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Full Length Research Paper

Evaluation of Myo-inositol Effects upon Frozen-Thawed Bovine Sperm Quality

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Abstract

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Despite great advances in standardization of bovine sperm cryopreservation, frozen-thawed sperm quality is commonly suboptimal. Semen supplementation with various compounds has been addressed to overcome the negative impact of cryopreservation upon sperm quality. While a mild enhancement in human semen parameters was reported after Myo-inositol (MI) supplementation, information is still scarce. This work aimed to evaluate the impact of MI supplementation (2mg/mL) upon sperm quality in frozen-thawed bovine sperm from 9 bulls (breeds: 5 dairy, 4 beef). Evaluations included assessment of total/progressively motile sperm and kinematic parameters (Computer-Assisted Sperm Analysis), sperm vitality, membrane osmotic competence, acrosomal status, chromatin compaction and DNA fragmentation. As a result, an increase ($P<0.05$) in sperm kinematic parameters (VSL, VCL, VAP, LIN, STR, ALH, BCF, WOB and MAD) was found in sperm suspensions after MI supplementation, although total or progressive motility did not significantly increase. Based on the MI Response Index, 3/9 bulls showed >60% improvement in progressive motility, and 6/9>30% improvement. MI supplementation did not alter sperm vitality, membrane osmotic competence, acrosome status, chromatin compaction and DNA fragmentation. In summary, MI supplementation of frozen-thawed bovine spermatozoa led to a significant improvement in flagellum movement, as evidenced by the increase in sperm kinematics values.

Key words: Bovine, cryopreservation, frozen-thawed, sperm, CASA, Myo-inositol.

INTRODUCTION

Demand for animal-based foods has been increasing worldwide, partly due to technological innovations and structural changes in livestock management. The outlook for the international dairy and beef market shows substantial growth in recent years based on statistical reports of the Food and Agriculture Organization of the United Nations (FAO, 2020). Specifically, bovine meat production has increased 1.8 million tons between 2016 and 2018 (69.7 to 71.5 million tons), whereas bovine milk has increased 49.9 million tons in the same period (FAO, 2018). Therefore, the production system for bovine cattle aims to produce at least one calf per cow per year using reproductive biotechnologies, such as artificial insemination and semen cryopreservation. This set of techniques allows increasing reproductive efficiency, reflected as a greater livestock production (Palma et al., 2007).

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Semen cryopreservation allows storage, transport (Grötter et al., 2019), time optimization (Pini et al., 2018), the constant use of breeders with favorable genetic characteristics, and a lower probability of introducing diseases during reproductive management (Bailey et al., 2000). Despite formulations and methodologies standardization for bovine semen cryopreservation, around 40-50% of the sperm population does not survive the process (Curry, 2000; Watson 2000). Cell death is, at least in part, due to changes caused by low temperatures, osmotic imbalances (Sieme et al., 2015), ice crystals formation, oxidative stress, and lipid-protein reorganizations in cellular membranes (Watson 1995; Bailey et al., 2000). In addition, those spermatozoa that remain alive after the freezing-thawing process undergo biochemical and molecular alterations (Pini et al., 2018), mainly reflected by a motility decrease, changes in the plasma membrane, acrosome deterioration and genetic damage (Watson, 2000; Bucak et al., 2010; Ortega Ferrusola et al., 2010), which negatively affect the sperm fertilizing potential. Several attempts have been made to reduce the damage after sperm cryopreservation by modifying the components of the cryopreservation medium. The addition of egg yolk, milk, glycerol, bovine serum albumin, polyvinyl alcohol and liposomes has been reported to mitigate the negative effects generated during sperm freezing (Layek et al., 2016). Some studies reported discrete and heterogeneous results in sperm quality by supplementing semen cryopreservation diluents with lycopene and resveratrol (Bucak et al, 2015) α-linoleic acid (Kaka et al, 2014), arginine and trehalose (Öztürl et al., 2017), cysteine and glutathione (Tuncer et al., 2010), ascorbic acid (Mirzoyan et al., 2006), vitamin E analogs (Peña et al., 2003), ethanol (Dodaran et al., 2015), and natural herbs (Zhao et al., 2009; Daghigh-Kia et al., 2014). On the other hand, semen supplementation after thawing also depicted similar results (i.e. crocin (Sapanidou et al., 2015); melatonin (Pang et al., 2016); MI, caffeine, and pentoxifylline, penicillamine + hypotaurine + epinephrine mixture (Boni et al., 2016); coenzyme Q10+ zinc + D-aspartate mixture (Boni et al., 2016; Barbato et al., 2017); cyclodextrins (Ferré et al., 2018).

Myo-inositol (MI) is a polyalcohol present in the male reproductive tract fluids of humans and other animal species, with levels up to 100 times higher in human seminal plasma than in blood. In recent years, the impact of MI supplementation *in vivo* and *in vitro* upon male infertility treatment has been investigated (Vazquez-Levin and Verón, 2020). In particular, some studies have evaluated the effect of MI supplementation before (Paternostro et al., 2015; Saleh et al., 2017) and after (Palmieri et al., 2016) sperm cryopreservation, showing an increase in sperm motility in MI-treated samples. Nevertheless, information on MI effect on bovine semen sperm quality is still scarce. Two previous studies reported MI supplementation before (Bucak et al., 2010) and after (Boni et al., 2016) bovine semen cryopreservation. In the first case, Bucak and collaborators reported an increase in sperm motility after supplementing the freezing medium with 7.5mM MI (Bucak et al., 2010). On the other hand, Boni and collaborators assessed the impact of 1.8 mg/mLMI supplementation upon sperm quality using frozen-thawed bovine semen; samples were devoid of the cryoprotectant, pooled, and supplemented with MI, resulting in a higher sperm motility and kinematics after 4 hours.

Based on these findings, the present study aimed to evaluate the effect of MI supplementation (2mg/mL) upon in frozen-thawed bovine sperm quality by assessing sperm motility, vigor, and a set of kinematic parameters, as well as sperm vitality, membrane osmotic competence, acrosomal and nuclear status.

MATERIALS AND METHODS

Reagents

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise noted. All procedures were performed following international biosafety standards for the operators and the environment.

Biological Samples

Semen samples from 9 breeding bulls, 5 of the Holstein dairy breed (bulls 1, 3, 4, 7, 8), and 4 of the Brangus and Braford beef breeds (Brangus: bulls 2, 5, 6; Braford: bull 9) were kindly provided by the Venado Tuerto Artificial Insemination Center (CIAVT; Santa Fe, Argentina). Semen samples were processed using the commercial reagent AndroMed® (containing phospholipids, citric acid, sugars, antioxidants, glycerol, and antibiotics in TRIS buffer; Minitube; Tiefenbach, Germany) following the protocol suggested by the manufacturer, loaded in 0.5 mL straws and subjected to cryopreservation using a standard procedure. In all cases, samples were transferred to the IBYME laboratory in a liquid nitrogen tank until analysis.

