

## RESEARCH ARTICLE

# Functional Variables of Bull Sperm Associated with Cryotolerance

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## Abstract

The objective of this study was to ascertain sperm population and cellular characteristics as well as total antioxidant capacity in spermatozoa from Holstein bulls with Good (11 bulls) and Poor (5 bulls) cryotolerance. Post-thaw sperm kinetics were evaluated using CASA, membrane integrity was assessed via HOS test, and DNA fragmentation was measured using the HaloSperm kit. Data were analyzed using principal component analysis. The spermatozoa from Good bulls had a higher number of cells with intact membranes ( $P=0.029$ ), non-fragmented DNA ( $P=0.018$ ), and post-thaw viability ( $P<0.001$ ) compared to sperm cells from Poor cryotolerance bulls. Sperm cells from Good bulls also had a faster average path velocity ( $P=0.017$ ) and straight-line velocity ( $P=0.036$ ), along with a greater distance average path ( $P=0.006$ ) and distance straight line ( $P=0.011$ ). However, total antioxidant capacity, number of live cells, and other kinetic parameters between spermatozoa from Good and Poor groups were not different. There is no one specific sperm function variable alone that can accurately predict cryotolerance of bull spermatozoa, and thus, a combination of sperm cell attributes and kinematics needs to be utilized by the AI industry in differentiating between freezability of spermatozoa between bulls.

**Keywords:** Sperm cryotolerance, Sperm freezability, Sperm parameters

## Boğa Sperminin Kriyotolerans İle İlişkili Fonksiyonel Değişkenleri

### Öz

Bu çalışmanın amacı, iyi (11 boğa) ve zayıf (5 boğa) kriyotoleransa sahip Holstein boğalardan sperm popülasyonun ve hücresel özelliklerinin yanı sıra toplam antioksidan kapasitesini belirlemektir. Dondurma çözündürme sonrası sperm kinetiği CASA kullanılarak değerlendirildi, membran bütünlüğü HOS testi ile değerlendirildi ve DNA fragmentasyonu HaloSperm kiti kullanılarak ölçüldü. Veriler, temel bileşen analizi kullanılarak değerlendirildi (PCA). İyi kriyotoleransa sahip boğalardan elde edilen spermatozoa, zayıf kriyotoleranslı boğalardan alınan sperme kıyasla daha fazla sayıda intakt membran ( $P=0.029$ ), fragmente olmamış DNA'ya ( $P=0.018$ ) ve dondurma çözündürme sonrası canlılığa ( $P<0.001$ ) sahip hücre bulunmaktadır. İyi kriyotoleransa sahip boğa sperm hücreleri daha fazla ortalama yol ( $P=0.017$ ) ve ileri doğru doğrusal hareket hızına sahiptir ( $P=0.036$ ), bunula birlikte daha büyük bir ortalama mesafe hızı ( $P=0.006$ ) ve ileri doğru doğrusal mesafe hızına ( $P=0.011$ ) sahiptir. Ancak, toplam antioksidan kapasitesi, canlı hücre sayısı ve kinetik parametreler iyi ve zayıf kriyotoleransa sahip spermatozoa grupları arasında farklı değildir. Boğa spermatozoanın kriyotoleransını doğru bir şekilde tahmin edebilecek tek bir spesifik sperm fonksiyon değişkeni yoktur ve bu nedenle, sperm hücresi özelliklerinin ve kinematiklerin bir kombinasyonunun, boğalar arasındaki spermelerin dondurulabilirliğini ayırt etmede suni tohumlama endüstrisi tarafından kullanılması önerilmektedir.

**Anahtar sözcükler:** Sperm kriyotolerans, Sperm dondurulabilirliği, Sperm parametreleri

## INTRODUCTION

The spermatozoon is composed of several membrane-bound

sections, consisting of the plasma membrane, acrosome membrane, and mitochondrial membrane, that must be intact to ensure the viability of the spermatozoa to fertilize

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an oocyte. Any damage incurred by these membranes is detrimental to the sperm cell health, freezability, and fertilizing ability [1,2]. Over the years, numerous studies have shown that any abnormalities to the structure of the sperm cell will assist with predicating infertility in males [3-5].

Sperm motility and morphology are intricately linked because any anatomical abnormalities will cause sperm to swim slower and less effectively [6,7]. Motility is crucial for sperm transportation in the female reproductive tract and penetration of the oocyte. The numbers of spermatozoa that show forward progressive motility and navigate the barriers of the female reproductive tract is positively associated with fertility and freezability [8,9]. Using computer assisted sperm analyses (CASA), it was revealed that sperm cells with the highest velocity and progressive motion were positively correlated with their resilience post-cryopreservation [10], indicating the importance of motility to determining fertility.

The process of cryopreservation involves extension, temperature reduction, addition of cryoprotectants, and freezing and thawing of sperm cells [11]. The rapid change in temperature alters the physical characteristics of the sperm plasma membrane [12] by forming water crystals within the cell, which causes physical damage and the loss of the acrosomal cap [13,14]. Spermatozoa are not designed to withstand rapid changes in temperature and experience cold shock during freezing. This causes disruption and rearrangement of membrane constituents, resulting in loss of plasma membrane integrity [15]. The thawing process requires the sperm cell to rapidly recover, rehydrate, and expand back into its normal shape in a brief timeframe, resulting in alteration of membrane function [1,16].

