 5 min	30 min	90 min	SEM[2]	Group	Time	G*T[4]
% Motile	Hyperketonemic cows (6)	28.0	25.8a	24.7	12.52	.042	.120	.879
	Healthy cows (25)	34.1	33.8b	28.2	8.59			
	Control (13)	35.0	29.7ab	25.1	6.25			
% Prog Mot	Hyperketonemic cows (6)	25.1	30.7a	23.7	3.28	.006	.275	.855
	Healthy cows (25)	24.4	20.3ab	20.2	3.35			
	Control (13)	16.1	14.3b	15.4	5.08			
VAP	Hyperketonemic cows (6)	48.5a	37.1a	38.4	3.32	.010	.131	.301
	Healthy cows (25)	50.0a	48.3b	41.9	3.25			
	Control (13)	34.3b	37.3a	37.8	5.04			
VCL	Hyperketonemic cows (6)	89.7a	71.0a	70.9	6.54	.003	.105	.389
	Healthy cows (25)	99.4a	92.9b	78.9	6.68			
	Control (13)	63.1b	66.1a	71.3	10.35			
VSL	Hyperketonemic cows (6)	32.3a	24.4a	24.3	2.56	.002	.253	.324
	Healthy cows (25)	32.1a	33.5b	28.2	2.61			
	Control (13)	20.3b	23.1a	22.7	3.96			
STR	Hyperketonemic cows (6)	0.59	0.57	0.57	0.02	.096	.522	.513
	Healthy cows (25)	0.58	0.62	0.59	0.02			
	Control (13)	0.50	0.58	0.55	0.03			
LIN	Hyperketonemic cows (6)	0.37	0.35	0.36	0.02	.532	.304	.477
	Healthy cows (25)	0.32	0.39	0.37	0.02			
	Control (13)	0.30	0.37	0.34	0.03			
WOB	Hyperketonemic cows (6)	0.56	0.55	0.56	0.01	.379	.409	.694
	Healthy cows (25)	0.53	0.56	0.56	0.01			
	Control (13)	0.55	0.59	0.57	0.02			
ALH	Hyperketonemic cows (6)	2.54a	2.11a	2.12	0.15	.004	.138	.265
	Healthy cows (25)	2.77a	2.61b	2.27	0.15			
	Control (13)	1.88b	1.96a	2.18	0.24			
BCF	Hyperketonemic cows (6)	8.91	6.85a	6.32	0.62	.064	.003	.210
	Healthy cows (25)	8.60	9.26b	6.64	0.61			
	Control (13)	7.18	6.88a	6.31	0.94			

Note: Different letters (a, b and c) within a column indicate significant differences between treatments ($p < .05$).

Abbreviations: % Motile, percentage of motile sperm %; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity; Prog Mot, percentage of progressive sperm; STR, straightness; VAP, velocity average path; VCL, velocity curved line; VSL, velocity straight line; WOB, wobble.

[1]*N*: number of plasma (from healthy or hyperketonemic cows) or straws used in experiment 1.

[2]Standard error of the mean.

[3]Values of $p < .05$ were considered statistically significant.

[4]Group*time interaction.

at the same time, no significant differences were observed for these kinematic parameters when sperm were coincubated with plasma of hyperketonemic cows compared to the control.

Only BCF changed over time ($p = .003$). However, there was no group-by-time interaction for this parameter. There were no significant changes in total or progressive motility or the other kinematic parameters, either through time or in a group-by-time interaction (Table 1).

3.2 | Experiment 2

3.2.1 | Sperm motility variables

The effects of BHB on frozen bovine sperm are presented in Table 2. Overall, the addition of 1.2 and 3 mM of BHB did not have a significant effect on total and progressive motility, or kinematic

parameters, compared to the controls. However, all kinematics were significantly changed over time ($p < .05$; Table 2). An interaction ($p < .01$) between treatment and time was observed only for LIN and STR.

