p-ISSN 2615-787X e-ISSN 2615-790X
Accredited by Directorate General of Strengthening for Research and Development No: 30/E/KPT/2018

Tropical Animal Science Journal, December 2022, 45(4):381-388
DOI: https://doi.org/10.5398/tasj.2022.45.4.381
Available online at http://journal.ipb.ac.id/index.php/tasj

Post-Thaw Characteristics of the Simmental Sperm Function in Different Ages of Bulls

F. A. Satrio[a], N. W. K. Karja[b], M. A. Setiadi[b], E. M. Kaiin[c], M. Gunawan[c], & B. Purwantara[b,*]
[a]Study Program of Reproductive Biology, Graduate School, IPB University
[b]Division of Reproduction and Obstetrics, Department of Veterinary Clinic, Reproduction, and Pathology, School of Veterinary Medicine and Biomedical Sciences, IPB University
Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia
[c]Research Center for Applied Zoology, National Research and Innovation Agency
Jalan Raya Jakarta-Bogor KM 46, Cibinong, Bogor 16911, West Java, Indonesia
*Corresponding author: purwantara@apps.ipb.ac.id
(Received 09-11-2021; Revised 02-02-2022; Accepted 14-02-2022)

ABSTRACT

This study aims to determine the effect of the age difference of Simmental bulls on motion characteristics, capacitation status, and DNA fragmentation of post-thawing sperms. The frozen semen used was collected from twelve bulls, which were divided into four groups of age, which include a group of two, four, ≥ 10 years old with high semen rejection (≥ 10 HR), and ≥ 10 years old with low semen rejection (≥ 10 LR). Computer Assisted Sperm Analysis (CASA) was used to determine sperm motion characteristics, capacitation status by chlortetracycline (CTC) staining, plasma membrane integrity, and viability using eosin-nigrosine staining. In contrast, the DNA fragmentation index was determined using the Sperm-Bos-halomax kit. The results showed that the four year old group had a higher total and progressive motility percentage than the others ($p<0.05$). In all groups, there was no significant difference among sperm kinematics such as VAP, VSL, VCL, STR, and ALH. However, the LIN, WOB, and BCF of the ≥ 10 HR year old groups were significantly lower ($p<0.05$) than those of the other groups. However, un-capacitated sperm was higher ($p<0.05$) in the two years and four years old groups compared to the ≥ 10 years old, while the four years old group had lower capacitated and acrosome-reacted ($p<0.05$) than the other groups. Furthermore, the sperm membrane integrity, viability, and DNA fragmentation index of the ≥ 10 years old groups were significantly higher ($p<0.05$) than those of the other groups. The research concludes that aging in the Simmental bull affects motion characteristics, capacitation status, and DNA fragmentation of post-thawing sperm. However, the semen rejection rate in the older bull did not directly affect the post-thawing sperm quality.

Keywords: bull age; capacitation; DNA fragmentation; sperm motility

INTRODUCTION

Artificial insemination (AI) technology is widely used in the cattle farming industry. According to Rahman *et al.* (2017), approximately 70% of the cattle farming industry uses AI for breeding globally. Bull fertility is an essential factor in the success of AI-assisted breeding programs; however, its incidence is responsible for 50% of the total failures in animal breeding (Park *et al.*, 2012; Rahman *et al.*, 2017). The bull's age affects fertility as the rates decrease with age (Collins *et al.*, 1962; Bhakat *et al.*, 2011) because the bull has a peak age for obtaining the best fertility. Collins *et al.* (1962) reported that Holstein and Guernsey's bulls have the highest fertility at 3-4 years old, while Bhakat *et al.* (2011) stated that the best semen quality in Sahiwal bull occurred for almost 5 years. A previous study has shown that fertility decreases slowly after passing the peak age (Collins *et al.*, 1962; Bhakat *et al.*, 2011). Collins *et al.* (1962) also added that the non-return rate decreased in Holstein bull by 0.31% and in Guernsey bull by 0.5% per year after passing the highest fertility age. An increase in age correlates with the decreased reproductive function (Johnson *et al.*, 1990; Belloc *et al.*, 2014), which is associated with oxidative stress (Gunes *et al.*, 2016; Bhanmeechao *et al.*, 2018; Aitken, 2020). Clinically, aging in bulls leads to the increased follicle-stimulating hormone (FSH) and the decreased serum testosterone (Johnson *et al.*, 1990). Furthermore, bull's aging also causes degeneration of the seminiferous tubules and reduces the number of Leydig cells, Sertoli cells, and germ cells (Jiang *et al.*, 2013). These conditions can interfere with the process of spermatogenesis (Zhao *et al.*, 2019; Tesi *et al.*, 2020) and affect semen quality (Carreira *et al.*, 2007; Bhakat *et al.*, 2011; Belloc *et al.*, 2014; Kipper *et al.*, 2016).

Oxidative stress occurs due to the overproduction of reactive oxygen species (ROS) and the decreased antioxidant production capacity (Gunes *et al.*, 2016). Meanwhile, the presence of oxidative stress can cause an

increase in lipid peroxidation (Gunes *et al.*, 2016; Dutta *et al.*, 2019) which disrupts plasma membrane permeability and mitochondrial dysfunction. These conditions cause a decrease in ATP production and efflux, which influences the failure of sperm motility (Dutta *et al.*, 2019). Sloter *et al.* (2006) also showed that an increase in male age positively correlated with a decrease in several sperm motility parameters each year, such as total and progressive motility, as well as sperm kinematics. Moreover, sperm kinematics describes spermatozoa's movement pattern, which is assessed based on the speed of sperm motility, velocity ratio, and characteristics of head vibration (Kathiravan *et al.*, 2011).

A previous study has reported that impaired membrane permeability leads to an uncontrolled calcium influx that initiates the occurrence of early sperm capacitation (Rahman *et al.*, 2014; Ostermeier *et al.*, 2018). Sperm capacitation is the main requirement for fertilizing oocytes (Jin & Yang, 2017); however, the sperms lose their viabilities before reaching the site of fertilization when capacitation occurs early (Dalal *et al.*, 2019).

