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Effect of extender on the quality and incubation resilience of cryopreserved Holstein bull semen

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Abstract: There are still concerns over sperm quality when plant-based diluents are used instead of animal-based semen extenders. Therefore, in our study we compared the effects of one soybean lecithin-based (AndroMed®) and two egg yolk-based (BullXcell® and Optidyl®) commercially available extenders on post-thaw *in vitro* sperm quality. Fifty ejaculates collected from ten bulls were aliquoted into three parts and diluted with the above-mentioned extenders. Post-thaw sperm viability, mitochondrial membrane potential (MMP), plasma membrane integrity, and acrosome status were analysed immediately after thawing (0 h) and at an hourly interval during 2-h incubation at 38 °C. Sperm functionality variables were assessed by simultaneous quadruple staining using flow cytometry. Semen stored in Optidyl® had a greater viability, plasma membrane and acrosome integrity than that stored in AndroMed® and BullXcell® ($P < 0.05$). With the use of BullXcell® there was a higher percentage of sperm with high MMP ($P < 0.05$) when compared with the other extenders. The incubation affected the development of sperm quality parameters differently as the variables related to the plasma membrane showed an increase, while MMP and acrosome integrity showed a decline. Although the semen from all bulls responded to treatments in a similar manner, significant intra- and inter-male differences were found. In conclusion, the results clearly displayed the beneficial effects of egg yolk over soybean lecithin supplementation on *in vitro* sperm quality.

Keywords: bovine; frozen-thawed semen; flow cytometry; thermoresistance

Artificial insemination and the associated semen cryopreservation are among the most commonly applied assisted reproductive technologies in farm animals worldwide. Despite its undeniable importance, sperm quality and fertilizing ability are severely compromised after cooling, freezing, and thawing in terms of sperm membrane disruption, mitochondrial and nuclear DNA impairment, and ROS (reactive oxygen species) production (Ugur et al. 2019). The most cryosensitive organelle is the plasma membrane, since about half of the total sperm population possesses some type of cell

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membrane injury. Other cellular components such as the acrosome, mitochondria, and chromatin tend to exhibit a lower incidence of damage (Khalil et al. 2018). The total sperm recovery after freezing and thawing is therefore quite low – just over 50% (Layek et al. 2016).

During cryopreservation, male gametes are kept in various aqueous media, i.e. semen extenders of a specific man-made composition (Hashem and Gonzalez-Bulnes 2020). The typical extender for sperm cryopreservation is composed of the following: permeating protectant (mostly glycerol), non-permeating protectant (egg yolk, egg yolk derivatives, milk, or milk by-products), organic buffer [such as Tris(hydroxymethyl)aminomethane], sugar for energy or osmotic balance (glucose, fructose, lactose, raffinose, sucrose or trehalose), solute for pH and osmolarity adjustment (sodium citrate, citric acid), and antibiotics (penicillin, streptomycin) (Layek et al. 2016). In an effort to ameliorate protection against cold or freezing shock, improve the uptake of nutrients, reduce the microbial attack, lower the negative impacts on sperm function and structure, intensive attempts at the modification of chemical composition of diluents have been made in recent years (Hashem and Gonzalez-Bulnes 2020). In order to reduce the above-mentioned detrimental effects of cryopreservation, various substances such as antioxidants, antifreeze proteins, fatty acids, vitamins, animal serum, nanoparticles, plant oils, soybean lecithin, and low-density lipoproteins have been added to the extenders (Ugur et al. 2019).

Although the egg yolk is traditionally used as a sperm cryoprotectant, it is not without its disadvantages and negative effects on semen processing. The most frequently cited deficiencies are as follows: egg yolk composition variability, risk of microbial contamination or pathogen transmission, presence of harmful metabolites, endotoxins or steroid hormones, and hampered quality evaluation. These drawbacks have led to an increased demand for alternatives to conventional extenders free of compounds of animal origin (Murphy et al. 2018; Singh et al. 2018; Miguel-Jimenez et al. 2020). A large body of recent research has been dedicated to the possible replacement of animal-based, non-permeating protectants with plant-based ones, primarily in order to lower the potential risk of microbial contamination or disease transmission (e.g. avian influenza or bovine spongiform encephalopathy) and to improve standardisation, product traceability, and biosecurity (Aires et al. 2003; Layek et al. 2016). Nonetheless, there are still disputes over contribution to *in vitro* and *in vivo* sperm quality, when soy by-products, e.g. soybean lecithin, are added to semen diluents (Leite et al. 2010).

Many studies reported comparable *in vitro* and/or *in vivo* quality assessment results in cattle or buffalo (Aires et al. 2003; Murphy et al. 2018) when soybean lecithin-based extenders were used as a substitute for egg yolk-based diluents. Yet, some authors reported superior results in preserving sperm quality or fertilizing capacity of sperm doses diluted in soybean-based rather than in egg yolk-based extenders (Beran et al. 2012; Miguel-Jimenez et al. 2020). However, other studies declared worsening results of *in vitro* (Muino et al. 2007) and *in vivo* sperm quality (Crespilho et al. 2014; Singh et al. 2018) after dilution in soybean lecithin-based extenders compared to animal-based ones.