3.2.2 | Flow cytometric analysis

As shown in Table 3, the overall reactive oxygen species (ROS) status was not significantly different between treatment and control except for the proportion of live superoxide-positive sperm, which was higher ($p < .05$) in BHB (3 mM) and (1.2 mM)-supplemented samples compared with control at 30 min of incubation. The addition of 1.2 or 3 mM of BHB in media had no effect on the proportion of live, LAR and LAI sperm (Figure 1a,c,d). However, coincubation of thawed bull semen with 3 mM of BHB significantly reduced ($p = .031$) the DNA fragmentation index compared with 1.2 and control groups (Figure 1b). At 30 min of incubation, there was an increase in the proportion of sperm with high mitochondrial potential ($p < .05$) in BHB-supplemented samples compared with control (Figure 1e). There was a decrease in the proportions of live sperm ($p < .0001$), live acrosome intact cells ($p = .013$) and cells with high mitochondrial potential ($p < .0001$) with time. However, no interaction ($p > .1$) between treatment and time was observed for all these analyses.

4 | DISCUSSION

Motility is considered to be an important parameter for the evaluation of frozen–thawed bovine sperm quality (Contri et al., 2010). However, motility and kinematic parameters should not be considered as a solid indication of the fertilizing ability of a given semen sample without accounting for the interaction of the sperm with the microenvironments it encounters in the cow (McKnight et al., 2014). Sperm motility can be affected by different conditions, including the type of CASA instrument (Amann & Waberski, 2014) and settings, the temperature at which the analysis is done (Broekhuijse et al., 2011) and, finally, the media (Anbari et al., 2016). In particular, different compounds present in the fluids of the female genital tract can affect sperm motility characteristics (Åkerlöf et al., 1987).

The total motility was lower in the media containing plasma from cows with high β-hydroxybutyrate (BHB) than from cows with low BHB concentrations. This is in agreement with the negative association between conception and high BHB concentrations (Abdelli et al., 2017). This consistency suggests that some of the low postpartum fertility of dairy cows may arise from poorer sperm motility due to metabolic alteration and altered oviductal (Desmet et al., 2016) and uterine environments (Wathes et al., 2003) in hyperketonemic cows. Hyperketonaemia is commonly reported in the first two months of lactation (Dohoo & Martin, 1984; Duffield, 2000) and an episode could last for at least 8 days (Dohoo & Martin, 1984). Together, this evidence supports the hypothesis that high levels of BHB may occur during the insemination period.

At insemination, it is obvious that fertility is affected by co-occurring environmental conditions in cows (e.g. altered uterine and oviductal environment) but we can assume, as has been suggested by Britt (1991), that it is even more affected by the conditions occurring during early lactation. In support of this hypothesis, previous studies reported a negative effect of elevated NEFA concentrations during the transition period on subsequent cow fertility. Garverick et al. (2013) reported an association between conception rate at first insemination and increased NEFA concentrations a few days after calving. Somewhat similar results were found by Ospina et al. (2010b), who reported that cows with elevated NEFA concentrations during the transition period had lower conception rates within 70 days of the post-voluntary waiting period. These authors also observed, in another study, an association between pre-partum and post-partum elevated NEFA concentrations and the herd-level incidence of post-partum hyperketonaemia (Ospina et al., 2010a). Other reports indicated that cows diagnosed with high BHB in the first week postpartum were less likely to become pregnant after the first insemination (Walsh et al., 2007). It has been suggested that there is a clear relationship between NEFA concentrations and the proportion of body weight change (Carvalho et al., 2014). According to these authors, an association between body condition score (BCS) changes during the first 3 weeks postpartum and pregnancy per artificial insemination (AI) to first-timed AI was reported. However, the authors failed to find an association between BCS loss and in vivo fertilization of oocytes (Carvalho et al., 2014). In contrast, a negative effect of high NEFA concentrations, which is the result of a significant BCS loss, on in vitro fertilization of oocytes was observed by Leroy et al. (2005).