One way that sperm cells can become damaged and negatively influence the fertility of a bull is through oxidative stress [17,18], which is defined as imbalance between higher levels of reactive oxygen species (ROS) production and low antioxidant activity in sperm that leads to DNA damage by base oxidation, chromatic dispersion and DNA protamine complex, and apoptosis, all of which then impair sperm viability [19,20]. Integrity of DNA is critical during freezing in several species, such as humans, stallions, and bulls, as cryopreservation alters mitochondrial membrane that induce the generation of ROS, which may consequently undergo oxidation of DNA, generating double and single-strand DNA breaks [21-23].

While research has improved cryopreservation over the years, today's beef producers are commonly facing post-thaw viability of less than 50% [24]. The goal of this study was to ascertain sperm population and cellular characteristics as well as total antioxidant capacity of bull sperm associated with cryotolerance to better understand and improve the post-thaw viability.

## MATERIAL AND METHODS

### Determination of Sperm Freezability and Sample Processing

Cryopreserved sperm samples and bull fertility data from 16 mature Holstein bulls that had satisfactory semen quality were provided by Alta Genetics (Watertown, WI, USA). Thus, experiments performed in our laboratories did not involve any live animals for this study. All bulls were housed and fed identically during the collection period. Bulls had contrasting freezability phenotypes based upon post-thaw viability with 11 bulls categorized as having good freezability (average postthaw viability 62.16%; Good) and 5 bulls with poor freezability (average post-thaw viability 52.59%; Poor). Bull semen freezability was determined using methods as previously described [25]. Briefly, sperm collection was done using an artificial vagina after false mounting of a teaser animal. Semen was extended with a commercial egg-yolk-tris-based extender and frozen at Alta Genetics as described methods [26]. Frozen sperm were packaged into 250  $\mu$ L straws and stored in liquid nitrogen. Post-thaw viability of sperm was evaluated using flow-cytometry (CyFlow SL, Partec, Germany). The proportion of the live and dead sperm were quantified through the dual staining with SYBR-14 and propidium iodide (PI) (SYBR-14/PI, Live/Dead Sperm Viability Kit L-7011, Thermo Fisher Scientific) [27]. The percentage of live sperm (stained green) were considered as the freezability parameter for Good and Poor bulls.

The post-thaw viability database used in this study included 100,448 ejaculates from 860 Holstein bulls that were collected at least 20 different times over a 3-month period. The average of population post-thaw viability was the threshold value to determine freezability phenotype. Bulls with greater post-thaw viability than population average considered as good freezability (Good), and those lower than population average considered as poor freezability (Poor) *Table 1*.

### Computer Assisted Sperm Analyses (CASA)

CASA was used to evaluate sperm cell motility and kinetic parameters. Cryopreserved sperm cells were thawed in a 37°C water bath for 30 sec. Five  $\mu$ L of each sample was loaded into a prewarmed chamber slide and 400 spermatozoa were evaluated per chamber immediately. A total of 12 parameters were assessed [28]. These parameters included the following: total motility (TM), progressive motility (PM), linearity (LN), straightness (ST), wobble (WB), curvilinear velocity (VCL), straight line velocity (VSL), beat cross frequency (BCF), average path velocity (VAP), amplitude of lateral head displacement (ALH), distance average path (DAP), distance straight line (DSL), and distance curved line (DCL).

### Hypoosmotic Swelling (HOS) Test

The HOS test was used to analyze the membrane integrity

**Table 1.** Average sperm population characteristic values of bulls with varying cryotolerance

Variable	Phenotype		Standard Error	P-value
	Good	Poor		
Intact membranes	27.95 <sup>a</sup>	19.00 <sup>b</sup>	3.059	0.02
Live cells	43.63 <sup>a</sup>	37.95 <sup>a</sup>	4.85	0.34
Non-fragmented DNA	63.45 <sup>a</sup>	43.26 <sup>b</sup>	6.27	0.01
Total Motility	44.95 <sup>a</sup>	45.56 <sup>a</sup>	4.73	0.91
Progressive motility	29.48 <sup>a</sup>	29.24 <sup>a</sup>	3.40	0.95
Linearity	58.37 <sup>a</sup>	56.70 <sup>a</sup>	1.63	0.41
Straightness	88.16 <sup>a</sup>	86.34 <sup>a</sup>	1.05	0.17
Wobble	64.95 <sup>a</sup>	64.26 <sup>a</sup>	1.19	0.63
Post-thaw viability	62.16 <sup>a</sup>	52.15 <sup>b</sup>	1.13	<.0001

<sup>a,b</sup> Means within a row with different superscripts differ ( $P \leq 0.05$ )

of the sperm cells as reported [29]. The sperm pellet was resuspended in 250  $\mu$ L of PBS. Fifty  $\mu$ L of the sperm sample was transferred into 450  $\mu$ L of HOS test solution (150 mOsm/kg pre-equilibrated at 37°C for 1 h) and gently mixed by hand. The mixture was then incubated in a 37°C water bath for 30 min upon when 10  $\mu$ L of sample were transferred onto a clean glass slide. Each slide was evaluated for HOS-positive (presence of coiled tail) or HOS-negative (absence of coiled tail) sperm by counting a total of 200 spermatozoa/sample using 40 x objective of a phase-contrast microscope.