MI Supplementation

Frozen semen samples were thawed at 37°C for 30 sec in water baths. Each straw was divided in 3 aliquots (Thawing Control, MI treatment, and Placebo Treatment). The Thawing Control aliquot was used to assess sperm concentration, vitality, and morphology, and initial sperm motility and kinematics. MI and Placebo Treatment (Control) aliquots were incubated for 1 h at room temperature with 2 mg/mL of MI or physiological solution, respectively. At the end of the incubation, sperm motility, kinematics, vitality, plasma membrane osmotic competence, acrosome (intact or reacted) and nucleus (chromatin

condensation and DNA fragmentation) status were assessed. All evaluations were done in triplicates (except for motility and kinematics assessment, which were performed in 5 replicates) and by two operators to increase quality and reliability.

Routine and Functional Sperm Parameters Assessment

Sperm Motility and Kinematics

One second videos were obtained from at least 5 fields per sample at a rate of 30 frames per second (FPS) using the Proiser ISAS v1. (BIO-OPTIC, S.R.L.) CASA system containing a heated stage set at 37°C. The system provides objective sperm motility parameters: straight-line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN), straightness (STR), beat cross frequency (BCF), wobble (WOB), amplitude of lateral head displacement (ALH), and mean angular displacement (MAD). Total and progressive sperm motility, as well as vigor was subjectively evaluated using videos registered with a CASA system. Vigor, a measure of the rectilinear progressive movement intensity, was rated following the Salisbury criteria (Salisbury and Van Demark, 1961) on a scale of 1 to 5, 5 being the most vigorous. At least 200 spermatozoa were recorded in each condition. Frozen-thawed sperm suspensions were considered normal for motility and vigor with a ≥30% progressive motility and ≥3 vigor. Considering the heterogeneity in motility values among samples from the same bull, the rate of change (ΔPM) was estimated. This parameter was defined as the change in progressive motility between control and treated samples ($\Delta PM = PM_{MI} - PM_C$). Next, a response index (RI) was estimated as the ratio between ΔPM and control progressive motility ($RI = \Delta PM/PM_C$). Therefore, samples were classified according to no ($RI \leq 0$), low ($0 < RI \leq 0.3$), medium ($0.3 < RI \leq 0.6$), or high ($RI \geq 0.6$) responses.

Sperm Morphology

Sperm suspensions were fixed using 4% paraformaldehyde (PFA). Fixed sperm were incubated with0.6% Giemsa solution in methanol (Biopack, Buenos Aires-Argentina) for 1 h. Normal sperm morphology was assessed considering head, acrosome, midpiece and flagellum abnormalities. At least 200 sperm were evaluated per sample, and samples were considered morphologically normal with≥70%spermatozoa with normal morphology (Rutter and Russo, 2006).

Sperm Vitality and Osmotic Competence of the Plasma Membrane

Sperm vitality was assessed by means of 0.5% Eosin Y staining and considered normal with≥50% live spermatozoa (Watson, 2000). Osmotic competence was assessed by means of the hypo-osmotic swelling (HOS) test. Briefly, sperm suspensions were incubated with 150 mOsm/mL hypoosmotic solution (28.5 mM sodium citrate, 75 mM fructose in distilled water) in a 1:10 ratio (semen: hypoosmotic solution) for 30 min at 37°C. At least 200 spermatozoa were counted per sample. Samples were considered osmotically competent with≥35% HOS test scores (Padrik et al., 2012).

Sperm Acrosomal Status

PFA-fixed sperm were stained using 0.22% Coomassie Brilliant Blue-G in 50% methanol and 10% acetic acid for 2 min. At least 200 spermatozoa were evaluated per sample. Samples were considered normal for acrosomal status with ≥50% acrosome intact spermatozoa (Bernardi et al., 2011).

Sperm Chromatin Condensation

Spermatozoa were fixed and permeabilized with methanol (J.T. Baker, Xalostoc, México) at 4°C for 3 min, and stained using 5% aniline blue (Biopack, Buenos Aires, Argentina) in 10% glacial acetic acid for 7 min. Sperm chromatin condensation was assessed under light microscopy at 1000X magnification, and at least 200 spermatozoa were evaluated per condition. Samples were considered normal for chromatin condensation with ≥90% spermatozoa with condensed chromatin (Khalifa et al., 2008).

Sperm DNA Fragmentation

Sperm DNA fragmentation was performed by means of the commercial In Situ Cell Death Detection Kit (Roche; Basel, Switzerland) using samples from high performance and subfertile bulls (bulls 2 and 4, respectively). Briefly, sperm suspensions were fixed using 2% formaldehyde for 4 min at room temperature. Fixed samples were loaded onto glass slides and permeabilized using 0.1% Triton X-100 in 0.1% sodium citrate) for 2 min at 4°C. Next, permeabilized sperm were stained using TUNEL (Terminal deoxynucleotidyl Transferased UTP Nick End Labeling) solution and counterstained with propidium iodide. Negative (no TUNEL solution), and positive (DNase I-treated samples) controls were added for each condition. DNA fragmentation was determined in at least 300 spermatozoa. Samples were considered normal for DNA fragmentation with≤8% spermatozoa with fragmented DNA (Takeda et al., 2015).

Statistical Analysis

Results were expressed as Mean ± Standard Error of the Mean (SEM), unless otherwise stated. Mann-Whitney or Wilcoxon tests were used to compare results between groups for paired (control versus MI-treated samples) or unpaired samples (beef versus dairy breed samples). Chi-squared test was used to compare results between groups when considering rates. All evaluations were performed using GraphPad InStat (GraphPad Software; California, United States). Differences between groups were considered statistically significant at $P<0.05$.

Table 1. Semen characteristics in frozen-thawed spermatozoa. Sperm concentration, total and progressive motility, vigor, vitality, osmotic competence, normal morphology, acrosome status and chromatin compaction for dairy and beef bull semen samples. Results are expressed as mean ± standard error of the mean.

Bull	Breed	Concentration (million/mL)	Total Motility (%)	Progressive Motility (%)	Vigor
1	Dairy	68.8 ± 3.6	59.7 ± 6.1	59.3 ± 6.1	3.8 ± 0.2
2	Beef	47.0 ± 3.7	61.4 ± 4.3	60.3 ± 4.7	3.8 ± 0.2
3	Dairy	48.2 ± 7.1	26.5 ± 4.0	22.6 ± 3.9	2.6 ± 0.2
4	Dairy	38.6 ± 1.4	62. 5 ± 3.0	58.6 ± 3.2	3.4 ± 0.2
5	Beef	41.2 ± 8.6	50.5 ± 2.8	47.5 ± 3.4	3.4 ± 0.2
6	Beef	73.4 ± 3.7	34.5 ± 3.4	24.2 ± 5.5	3.2 ± 0.4
7	Dairy	49.4 ± 4.0	50.1 ± 6.7	47. 6 ± 7.0	3.2 ± 0.4
8	Dairy	31.2 ± 2.7	47.7 ± 4.2	46.6 ± 4.1	3.8 ± 0.2
9	Beef	48.2 ± 7.1	40.9 ± 3.2	36.2 ± 4.1	3.0 ± 0.4

Table 1 (cont).