Previous studies comparing effects of non-animal and animal based extenders on post-thaw sperm quality relied on subjective sperm analysis to varying degrees. Therefore, the aim of the present study was to determine objectively the post-thaw quality and incubation-related changes in cryopreserved bull spermatozoa subpopulations using three commercially available bull semen extenders: soybean lecithin-based extender AndroMed® and two egg yolk-based extenders BullXcell® and Optidyl®.

MATERIAL AND METHODS

Suppliers

Chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA), unless indicated otherwise.

Animals

The bull semen used in this experiment was collected from Holstein Friesian breeding bulls (n = 10) kept in a private Artificial Insemination Centre (Hradištko, Central Bohemian Region, Czech Republic). The bulls were healthy and sexually mature (3.5 ± 0.5 years old), routinely used for semen collection. Sires were stabled under the identical

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and optimal conditions of handling, housing, feeding, watering, and management during the entire duration of the study.

Semen collection

The semen samples were collected by a standard operating procedure at weekly intervals for a five-week period (five ejaculates per bull in total). The collection of the semen was provided with a warmed (38 °C) and sterilised artificial bovine vagina. The obtained ejaculates were evaluated immediately after the semen collection process using an electronic precision scale and an AndroVision® computer-assisted sperm analysis system (Minitübe GmbH, Tiefenbach, Germany). All ejaculates failing to meet the minimal limits of fresh semen volume (≥ 1.5 g), sperm concentration (≥ 0.7×10^9 spermatozoa/ml), and percentage of motile sperm (≥ 70%) were excluded from the study.

Semen processing, freezing, and thawing

After successful initial quality control, each collected semen sample was divided into three aliquots and diluted with three commercial extenders: AndroMed® containing 1% soybean lecithin (Minitübe GmbH, Tiefenbach, Germany), BullXcell® containing 25% (v/v) of fresh egg yolk (IMV Technologies, L'Aigle, France), and Optidyl® containing 20% (v/v) of ionised egg yolk (IMV Technologies, L'Aigle, France) to the final sperm concentration of 10×10^6 per one insemination dose. The diluted semen was then automatically placed into 0.25 ml French straws (IMV Technologies, L'Aigle, France) and sealed. The straws were equilibrated at 4 °C for two hours and frozen at a two-phase freezing rate (Januskauskas et al. 1999) by using a programmable DigitCool® freezer (IMV Technologies, L'Aigle, France). Frozen doses were subsequently plunged into liquid nitrogen and stored in this medium for two years until the thawing process. The thawing was performed in a water bath at 38 ± 1 °C for 30 seconds. The content of the insemination dose was then transferred into the Eppendorf tube and stored in an INB 400 incubator (Memmert GmbH, Schwabach, Germany) in the dark at 38 °C for up to 2 hours.

Flow cytometry

Flow cytometry evaluation was performed immediately after thawing and following 1 h or 2 h incubation according to Savvulidi et al. (2021). Specifically, 25 µl of frozen-thawed sperm was pipetted to 200 µl of Dulbecco's phosphate-buffered saline without divalent cations (Biosera Europe, Nuaille, France) and stained for 10 min at 38 °C in the dark with the following fluorescent probes given at a final concentration: 16.2 µM Hoechst-33342 (H-342) for discriminating debris; 12 µM propidium iodide (PI) for assessing plasma membrane damage; 0.5 µg/ml fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) (Thermo Fisher Scientific, Waltham, MA, USA) for assessing the acrosomal status; and 80 nM Mitotracker Deep Red (MTR DR) (Thermo Fisher Scientific, Waltham, MA, USA) for assessing the mitochondrial membrane potential (MMP).

Subsequently, sperm samples were analysed using a NovoCyte 3000 digital flow cytometer (Acea Biosciences, part of Agilent, Santa Clara, CA, USA). The flow cytometer was equipped with violet (405 nm), blue (488 nm), and red (640 nm) lasers and appropriate optical filters for the detection of emitted fluorescence signals. The fluorescence was collected using optical filters 445/45 (violet laser, H342), 530/40 (blue laser, FITC-PNA), 675/30 (blue line, red fluorescence: PI) and 675/30 (red line, red fluorescence: MTR DR).

Each thawed straw was analysed with a flow cytometer twice. The final concentration of cells during analysis was 0.8×10^6 spermatozoa/ml. The samples were run at a low speed (14 µl/min), and the fluorescence from 30 000 events was recorded for each sample. A rinse procedure was performed after every sample acquisition. NovoExpress software, v1.3.0 (Acea Biosciences, part of Agilent, Santa Clara, CA, USA) was used for the automated cytometer setup and performance tracking, as well as for data acquisition. The same software was also used to analyse the acquired flow cytometry data. No compensation was required with the optical filter setup used. The flow cytometer was quality-controlled on a daily basis using polystyrene microspheres (ACEA NovoCyte QC Particles; Agilent, Santa Clara, CA, USA) providing the routine calibration control.