During early lactation, cows experience a negative energy balance (NEB), which causes excessive lipid mobilization, and consequently an increased concentration of NEFA (Roche et al., 2013). When released, NEFA are taken up by the liver, where they may be oxidized to carbon dioxide to provide energy or partially oxidized to produce acetoacetate, subsequently producing BHB (Allen et al., 2009) to be transported for use elsewhere in the body.

This migration of BHB implies that the uterine and oviductal environments can be altered by metabolic disorders and may influence sperm motility. It may partly explain the low fertility rates reported in cows with higher BHB concentrations (Ospina et al., 2010b). Likewise, the poor fertility associated with diabetic ketoacidosis in women was suspected to be linked to a hostile intrauterine environment (Kamalakannan et al., 2003). Furthermore, BHB administered to pregnant sheep crossed into the ovine uterus in small but significant amounts (Miodovnik et al., 1986). The negative effect of high BHB on total sperm motility may be explained by a direct effect of these molecules on the ability of sperm to move. Many studies have reported a negative effect of such high BHB concentrations on several cell types. Hoeben et al. (1997) reported a toxic effect of high BHB concentrations (>1 mM) on neutrophil function, concluding that high BHB concentrations are a causal link between NEB and the immune depression in early postpartum cows. Similarly, other authors reported that high BHB concentrations were noted to

TABLE 2 Least square means ± SEM of sperm motility descriptors in different concentrations of BHB [0 (control), 1.2 and 3 mM] within 5, at 30, 90 and 180 min ($N = 9$).

		Time					*p*-Value[2]		
Sperm motility descriptors	Concentration of BHB (mM)	Within 5 min	30 min	90 min	180 min	SEM[1]	Treatment	Time	G*T[3]
% Motile	Control	57.0	70.3	70.0	54.0	6.70	.131	.004	.502
	1.2	56.5	66.7	55.7	58.0	5.47			
	3	48.0	70.7	54.3	44.0	5.47			
% Prog Mot	Control	54.5	67.7	65.0	50.0	6.98	.214	.005	.491
	1.2	54.5	64.0	51.0	55.7	5.70			
	3	47.0	68.3	51.3	40.0	5.70			
VAP	Control	79.5	82.0	69.7	56.7	5.15	.239	<.0001	.239
	1.2	77.5	85.0	76.3	64.3	4.21			
	3	82.5	80.3	70.0	53.7	4.21			
VCL	Control	166	152	130	105	8.72	.265	<.0001	.665
	1.2	158	156	142	121	7.12			
	3	166	144	135	105	7.12			
VSL	Control	63,5	66	52,3	44.3ab	4.42	.217	<.0001	.391
	1.2	60.0	70.0	60.0	50.7a	3.61			
	3	69.0	65,7	54.0	40.0b	3.61			
STR	Control	0.79	0.78	0.77	0.78a	0.01	.505	.0001	.006
	1.2	0.77	0.81	0.77	0.78a	0.01			
	3	0.83	0.81	0.76	0.73b	0.01			
LIN	Control	0.37	0.43	0.40	0.41a	0.01	.653	.0001	.048
	1.2	0.37	0.44	0.41	0.41a	0.01			
	3	0.41	0.45	0.39	0.37b	0.01			
WOB	Control	0.47	0.53	0.53	0.54	0.01	.861	<.0001	.168
	1.2	0.48	0.54	0.53	0.52	0.01			
	3	0.49	0.55	0.51	0.50	0.01			
ALH	Control	4.98	4.8a	4.64	3.57	0.21	.105	<.0001	.489
	1.2	4.91	4.47ab	4.58	3.85	0.17			
	3	4.45	4.24b	4.40	3.72	0.17			
BCF	Control	27.8	29.2	26.1	25.6	0.96	.767	<.0001	.469
	1.2	28.1	30.0	25.9	25.9	0.78			
	3	30.4	29.4	26.5	24.8	0.78			

Note: Different letters (a, b and c) within a column indicate significant differences between treatments ($p < .05$).