Bull	Vitality (%)	Osmotic Competence (%)	Normal Morphology (%)	Acrosome Status (%)	Chromatin Compaction (%)
1	55.5 ± 4.1	52.9 ± 7.3	88.2	95.8 ± 1.5	97.5 ± 0.3
2	61.2 ± 2.6	54.9 ± 13.1	33.5	85.4 ± 3.9	93.5 ± 0.9
3	69.3 ± 14.4	17.5 ± 3.6	66.1	93.5 ± 0.7	97.8 ± 0.6
4	76.1 ± 1.1	48.7 ± 2.8	94.8	88.8 ± 10.2	98.2 ± 0.4
5	48.0 ± 1.8	38.1 ± 3.2	91.6	88.0 ± 4.7	98.5 ± 0.5
6	37.2 ± 2.0	16.1 ± 1.6	76.0	94.1 ± 0.4	97.7 ± 0.2
7	64.1 ± 7.7	37.0 ± 7.4	79.4	84.6 ± 3.7	98.4 ± 0.4
8	40.9 ± 5.1	42.4 ± 7.6	85.8	90.4 ± 2.9	97.7 ± 0.1
9	75.9 ± 1.6	48.1 ± 3.6	84.2	86.7 ± 3.9	97.9 ± 1.1

RESULTS

Comparison between Dairy and Beef Breeds Sperm Quality

Frozen-thawed semen samples from 9 bulls were analyzed for sperm concentration, motility, vigor, morphology, vitality, osmotic competence, acrosomal status and chromatin compaction (Table 1). Sperm concentration was similar for dairy (47.24 ± 14.16 million/mL; range: 31.20-68.8) and beef (52.45 ± 14.30 million/mL; range: 41.20-73.40) bull semen samples (P=0.81; Mann-Whitney test). Sperm motility, vigor, morphology, vitality, osmotic competence, acrosomal status and chromatin compaction were found within normal values in most cases, and no differences were found between dairy and beef bull samples (Table 1;P>0.05; Mann-Whitney test).

Effect of MI Supplementation Upon Sperm Quality

Sperm Motility and Vigor

A variable response among bulls was recorded after MI supplementation (Figures 1-2). When considering the breed, at least 40% of the dairy breed bull samples supplemented with MI showed a trend toward higher total and progressive sperm motility, whereas the beef breeds bull samples depicted a trend towards a motility improvement in at least 20% of the cases (Table 2). Similarly, sperm vigor depicted similar values between control an MI-treated samples (Wilcoxon test; Table 3). Next, the proportion of total and progressively motile spermatozoa was estimated for each bullby calculating the ratio between the number of motile sperm (total or progressive) and the total number of sperm evaluated

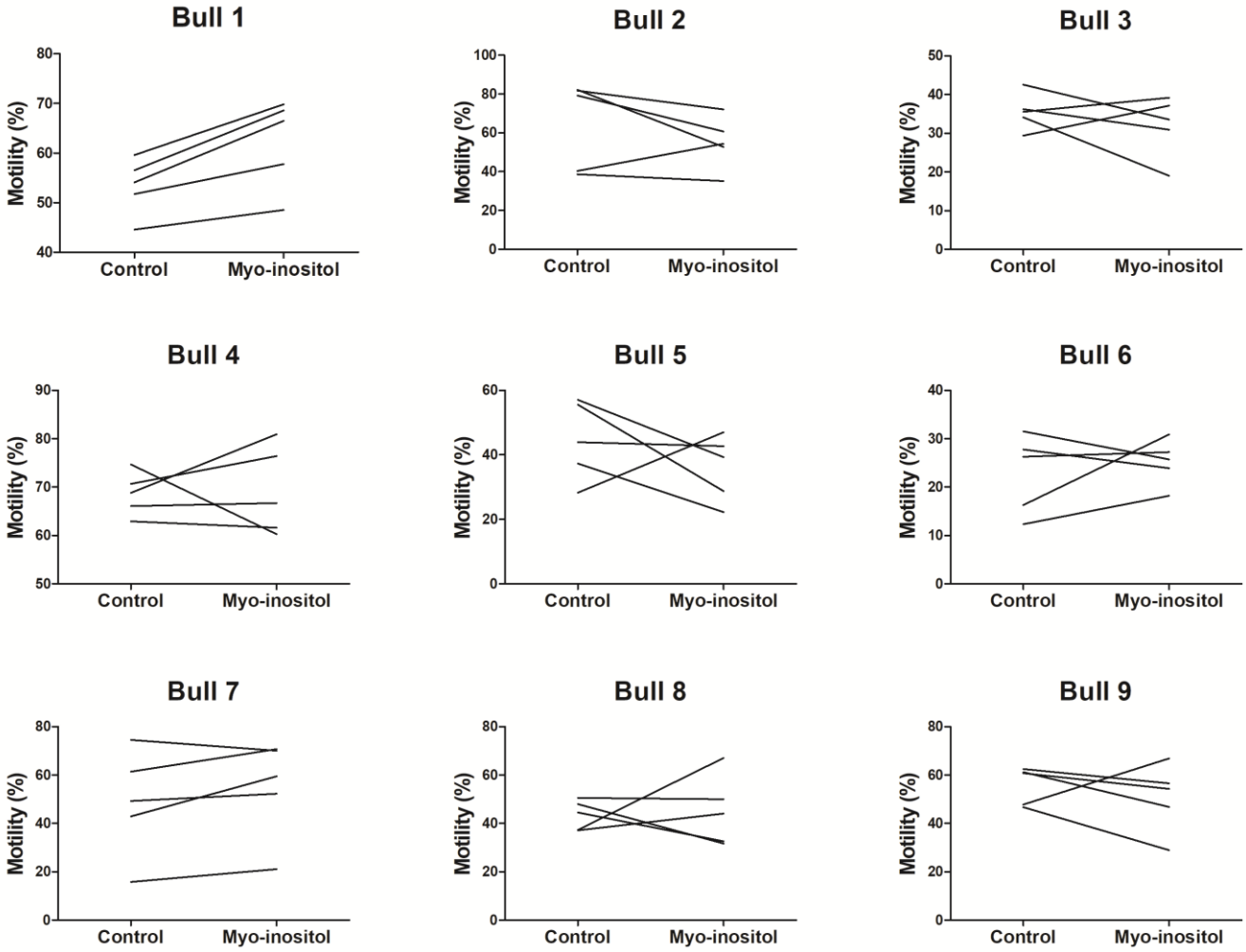

Figure 1. Effect of Myo-inositol supplementation upon total sperm motility in frozen-thawed bovine semen samples. Total sperm motility depicted a mixed response to Myo-inositol supplementation among different bull samples and among the same bull straws. Results are expressed as total motility (%), and each line corresponds to the same straw subjected to control or Myo-inositol supplementation.

for all straws. This analysis revealed a significant increase in total motility in 4 bulls (bulls 1, 6, 7 and 8) and a decrease in 3 bulls (bulls 2, 5 and 9) (Chi-squared test; Table 4).