The gating strategy used for this research is presented in Figure 1. Briefly, the events were

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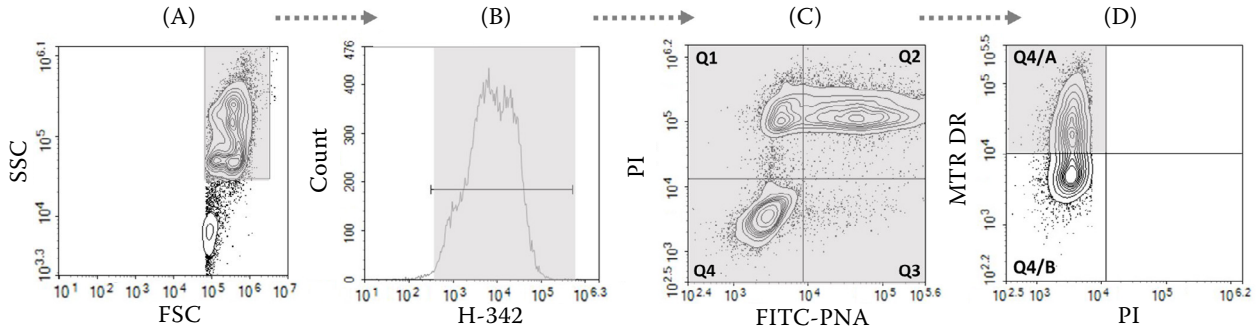

Figure 1. The gating strategy for quadruple staining analysis by flow cytometry

Q1 = deteriorated plasma membrane and intact acrosome; Q2 = both plasma membrane and acrosome damaged; Q3 = intact plasma membrane and damaged acrosome; Q4 = both plasma membrane and acrosome integrity undamaged (viable spermatozoa); Q4/A = viable sperm with high mitochondrial membrane potential (MMP); Q4/B = viable sperm with low MMP

The sample was stained with Hoechst-33342 (H-342) for discriminating DNA free events, propidium iodide (PI) for assessing plasma membrane integrity, peanut agglutinin-conjugated fluorescein isothiocyanate (FITC-PNA) for assessing the acrosomal status, and Mitotracker Deep Red (MTR DR) for evaluating the mitochondrial membrane potential (MMP). Events were distinguished regarding the size and orientation of cells in the laser beam (SSC) (A), followed by gating based on DNA content (B). Gated spermatozoa were divided into four groups (Q1–Q4) by plasma membrane and acrosome integrity (C). The viable sperm subpopulation was further identified based on the MMP into two groups (Q4/A–B) (D)

initially identified using an SSC (side scatter, log scale) versus FSC (forward scatter, log scale) bivariate histogram plot. Spermatic events were identified based on the threshold and gating set with the Hoechst-33342 stain (DNA content). The sperm subpopulations were categorised as follows: with damaged plasma membrane and intact acrosome in the upper left (Q1) quadrant (H-342$^+$/PI$^+$/FITC-PNA$^-$), with both plasma membrane and acrosome damaged in the upper right (Q2) quadrant (H-342$^+$/PI$^+$/FITC-PNA$^+$), with intact plasma membrane and ruptured acrosome in the lower right (Q3) quadrant (PI$^-$/FITC-PNA$^+$), and with viable sperm (both plasma membrane and acrosome intact) (H-342$^+$/PI$^-$/FITC-PNA$^-$) in the lower left quadrant (Q4). Furthermore, the viable subpopulation was divided into subpopulations with high (H-342$^+$/PI$^-$/FITC-PNA$^-$/MTR DR$^+$) and low MMP.

Statistical analysis

Statistical analyses were performed using the SAS statistical package (v9.3; SAS Institute Inc., Cary, NC, USA). Data for sperm variables were examined for normal distribution (Shapiro-Wilk test) and homogeneity of variance (Bartlett's test), and were subsequently analysed using the generalised linear model (PROC GLM). The best model evaluation was selected based on the procedure REG (STEPWISE method). The dependent variables in the study were sperm viability percentage (originates from Q4 subpopulation), percentage of viable sperm with high MMP (Q4/A subpopulation), plasma membrane damage percentage (summed Q1 and Q2 subpopulations), and acrosome damage percentage (summed Q2 and Q3 subpopulations). The fixed effects of bull, extender, incubation time, and interactions between those effects were included in the final model. The differences between least squares means were evaluated by the Tukey-Kramer method. Results are presented as the least squares means [± the standard error of the mean (SEM)]. Data were considered to differ significantly at $P < 0.05$.

RESULTS

Spermatozoa viability

The preservation of sperm viability significantly differed ($P < 0.05$) depending on the extender used (Table 1). The superiority of egg yolk supplementation over soybean lecithin was proved on an individual bull level. The exception was bull No. 8, where no statistically significant differences between extender treatments were proved.

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Table 1. Effect of different semen extenders on post-thaw sperm viability, viable sperm showing high mitochondrial membrane potential (MMP), plasma membrane damage and acrosome damage of bull (n = 10) semen (n = 50) exposed to 2-h incubation