Abbreviations: % Motile, percentage of motile sperm; % Prog Mot, percentage of progressive sperm; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity; STR, straightness; VAP, velocity average path; VCL, velocity curved line; VSL, velocity straight line; WOB, wobble.

[1]Standard error of the mean.

[2]Values of $p < .05$ were considered statistically significant.

[3]Group*time interaction.

impair the proliferation of bone marrow cells (Hoeben et al., 1999), the in vitro chemotactic differentials of leukocytes (Suriyasathaporn et al., 1999), and the proliferation of lymphocytes (Franklin et al., 1991) in cattle.

In experiment 2, when sperm were incubated with BHB, neither total or progressive motility, nor any kinematic parameters were affected by either 1.2 mM or 3 mM BHB. This result is not consistent with evidence that sperm may be affected by the plasma of hyperketonemic cows. These findings are consistent with those of Leroy et al. (2006), who reported that the negative effect of high BHB concentrations on oocyte quality was due to other concomitant conditions in dairy cows (hypoglycaemia). In line with this observation, it was demonstrated in previous studies that hyperketonaemia and NEB are associated with high levels of oxidative stress markers such as H_2O_2, low levels of enzymatic antioxidants such as superoxide dismutase (SOD) and antioxidants such as vitamin C, E and uric acid in dairy cows (Li et al., 2016). In a series of studies, it was reported that thawed sperm are highly sensitive to oxidative stress, which occurs as a result of the cryopreservation process (Bucak et al., 2010; Gürler et al., 2016) and may affect their motility in an inadequate condition such as hyperketonaemia.

In addition, the possibility that there is a localized uterine influx of overall reactive oxygen species (ROS) was established (Sordillo et al., 2009). These ROS, in turn, could contribute to the reduction in sperm motility (Tuncer et al., 2010), damage of cellular membranes (Bucak et al., 2010) and DNA in thawed bull sperm (Bucak et al., 2010; Gürler et al., 2016), thus decreasing their fertilizing capacity (Tuncer et al., 2010) and embryo viability (Rienzi et al., 2019). The results of experiment 2 show that during a 3-h incubation, there was no detectable effect of treatment on all categories of ROS except for the live superoxide-positive population at 30 min. This was higher in BHB-supplemented samples (1.2 and 3 mM) compared with control, indicating a high mitochondrial activity in BHB groups (Figure 1e) that could be the source of ROS such as superoxide anion (Table 3).

In contrast to total sperm motility, sperm showed higher values for progressive motility and straightness in plasma containing high BHB compared to control. Because of this difference, we hypothesized that, overall, sperm are sensitive to conditions resulting from

TABLE 3 Reactive oxygen species-production (LSM ± SEM) at 30, 90 and 180 min in dairy bull semen incubated in 0 (control), 1.2 and 3 mM of BHB ($N = 9$).

		Time				*p*-Value[2]		
ROS category	Concentration of BHB (mM)	30 min	90 min	180 min	SEM[1]	Group	Time	G*T[3]
Dead hydrogen peroxide negative (%)	Control	42.1	46.6	52.2	6.23	.894	.030	.952
	1.2	41.9	42.1	51.6	5.02			
	3	38.0	44.8	53.4	5.02			
Dead hydrogen peroxide positive (%)	Control	0.32	0.35	0.38	0.71	.620	.980	.743
	1.2	0.84	0.13	0.17	0.57			
	3	0.22	1.14	0.80	0.57			
Live hydrogen peroxide negative (%)	Control	56.4	52.5	47.0	6.89	.822	.263	.996
	1.2	55.8	57.2	48.1	6.58			
	3	52.9	53.0	45.2	6.58			
Live hydrogen peroxide positive (%)	Control	0.77	0.56	0.36	0.53	.685	.501	.907
	1.2	0.90	0.54	0.11	0.55			
	3	0.62	1.12	0.59	0.55			
Dead superoxide positive (%)	Control	42.5	47.1	52.1	4.23	.912	.085	.980
	1.2	42.3	42.4	51.1	5.66			
	3	39.8	45.3	53.6	5.66			
Live superoxide positive (%)	Control	15.9a	19.7	20.7	1.39	.151	.080	.212
	1.2	18.9b	20.7	21.4	1.38			
	3	21.6b	19.2	21.9	1.38			
Live superoxide negative (%)	Control	31.3	31.4	25.5	4.86	.799	.189	.993
	1.2	36.6	35.1	25.3	6.61			
	3	32.3	33.1	22.1	6.61			

Note: Different letters (a, b and c) within a column indicate significant differences between treatments ($p < .05$).