Considering the response index (RI), samples were classified according to no ($RI \leq 0$), low ($0 < RI \leq 0.3$), medium ($0.3 < RI \leq 0.6$), or high ($RI \geq 0.6$) responses. As a result, a MI response was determined in at least one sample per animal. Moreover, samples from one third of the bulls depicted a high response; samples from other third up to a medium response; and samples from the last third up to a low response (Figure 3).

Sperm Kinematics

Sperm kinematics was found significantly improved in MI-treated samples when compared to controls (Tables 5-6). Specifically, a significant increase in VCL, and VAP was found in 100% (9/9) of bull samples. Moreover, VSL and LIN were significantly higher in 89% (8/9) of bull samples. Finally, STR, BCF and WOB values were significantly higher in 78% (7/9) of the bull semen samples. Results are presented according to the increase/decrease (Table 5) or mean values (Table 6). The increase in sperm kinematics was significant in samples from both breeds.

Sperm Vitality, Osmotic Competence, Acrosomal Status and Chromatin Condensation

MI supplementation did not exert a significant effect on sperm vitality, osmotic competence of the plasma membrane (Table 7), or acrosomal status when compared to controls (Table 8). Likewise, similar values were

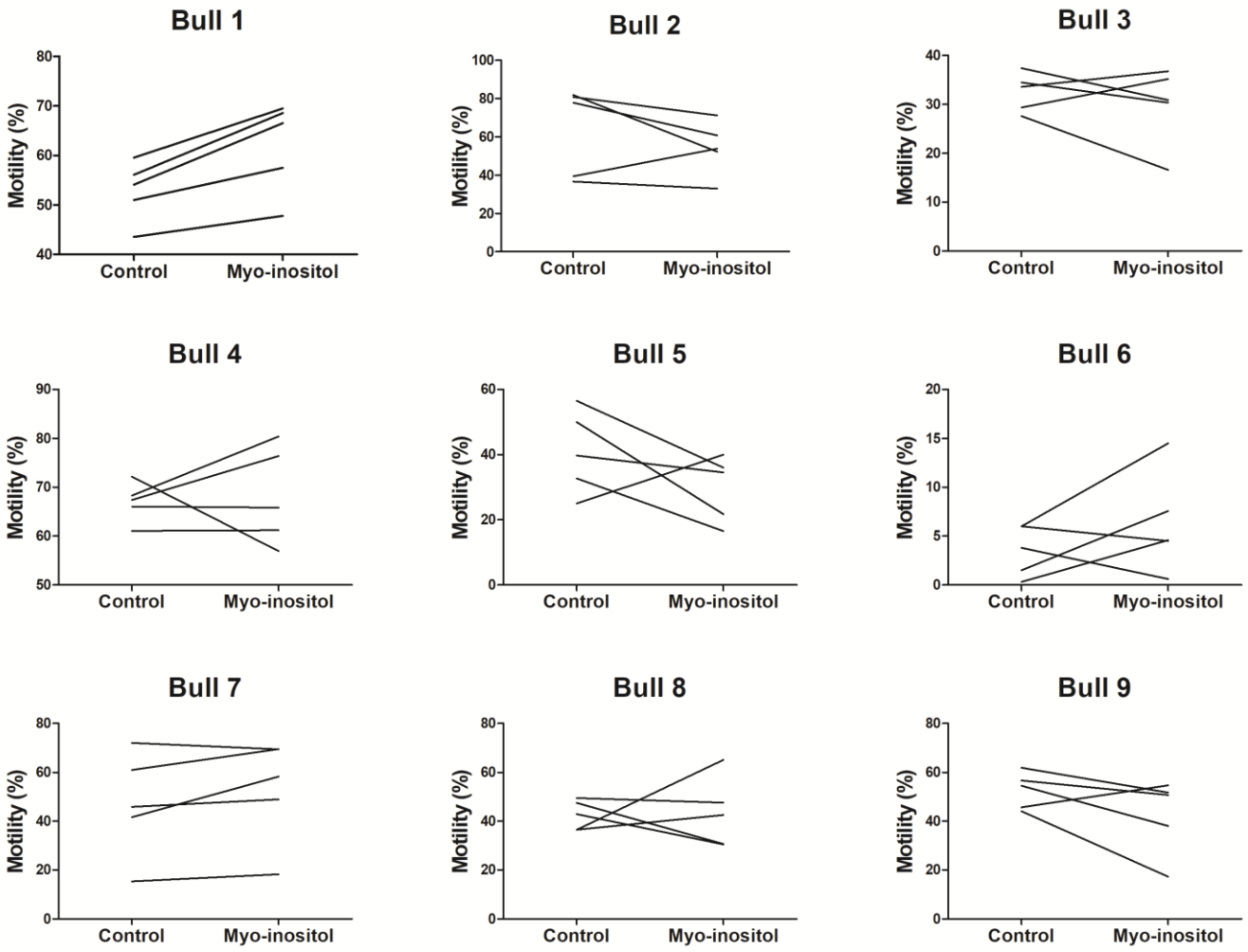

Figure 2. Effect of Myo-inositol supplementation upon progressive sperm motility in frozen-thawed bovine semen samples. Progressive sperm motility depicted a mixed response to Myo-inositol supplementation among different bull samples and among the same bull straws. Results are expressed as progressive motility (%), and each line corresponds to the same straw subjected to control or Myo-inositol supplementation.

observed for chromatin condensation between control and MI-treated samples (Table 9).

DNA Fragmentation

DNA fragmentation was determined by means of the TUNEL assay using samples from one high performance and one subfertile bull (bulls 2 and 4, respectively). As a result, a significant difference in DNA fragmentation was determined between bulls 2 and 4 (22.2 ± 2.4% vs. 1.8 ± 0.6%; $P<0.0001$; Mann-Whitney test). MI supplementation did not exert a significant effect upon DNA fragmentation, as depicted by similar DNA fragmentation between control and MI-treated samples for both bulls (Wilcoxon test; Figure 4).

DISCUSSION

The present study aimed to characterize the impact of MI supplementation on frozen-thawed bull sperm quality. MI was selected based on the results of studies using human semen (Condorelli et al., 2011, 2012; Calogero et al., 2015; Capece et al., 2016; Gulino et al., 2016; Montanino et al., 2016; Palmieri et al., 2016; Park et al., 2016; Scarselli et al., 2016; Artini et al., 2017; Dinkova et al., 2017; Canepa et al., 2018), as well as some evidence obtained on its use in bovine semen (Bucak et al., 2010; Boni et al., 2016). The study population consisted of 9 bulls, 5 from the dairy breed and 4 from beef breeds. A set of parameters related to sperm functionality were evaluated, namely sperm motility, vigor, vitality, osmotic competence of the plasma membrane, acrosomal and chromatin/DNA status. Frozen-thawed semen samples from most bulls (7/9) presented normal values (CIAVT standards: at least 30% progressive motility and 70% normal morphology; 6 million progressively motile spermatozoa per dose) (Salisbury and Van Demark, 1961; Watson, 2000; Rutter and Russo; 2006; Khalifa et

Table 2. Effect of Myo-inositol supplementation on total and progressive sperm motility (percentage). Sperm total and progressive motility depicted similar results in control and Myo-inositol-treated frozen-thawed bovine semen samples. Results are expressed as mean ± standard error of the mean (*n* = 5 straws per animal).