Bull	Extender	Viability (%)	High MMP (%)	Plasma membrane damage (%)	Acrosome damage (%)
1	AndroMed®	39.72 ± 0.889[a]	35.17 ± 1.923[a]	59.20 ± 0.888[a]	31.00 ± 0.770
	BullXcell®	48.32 ± 0.889[b]	60.43 ± 1.923[b]	50.50 ± 0.888[b]	28.10 ± 0.770
	Optidyl®	49.76 ± 0.889[b]	54.06 ± 1.923[b]	48.98 ± 0.888[b]	29.39 ± 0.770
2	AndroMed®	40.76 ± 0.889[a]	54.86 ± 1.923[a]	57.80 ± 0.888[a]	41.73 ± 0.770[a]
	BullXcell®	48.74 ± 0.889[b]	65.23 ± 1.923[b]	49.59 ± 0.888[b]	27.28 ± 0.770[b]
	Optidyl®	52.85 ± 0.889[b]	59.19 ± 1.923	45.03 ± 0.888[b]	25.89 ± 0.770[b]
3	AndroMed®	49.35 ± 0.889[a]	66.56 ± 1.923	49.21 ± 0.888[a]	33.36 ± 0.770[a]
	BullXcell®	52.92 ± 0.889	69.90 ± 1.923	45.33 ± 0.888	29.06 ± 0.770[b]
	Optidyl®	55.58 ± 0.889[b]	64.33 ± 1.923	42.46 ± 0.888[b]	26.68 ± 0.770[b]
4	AndroMed®	40.35 ± 0.889[a]	61.67 ± 1.923[a]	58.43 ± 0.888[a]	38.44 ± 0.770[a]
	BullXcell®	47.70 ± 0.889[b]	75.60 ± 1.923[b]	51.20 ± 0.888[b]	32.22 ± 0.770[b]
	Optidyl®	50.05 ± 0.889[b]	62.54 ± 1.923[a]	48.54 ± 0.888[b]	30.43 ± 0.770[b]
5	AndroMed®	40.72 ± 0.889[a]	54.10 ± 1.923[a]	58.31 ± 0.888[a]	36.03 ± 0.770[a]
	BullXcell®	42.37 ± 0.889[a]	83.50 ± 1.923[b]	56.34 ± 0.888[a]	30.07 ± 0.770[b]
	Optidyl®	49.43 ± 0.889[b]	61.51 ± 1.923[a]	49.14 ± 0.888[b]	28.93 ± 0.770[b]
6	AndroMed®	40.87 ± 0.889[a]	52.50 ± 1.923[a]	58.20 ± 0.888[a]	36.57 ± 0.770[a]
	BullXcell®	46.40 ± 0.889[b]	91.21 ± 1.923[b]	52.64 ± 0.888[b]	32.08 ± 0.770[b]
	Optidyl®	47.86 ± 0.889[b]	60.25 ± 1.923[a]	50.88 ± 0.888[b]	32.11 ± 0.770[b]
7	AndroMed®	42.37 ± 0.889[a]	61.03 ± 1.923[a]	56.88 ± 0.888[a]	38.08 ± 0.770[a]
	BullXcell®	39.94 ± 0.889[a]	89.28 ± 1.923[b]	59.10 ± 0.888[a]	31.91 ± 0.770[b]
	Optidyl®	49.11 ± 0.889[b]	61.19 ± 1.923[a]	49.83 ± 0.888[b]	28.26 ± 0.770[b]
8	AndroMed®	48.83 ± 0.889	56.87 ± 1.923[a]	50.10 ± 0.888	30.39 ± 0.770
	BullXcell®	46.13 ± 0.889	92.61 ± 1.923[b]	52.81 ± 0.888	29.99 ± 0.770
	Optidyl®	50.38 ± 0.889	61.63 ± 1.923[a]	48.35 ± 0.888	27.80 ± 0.770
9	AndroMed®	37.40 ± 0.889[a]	47.29 ± 1.923[a]	62.04 ± 0.888[a]	37.62 ± 0.770[a]
	BullXcell®	48.96 ± 0.889[b]	84.73 ± 1.923[b]	49.96 ± 0.888[b]	27.26 ± 0.770[b]
	Optidyl®	51.16 ± 0.889[b]	58.19 ± 1.923[c]	47.77 ± 0.888[b]	29.05 ± 0.770[b]
10	AndroMed®	36.26 ± 0.889[a]	48.83 ± 1.923[a]	63.08 ± 0.888[a]	35.19 ± 0.770[a]
	BullXcell®	51.43 ± 0.889[b]	92.60 ± 1.923[b]	47.62 ± 0.888[b]	25.49 ± 0.770[b]
	Optidyl®	51.91 ± 0.889[b]	58.83 ± 1.923[a]	46.96 ± 0.888[b]	26.19 ± 0.770[b]
All bulls	AndroMed®	41.66 ± 0.281[a]	53.89 ± 0.608[a]	57.32 ± 0.281[a]	35.84 ± 0.244[a]
	BullXcell®	47.29 ± 0.281[b]	80.51 ± 0.608[b]	51.51 ± 0.281[b]	29.35 ± 0.244[b]
	Optidyl®	50.81 ± 0.281[c]	60.17 ± 0.608[c]	47.79 ± 0.281[c]	28.47 ± 0.244[c]

[a–c]Different letters indicate differences between groups within a column ($P < 0.05$)

Results are presented as least squares means ± SEM

Other deviations from average variable development were found in bulls No. 5 and No. 7, in which the viability between AndroMed® and BullXcell® did not vary. Sperm variables, including viability, varied not only within a bull, but also between bulls significantly ($P < 0.05$). Bull No. 3, for instance, statistically differed from all other animals in the viability parameter for the highest detected values during the entire assessment (data not shown). On average for all bulls,

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the greatest percentage of viable spermatozoa was observed with the use of Optidyl® (50.81%) compared to BullXcell® or AndroMed® (47.29% and 41.66%, respectively). Incubation time affected ($P < 0.05$) the sperm viability parameter within bulls differently (Table 2). In the majority of bulls, significant differences between incubation times were not proved, while some animals exhibited ($P < 0.05$) the lowest viability values immediately after thawing, which is in accord-