DNA integrity is an essential parameter for evaluating sperm quality because it carries genetic information from the bulls (Bhanmeechao *et al.*, 2018; Fuente-Lara *et al.*, 2019). Sperm DNA fragmentation is caused by oxidative stress (Amaral *et al.*, 2013; Gunes *et al.*, 2016), associated with aging in bulls (Balic *et al.*, 2012; Belloc *et al.*, 2014) and also has a significant impact on fertility. Gunes *et al.* (2016) and Bhanmeechao *et al.* (2018) showed that sperm DNA fragmentation affected fertilization, pregnancy, and fetal development. However, only a few reports on the sperm motion characteristics, capacitation status, and DNA fragmentation index of the Simmental bulls with different ages in Indonesian National Artificial Insemination Centers (NAIC). Therefore, this study aims to identify the effect of age on the sperm motion characteristics, capacitation status, and DNA fragmentation index of Simmental bulls. The results are expected to be one of the primary references in determining bull selection and culling policies in Indonesian NAIC in the future.

MATERIALS AND METHODS

Ethical Approval

The study did not involve the handling of a live animal. Frozen semen of Simmental bull was produced by one of the AI Centers in Indonesia. The semen collection and processing in the AI Center were conducted based on the standard operating procedure, namely SNI ISO 9001: 2015 No. 824 100 16072, and under supervision by a veterinarian. Moreover, the semen collection uses an artificial vagina by an experienced bull master technician.

Experimental Design

This study used frozen semen of Simmental bull with a skim-milk extender. The frozen semen from twelve bulls was obtained from two, four, ≥ 10 years old with > 30% of total semen rejection (high semen rejection; ≥ 10 HR), and ≥ 10 years old with < 30% of total semen rejection (low semen rejection; ≥ 10 LR). The age grouping of bulls was based on the secondary data on semen rejection and production from January to December 2020 (Table 1).

Sperm Motion Characteristic Evaluation

Sperm motion characteristics were evaluated using computer-assisted sperm analysis (CASA SpermvisionTM 3.7 Minitub, Germany) under Olympus BX 51 microscope (Olympus, Tokyo, Japan) on a heating stage (37 °C). Subsequently, the frozen semen was thawed at 37 °C for 30 seconds, and 10 uL of sperm samples were diluted with 90 uL of prewarmed physiological saline solution. Sperm samples aliquot (10 μL) was placed on the glass slide and then covered with a prewarm (37 °C) coverslip. The microscope was set with a 20x objective, and the camera operates at 60 frames per second. The CASA was also developed to evaluate the particle size range of 10-200 μm^2 and 50-250 sperm per field. Sperm motion characteristics were determined by the sperm's total motility, progressive motility, and sperm kinematics. The sperm kinematics was defined based on average path velocity (VAP: μm/s), straight-line velocity (VSL: μm/s), curvilinear velocity (VCL: μm/s), linearity (LIN; VSL.VCL), wobble (WOB; VAP/VCL), straightness (STR; VSL/VAP), the amplitude of lateral head displacement (ALH: μm), and the beat cross frequency (BCF: Hz) (Valverde *et al.*, 2020).

Capacitation Status Determination

Sperm capacitation status was determined using *chlortetracycline* (CTC) assay, a modified method by Fraser *et al.* (1995). A total of 750 mM CTC (Sigma,

Table 1. Semen rejection and average total semen production per year of Simmental bull with different ages from January to December 2020

Bull's age (years)	Total of semen collection (times/year)*	Semen rejection**	Processed**	Average total semen production per year (straw/year)
2	249	21 (9.32±7.53[a])	228 (90.68±7.53[a])	15.345±5.214[a]
4	274	37 (13.26±4.39[a])	237 (86.74±4.39[a])	24.125±4.021[a]
≥ 10 HR	243	94 (35.79±14.41[b])	149(64.21±1442[b])	12.558±1.148[b]
≥ 10 LR	251	38 (15.03±8.96[a])	213 (84.97±8.96[a])	18.558±2.987[a]

Note: *The data were obtained from three bulls of each group. **The data were presented by number of semen collections (the mean of percentage ± standard deviation). Data of average total semen production per year was presented by the mean of frozen semen production per year ± standard deviation. HR (High semen rejection rate) and LR (Low semen rejection rate). [a,b] Means in the same column with different superscripts differ significantly ($p<0.05$).

C4881) and 5 mM l-cysteine (Sigma, C7352) were diluted in a buffer medium containing 130 mM NaCl (Sigma, S9888) and 20 mM tris-HCl (Sigma, T5941) (pH: 7.8). The CTC solution was prepared daily, stored at 4 °C, and covered with aluminum foil. Sperm aliquots of 50 mL were mixed with 200 mL of the CTC solution and incubated at 38.5 °C and 5% CO_2 for seven minutes. The solution (15 mL) were mixed with 10 mL of fixative solution (12.5% (w/v) paraformaldehyde (Sigma, P6148) and 0.5 M tris-HCl (Sigma, T5941, pH 7.4). Subsequently, the sample solution (10 mL) was pipetted on a glass slide containing an 8 mL antifade and covered by coverslips. The slide can be stored at 4 °C in the dark and evaluated 24 hours. A total of 200 sperm cells were evaluated under Zeiss Confocal Laser Scanning Microscope 710 with an excitation filter of 400-440 nm and an emission filter of 470 nm. Sperm capacitation status was determined using the CTC patterns, which were F pattern for uncapacitated sperm, B pattern for capacitated sperm, and AR pattern for acrosome-reacted sperm.

Sperm Plasma Membrane Integrity and Viability Analysis

Sperm plasma membrane integrity was analyzed using the hypoosmotic swelling test (HOS-test) method. The HOS solution contains 0.179 g sodium chloride (Sigma, S9888), 0.0737 g tris-sodium citrate dihydrate (Merck, A0533848 304), 0.135 g fructose (Merck, K43171807 303) in 100 mL aquabidest. A total of 50 µL semen were mixed in 500 µL HOS solution and incubated at 37 °C for 30 minutes. Semen aliquots (10 µL) were placed on the glass slide and covered by coverslips. The slides were further evaluated using a 40x objective under a light microscope (Olympus CK2, ULWCD 0.30). Subsequently, the swelling ability of 200 sperm in HOS was assessed and those with an intact plasma membrane have coiling of the tail (swollen tail) (Ahmad *et al.*, 2011).

The sperm viability was assessed using 1% nigrosine-eosin staining, a modified method by Ahmad *et al.* (2011). A smear slide was prepared by mixing the semen and dye in a ratio of 1:4. After air-drying the slide, 200 sperm cells were observed under a light microscope (Olympus CK2, ULWCD 0.30) at 40x objective for unstained (life) heads and stained/partially stained heads of sperm (dead).