### Eosin-Nigrosine Staining

Eosin-Nigrosin staining was used to assess the viability of sperm cells according to the method as reported [30]. This assessment of sperm vitality is used to distinguish between immotile dead sperm and immotile live sperm. Frozen sperm cells were thawed in a 37°C water bath for 30 sec, transferred to a 1.5 mL tube that contained 1 mL of PBS, and centrifuged at 3700 rpm for 10 min. The supernatant was aspirated, and this process was repeated 3 times. One mL of pre-equilibrated PBS was added to the tube and gently agitated by hand. 10  $\mu$ L of sperm suspension and 10  $\mu$ L of eosin-nigrosin solution (0.67 g eosin Y and 0.9 g of sodium chloride in 100 mL of distilled water) was transferred into another tube and gently mixed together by hand. 10  $\mu$ L of this solution was smeared on a glass slide using a coverslip and air dried for evaluation of 200 sperm cells per slide via light microscopy. The underlying principle of this assay is that spermatozoa with structurally intact cell membranes (live spermatozoa) are not stained, while dead spermatozoa with disintegrating cell membranes take up the eosin stain.

### HaloSperm Experiment

The HaloSperm G2 test kit (Halotech DNA, SL San Diego, CA) is an *in vitro* diagnostic kit that measures DNA fragmentation in sperm cells. The experiment was carried out according to the manufacturer's instructions. An

agarose screw tube (ACS) was melted using a 100°C water bath for 5 min. One hundred  $\mu$ L of the melted agarose was transferred into a 1.5 mL tube. Sperm cells were thawed in a 37°C water bath for 30 sec, transferred to a 1.5 mL tube that contained 1 mL of PBS, and centrifuged at 3700 rpm for 10 min. The supernatant was removed, and 1 mL of pre-equilibrated PBS was added to the tube and mixed gently by hand. Fifty  $\mu$ L of the sperm sample was transferred to an Eppendorf tube containing 100  $\mu$ L of the agarose and mixed with a micropipette. Eight  $\mu$ L of the cell suspension was placed in the center of a sample well and covered with a coverslip. Next, slides were placed on a plate precooled to 4°C, and then, put into the fridge for 5 min. to solidify the agarose. Slides were kept in a horizontal position throughout the entire process. Solution 2 (LS) was applied until the sample well was fully immersed and incubated for 20 min, and then, washed with distilled water. The slides were dehydrated by flooding slides with 70% ethanol and incubating for 2 min. The 70% ethanol was drained off and 100% ethanol was applied for 2 min. Excess ethanol was drained off slides and slides were allowed to air dry horizontally on filter paper. Slides were then transferred into a petri dish and Solution 3 (SSA) was applied until sample well was completely immersed, incubated for 7 min. and then, the excess stain was drained off. Solution 4 (SSB) was then applied until sample wells were fully immersed, incubated for another 7 min, and then, the excess stain was drained off. Slides were dried at room temperature, and then, evaluated under bright field microscopy, counting 300 cells per slide.

### Trolox Equivalent Antioxidant Capacity (TEAC)

The TEAC experiment was conducted according to the method as reported [31] to measure the total antioxidant capacity of sperm cells utilizing the Total Antioxidant Capacity (TAC) Colorimetric Assay kit (Cat # 709001; Cayman Chemical, Ann Arbor, Michigan). Frozen sperm cells were thawed in a 37°C water bath for 30 sec, transferred to a 1.5 mL tube containing 1 mL of PBS, and centrifuged at 3700

rpm for 10 min. The supernatant was aspirated, and this was repeated 3 times. One mL of pre-equilibrated PBS was added to the tube and gently agitated. 0, 4, 8, 12, 16, 20  $\mu$ L of the Trolox standard were added to individual wells of the plate. Fifty  $\mu$ L of samples were added to individual wells. 100  $\mu$ L of  $\text{Cu}^{2+}$  working solution was added to all wells on the plate. The plate was then covered and incubated at room temperature for 90 min. Following the incubation, the absorbance was read at 750 nm using a microplate reader.

**Statistical Analysis**

Sixteen Holstein bulls (n=11 Good, n=5 Poor) were used for statistical analysis. Principal component analysis (PCA) performed by the FACTOR procedure of SAS 9.4 was used to reduce sperm population variables (POP). The number of live cells, cells with intact membranes, cells without fragmented DNA, total motility, progressive motility, linearity (LIN), straightness (STR), wobble (WOB), and post-thaw viability (PTV) were reduced to 2 principal components (POP1 and POP2), while preserving total variance in the data. The correlation coefficients of POP variables with POP1 and POP2 were used to map these variables in a biplot. The principal component analysis was also used to reduce sperm cell characteristic variables (CELL), including curvilinear velocity (VCL  $\mu$ m/s), straight line velocity (VSL  $\mu$ m/s), beat cross frequency (BCF Hz), average path velocity (VAP  $\mu$ m/s), amplitude of lateral head (ALH  $\mu$ m), distance average path (DAP  $\mu$ m), distance straight line (DSL  $\mu$ m), and distance curved line (DCL  $\mu$ m) to two principal components CELL1 and CELL2, while preserving the total variance within the data. The