Table 2. Comparison of incubation-mediated development of sperm variables [sperm viability, viable sperm showing high mitochondrial membrane potential (MMP), plasma membrane damage and acrosome damage in bull (n = 10) semen (n = 50) as assessed by flow cytometry immediately (0), 1 and 2 h after thawing

Bull	Incubation time (h)	Viability (%)	High MMP (%)	Plasma membrane damage (%)	Acrosome damage (%)
1	0	46.04 ± 0.889	57.41 ± 1.923^{a}	53.11 ± 0.888	24.24 ± 0.770^{a}
	1	48.67 ± 0.889^{a}	50.80 ± 1.923	50.22 ± 0.888^{a}	28.28 ± 0.770^{a}
	2	43.09 ± 0.889^{b}	41.45 ± 1.923^{b}	55.35 ± 0.888^{b}	35.97 ± 0.770^{b}
2	0	46.83 ± 0.889	61.83 ± 1.923	52.32 ± 0.888^{a}	23.30 ± 0.770^{a}
	1	51.19 ± 0.889^{a}	60.15 ± 1.923	47.26 ± 0.888^{b}	30.74 ± 0.770^{b}
	2	44.32 ± 0.889^{b}	57.29 ± 1.923	52.84 ± 0.888^{a}	40.86 ± 0.770^{c}
3	0	49.61 ± 0.889	71.58 ± 1.923^{a}	49.12 ± 0.888^{a}	25.23 ± 0.770^{a}
	1	56.05 ± 0.889	71.24 ± 1.923^{a}	42.54 ± 0.888^{b}	28.42 ± 0.770^{a}
	2	52.19 ± 0.889	57.98 ± 1.923^{b}	45.34 ± 0.888	35.45 ± 0.770^{b}
4	0	44.41 ± 0.889^{a}	72.36 ± 1.923^{a}	54.45 ± 0.888^{a}	29.31 ± 0.770^{a}
	1	49.22 ± 0.889^{b}	66.41 ± 1.923	49.67 ± 0.888^{b}	32.39 ± 0.770^{a}
	2	44.48 ± 0.889^{a}	61.03 ± 1.923^{b}	54.06 ± 0.888	39.39 ± 0.770^{b}
5	0	41.99 ± 0.889	73.76 ± 1.923^{a}	57.13 ± 0.888	29.02 ± 0.770^{a}
	1	45.46 ± 0.889	63.46 ± 1.923^{b}	53.53 ± 0.888	32.04 ± 0.770
	2	45.08 ± 0.889	61.88 ± 1.923^{b}	53.13 ± 0.888	33.97 ± 0.770^{b}
6	0	44.62 ± 0.889	72.17 ± 1.923	54.68 ± 0.888	30.41 ± 0.770^{a}
	1	45.26 ± 0.889	68.21 ± 1.923	53.73 ± 0.888	34.79 ± 0.770^{b}
	2	45.24 ± 0.889	63.58 ± 1.923	53.31 ± 0.888	35.56 ± 0.770^{b}
7	0	42.57 ± 0.889	75.49 ± 1.923	56.82 ± 0.888	30.88 ± 0.770
	1	45.28 ± 0.889	70.19 ± 1.923	53.88 ± 0.888	32.90 ± 0.770
	2	43.58 ± 0.889	65.82 ± 1.923	55.11 ± 0.888	34.47 ± 0.770
8	0	44.39 ± 0.889^{a}	76.16 ± 1.923	54.73 ± 0.888^{a}	26.76 ± 0.770^{a}
	1	51.26 ± 0.889^{b}	68.77 ± 1.923	47.71 ± 0.888^{b}	29.88 ± 0.770
	2	49.70 ± 0.889^{b}	66.17 ± 1.923	48.81 ± 0.888^{b}	31.55 ± 0.770^{b}
9	0	45.14 ± 0.889	67.24 ± 1.923	54.28 ± 0.888	27.82 ± 0.770^{a}
	1	45.91 ± 0.889	59.70 ± 1.923	53.19 ± 0.888	32.91 ± 0.770^{b}
	2	46.46 ± 0.889	63.27 ± 1.923	52.29 ± 0.888	33.21 ± 0.770^{b}
10	0	45.41 ± 0.889	73.66 ± 1.923^{a}	53.99 ± 0.888	23.69 ± 0.770^{a}
	1	46.09 ± 0.889	62.39 ± 1.923^{b}	52.98 ± 0.888	30.94 ± 0.770^{b}
	2	48.10 ± 0.889	64.22 ± 1.923	50.69 ± 0.888	32.24 ± 0.770^{b}
All bulls	0	45.1 ± 0.281^{a}	70.17 ± 0.608^{a}	54.06 ± 0.281^{a}	27.07 ± 0.244^{a}
	1	48.44 ± 0.281^{b}	64.13 ± 0.608^{b}	50.47 ± 0.281^{b}	31.33 ± 0.244^{b}
	2	46.22 ± 0.281^{c}	60.27 ± 0.608^{c}	52.09 ± 0.281^{c}	35.27 ± 0.244^{c}

$^{a–c}$Different letters indicate differences between groups within a column ($P < 0.05$)

Results are presented as least squares means ± SEM

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ance with the overall findings, where the greatest average sperm viability was reached after one hour post-thawing (48.44%; $P < 0.05$, compared to evaluation immediately after thawing and after a two-hour incubation (45.1% and 46.22%, respectively). This time dependent variability was confirmed on an extender level (Figure 2A), with the exception of AndroMed® where the values detected between one- and two-hour incubation did not differ ($P < 0.05$). Besides, AndroMed® exhibited the lowest decrease from the viability peak (–0.23%) compared to both egg yolk-based extenders (–2.39% and –4.02% for Optidyl® and BullXcell®, respectively).