Sperm DNA Fragmentation Analysis

Sperm DNA fragmentation was assessed using the Sperm-Bos-halomax® kit (Halotech DNA, SL; Campus de Cantoblaco, Madrid, Spain) and the procedure was carried out based on the manufacturer's instructions. A total of 15 million sperm/mL was mixed with the solvent and agarose gel was diluted at 95-100 °C for five minutes and equilibrated at 37 °C for five minutes. Furthermore, 25 µL of sperm was mixed with 50 µL of agarose solution while the aliquots solution (2 µL) was pipetted onto coated slides, covered by coverslips, and incubated at 4 °C for five minutes. The coverslip was separated from the slide and incubated using a lysis solution for five minutes. The slide was washed with distilled water, dehydrated with 70% and 100% alcohol for two minutes. The preparation was further stained with Wright's eosin methylene blue solution. After air-drying the slide, 200 sperm cells were observed under a light microscope (Olympus CK2, ULWCD 0.30) at 40x objective for a large halo and dispersion, which were sperm with DNA fragmentation and a small and compact halo of sperm head or sperm with intact DNA (Fernandez *et al.*, 2003).

Data Analysis

This study consisted of three bulls, and each was repeated five times for the total repetitions in one treatment to be 15 replications. The data obtained were presented by the mean ± standard deviation and analyzed using one-way analysis of variance (ANOVA) at $p<0.05$. Duncan's Multiple Range Test (DMRT) was carried out when there were any significant differences among the ages, and the data were estimated using IBM® SPSS® Statistics version 24.0 (IBM Corp., Armonk, NY, US).

RESULTS

Figure 1 showed that the four-year-old groups had a higher ($p<0.05$) total and progressive motility percentage than the other groups. Furthermore, there were no significant differences in the total and progressive motility percentage between the ≥ 10 years old and the other groups. The post-thawing sperm kinematics of Simmental bulls of different ages was shown in Table 2. Sperm kinematics such as VAP, VSL, VCL, STR, and ALH were also not different in all groups. However, the LIN and WOB of the ≥ 10 HR years old group were significantly lower ($p<0.05$) than the four years old group and showed no difference compared to the ≥ 10 LR years old group. The BCF of the ≥ 10 HR-year-old groups was lower ($p<0.05$) than the other groups.

Sperm capacitation status was described by the difference in the distribution pattern of the fluorescent CTC on the head of the sperm (Figure 2). Based on Figure 3, the un-capacitated sperm were significantly higher ($p<0.05$) in the two and four years old groups than in the ≥ 10 years old groups. However, the four-year-old group had lower capacitated and acrosome-reacted ($p<0.05$) than the other groups. The percentages of sperm plasma membrane integrity and viability were significantly lower ($p<0.05$) in the ≥ 10 years old groups than in the others (Figure 4). The sperm DNA fragmentation was also characterized by forming a halo on the head of the sperm (Figure 5). Furthermore, the sperm DNA fragmentation index was significantly higher ($p<0.05$) in the ≥ 10 years old groups compared to the other groups (Figure 6).

DISCUSSION

The result showed that the bull's age could affect the post-thawing semen quality in Simmental bulls. Furthermore, the four-year-old group had the highest percentage of total and progressive motility compared

Table 2. Post thawing sperm kinematics of Simmental bull with different ages

Bull's age (years)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	LIN (VSL/VAP)	WOB (VAP/VCL)	STR (VSL/VAP)	ALH (μm)	BCF (Hz)
2	86.25±14.34	67.41±12.37	123.89±23.01	0.54±0.02^{ab}	0.70±0.02^{ab}	0.77±0.03	4.62±0.88	32.83±2.63^{a}
4	87.09± 8.69	69.05± 8.28	124.26±14.25	0.55±0.03^{b}	0.70±0.03^{a}	0.79±0.03	4.82±0.64	32.65±3.56^{a}
≥ 10 HR	81.69±11.95	63.96±10.22	121.28±19.54	0.52±0.03^{a}	0.67±0.02^{c}	0.78±0.04	4.70±0.62	28.58±6.69^{b}
≥ 10 LR	85.65±12.63	68.86±12.38	125.36±16.78	0.54±0.04^{ab}	0.68±0.03^{bc}	0.80±0.03	4.43±0.75	33.04±2.92^{a}
p Value	0.624	0.560	0.388	0.151	0.005	0.237	0.520	0.016

Note: VCL (Curve linear velocity), VAP (Average path velocity), VSL (Straight line velocity), LIN (Linearity=VSL/VCL), WOB (Wobble=VAP/VCL), STR (Straightness), BCF (Beat cross frequency), ALH (Amplitude of lateral head movement), HR (High semen rejection rate), and LR (Low semen rejection rate). The data were presented by the mean ± standard deviation. ^{a,b,c} Means in the same column with different superscripts differ significantly ($p<0.05$).

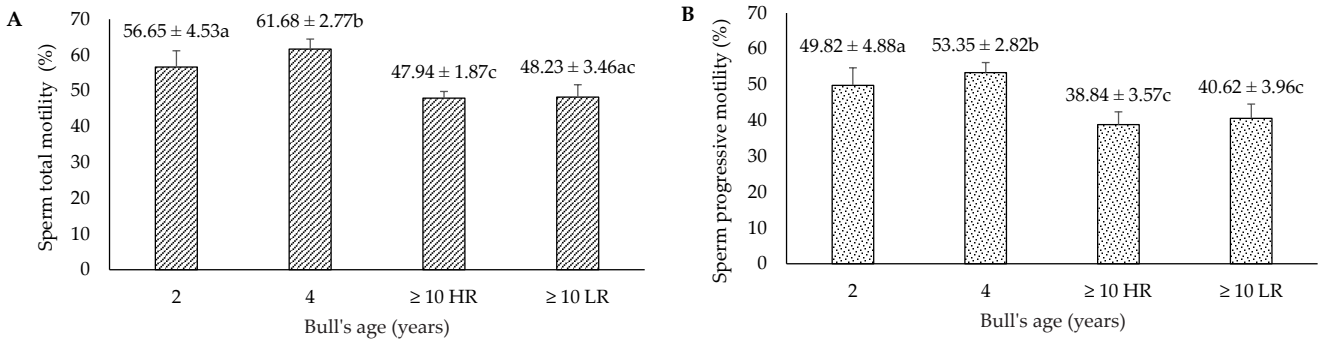

Figure 1. Post thawing sperm total motility (A) and progressive motility (B) of Simmental bull with different ages. The data were shown as the mean ± standard deviation. The symbols (a, b, and c) were indicated a significance difference with $p<0.05$.