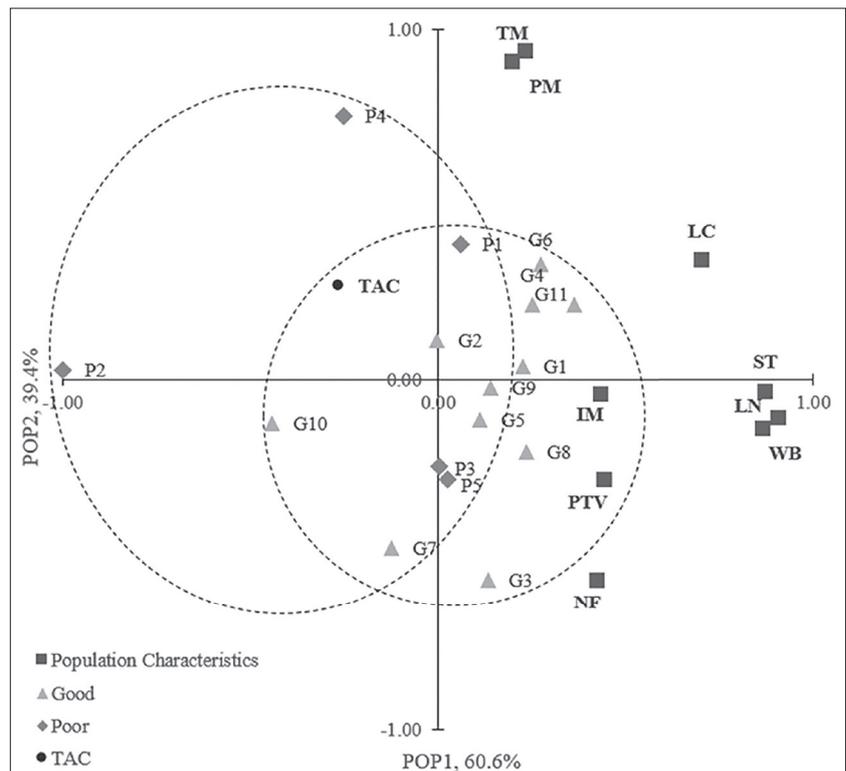
correlation coefficients of CELL variables with CELL1 and CELL2 were used to map these variables in another biplot. The scores of bulls calculated from the POP and CELL variables were also used to map the bulls in both biplots. Additionally, correlation coefficients of total antioxidant capacity (TAC) with the scores were determined by the CORR procedure (SAS version 9.4; SAS Inst. Inc., Cary, NC) and were used to map TAC variable on both biplots. The CORR procedure was also used to determine Spearman's correlation coefficients between the POP and CELL variables. Univariate analysis of variance was performed by the GLIMMIX procedure of SAS 9.4 with freezability phenotype being the fixed effect in a generalized linear mixed model. The degree of freedom was estimated by the Kenward-Roger approximation method and means were separated by a protected *t*-test. Actual probability values were reported with statistical comparisons ( $\alpha \leq 0.05$ ).

**RESULTS**

**Sperm Population Dynamics**

Principal component analysis designated that the total variance of sperm population characteristics was largely explained by two principal components, POP1 (60.6% of total variance) and POP2 (39.4% of total variance; Fig. 1). Factor pattern analysis indicated a strong correlation between POP1 and the number of live cells (LC;  $r = 0.70$ ;  $P=0.002$ ), LIN ( $r = 0.91$ ;  $P<0.001$ ), STR ( $r = 0.87$ ;  $P<0.001$ ), and WOB ( $r = 0.87$ ;  $P<0.001$ ); whereas POP2 was correlated with cells with non-fragmented DNA (NF;  $r = -0.57$ ;  $P=0.020$ ), total motility (TM;  $r = 0.91$ ;  $P<0.001$ ), progressive motility