Mitochondrial membrane potential

The largest proportion of viable sperm with high MMP was observed ($P < 0.05$) in the BullXcell® extender in most bulls (Table 1). The exception from this finding was bull No. 3, where no differences between diluent treatments were assessed. The lowest values in this variable were observed in bull No. 1, which varied from all other bulls significantly (data not shown). In total, considering all bulls, the greatest proportion of sperm showing high MMP was detected in BullXcell® (80.51%), compared to significantly lower values for Optidyl® (60.17%) or AndroMed® (53.89%). A negative impact of incubation length on this variable was confirmed (Table 2), as the proportion of viable spermatozoa with high MMP decreased with incubation time ($P < 0.05$) although this change in the mitochondrial activity of several bulls was not statistically significant (Table 2). On average for all bulls, a continuous decline from 70.17% (immediately after thawing) to 60.27% (two hours after thawing) was observed. The greatest incubation-mediated drop in high MMP was noticed in AndroMed® (–13.62%) compared to BullXcell® (–12.51%) and Optidyl® (–3.55%; Figure 2B), where no statistical differences between evaluated times were observed.

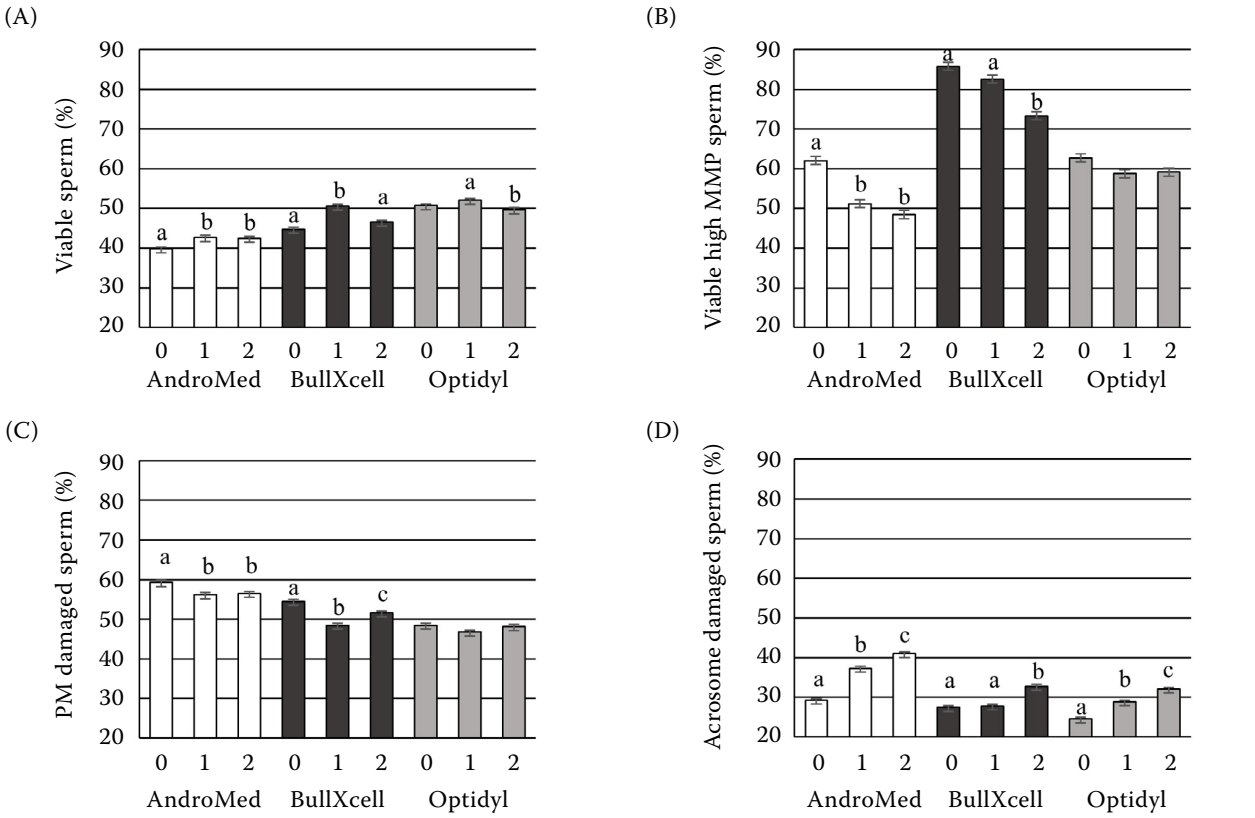

Figure 2. Proportion of viable sperm (A), viable sperm with high mitochondrial membrane potential (MMP) (B), plasma membrane (PM) damage (C), and acrosome damage (D) in frozen-thawed bull semen extended in different diluents immediately (0), one (1) and two hours (2) after thawing

[a–c]Values with different letters differ significantly within the extender ($P < 0.05$)