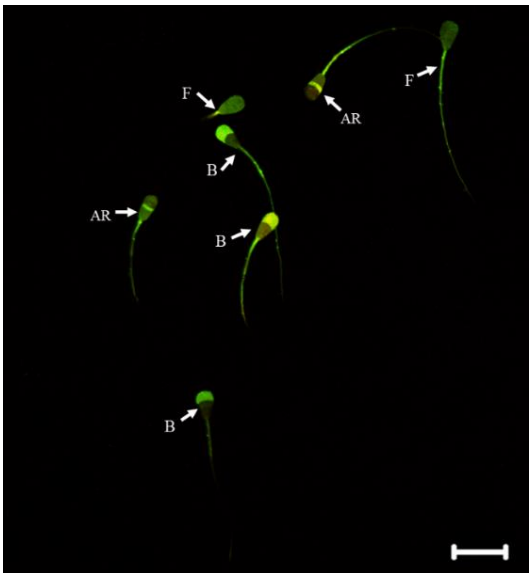

Figure 2. Fluorescent patterns of sperm stained with CTC. F pattern (un-capacitated sperm), B pattern (capacitated sperm), and AR pattern (acrosome-reacted sperm). Scale bar: 10 μm.

to the other groups. The high percentage of motility is associated with the high ability of mitochondria to produce ATP, which supports the movement of sperm flagella (Hallap *et al.*, 2004; Carreira *et al.*, 2007). It was also shown that sperm are directly dependent on the availability of sufficient energy to move. This energy is obtained by dynein ATPase hydrolysis of ATP along the axenome-microtubule pathway (Piomboni *et al.*, 2012; Tourmente *et al.*, 2015). Meanwhile, previous studies have shown that bovine sperm provide their energy requirements via two major metabolic pathways, namely glycolysis and oxidative phosphorylation/ OXPHOS (Moraes & Meyers, 2018; Piomboni *et al.*, 2012; Tourmente *et al.*, 2015; Magdanz *et al.*, 2019). The products of ATP formation via these two pathways are transferred to microtubules to regulate sperm motility (Oberoi *et al.*, 2014). However, aging in bulls causes mitochondrial dysfunction (Amaral *et al.*, 2013; Oberoi *et al.*, 2014), which disrupts the OXPHOS pathway (Amaral *et al.*, 2013) and reduces ATP production (Amaral *et al.*, 2013; Ryu *et al.*, 2019), causing sperm motility failure (Hallap *et al.*, 2004; Carreira *et al.*, 2007). This study also obtained similar results, where the two groups of ≥ 10 years old had the same percentage of total and progressive motility but appeared the lowest compared to the other age groups.

Based on sperm kinematics parameters, the two groups of ≥ 10 years old showed the presence of hyperactive motility that was too early. Sperm hyperactivity is non-progressive, non-linear, and vigorous movements (Kathiravan *et al.*, 2011). According to Hallap *et al.* (2004), hyperactive motility is characterized by low LIN values and progressive motility, and high VCL values. Although the VCL values in this study were the same, the groups ≥ 10 years old had lower LIN values and progressive motility than the other groups. Furthermore, the prevalence of hyperactivity is characterized by low WOB values (Shojaei *et al.*, 2012), which indicates that the sperm are experiencing irregular vibrations. The WOB value also indicates a repetitive motion (vibration) level until the sperm returns to a stationary position (Valverde *et al.*, 2020). The irregular movement patterns can also be seen in the low BCF value that indicates

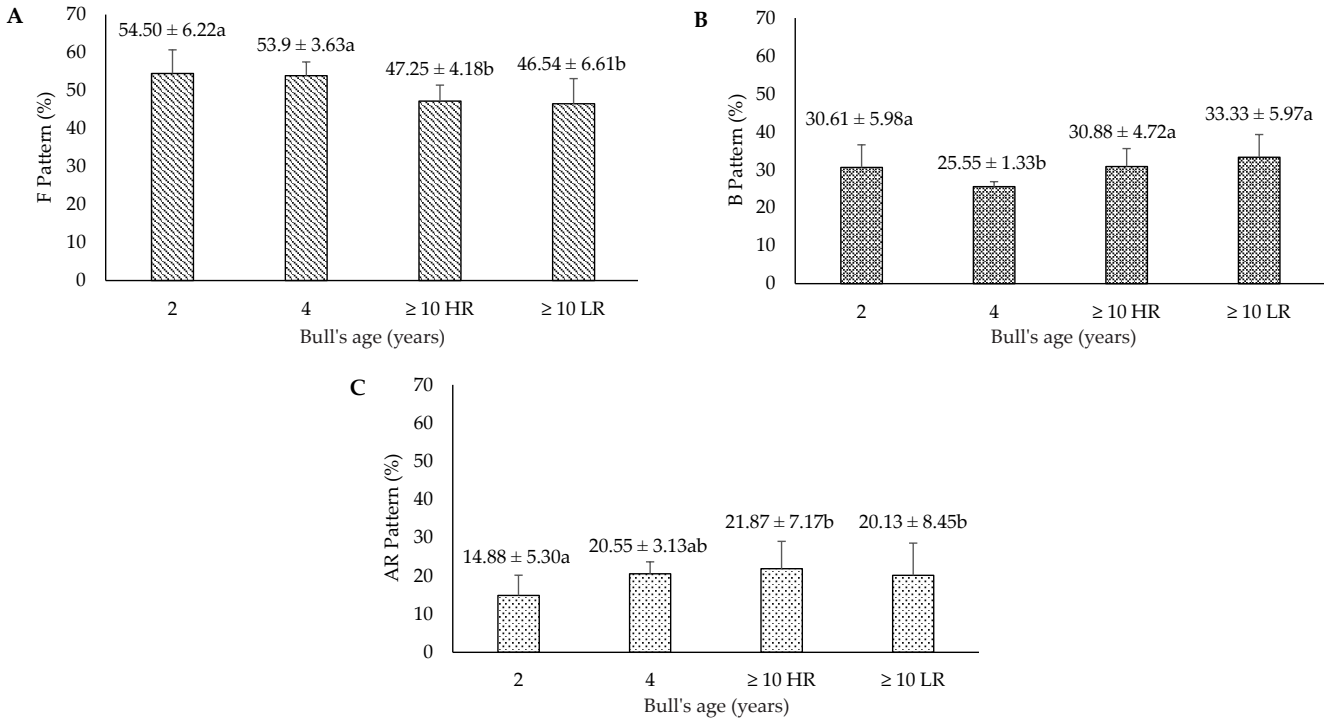

Figure 3. Percentage of post-thawing sperm capacitation status of Simmental bull with different ages. F pattern (un-capacitated sperm; A), B pattern (capacitated sperm; B), and AR pattern (acrosome-reacted sperm; C). The data were shown as the mean ± standard deviation. The symbols (a and b) were indicated a significance difference with $p<0.05$.