	Total motility (%)			Progressive motility (%)		
Bull	**Control**	**Myo-inositol**	**Wilcoxon test *P*-value**	**Control**	**Myo-inositol**	**Wilcoxon test *P*-value**
1	53.3 ± 2.54	62.2 ± 4.0	0.0625	52.9 ± 2.7	62.0 ± 40.1	0.0625
2	64.4 ± 10.2	55.0 ± 6.0	0.3125	63.3 ± 10.4	54.2 ± 6.3	0.3125
3	35.7 ± 2.3	31.9 ± 3.5	0.4375	32.5 ± 1.8	30.0 ± 3.6	0.4375
4	68.6 ± 2.0	69.2 ± 4.1	1.0000	67.0 ± 1.8	68.1 ± 4.5	1.0000
5	44.4 ± 5.4	36.0 ± 4.6	0.4375	40.8 ± 5.7	29.7 ± 4.5	0.1875
6	22.8 ± 3.6	25.2 ± 2.1	0.6250	3.5 ± 1.2	6.4 ± 2.3	0.3125
7	48.8 ± 9.9	54.7 ± 9.1	0.1875	47.2 ± 9.6	52.9 ± 9.5	0.1250
8	43.5 ± 2.7	45.0 ± 6.5	1.0000	42.6 ± 2.7	43.3 ± 6.4	1.0000
9	55. 8 ± 3.5	50.7 ± 6.3	0.6250	52.6 ± 3.4	42.5 ± 6.9	0.1875

Table 3. Effect of Myo-inositol supplementation on sperm vigor. Sperm vigor depicted similar values between control and Myo-inositol-treated samples. Results are expressed as mean ± standard error of the mean (*n* = 5 straws per bull).

	Vigor		
Bull	**Control**	**Myo-inositol**	**Wilcoxon test *P*-value**
1	3.8 ± 0.2	3.8 ± 0.2	1.0000
2	3.8 ± 0.2	3.8 ± 0.2	1.0000
3	3.4 ± 0.4	3.4 ± 0.4	1.0000
4	3.6 ± 0.4	3.8 ± 0.2	0.3458
5	2.8 ± 0.5	3.2 ± 0.4	1.0000
6	1.2 ± 0.2	1.2 ± 0.2	1.0000
7	3.6 ± 0.2	3.8 ± 0.2	1.0000
8	3.8 ± 0.2	3.8 ± 0.2	1.0000
9	3.2 ± 0.2	2.8 ± 0.2	1.0000

Table 4. Effect of Myo-inositol upon total and progressive motility (rates). Sperm total and progressive motility was significantly different in Myo-inositol-treated samples when compared to the controls. Results are expressed as motility rates (*n* = 5 straws per animal).

Bull	**Total Sperm motility**			**Progressive Sperm Motility**		
	Control	**Myo-inositol**	**Chi-squared test *P*-value**	**Control**	**Myo-inositol**	**Chi-squared test *P*-value**
1	0.53	0.63	<0.0001	0.53	0.63	<0.0001
2	0.65	0.55	<0.0001	0.66	0.55	<0.0001
3	0.35	0.32	0.1929	0.33	0.30	0.2533
4	0.67	0.68	0.6435	0.67	0.68	0.6435
5	0.44	0.38	0.0032	0.41	0.31	<0.0001
6	0.22	0.25	0.0496	0.03	0.07	0.0003
7	0.47	0.53	0.0068	0.46	0.52	0.0122
8	0.44	0.49	0.0182	0.41	0.44	0.1100
9	0.55	0.49	0.0104	0.51	0.40	<0.0001

al., 2008; Bernardi et al., 2011; Padrik et al., 2012; Takeda et al., 2015; Gliozzi et al., 2017). Noteworthy, total and progressive sperm motility ranged between 27-63% and 23-60%, respectively; as opposed to previous reports on MI supplementation using a cohort of frozen-thawed semen samples with high total and progressive sperm motility (>70% and >60%, respectively; Boni et al., 2016). In addition, while in the present study the cryopreser

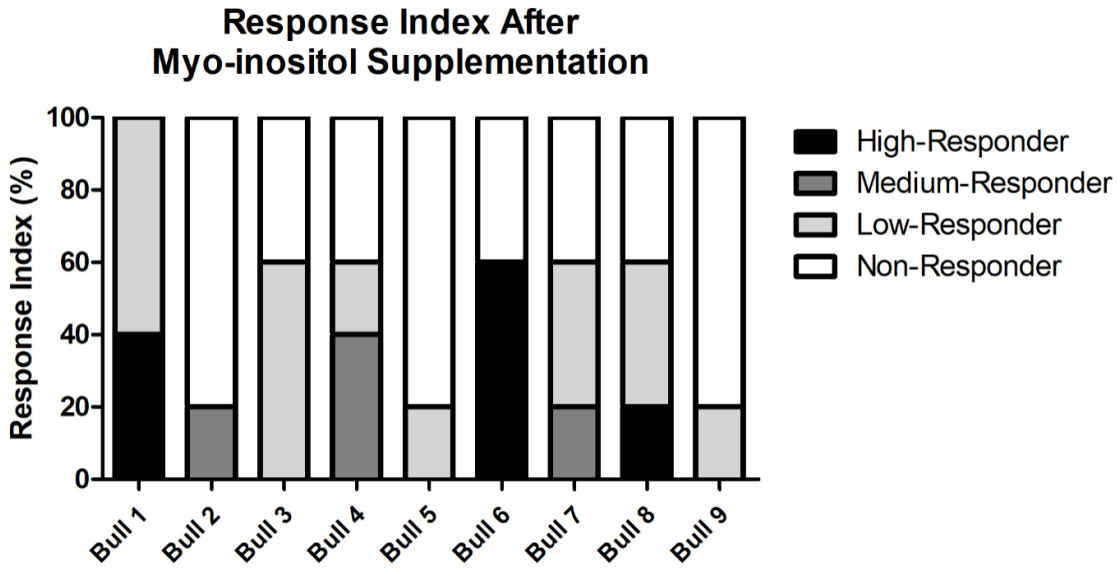

Figure 3. Sperm Response Index to Myo-inositol supplementation. Samples were classified according to no (Response Index; RI ≤ 0), low (0 <RI ≤ 0.3), medium (0.3 <RI ≤ 0.6), or high (RI ≥ 0.6) responses. A Myo-inositol response was determined for at least one sample per animal.