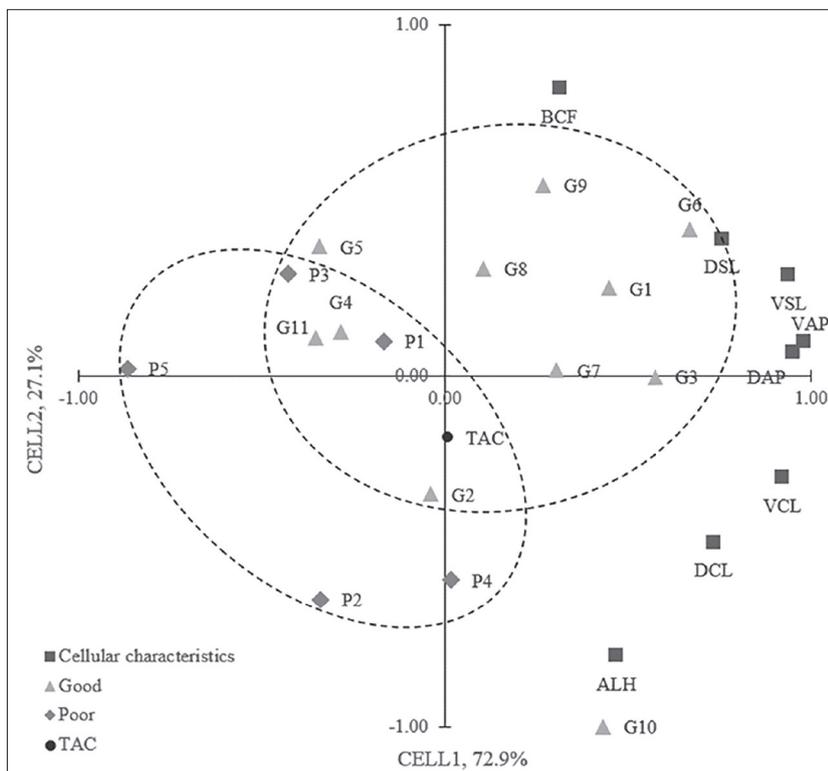
**Fig 1.** Sperm Population Variables PCA. Principal component analysis of sperm population variables (POP) of bulls with good and poor cryotolerance: percentage of total motility (TM), percentage of progressive motility (PM), percentage of live sperm (LC), percentage of sperm that travel straight (ST), percentage of sperm with linear movement (LN), percentage of deviation of the sperm head from the path of progression (WB), percentage of sperm with intact membranes (IM), post-thaw viability (PTV), percentage of sperm with non-fragmented DNA, and total antioxidant capacity (TAC). The coordinates are correlation coefficients of POP with principal component 1 (POP1; explaining 60.6% of variance) and principal component 2 (POP2; 39.4% of variance) and scores of individual bulls



**Table 2.** Average sperm cellular characteristic values of bulls with varying cryotolerance

Variable	Phenotype		Standard Error	P-value
	Good	Poor		
Curvilinear velocity (VCL; $\mu\text{m/s}$ )	118.25 <sup>a</sup>	108.40 <sup>a</sup>	4.70	0.10
Straight line velocity (VSL; $\mu\text{m/s}$ )	67.54 <sup>a</sup>	59.38 <sup>b</sup>	2.50	0.01
Beat cross frequency (BCF; Hz)	30.98 <sup>a</sup>	28.80 <sup>a</sup>	1.19	0.15
Average path velocity (VAP; $\mu\text{m/s}$ )	75.14 <sup>a</sup>	67.82 <sup>b</sup>	2.61	0.03
Amplitude of lateral head (ALH; $\mu\text{m}$ )	4.92 <sup>a</sup>	4.84 <sup>a</sup>	0.26	0.79
Distance average path (DAP; $\mu\text{m}$ )	31.52 <sup>a</sup>	27.32 <sup>b</sup>	1.07	<.01
Distance straight line (DSL; $\mu\text{m}$ )	29.19 <sup>a</sup>	23.90 <sup>b</sup>	1.50	0.01
Distance curved line (DCL; $\mu\text{m}$ )	47.78 <sup>a</sup>	43.84 <sup>a</sup>	2.66	0.24

<sup>a,b</sup> Means within a row with different superscripts differ ( $P \leq 0.05$ )



**Fig 2.** Sperm Cellular Characteristic Variable PCA. Principal component analysis of sperm cellular characteristic variables (CELL) of bulls with good and poor cryotolerance: curvilinear velocity (VCL;  $\mu\text{m/s}$ ), straight line velocity (VSL;  $\mu\text{m/s}$ ), beat cross frequency (BCF; Hz), average path velocity (VAP;  $\mu\text{m/s}$ ), amplitude of lateral head (ALH;  $\mu\text{m}$ ), distance average path (DAP;  $\mu\text{m}$ ), distance straight line (DSL;  $\mu\text{m}$ ), distance curved line (DCL;  $\mu\text{m}$ ), and total antioxidant capacity (TAC). The coordinates are correlation coefficients of CELL with principal component 1 (CELL1; explaining 72.9% of variance) and principal component 2 (CELL2; 27.1% of variance) and scores of individual bulls

(PM;  $r = 0.94$ ;  $P < 0.001$ ; Table 1). The PCA scores of individual bulls on the population characteristic biplot (Fig. 1) showed that there was slight separation between Good and Poor groups; however, there was an overlap between the two populations. The Good bulls clustered within the proximity of the center of the biplot in quadrant II and III with two bulls in quadrant IV. The Poor bulls did not cluster well, locating in quadrant I and II. The Good bulls were in the close proximity of IM, NF, and PTV and scored positively by POP1 variables (ST, LN, and WB); whereas the Poor bulls were in the close proximity of TM, PM, and PTV. One Poor bull was scored negatively by POP1 variables. Univariate analysis revealed that the Good bulls had 9% greater IM ( $P = 0.029$ ), 20% greater NF ( $P = 0.018$ ), and 10% greater PTV ( $P < 0.001$ ).