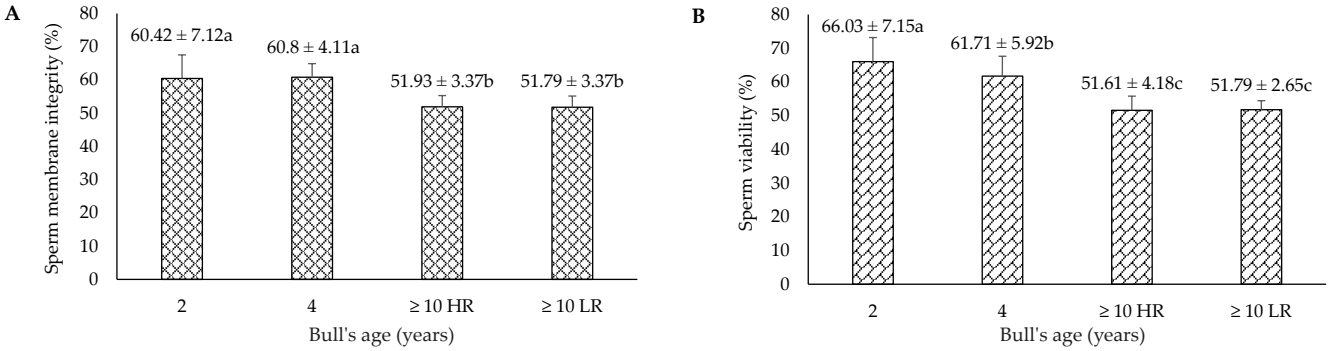

Figure 4. Post thawing sperm membrane integrity (A) and viability (B) of Simmental bull with different ages. The data were shown as the mean ± standard deviation. The symbols (a, b, and c) were indicated a significance difference with $p<0.05$.

the average frequency of sperm passing through the track (Oliveira *et al.,* 2013). According to Perumal *et al.* (2014), the LIN and BCF values are used to determine whether sperm moves linearly progressively or straight ahead regularly. Sperm hyperactivation occurs due to uncontrolled intracellular calcium influx (Rahman *et al.,* 2014; Ostermeier *et al.,* 2018). Pereira *et al.* (2017) also stated that low intracellular calcium influx caused the flagellar movement to be symmetrical. However, when it is excessive, it can lead to asymmetric movement. Uncontrolled intracellular calcium influx occurs due to the disintegration of the sperm plasma membrane (Rahman *et al.,* 2014; Ostermeier *et al.,* 2018). This is similar to the results, which showed that the two groups of ≥ 10 years old had the lowest plasma membrane integrity compared to the other groups. Plasma membrane disintegration occurs due to lipid peroxidation from sperm oxidative stress, associated with aging (Amin *et al.,* 2018; Dalal *et al.,* 2019). Oxidative stress occurs due to ROS overproduction and the decreased production of antioxidants because of mitochondrial dysfunction in older animals (Amaral *et al.,* 2013).

Hyperactive motility is the main characteristic of sperm that has passed through capacitation (Rahman *et al.,* 2014; Ostermeier *et al.,* 2018). Sperm capacitation is initiated by intracellular calcium influx, which activates the second messenger (sAC), causing it to produce cAMP and activate protein kinase A (PKA). The PKA also stimulates tyrosine kinase during the inhibition of serine/tyrosine phosphatase, which leads to an increase in protein phosphorylation (Jin & Yang, 2017; Pereira *et al.,* 2017; Ostermeier *et al.,* 2018). However, the uncontrolled calcium influx is due to the disintegration of plasma membranes in older bulls (Rahman *et al.,* 2014; Ostermeier *et al.,* 2018), which causes sperm premature capacitation and acrosome reaction (Thundathil *et al.,* 1999; Ostermeier *et al.,* 2018). In the present study, the groups of ≥ 10 years old had the highest sperm

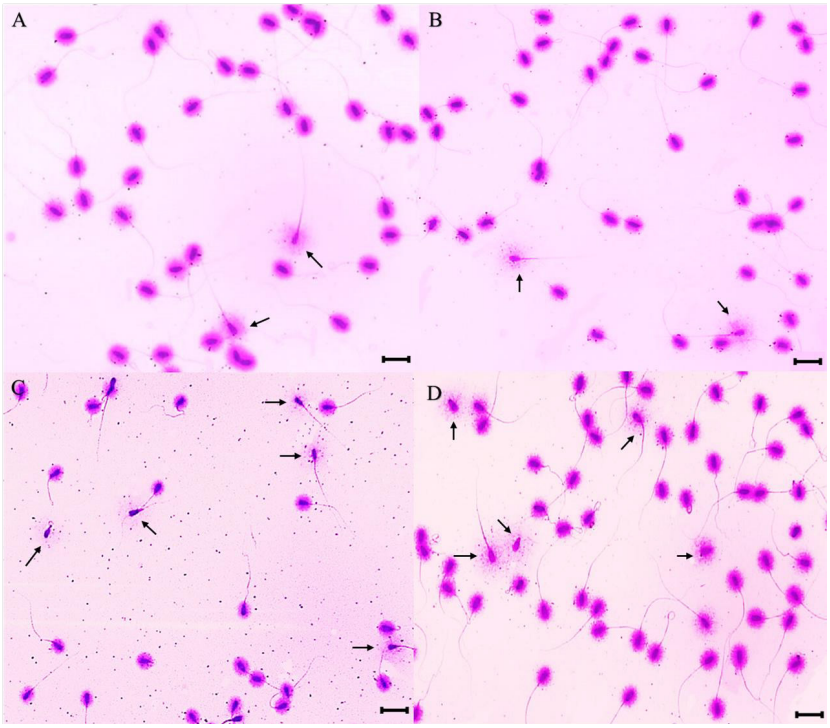

Figure 5. Post-thawing sperm DNA fragmentation of simmental bull with different ages. Black arrow means sperm with DNA fragmentation (large halo and dispersion) and without black arrow means sperm with intact DNA (small and compact halo). A) 2 years old, B) 4 years old, C) ≥ 10 years old with high semen rejection, and D) ≥ 10 years old with low semen rejection. Scale bar: 10 μm.