Table 5. Effect of MI supplementation on sperm kinematic parameters. Sperm kinematics were found significantly altered between control and Myo-inositol-treated samples. ↑: Increase; ↓: Decrease (P<0.005; Mann-Whitney test); ns: not significant.

Bull	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	ALH (µm)	BCF (Hz)	WOB (%)	MAD (Degrees)
1	↑	ns	↑	↓	↓	↑	ns	↓	↑
2	↑	↑	↑	↑	↑	↑	↑	↑	ns
3	↑	↑	↑	↑	↑	↑	↑	↑	ns
4	↑	↑	↑	↑	↑	↑	↑	↑	ns
5	↑	↑	↑	↑	↑	↑	↑	↑	ns
6	↑	↑	↑	↑	↑	↑	↑	ns	ns
7	↑	↑	↑	↑	ns	ns	ns	↑	ns
8	↑	↑	↑	↑	↑	↑	↑	↑	ns
9	↑	↑	↑	↑	↑	ns	↑	↑	↓
↑	**9/9**	**8/9**	**9/9**	**8/9**	**7/9**	**7/9**	**7/9**	**7/9**	**1/9**

vation agent was left during the 1h-incubation with MI, it was removed prior to MI supplementation in the other study.

MI supplementation of frozen-thawed bovine semen samples using 2 mg/mL of MI resulted in a 10% increase in sperm motility in semen samples of 4/9 bulls studied, and a 10% decrease in samples of 3/9 bulls, accompanied by similar results for progressive motility, as reported in studies using fresh human semen samples (Condorelli et al., 2011; Artini et al., 2017). On the other hand, studies using frozen-thawed human semen (*n* = 25; 15 min incubation with 3.2 mg/mL MI) reported a 5% increase in progressive motility (Palmieri et al., 2016). In contrast, no differences were found by Boni et al. (2016) in total sperm motility after 1 h of MI incubation in bulls, accompanied by a significant decrease in progressive motility. In this sense, evaluating individual samples instead of a pool revealed differences along individual initial motility, which may affect the outcome, as reported for human samples (Artini et al., 2017).

The use of a cohort of 9 bulls of beef and dairy breeds and 5 semen replicates of each bull led to the identification of a variable behavior among bull semen samples as well as within replicates of the same bull, when comparing total and progressive sperm motility before and after 1h incubation with MI. Part of these differences maybe associated to several biophysical and biochemical factors that are known to impact in sperm performance after freezing/thawing procedures, among them reorganization and/or loss of sperm membrane lipids

Table 6. Sperm kinematics in control and Myo-inositol-treated frozen-thawed bovine semen samples. Sperm kinematics were found significantly altered between control and Myo-inositol-treated samples. Results are expressed as mean ± standard error of the mean (*n* = 5 straws per bull). *P<0.05; **P<0.005; ***P<0.0005 (Mann-Whitney test).

Bull	VCL (µm/s)		VSL (µm/s)		VAP (µm/s)	
	Control	Myoinositol	Control	Myo-inositol	Control	Myo-inositol
1	157.80 ± 2.57	176.70 ± 1.95 ***	73.63 ± 1.88	75.32 ± 1.55	84.27 ± 1.60	90.12 ± 1.28 **
2	137.00 ± 2.18	178.20 ± 2.46 ***	81.21 ± 1.95	112.80 ± 2.50 ***	89.79 ± 1.72	111.60 ± 1.78 ***
3	127.30 ± 2.95	168.90 ± 3.51 ***	53.53 ± 1.80	81.26 ± 2.57 ***	66.77 ± 1.81	87.64 ± 2.11 ***
4	166.20 ± 2.51	212.10 ± 2.65 ***	71.40 ± 1.66	108.00 ± 2.44 ***	87.27 ± 1.57	116.90 ± 1.78 ***
5	120.60 ± 2.56	155.40 ± 4.15 ***	61.12 ± 1.88	90.68 ± 3.15 ***	69.66 ± 1.72	94.53 ± 2.74 ***
6	59.30 ± 1.78	81.91 ± 2.07 ***	20.08 ± 0.93	29.24 ± 1.36 ***	27.13 ± 1.04	37.06 ± 1.23 ***
7	149.30 ± 2.67	158.50 ± 3.27 *	74.60 ± 1.99	86.40 ± 2.75 **	84.72 ± 1.81	92.63 ± 2.19 **
8	169.00 ± 4.69	213.80 ± 3.73 ***	89.96 ± 3.67	134.10 ± 4.74 ***	92.91 ± 2.97	117.50 ± 2.96 ***
9	101.60 ± 1.81	108.40 ± 2.43 *	55.58 ± 1.49	64.76 ± 1.95 ***	63.52 ± 1.36	70.30 ± 1.76 ***

Table 6 (cont.)

Bull	LIN (%)		STR (%)		ALH (µm)	
	Control	Myo-inositol	Control	Myo-inositol	Control	Myo-inositol
1	47.23 ± 0.87	42.97 ± 0.66 ***	75.50 ± 0.97	71.11 ± 0.79 ***	5.33 ± 0.09	5.94 ± 0.07 ***
2	55.98 ± 0.94	63.88 ± 0.99 ***	80.8 ± 0.8	84.09 ± 1.02 ***	4.02 ± 0.06	4.73 ± 0.09 ***
3	40.62 ± 0.94	49.29 ± 1.18 ***	73.27 ± 1.12	80.38 ± 1.28 ***	4.71 ± 0.10	5.42 ± 0.14 ***
4	41.69 ± 0.68	50.94 ± 0.80 ***	70.89 ± 0.79	77.6 ± 0.91 ***	5.70 ± 0.08	6.34 ± 0.10 ***
5	48.29 ± 1.01	58.44 ± 1.36 ***	78.14 ± 1.09	86.66 ± 1.28 ***	4.39 ± 0.08	4.97 ± 0.13 ***
6	31.24 ± 1.21	35.52 ± 1.28 *	66.16 ± 1.83	72.46 ± 1.70 *	3.03 ± 0.09	3.83 ± 0.10 ***
7	48.00 ± 0.87	51.70 ± 1.08 **	80.11 ± 0.96	82.00 ± 1.09	5.09 ± 0.09	5.07 ± 0.11
8	50.60 ± 1.11	60.40 ± 1.19 ***	77.46 ± 1.19	79.84 ± 30.42 ***	4.95 ± 0.12	5.37 ± 0.13 **
9	50.99 ± 0.84	58.72 ± 1.08 ***	78.49 ± 0.87	86.42 ± 0.98 ***	3.74 ± 0.06	3.80 ± 0.09

Table 6 (cont.)