### Sperm Cellular Characteristics

The total variance of sperm cellular characteristics was also explained by two principal components, CELL1 (72.9%) and CELL2 (27.1%; Fig. 2). Factor loadings on the biplot revealed a strong correlation between CELL1 and VCL ( $r = 0.92$ ;  $P < 0.0001$ ), VSL ( $r = 0.94$ ;  $P < 0.0001$ ), VAP ( $r = 0.98$ ;  $P < 0.0001$ ), DAP ( $r = 0.95$ ;  $P < 0.0001$ ), DSL ( $r = 0.76$ ;  $P = 0.001$ ), and DCL ( $r = 0.73$ ;  $P = 0.001$ ); whereas, CELL2 was correlated with BCF ( $r = 0.82$ ;  $P < 0.001$ ) and ALH ( $r = -0.80$ ;  $P < 0.001$ ; Table 2). The PCA scores of each bull on the cellular characteristic biplot displayed a slight partition between the Good and Poor groups, although there was an overlap among the two groups. The Good bulls clustered into quadrants I, II, and III (Fig. 2). The Poor bulls did not cluster very well,

localizing into quadrants I and IV. The majority of the Good bulls were in close proximity and scored positively with CELL1 variables (DSL, VSL, VAP, and DAP); whereas the Poor bulls were not in close proximity to any of the sperm cellular characteristic variables. Four of five Poor bulls were negatively scored by the CELL1 variables. The univariate analysis showed that spermatozoa from Good bulls was faster for VSL (8.17  $\mu\text{m/s}$ ;  $P=0.017$ ) and VAP (7.33  $\mu\text{m/s}$ ;  $P=0.036$ ) compared to spermatozoa from Poor bulls. In addition, spermatozoa from Good bulls traveled 4.21  $\mu\text{m}$  further as measured by DAP ( $P=0.006$ ) and 5.29  $\mu\text{m}$  further as measured by DSL ( $P=0.011$ ) compared to spermatozoa from Poor bulls.

### Total Antioxidant Capacity

The TAC was in close proximity to the origin of both POP and CELL biplots (Fig. 1, Fig. 2), but not in close proximity to any sperm population or cellular characteristics. This indicated that TAC is not correlated with POP1 and POP2 or CELL1 and CELL2. Univariate analysis of variance indicated that TAC was similar between Good (0.182 nm) and Poor (0.260 nm) groups.

## DISCUSSION

We hypothesized that a combination of sperm cell attributes and kinematics of bull sperm are associated with cryotolerance. To test our hypothesis, we carried out a study evaluating sperm kinematics, viability, plasma membrane and DNA integrity, and TAC. In this study, we used the bull model which is directly relevant to human reproductive mechanisms to examine a fundamental issue of predicting sperm freezability. In addition, significant similarities exist between the bovine and the other mammals including humans both in genetics and reproductive physiology. Rather than relying on anecdotal records, for the bulls, there is a wealth of valuable reliable fertility data for the discovery of biomarkers. Through an existing partnership with the beef industry, semen samples from bulls with well-documented sperm freezability phenotypes were used for this project. These results have exceptional importance because the findings shed light onto population and cellular underpinnings of sperm cryopreservation, ultimately mammalian reproduction and development.

Among the CELL parameters measured, membrane motility is one of the most crucial sperm characteristics linked to the fertility of spermatozoa, signifying its importance in sperm viability and membrane integrity. Motility is essential for successful sperm transport and fertilization *in vivo* and *in vitro*. Apart from motility analysis, our study as well as others have validated that the velocity parameters, such as VSL and VAP, are linked with the fertilizing capacity of frozen-thawed sperm [32,33]. Therefore, velocity of VSL and VAP rather than post thaw total and progressive motility was used to predict cryotolerance

of human and bull sperm [34,35]. Our results were found to be in accordance with that the higher velocity can in part be attributed to the higher number of cells with intact plasma membranes in the Good bulls [36,37], which ensures the viability of the cell. In our study, of the CELL parameters analyzed, the VSL and VAP were significantly faster in the Good bulls versus the Poor bulls, showing that the speed in which a spermatozoon travels aids in its post-thaw viability. It was also in line with our study that VSL could be better utilized as a determining component of sperm which help distinguish quality of frozen bull semen [38]. Accordingly, the velocity parameters could be the outcome of intact plasma membranes that maintain sperm cell viability [39] which, in our study, VSL and VAP had strong correlation with cellular characteristics of Good bull sperm from differing cryotolerance. In addition, VSL and VAP had proximity and scored positively with CELL1 variables, allowing that velocity parameters may be used to narrow down cryotolerance parameters. In addition to VSL and VAP, there was also an increase in the DAP and the DSL for spermatozoa from Good bulls compared to Poor bulls (Table 2). These results confirm that bulls with good freezability have a higher number of spermatozoa that traveled longer distances and at higher speeds in comparison to the sperm from Poor freezability bulls.

Among the POP parameters measured, membrane and DNA integrity, along with post-thaw viability, differed among the bulls with different cryotolerance and showed a significant relationship with sperm cryo-survival (Table 1). Although sperm quality is generally assessed based on sperm motility characteristics, other parameters can be considered, such as viability and sperm membrane integrity post-thaw [40]. When sperm cells are cryopreserved, they undergo thermal stress, which results in protein denaturation, shrinkage, and collapse of the plasma membrane, gravely damaging the viability of the spermatozoon [15,41]. This is consistent with reports that the spermatozoa from Good bulls had a higher number of cells with post-thaw viability in comparison to those of the Poor cryotolerance bulls. Because the sperm membrane is known to be the primary site of cryodamage during cryopreservation [42], it has been proposed to be linked with alterations in membrane dynamics including cholesterol content and phospholipid compound, as well as membrane permeability. As such, procedures of cryopreservation cause dramatic changes in the cell which cause injuries to the sperm membrane, thereby reducing sperm quality [14,43]. In our study, spermatozoa from Good cryotolerance bulls have higher percentage of cells with intact plasma membrane and non-fragmented DNA. Therefore, the Good bulls were in the close proximity of IM, NF, and PTV and scored positively by POP1 variables which may highlight the clear associations of cryotolerance.