[1]Standard error of the mean.

[2]Values of $p < .05$ were considered statistically significant.

[3]Group*time interaction.

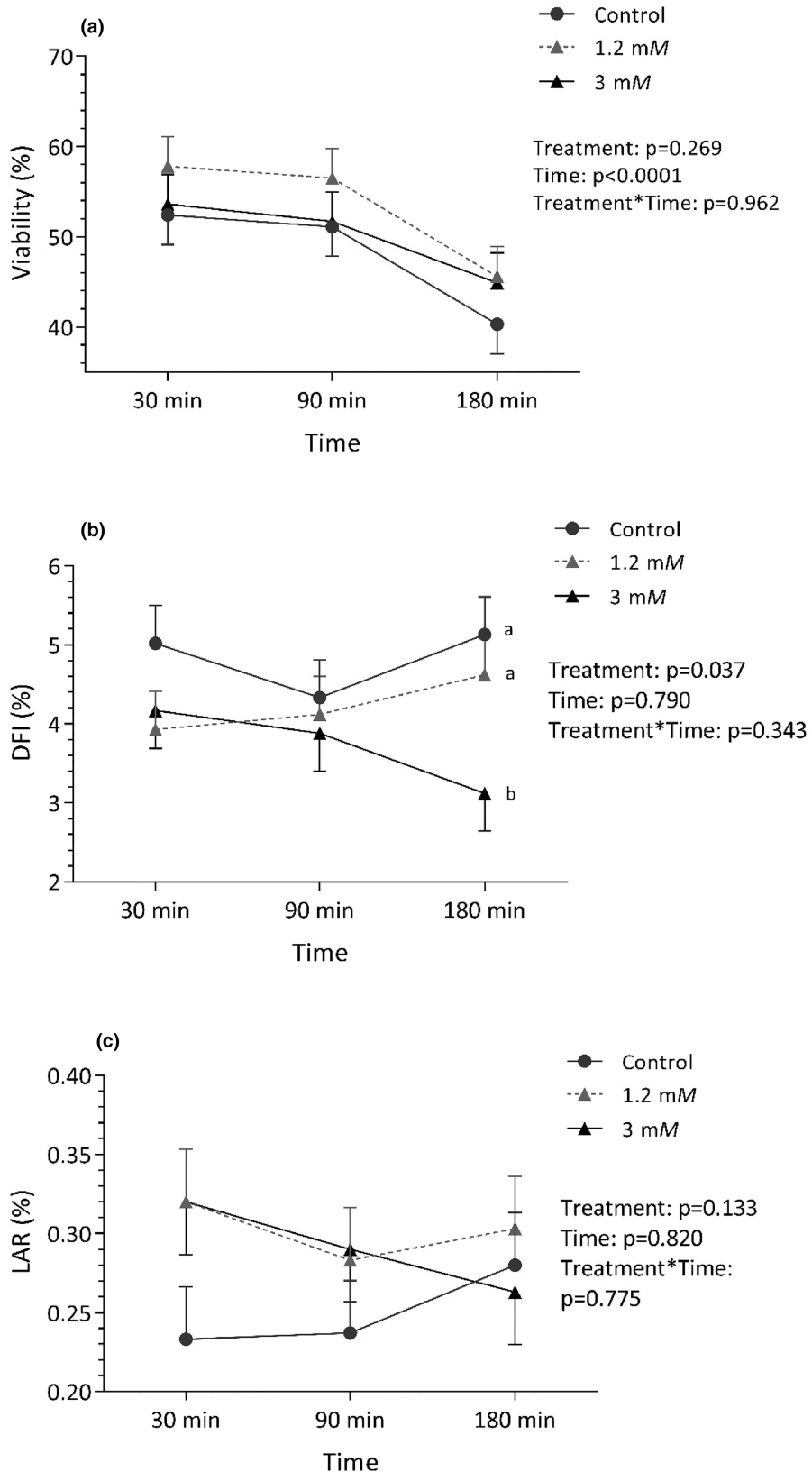

FIGURE 1 Proportion of sperm cell characteristics using flow cytometry. (a) Viability; (b) DNA fragmentation index (DFI); (c) live acrosome reacted (LAR); (d) live acrosome intact (LAI) and (e) high mitochondrial membrane potential (MMP) of spermatozoa incubated in 0 (control), 1.2 and 3 mM of BHB at 30, 90 and 180 min. Significant difference between groups ($p < .05$). Values are presented as LSMEANS ± SE.

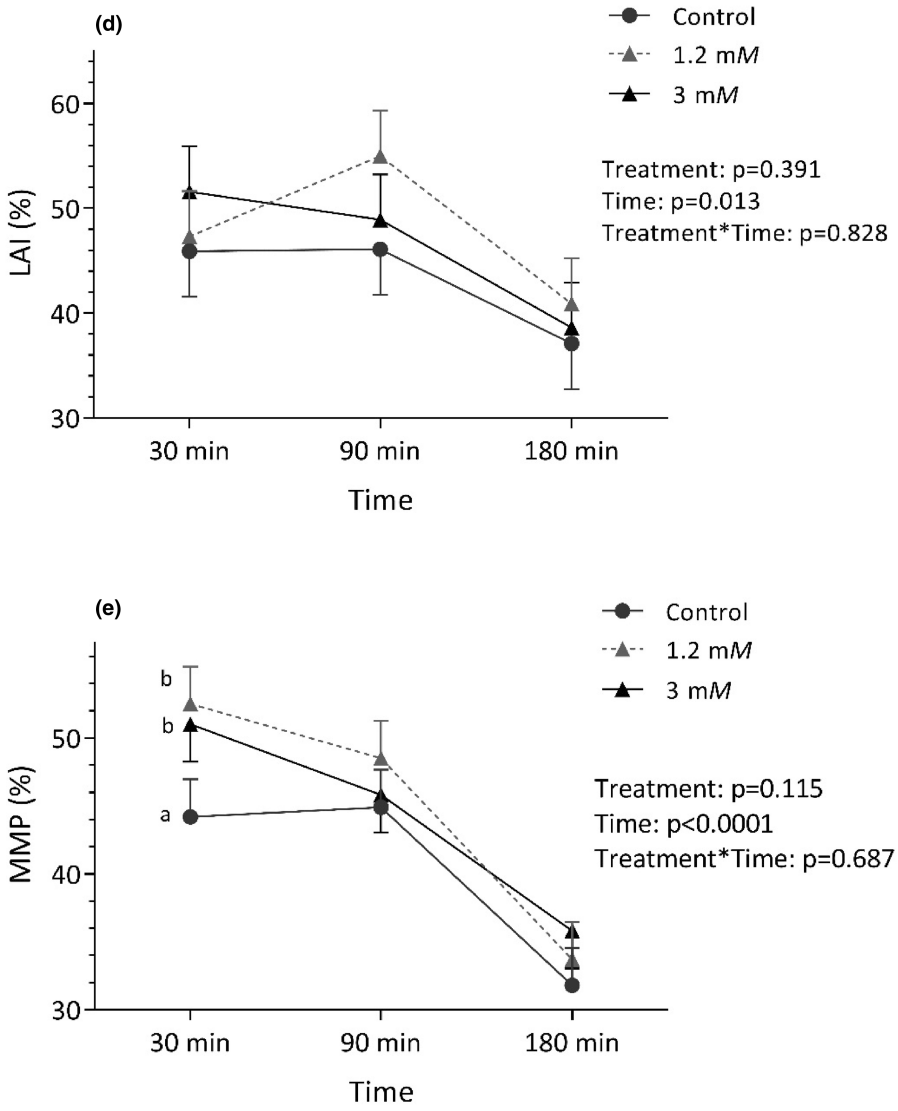

FIGURE 1 (Continued)