Bull	BCF (Hz)		WOB (%)		MAD (degrees)	
	Control	Myo-inositol	Control	Myo-inositol	Control	Myo-inositol
1	7.61 ± 0.14	7.88 ± 0.12	54.18 ± 0.63	51.21 ± 0.47 ***	106.20 ± 1.42	113.90 ± 1.12 ***
2	9.28 ± 0.15	10.49 ± 0.18 ***	64.06 ± 0.64	65.20 ± 0.80 *	105.50 ± 1.72	103.60 ± 2.09
3	8.38 ± 0.19	9.43 ± 0.24 ***	51.73 ± 0.70	53.90 ± 0.93 *	121.20 ± 1.52	117.80 ± 2.22
4	7.60 ± 0.11	9.15 ± 0.16 ***	52.11 ± 0.49	56.47 ± 0.61 ***	114.50 ± 1.09	113.70 ± 1.55
5	8.42 ± 0.17	9.94 ± 0.25 ***	56.78 ± 0.70	61.84 ± 1.03 ***	111.20 ± 1.59	113.00 ± 2.79
6	5.09 ± 0.21	6.45 ± 0.22 ***	43.35 ± 1.29	45.46 ± 0.98	106.20 ± 1.90	107.10 ± 2.01
7	9.93 ± 0.16	10.23 ± 0.22	55.86 ± 0.59	58.38 ± 0.73 **	119.00 ± 1.56	120.30 ± 2.22
8	7.84 ± 0.20	9.08 ± 0.23 ***	55.39 ± 0.86	56.79 ± 1.05 *	109.40 ± 1.95	112.10 ± 2.53
9	8.57 ± 0.14	9.61 ± 0.20 ***	61.02 ± 0.58	64.94 ± 0.77 ***	110.90 ± 1.48	103.90 ± 2.16 ***

and proteins, as well as distinctive responses to osmotic changes, cooling, cold shock, ice oxidative stress and addition of cryoprotective agents, as recently summarized (Grötter et al, 2019). In particular, studies have previously shown differences in sperm membrane permeability to water and glycerol (Chaveiro et al., 2006), and in sperm and seminal plasma protein composition (Rego et al., 2016; Gomez et al., 2020) that may explain heterogeneity in sperm response to MI treatment. Moreover, initial endogenous MI concentration may also play an important role, since it was found significantly lower in seminal fluids of oligozoospermic patients when compared to normozoospermic ones (Murgia et al., 2020). Given the heterogeneity in sperm motility among samples, a response index (RI) to MI supplementation was assessed. As a result, a high RI was determined for one

Table 7. Effect of Myo-inositol supplementation on Sperm Vitality and Osmotic Competence. Sperm vitality and plasma membrane osmotic competence depicted similar results between control and Myo-inositol-treated frozen-thawed bovine semen samples. Results are expressed as mean ± standard error of the mean (*n* = 5 straws per animal).

Bull	Sperm Vitality (%)			Sperm Osmotic Competence (%)		
	Control	Myo-inositol	Wilcoxon Test *P*-value	Control	Myo-inositol	Wilcoxon Test *P*-value
1	56.4 ± 4.4	57.2 ± 5.5	1.0000	35.2 ± 3.1	36.8 ± 1.9	1.0000
2	51.3 ± 6.8	55.3 ± 3.5	0.2500	60.8 ± 6.6	57.7 ± 5.3	0.1736
3	57.0 ± 4.9	54.8 ± 3.7	0.1000	17.5 ± 0.9	18.9 ± 3.2	0.1000
4	69.5 ± 1.1	69.8 ± 2.5	1.0000	50.3 ± 4.8	47.3 ± 7.6	1.0000
5	43.1 ± 2.3	51.3 ± 6.3	0.5000	23.43± 6.1	27.7 ± 6.7	0.5000
6	26.7 ± 1.7	26.4 ± 5.4	0.7500	15.9 ± 3.4	7.7 ± 3.1	0.1736
7	58.0 ± 4.5	48.4 ± 3.7	0.5000	27.7 ± 4.8	29.8 ± 0.9	1.0000
8	39.3 ± 3.6	48.8 ± 1.4	0.7500	48.3 ± 5.7	53.5 ± 3.5	0.2500
9	76.5 ± 2.6	68.7 ± 5.5	0.2500	46.8 ± 2.0	45.8 ± 2.9	1.0000

Table 8. Effect of Myo-inositol supplementation on Acrosomal Status. Sperm acrosomal status depicted similar results between control and Myo-inositol-treated frozen-thawed bovine semen samples. Results are expressed as mean ± standard error of the mean (*n* = 5 straws per animal).

Bull	Acrosomal Status (%)		
	Control	Myo-inositol	Wilcoxon Test *P*-value
1	92.2 ± 2.0	93.3 ± 1.5	0.5000
2	83.6 ± 2.5	80.0 ± 6.4	0.5000
3	90.4 ± 3.2	86.8 ± 3.6	0.1000
4	88.5 ± 8.4	86.9 ± 7.8	0.5862
5	78.5 ± 12.7	77.9 ± 13.8	1.0000
6	95.1 ± 0.6	96.1 ± 0.1	1.0000
7	83.5 ± 6.1	81.2 ± 8.1	0.5000
8	88.6 ± 1.2	90.0 ± 2.1	1.0000
9	85.9 ± 4.4	85.6 ± 3.8	0.7500

Table 9. Effect of Myo-inositol supplementation on Chromatin Compaction. Sperm chromatin compaction depicted similar results between control and Myo-inositol-treated frozen-thawed bovine semen samples. Results are expressed as mean ± standard error of the mean (*n* =5 straws per animal).

Bull	Chromatin Compaction (%)		
	Control	Myo-inositol	Wilcoxon Test *P*-value
1	97.8 ± 0.3	99.0 ± 0.0	0.2500
2	91.4 ± 0.4	90.4 ± 1.6	0.7500
3	97.2 ± 0.4	97.9 ± 0.7	0.1000
4	98.9 ± 0.4	98.5 ± 0.3	0.7728
5	98.8 ± 0.4	98.7 ± 0.6	0.4142
6	98.0 ± 0.6	98.5 ± 0.5	0.5000
7	99.0 ± 0.0	99.0 ± 0.5	0.5862
8	98.4 ± 0.4	99.0 ± 0.0	0.2500
9	97.7 ± 0.7	98.0 ± 0.3	0.7500

third of bulls, whereas up to a medium and low RI were determined for the remaining thirds. Specifically regarding the breed, dairy bulls responded in 60% of their samples, while beef bulls responded in 20%. Differences between breeds may be attributed, at least in part, to the presence of abnormalities in the sperm midpiece, reported to be higher in beef breeds and proposed to result from gamete freezing and thawing; these alterations