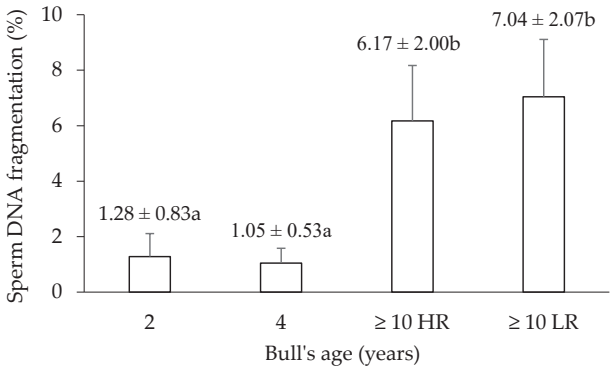

Figure 6. Percentage of post-thawing sperm DNA fragmentation of Simmental bull with different ages. The data were shown as the mean ± standard deviation. The symbols (a and b) were indicated a significance difference with $p<0.05$.

capacitation and acrosomal reactions compared to the other groups. Sperm premature capacitation depletes the energy and causes sperm's death before fertilizing the oocyte (Thundathil *et al.*, 1999). This incident is also supported by results, which showed that the groups of ≥ 10 years old had the lowest sperm viability compared to the other groups; therefore, the viability and fertility of the sperm's premature capacitation is low (Thundathil *et al.*, 1999). Aslam *et al.* (2018) also stated that a high percentage of capacitation and acrosome-reacting sperm had a low fertilization success rate.

The mitochondrial dysfunction due to aging in bulls (Amaral *et al.*, 2013; Oberoi *et al.*, 2014) caused irregular electron leakage from the mitochondria (Amaral *et al.*, 2013; Aitken, 2020). The main product of mitochondrial leakage is the superoxide anion ($O2.^-$) which is dismutated to hydrogen peroxide under the control of superoxide dismutase (SOD), causing the excess of ROS productiom (Amaral *et al.*, 2013; Aitken, 2020). However, the decreased mitochondrial function reduces the production of its antioxidants, such as glutathione peroxidase and superoxide dismutase (Ozkosem *et al.*, 2015). Oxidative stress also occurs due to an imbalance between the production of oxidants and antioxidants, while sperm DNA fragmentation is a form of oxidative damage caused by oxidative stress (Amaral *et al.*, 2013; Aitken, 2020). Previous studies have shown that aging is molecularly linked to DNA repair pathways, specifically a decrease in the base excision repair (BER) pathway, which leads to the inadequate repair of 8-oxodG lesions in germ cells and sperm. The lesion decreased the capacity to repair DNA damage of germ cells and caused the high production of sperm with DNA fragmentation (Paul *et al.*, 2011). This study also obtained similar results, where the groups of ≥ 10 years had the highest level of DNA fragmentation compared to the other groups.

Semen rejection is associated with low sperm freeze ability due to high cryodamage (Rego *et al.*, 2016; Nagata *et al.*, 2019), while cryodamage is caused by the increased oxidative stress of spermatozoa (Nagata *et al.*, 2019; Peris-Frau *et al.*, 2020). The oxidative stress that occurs during the cryopreservation to thawing process influences cellular changes such as membrane destabilization, loss of cholesterol, and decreased fluidity, which makes it susceptible to cryodamage in spermatozoa (Castro *et al.*, 2016; Amin *et al.*, 2018; Nagata *et al.*,

2019; Peris-Frau *et al.,* 2020). Bull aging also causes the increased production of ROS that can initiate oxidative stress (Gunes *et al.,* 2016; Ahmed *et al.,* 2018; Aitken, 2020), which can exacerbate the occurrence of cryodamage in old bull sperm. High sperm cryodamage was shown in both groups aged over ten years with premature capacitation and acrosomal reaction, hyperactive motility, low membrane integrity and sperm viability, and a high percentage of spermatozoa DNA fragmentation. Aslam *et al.* (2018) stated that the high incidence of cryodamage can cause changes in various parameters of sperm function, such as the high incidence of premature capacitation and acrosomal reactions. Further study is recommended to evaluate the *in vitro* or *in vivo* fertility rates in all age groups and determine the protein profile expressed in different age groups

CONCLUSION

This study showed that aging in Simmental bull affects motion characteristics, capacitation status, and DNA fragmentation of post-thawing sperm. Furthermore, the semen rejection rate in the old bull does not directly affect the post-thawing sperm quality. This showed that the Simmental bull could be culled at least ten years old with a high or low semen rejection rate.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENT

This study was funded and supported by the Ministry of Research and Technology/ National Research and Innovation Agency of the Republic of Indonesia through PMDSU program Grant Number 1/E1/ KP.PTNBH/2021.

REFERENCES

Ahmad, E., N. Ahmad, Z. Naseer, M. Aleem, M. S. Khan, M. Ashiq, & M. Younis. 2011. Relationship of age to body weight, scrotal circumference, testicular ultrasonograms, and semen quality in Sahiwal bulls. Trop. Anim. Health. Prod. 43:159-164. https://doi.org/10.1007/s11250-010-9668-1

Ahmed, S., M. I-ur-R. Khan, M. Ahmad, & S. Iqbal. 2018. Effect of age on lipid peroxidation of fresh and frozen-thawed semen of Nili-Ravi buffalo bulls. Ital. J. Anim. Sci. 17:730-735. https://doi.org/10.1080/1828051X.2018.1424569

Aitken, R. J. 2020. Impact of oxidative stress on male and female germ cells: implications for fertility. Reproduction 159:189-201. https://doi.org/10.1530/REP-19-0452

Amaral, S., A. Amaral, & J. Ramalho-Santos. 2013. Aging and male reproductive function: a mitochondrial perspective. Front. Biosci. (Schol. Ed.). 5:181–197. https://doi.org/10.2741/S365

Amin, B. Y., J. K. Prasad, S. K. Ghosh, S. A. Lone, A. Kumar, A. R. Mustapha, O. Din, & A. Kumar. 2018. Effect of various levels of dissolved oxygen on reactive oxygen species and cryocapacitation-like changes in bull sperm. Reprod. Domest. Anim. 53:1-8. https://doi.org/10.1111/rda.13200

Aslam, M. K. M., V. K. Sharma, S. Pandey, A. Kumaresan, A. Srinivasan, T. K. Datta, T. K. Mohanty, & S. Yadav. 2018. Identification of biomarker candidates for fertility in spermatozoa of crossbred bulls through comparative proteomics. Theriogenology 119:43-51. https://doi.org/10.1016/j.theriogenology.2018.06.021

Balic, I. M., S. Milinkuvic-tur, M. Samardzija, & S. Vince. 2012. Effect age and environmental factors on semen quality, glutathione peroxidase activity and oxidative parameters in simmental bulls. Theriogenology 78:423-431. https://doi.org/10.1016/j.theriogenology.2012.02.022