hyperketonaemia and the response of individual sperm to plasma containing high BHB differed. The fact that sperm were more vigorous with high concentrations of BHB could be the result of an effective use of this substrate; in contrast, bovine epididymal sperm decreased their oxygen consumption while increasing their motility in BHB-supplemented samples when compared with other metabolic substrates (Lardy & Phillips, 1945).

Mouse sperm maintained vigorous motility for more than 60 min in the presence of BHB (Takei et al., 2014). In addition, the sperm penetration rate in media containing high BHB concentrations was significantly higher than that in media containing low or no BHB (Endo et al., 2010). This could be explained by ATP production being increased in the presence of BHB (experiment 2, Figure 1e), resulting in enhanced vigorous motility. Earlier studies found that BHB was able to sustain high ATP levels as a non-glycolysable substrate via secondary metabolic pathways in sperm (Visconti, 2012); hyperketonaemia conditions are accompanied by hypoglycaemia in cows (Kronfeld, 1971). Similarly, ketone bodies were reported to improve metabolic efficiency by increasing ATP synthesis in humans (Veech, 2004). This increase in metabolic efficiency was extensively studied in ram sperm by O'Shea and Voglmayr (1970). Mammalian sperm must metabolize extracellular energy substrates to produce ATP for a long period to accomplish fertilization (Murphy et al., 1986).

Surprisingly, in the current study, the DNA fragmentation in BHB (3 mM)-supplemented samples was lower than that of the control samples (Figure 1b). It is, however, noteworthy that the % DFI shown for the control, 1.2 and 3 mM-supplemented samples all have low levels of abnormalities (Figure 1b) and are below the threshold of concern for fertility (Evenson et al., 2002). Furthermore, H_2O_2 is described as the primary source of DNA damage in cryopreserved bull sperm (Gürler et al., 2016). In the present study, H_2O_2 was similar ($p > .5$) between the BHB-supplemented groups and the control group (Table 3). In contrast to experiment 1, sperm motility, progressivity and kinematic parameters changed over time in experiment 2. This could be explained by the time of incubation applied in

experiment 2 (up to 180 min in experiment 2 compared to 90 min in experiment 1). Previous studies showed that post-thaw bull semen motility as well as kinematic parameters were maintained up to 2 h of incubation (Muiño et al., 2009); they were, however, decreased after 2 h post-thaw (Muiño et al., 2008). This could be due to the energy availability in sperm which reflects on sperm motility (Botta et al., 2019).

5 | CONCLUSION

Based on the current results, the total motility of thawed bull sperm can be impaired in media containing plasma from hyperketonemic cows. Clearly, other factors than BHB in cow plasma can cause this impairment (e.g., oxidative stress). Results from the present study also indicate that the supplementation of 3 mM BHB increased the proportion of live superoxide-positive sperm and sperm with high mitochondrial potential, while the DNA fragmentation index decreased. These findings support the hypothesis that some of the low fertility of postpartum dairy cows with hyperketonaemia may be due to the negative effect of metabolic disorders, among others, on sperm motility. Further investigation of the interactions between hyperketonemic environment and sperm could enhance our understanding of the molecular mechanisms that regulate sperm metabolism and might uncover new targets for modulating sperm function.

AUTHOR CONTRIBUTIONS

AA, MI, JM, RK and DR were involved in the planning of the study. AA and IB were responsible for the blood collection. AA, IB, AJ, AK, MB and JM were responsible for performing the laboratory work. AA, MB and DR performed the data analysis. AA wrote the manuscript drafts of the study, all authors have contributed to the writing process. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

None of the authors have any conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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