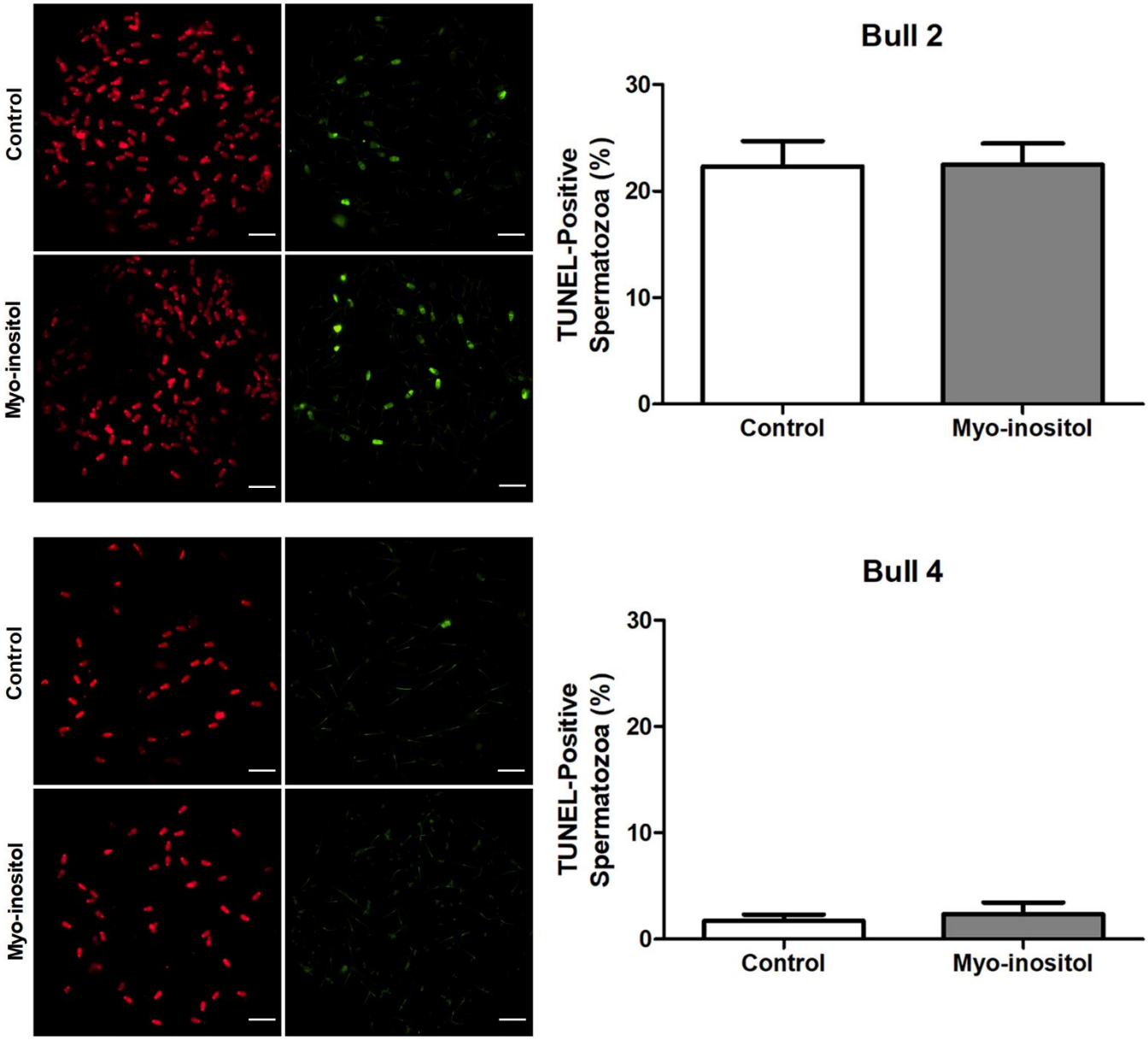

Figure 4. Effect of Myo-inositol supplementation on sperm DNA fragmentation. Representative images of spermatozoa stained with TUNEL label (green) and Propidium Iodide (red) corresponding to bulls 2 and 4 (600X magnification). DNA fragmentation was similar between control and Myo-inositol-treated samples. Results are presented as mean ± standard error of the mean (P>0.05; Wilcoxon test). Bar = 20µm.

could affect sperm motility and may not be overcome after MI treatment (Ghirardosi et al., 2018).

Considering the relevance of the objective evaluation of sperm motility, assessment of sperm kinematics in each replicate of sperm suspensions from all semen samples was also done. As a result of these evaluations, a significant increase was determined in most sperm kinematic parameters after 1h MI supplementation. The findings contrast with those by Boni et al. (2016), who reported a significant improvement in VCL, VAP and ALH, but not in VSL, at the second hour of incubation with MI. Our results are highly relevant considering the association between sperm kinematics and fertility. Specifically, VCL, VSL, VAP, LIN, and BCF have been associated to bull fertility potential, as shown by the correlation between these and the non-return rate (NRR) (Amann et al., 1989; Farrell et al., 1998; Kathiravan et al., 2011; Michos et al., 2013; Nagy et al., 2015; Gliozzi et al., 2017). Moreover, VCL, VSL, and VAP positively correlated to pregnancy rates (Nagy et al., 2015); whereas STR and ALH did not depict a significant association with bull fertility (Kathiravan et al., 2011). Also, CASA velocity parameters have also been suggested to associate with fertility in studies carried out in pigs (Broekhuijse et al., 2012),

rabbit, ram, dog, rat (Verstegen et al., 2002), and humans (Fetterolf and Rogers, 1990; Larsen et al., 2000; Hirano et al., 2001; Verstegen et al., 2002).

Regarding membrane integrity, an intact and biochemically active plasma membrane is essential for several sperm functions, such as motility, capacitation, acrosome reaction, and interactions with the oviductal epithelium and the oocyte vestments (Yániz et al., 2015); and it positively correlates with NRR (Petrunkina et al., 2001). In the present work, semen supplementation with MI during 1h did not lead to changes in sperm vitality when compared to the control group. Likewise, the percentages of osmotically competent spermatozoa or with an intact acrosome were similar between control and MI-treated samples.

The present study also evaluated chromatin condensation, considered a crucial factor for fertilization success. Chromatin condensation can be altered during cryopreservation (Waterhouse et al., 2010), with a concomitant fertility reduction (Januskauskas et al., 2003). In this sense, Oliveira et al. (2013) reported a significantly negative correlation between chromatin decondensation and fertility using Aniline blue staining. High percentages of spermatozoa with compact chromatin were determined in all samples, and no deleterious effects were recorded after MI supplementation.

Finally, assessment of DNA fragmentation has become a biomarker of male infertility, since sperm DNA fragmentation has been linked to embryonic development defects and low conception rates (Lewis and Simon, 2010; Takeda et al., 2015). Based on this background information, DNA fragmentation was evaluated by the TUNEL assay using frozen-thawed semen samples from one bull depicting normal fertility and from a subfertile bull. As a result, MI supplementation did not exert a significant effect on DNA fragmentation when compared to control conditions.

Overall, this is the first study that thoroughly evaluated the effect of supplementation with MI (2mg/mL) on frozen-thawed bovine semen. As a result, a positive impact of MI was found on a set of sperm quality biomarkers, especially in objective sperm kinematic parameters. Moreover, sperm motility depicted a trend towards an increase in the percentage of total and progressive motile sperm, and in the RI. Interestingly, some differences in sperm response were found between breeds, and among replicates of each bull. Noteworthy, supplementation with MI did not exert a deleterious effect on sperm vitality, plasma membrane osmotic competence, acrosome status, nuclear chromatin condensation, or DNA fragmentation.

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