Belloc, S., A. Hazouta, A. Zini, P. Merviel, R. Cabry, H. Chahine, H. Copin, & M. Benkhalifa. 2014. How to overcome male infertility after 40: Influence of paternal age on fertility. Maturitas 78:22–29. https://doi.org/10.1016/j.maturitas.2014.02.011

Bhakat, M., T. K. Mohanty, V. S. Raina, A. K. Gupta, H. M. Khan, R. K. Mahapatra, & M. Sarkar. 2011. Effect of age and season on semen quality parameters in Sahiwal bulls. Trop. Anim. Health. Prod. 43:1161–1168. https://doi.org/10.1007/s11250-011-9817-1

Bhanmeechao, C., S. Srisuwatanasagul, & S. Ponglowhapan. 2018. Age-related changes in interstitial fibrosis and germ cell degeneration of the canine testis. Reprod. Domest. Anim. 53:37–43. https://doi.org/10.1111/rda.13354

Carreira, J. T., J. T. Trevizan, I. R. Carvalho, B. Kipper, L. H. Rodrigues, C. Silva, S. H. V. Perri, J. R. drevet, & M. B. Koivisto. 2007. Does sperm quality and DNA integrity differ in cryopreserved semen samples from young, adult, and aged Nellore bulls?. Basic. Clin. Androl. 27:12. https://doi.org/10.1186/s12610-017-0056-9

Castro, L. S., T. R. S. Hamilton, C. M. Mendes, M. Nichi, V. H. Barnabe, J. A. Visintin, & M. E. O. A. Assumpção. 2016. Sperm cryodamage occurs after rapid freezing phase: Flow cytometry approach and antioxidant enzymes activity at different stages of cryopreservation. J. Anim. Sci. Biotechnol. 7:17. https://doi.org/10.1186/s40104-016-0076-x

Collins, W. E., E. K. Inskeep, W. H. Dreher, W. J. Tyler, & L. E. Casida. 1962. Effect of age on fertility of bulls in artificial insemination. J. Dairy. Sci. 45:1015-1018. https://doi.org/10.3168/jds.S0022-0302(62)89545-9

Dalal, J., P. Kumar, R. K. Chandolia, S. Pawaria, R. Rajendran, S. Sheoran, J. Andonissamy, & D. Kumar. 2019. A new role for RU486 (mifepristone): It protects sperm from premature capacitation during cryopreservation in buffalo. Sci. Rep. 9:6712. https://doi.org/10.1038/s41598-019-43038-4

Dutta, S., A. Majzoub, & A. Agarwal. 2019. Oxidative stress and sperm function: A systematic review on evaluation and management. Arab. J. Urol. 17:87-97. https://doi.org/10.1080/2090598X.2019.1599624

Fernandez, J. L., L. Muriel, M. T. Rivero, V. Goyanes, R. Vazquez, & J. G. Alvarez. 2003. The sperm chromatin dispersion test: A simple method for the determination of sperm DNA fragmentation. J. Androl. 24:59-66.

Fraser, L. R., L. R. Abeydeera, & K. Niwa. 1995. Ca^{2+} regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. Mol. Reprod. Dev. 40:233-241. https://doi.org/10.1002/mrd.1080400213

Fuente-Lara, A., A. Hesser, B. Christensen, K. Gonzales, & S. Meyers. 2019. Effects from aging on semen quality of fresh and cryopreserved semen in Labrador Retrievers. Theriogenology 132:164-171. https://doi.org/10.1016/j.theriogenology.2019.04.013

Gunes, S., G. N. T. Hekim, M. A. Arslan, & R. Asci. 2016. Effects of aging on the male reproductive system. J. Assisted Reprod. Genet. 33:441-454. https://doi.org/10.1007/s10815-016-0663-y

Hallap, T., M. Haard, U. Jaakma, B. Larsson, & H. Rodriguez-Martinez. 2004. Variations in quality of frozen–thawed semen from Swedish red and white AI sires at 1 and 4 years of age. Int. J. Androl. 27:166-171. https://doi.org/10.1111/j.1365-2605.2004.00470.x

Jiang, H., W-J. Zhu, J. Li, Q-J. Chen, W-B. Liang, & Y-Q. Gu. 2013. Quantitative histological analysis and ultrastructure of the aging human testis. Int. Urol. Nephrol. 46:879-885. https://doi.org/10.1007/s11255-013-0610-0

Jin, S-K. & W-X. Yang. 2017. Factors and pathways involved in capacitation: How are they regulated?. Oncotarget. 8:3600-3627. https://doi.org/10.18632/oncotarget.12274

Johnson, L., J. S. Grumbles, A. Bagheri, & C. S. Petty. 1990. Increased germ cell degeneration during postprophase of meiosis is related to increase serum follicle-stimulating hormone concentration and reduce daily sperm production in aged men. Biol. Reprod. 42:281-287. https://doi.org/10.1095/biolreprod42.2.281

Kathiravan, P., J. Kalatharan, G. Karthikeya, K. Rengarajan, & G. Kadirvel. 2011. Objective sperm motion analysis to assess dairy bull fertility using computer-aided system: A review. Reprod. Domest. Anim. 46:165-172. https://doi.org/10.1111/j.1439-0531.2010.01603.x

Kipper, B. H., J. T. Trevizan, J. T. Carreira, I. R. Carvalho, G. Z. Mingoti, M. E. Beletti, S. H. V. Perri, D. A. Franciscato, J. C. Pierucci, & M. B. de Koivisto. 2016. Sperm morphometry and chromatin condensation in Nelore bulls of different ages and their effects on *in vitro* fertilization. Theriogenology 87:154-160. https://doi.org/10.1016/j.theriogenology.2016.08.017

Magdanz, V., S. Boryshpolets, C. Ridzewski, B. Eckel, & K. Reinhardt. 2019. The motility-based swim-up technique separates bull sperm based on differences in metabolic rates and tail length. PLoS ONE 14:e0223576. https://doi.org/10.1371/journal.pone.0223576

Moraes, C. R. & S. Meyers. 2018. The sperm mitochondrion: organelle of many functions. Anim. Reprod. Sci. 194:71-80. https://doi.org/10.1016/j.anireprosci.2018.03.024

Nagata, M. P. B., J. Egashira, N. Katafuchi, K. Endo, K. Ogata, K. Yamanaka, T. Yamanouchi, H. Matsuda, Y. Hashiyada, & K. Yamashita. 2019. Bovine sperm selection procedure prior to cryopreservation for improvement of post-thawed semen quality and fertility. J. Anim. Sci. Biotechnol. 10:91. https://doi.org/10.1186/s40104-019-0395-9