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Plasma membrane integrity

The extender treatment affected plasma membrane integrity ($P < 0.05$), as semen extended in egg yolk-based diluents scored lower plasma membrane damage than semen samples extended in AndroMed® in the majority of bulls (Table 1). However, a minor portion of bulls responded divergently. For example, the proportion of plasma membrane damage in bull No. 8 was not affected by extender treatment, and thus the obtained results did not vary among the diluents used. Similarly, bulls No. 5 and No. 7 did not differ in the plasma membrane damage level when being diluted in AndroMed® or BullXcell®. Nevertheless, on average for all bulls, Optidyl® exhibited superior results (47.79%) as opposed to sperm diluted in BullXcell® or AndroMed® (51.51% and 57.32%, respectively). The length of incubation significantly influenced ($P < 0.05$) the percent change in this variable as well (Table 2). The greatest plasma membrane damage was observed immediately after thawing in the majority of bulls, although not significant in all animals. This finding was also confirmed by average values, as the highest plasma membrane damage was detected immediately after thawing (54.06%) in comparison with damage after one- (50.47%) and two-hour (52.09%) post-thawing. Similarly, the values detected at all evaluated times differed ($P < 0.05$) in samples diluted in BullXcell®, unlike those of AndroMed® or Optidyl® (Figure 2C).

Acrosome integrity

The least suitable extender in terms of maintaining the acrosome integrity status was AndroMed® in the vast majority of bulls, if compared with both egg yolk-based extenders (Table 1). The exceptions were bulls No. 1 and No. 8, where no statistical differences were observed between the evaluated extenders. On average for all bulls, the greatest ($P < 0.05$) acrosome integrity was preserved in Optidyl® (28.47%) compared to BullXcell® and AndroMed® (29.35% and 35.84%, respectively). The results in Table 2 show an incubation effect ($P < 0.05$) on the acrosome integrity in the majority of bulls. Deviation from this trend was observed in bull No. 8, which maintained a similar level of acrosome damage throughout the entire incubation, making differences statistically insignificant. In total, considering all bulls, the integrity of the acrosomal membrane was worsened significantly ($P < 0.05$) as the incubation progressed, with the lowest level immediately after thawing. The same was observed on the extender level (Figure 2D), where the highest percent change in acrosome integrity was detected in AndroMed® (–11.71%) followed by Optidyl® and BullXcell® (–5.3% and –7.58%, respectively).

DISCUSSION

The present study assessed some sperm parameters that determine the quality and ultimately the fertilising capacity of cryopreserved sperm, when only intact spermatozoa may penetrate the *zona pellucida* and fertilise an oocyte. Important indicators, including viability, mitochondrial activity status, plasma membrane integrity, and acrosome integrity, were evaluated in bull semen samples extended in three semen diluents: AndroMed®, BullXcell®, and Optidyl®.

The results of the present work are consistent with those from previous studies (Crespilho et al. 2014; Singh et al. 2018), where egg-yolk based extenders provide greater protection to sperm cell membrane integrity than with the use of soybean lecithin-based extenders. The opposite conclusions were documented by Ghareeb et al. (2017) and Miguel-Jimenez et al. (2020), who reported greater plasma membrane and acrosome integrity with the use of AndroMed® compared to Triladyl® or Tris-egg yolk extenders. In contrast, Kumar et al. (2015) found no difference between the use of Triladyl® or AndroMed® for bull sperm cryopreservation. These inconsistencies across studies could be related to alterations in freezing and thawing techniques, such as different sperm concentration, cooling and freezing rates, or extender choice and also due to evaluating the semen of various breeds (Morrell et al. 2018). To further scrutinise the dynamics of different quality parameters, sperm were subjected to 2-h incubation at 38 °C. Despite the expected decline in sperm viability, no incubation-mediated decrease was observed in our study, which is consistent with Anzar et al. (2011). We hypothesize that this phenomenon could be explained by the greater instability, i.e. phase changes, of plasma membrane structure and thus higher fluorescent probe permeation caused by the cellular stress

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environment directly after thawing. Nevertheless, our findings are in contrast to Bucher et al. (2019), who reported a 15% decrease in sperm viability throughout 3-h incubation. A decline in sperm viability was also observed in the study by Ansari et al. (2014), where a decrease amounted to 18% during 2-h incubation.

As discussed by Varela et al. (2020), sperm with lower MMP exhibit a higher level of cryocapacitation and as further concluded, a significant association exists between mitochondrial activity and plasma membrane stability and integrity. Therefore mitochondrial integrity, expressed in MMP parameter, is critically important for the cell functional state and viability, being also responsible for proper sperm metabolism and motility (Zorova et al. 2018). As evident from our study, egg yolk protected MMP more efficiently than soybean lecithin, which was seen not only on an individual bull level, but also more clearly in the average values for all bulls. The detrimental effect of soybean lecithin supplementation on post-thaw mitochondrial activity was also observed by Mata-Campuzano et al. (2015), while Fleisch et al. (2017) detected greater MMP in samples diluted with AndroMed® in comparison with those extended with Triladyl®. Our study showed that the ability of cryopreserved sperm to maintain high MMP was negatively affected by incubation stress. Regarding the incubation resilience, a similar decrease in a viable sperm population with unstained membranes and high MMP was recently reported (Gurler et al. 2016; Bucher et al. 2019), although after 3-h incubation; whereas a 3% increase in the high MMP of sperm population was shown in the study by Anzar et al. (2011). Our results show that the incubation-mediated decrease was more pronounced in MMP compared to the viability, from which we may assume that the MMP parameter more realistically reflects the functional state of the frozen-thawed sperm sample.

The plasma membrane is one of the most damaged cellular sperm structures due to cryopreservation and its deleterious effect on sperm phospholipid deterioration and cholesterol loss (Sieme et al. 2015). The significance of the plasma membrane integrity lies in its interaction between the external and internal cell environment, in the regulation of many functions related to the fertilisation potential, motility, and its resistance to various freezing-thawing challenges (Singh et al. 2018). Hence the plasma membrane integrity plays a critical role in the fertilisation process (Ansari et al. 2014). Our results showed that plasma membrane integrity was preserved unequally after cryopreservation with different extenders, as significant inter-individual variabilities were detected. In total, egg yolk-supplemented diluents more efficiently stabilised the plasma membrane in comparison with the extender with soybean lecithin supplementation, which is in accordance with previously published literature (Leite et al. 2010). Yet, these results disagree with Aires et al. (2003), who observed improved acrosome integrity when a soybean lecithin-based extender was used rather than egg yolk supplementation. The results of our study demonstrated an improvement in the plasma membrane integrity within 2-h incubation, unlike Anzar et al. (2011), who found no alteration and Ansari et al. (2014), who reported an incubation-mediated drop in this variable after an equally long incubation.

The acrosome membrane may be altered by acrosome reaction or capacitation-like changes, which are more pronounced in cryopreserved mammalian sperm (Varela et al. 2020). These changes are ultimately responsible for the frozen-thawed sperm longevity decrease in the female reproductive tract (Bucher et al. 2019). The occurrence of acrosome damage was lower with the use of egg yolk-based extenders compared with the use of soybean lecithin. Among the treatments, as it is apparent from the male-to-male variations, the statistically higher cryoprotective effects were observed for Optidyl®, followed by BullXcell® and AndroMed®. These findings are in line with Leite et al. (2010), who reported higher post-thaw acrosome integrity in spermatozoa extended with a Tris-egg yolk-based diluent compared to sperm extended with AndroMed® or Bioxcell®, respectively. On the contrary, Ghareeb et al. (2017) found a beneficial effect of soybean lecithin addition on acrosome integrity in comparison with egg-yolk extender supplementation. Oppositely, several other studies (Kumar et al. 2015; Singh et al. 2018) did not observe any significant differences in acrosome integrity between sperm samples cryopreserved with soybean lecithin or egg yolk. As evident from our study, the incubation had an adverse effect on the number of sperm with an intact acrosome. Our results are in close agreement with Sellem et al. (2015),

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who recorded a similar drop in acrosome integrity after 4-h incubation. Simultaneously, the decrease in acrosome integrity during incubation was more pronounced in AndroMed® compared to BullXcell® or Optidyl®. Rastegarnia et al. (2014) also reported a greater decline in acrosome integrity in sperm samples extended with AndroMed® and Bioxcell® rather than with Tris citrate-egg yolk diluent after 4-h incubation.

The viability, high MMP, and plasma membrane and acrosome integrity significantly varied between bulls in response to the extender treatment and incubation time. These findings are in accordance with the previous works of Beran et al. (2012), who documented inter-individual variability in post-thaw sperm characteristics, and Gurler et al. (2016), where different time-dependent alterations between ejaculates within bulls were reported. The observed differences are probably due to other factors like osmolarity or variation in seminal plasma protective proteins (Casas et al. 2009). The sire individuality should therefore be taken into serious consideration when deciding which diluent to use to extend the semen while improving the insemination dose production quality and ultimately the sperm fertilising ability (Dolezalova et al. 2015).

Based on our findings, it is evident that egg yolk preserved the sperm function better than soybean lecithin. Significant differences were found even between the egg yolk-based diluents. In more detail, most of the analysed parameters were better for Optidyl® (containing ionised egg yolk) than for BullXcell® (containing fresh egg yolk). A possible explanation may lie in the retention of some antioxidants, which are associated with improved cryopreservation of bovine semen. These results could also be attributed to the action of the egg yolk lipoproteins having better protective effects than soybean lecithin during the entire cryopreservation process, including cooling, equilibration, and freezing. Furthermore, we oppose that the aforementioned problematic microbial purity of egg yolk-based extenders is still relevant and represents a crucial weakness, as modern egg yolk processing for extender use eliminates any hygienic risk. The results of the present study and of many others therefore suggest that until soybean lecithin-based extenders undergo proven refinement in their ability to better protect vital sperm functions, we might recommend to continue using extenders of animal origin for the cryopreservation of bull semen.

CONCLUSION

Regarding the extenders' effects on post-thaw functionality variables, several conclusions can be drawn. Firstly, the results of the current work demonstrate that the specific composition of a diluent has an evident effect on sperm viability, mitochondrial status, and membrane integrity. Male-to-male differences were also detected, proving bull's individuality in successful adaptation to different extender compositions. Furthermore, incubation-mediated changes in sperm variables were observed. In general, it was assessed that egg yolk-based extenders were superior, in all analysed parameters, to a diluent containing soybean lecithin as determined by flow cytometry evaluation. We conclude that egg yolk is still the most suitable compound in bull semen cryopreservation despite the currently still claimed, although questionable, disadvantages. Based on the study results, we suggest that in the case of a choice between soybean lecithin-based or egg yolk-based semen extender, the latter is preferable in terms of maintaining better post-thaw *in vitro* sperm quality.

Conflict of interest

The authors declare no conflict of interest.

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