Oberoi, S. L. B., S. V. A. S. Kumar, & C. P. Talwar. 2014. Study of human sperm motility post cryopreservation. Med. J. Armed Forces India. 70:349-353. https://doi.org/10.1016/j.mjafi.2014.09.006

Oliveira, L. Z., R. P. de Arruda, A. F. C. de Andrade, E. C. C. Celeghini, P. D. Reeb, J. P. N. Martins, R. M. dos Santos, M. E. Beletti, R. F. G. Peres, F. M. Monteiro, & V. F. M. H. de Lima. 2013. Assessment of *in vitro* sperm characteristics and their importance in the prediction of conception rate in a bovine timed-AI program. Anim. Reprod. Sci. 137:145-155. https://doi.org/10.1016/j.anireprosci.2013.01.010

Ostermeier, G. C., C. Cardona, M. A. Moody, A. J. Simpson, R. Mendoza, E. Seaman, & A. J. Travis. 2018. Timing of sperm capacitation varies reproducibly among men. Mol. Reprod. Dev. 85:1-10. https://doi.org/10.1002/mrd.22972

Ozkosem, B., S. I. Feinstein, A. B. Fisher, & O'Flaherty. 2015. Advancing age increases sperm chromatin damage and impairs fertility in peroxiredoxin 6 null mice. Redox Biology. 5:15-23. https://doi.org/10.1016/j.redox.2015.02.004

Park, Y-J., W-S. Kwon, S-A. Oh, & M-G. Pang. 2012. Fertility-related proteomic profiling bull spermatozoa separated by percoll. J. Proteome. Res. 11:4162-4168. https://doi.org/10.1021/pr300248s

Paul, C., M. Nagano, & B. Robaire. 2011. Aging results in differential regulation of DNA repair pathways in pachytene spermatocytes in the brown norway rat. Biol. Reprod. 85:1269-1278. https://doi.org/10.1095/biolreprod.111.094219

Pereira, R., R. Sá, A. Barros, & M. Sousa. 2017. Major regulatory mechanisms involved in sperm motility. Asian. J. Androl. 19:5–14.

Peris-Frau, P., A. J. Soler, M. Iniesta-Cuerda, A. Martín-Maestro, I. Sánchez-Ajofrín, D. A. Medina-Chávez, M. R. Fernández-Santos, O. García-Álvarez, A. Maroto-Morales, V. Montoro, & J. J. Garde. 2020. Sperm cryodamage in ruminants: understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. Int. J. Mol. Sci. 21:2781. https://doi.org/10.3390/ijms21082781

Perumal, P., S. K. Srivastava, S. K. Ghosh, & K. K. Baruah. 2014. Computer-assisted sperm analysis of freezable and non-freezable mithun (*Bos frontalis*) semen. Animals 2014:1-6. https://doi.org/10.1155/2014/675031

Piomboni, P., R. Focarelli, A. Stendardi, A. Ferramosca, & V. Zara. 2012. The role of mitochondria in energy production for human sperm motility. Int. J. Androl. 35:109-125. https://doi.org/10.1111/j.1365-2605.2011.01218.x

Rahman, M. S., W-S. Kwon, & M-G. Pang. 2014. Calcium influx and male fertility in the context of the sperm proteome: An update. Biomed. Res. Int. 2014:1-13. https://doi.org/10.1155/2014/841615

Rahman, M. S., W-S. Kwon, & M-G. Pang. 2017. Prediction of male fertility using capacitation-associated proteins in spermatozoa. Mol. Reprod. Dev. 84. https://doi.org/10.1002/mrd.22810

Rego, J. P. A., J. M. Martins, C. A. Wolf, M. van Tilburg, F. Moreno, A. C. Monteiro-Moreira, R. A. Moreira, D. O. Santos, & A. A. Moura. 2016. Proteomic analysis of seminal plasma and sperm cells and their associations with semen freezability in Guzerat bulls. J. Anim. Sci. 94:5308-5320. https://doi.org/10.2527/jas.2016-0811

Ryu, D-Y., W-H. Song, W-K. Pang, S-J. Yoon, M. S. Rahman, & M-G. Pang. 2019. Freezability biomarkers in bull epididymal spermatozoa. Sci. Rep. 9:12797. https://doi.org/10.1038/s41598-019-49378-5

Shojaei, H., T. Kroetsch, R. Wilde, P. Blondin, J. P. Kastelic, & J. C. Thundathil. 2012. Moribund sperm in frozen-thawed semen, and sperm motion end points post-thaw and post-swim-up, are related to fertility in Holstein AI bulls. Theriogenology 77:940-951. https://doi.org/10.1016/j.theriogenology.2011.09.026

Sloter, E., T. E. Schmid, F. Marchetti, B. Eskenazi, J. Nath, & A. J. Wyrobek. 2006. Quantitative effects of male age on sperm motion. Hum. Reprod. 21:2868–2875. https://doi.org/10.1093/humrep/del250

Tesi, M., G. Lazzarini, C. Magliaro, F. Abramo, D. Fanelli, V. Miragliotta, & A. Rota. 2020. Age-related changes of seminiferous tubule morphology, interstitial fibrosis and spermatogenesis in dogs. Anim. Reprod. Sci. 219:106534. https://doi.org/10.1016/j.anireprosci.2020.106534

Thundathil, J., J. Gil, A. Januskauskas, B. Larsson, L. Soderquist, R. Mapletoft, & H. Rodriguez-Martinez. 1999. Relationship between the proportion of capacitated spermatozoa present in frozen-thawed bull semen and fertility with artifcial insemination. Int. J. Androl. 22:366-373. https://doi.org/10.1046/j.1365-2605.1999.00194.x

Tourmente, M., P. Villar-Moya, E. Rial, & E. R. S. Roldan. 2015. Differences in ATP generation via glycolysis and oxidative phosphorylation and relationships with sperm motility in mouse species. J. Biol. Chem. 290:20613-20626. https://doi.org/10.1074/jbc.M115.664813

Valverde, A., V. Barquero, & C. Soler. 2020. The application of computer-assisted semen analysis (CASA) technology to optimise semen evaluation. A review. J. Anim. Feed. Sci. 29:189-198. https://doi.org/10.22358/jafs/127691/2020

Zhao, H., N. Ma, Q. Chen, X. You, C. Liu, T. Wang, D. Yuan, & C. Zhang. 2019. Decline in testicular function in ageing rats: changes in the unfolded protein response and mithocondrial apoptotic pathway. Exp. Gerontol. 127:110271. https://doi.org/10.1016/j.exger.2019.110721