Sperm nuclear changes can be affected by the critical

procedure of freezing and thawing because distinct mechanisms lead to DNA damage owing to high levels of ROS production [44]. Accordingly, OS stress gives rise to impaired sperm function by causing DNA damage, thus remaining an important factor for male fertility and potential embryonic loss [45,46]. On the other hand, morphological abnormalities were attributed to poor DNA quality [47]. It has also been demonstrated that sperm with abnormal morphology are more vulnerable to DNA damage during cryopreservation [20]. Therefore, sperm DNA fragmentation was indicative of low AI success in bulls [48] and was the mostly affected marker of sperm cryopreservation [39,49]. In our study, DNA fragmentation was significantly higher in the Poor bull group in our study, and thus, we propose that assessment of sperm DNA, in addition to conventional semen analysis, may offer additional insight into identifying poor cryotolerance bulls. Our conclusions are supported by multiple studies where DNA fragmentation is much higher in cryopreserved bull sperm than non-fragmented DNA is greater in Good freezability than Poor freezability bull, thus seems to be related to those variables of which sperm intact membranes and post-thaw viability were explained by components of POP1.

Both spermatozoa and seminal plasma contain antioxidants to protect against oxidative stress [50], but due to the small size of spermatozoa, their antioxidant capacity is limited. The previous reports on TAC of semen are contradictory. Studies revealed that infertile men demonstrated a lower TAC than fertile men and lower levels of individual antioxidants [51,52]. It was shown that the TAC did not differ among fertile and infertile men [53]. Similarly, the TAC levels in our study did not differ significantly among the Good and Poor bulls. During the cryopreservation process the naturally occurring antioxidants lose their strength. The relationship between TAC and cryotolerance is highly variable partly due to the varying number of antioxidants in commercial semen extenders used for cryopreservation [54]. Thus, these inconsistencies between studies show that TAC alone may not be used to predict freezability phenotype of bulls. Instead, prediction of freezability phenotype of bulls needs at least one other independent variable that is more correlated with POP1 and CELL1 to better predict freezability of bull spermatozoa along with TAC.

In conclusion, the comprehensive assessment of varying sperm functions and the subsequent analysis of these functions indicated that semen from bulls with Good cryotolerance differed in post-thaw viability, plasma membrane and DNA integrity, VSL, VAP, DAP, and DSL in comparison to the bulls with Poor cryotolerance. The PCA also indicated that spermatozoa from Good cryotolerance bulls was strongly correlated with a higher percentage of cells with intact plasma membrane and DNA, and post-thaw viability, along with higher levels of certain sperm kinematic parameters (VSL, VAP, DAP, and DSL) compared

to spermatozoa from Poor bulls. There is no one specific sperm function variable alone that can accurately predict cryotolerance of bull spermatozoa, and thus, a combination of sperm cell attributes and kinematics needs to be utilized by the AI industry in differentiating between freezability of spermatozoa between bulls.

No study has shown that a single sperm parameter can be used to predict spermatid fertility or cryotolerance. The current study investigated sperm population and cellular dynamics as well as TAC levels in bull spermatozoa of Good and Poor cryotolerance. Sperm from Good cryotolerance bulls had more intact membranes and non-fragmented DNA with higher post-thaw viability and key kinematics, including VAP, VSL, DAP, and DSL, as compared to sperm from Poor cryotolerance bulls. However, there was no statistical difference in TAC levels between the groups. These results can be used to concentrate the focus on critical parameters that can be used to best predict cryotolerance of spermatozoa.

## AUTHOR CONTRIBUTIONS

Conceptualization: AG, MH, TTND, AK, EM; Data curation: AG, MH, TTND; Formal analysis: AG, MH, TTND; Investigation: AG, MH, MRU, TTND, WT, AK, EM; Methodology: AG, MH, MRU, TTND, WT, ET, AK, EM; Writing - original draft preparation: AG, MH, MRU, TTND, WT, DJ, MN, AK, EM; Writing - review and editing: AG, MH, MRU, TTND, WT, DJ, MN, AK, EM.

## CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## REFERENCES

- Graham JK, Kunze E, Hammerstedt RH:** Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. *Biol. Reprod.*, 43 (1): 55-64, 1990. DOI: 10.1095/biolreprod43.1.55
- Ugur MR, Saber Abdelrahman A, Evans HC, Gilmore AA, Hitit M, Arifiantini RI, Purwantara B, Kaya A, Memili E:** Advances in cryopreservation of bull sperm. *Front Vet Sci*, 6:268, 2019. DOI: 10.3389/fvets.2019.00268
- Barth AD, Oko RJ:** Abnormal morphology of bovine spermatozoa. (Iowa State University Press), 1989.
- Kastelic JP, Thundathil JC:** Breeding soundness evaluation and semen analysis for predicting bull fertility. *Reprod Domest Anim*, 43, 368-373, 2008. DOI: 10.1111/j.1439-0531.2008.01186.x
- Nagata MPB, Egashira J, Katafuchi N, Endo K, Ogata K, Yamanaka K, Yamanouchi T, Matsuda H, Hashiyada Y, Yamashita K:** Bovine sperm selection procedure prior to cryopreservation for improvement of post-thawed semen quality and fertility. *J Anim Sci Biotechnol*, 10:91, 2019. DOI: 10.1186/s40104-019-0395-9
- Katz DF, Diel L, Overstreet JW:** Differences in the movement of morphologically normal and abnormal human seminal spermatozoa. *Biol Reprod*, 26 (4): 566-570, 1982. DOI: 10.1095/biolreprod26.4.566
- Li Y, Kalo D, Zeron Y, Roth Z:** Progressive motility-a potential predictive parameter for semen fertilization capacity in bovines. *Zygote*, 24 (1): 70-82, 2016. DOI: 10.1017/s0967199414000720

- 8. Nadir S, Saacke RG, Bame J, Mullins J, Degelos S:** Effect of freezing semen and dosage of sperm on number of accessory sperm, fertility, and embryo quality in artificially inseminated cattle. *J Anim Sci*, 71 (1): 199-204, 1993. DOI: 10.2527/1993.7111199x
- 9. Pardede BP, Supriatna I, Yudi Y, Agil M:** Decreased bull fertility: age-related changes in sperm motility and DNA fragmentation. *E3S Web Conf*, 151:01010, 2020. DOI: 10.1051/e3sconf/202015101010
- 10. Muño R, Rivera MM, Rigau T, Rodríguez-Gil JE, Peña AI:** Effect of different thawing rates on post-thaw sperm viability, kinematic parameters and motile sperm subpopulations structure of bull semen. *Anim Reprod Sci*, 109 (1-4): 50-64, 2008. DOI: 10.1016/j.anireprosci.2007.11.028
- 11. Medeiros CMO, Forell F, Oliveira ATD, Rodrigues JL:** Current status of sperm cryopreservation: Why isn't it better? *Theriogenology*, 57, 327-344, 2002. DOI: 10.1016/S0093-691X(01)00674-4
- 12. Gunawan M, Kaiin EM, Mudita GS, Chaidir RRA:** Soybean phospholipids-based extender as an alternative for bull sperm cryopreservation. *IOP Conf Ser Earth Environ Sci*, 478:012014, 2020. DOI: 10.1088/1755-1315/478/1/012014
- 13. Yoon SJ, Kwon WS, Rahman MS, Lee JS, Pang MG:** A novel approach to identifying physical markers of cryo-damage in bull spermatozoa. *PLoS One*, 10 (5): e0126232, 2015. DOI: 10.1371/journal.pone.0126232
- 14. Peris-Frau P, Soler AJ, Iniesta-Cuerda M, Martín-Maestro A, Sánchez-Ajofrín I, Medina-Chávez DA, Fernández-Santos MR, García-Álvarez O, Maroto-Morales A, Montoro V, Garde JJ:** Sperm cryodamage in ruminants: Understanding the molecular changes induced by the cryopreservation process to optimize sperm quality. *Int J Mol Sci*, 21 (8): 2781, 2020. DOI: 10.3390/ijms21082781
- 15. Khalil WA, El-Harairy MA, Zeidan AE, Hassan MA, Mohey-Elsaeed O:** Evaluation of bull spermatozoa during and after cryopreservation: Structural and ultrastructural insights. *Int J Vet Sci Med*, 6, S49-S56, 2018. DOI: 10.1016/j.ijvsm.2017.11.001
- 16. Grötter LG, Cattaneo L, Marini PE, Kjelland M, and Ferré LB:** Recent advances in bovine sperm cryopreservation techniques with a focus on sperm post-thaw quality optimization. *Reprod Domest Anim*, 54 (4): 655-665, 2019. DOI: 10.1111/rda.13409
- 17. Aitken RJ, Mark AB, Brett Nixon:** Are sperm capacitation and apoptosis the opposite ends of a continuum driven by oxidative stress? *Asian J Androl*, 17 (4): 633-639, 2015. DOI: 10.4103/1008-682X.153850
- 18. Diether N, Dyck MK:** Male fertility evaluation using biomarkers in livestock. *JSM Biomar*, 3 (1): 1011, 2017.
- 19. Aitken RJ, De Iulius GN:** On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod*, 16 (1): 3-13, 2010. DOI: 10.1093/molehr/gap059
- 20. Di Santo M, Tarozzi N, Nadalini M, Borini A:** Human sperm cryopreservation: Update on techniques, effect on DNA integrity, and implications for ART. *Adv Urol*, 2012:854837, 2012.
- 21. Evenson DP, Larson KL, Jost LK:** Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J Androl*, 23, 25-43, 2002. DOI: 10.1002/j.1939-4640.2002.tb02599.x
- 22. Said TM, Gaglani A, Agarwal A:** Implication of apoptosis in sperm cryoinjury. *Reprod Biomed Online*, 21 (4): 456-462, 2010. DOI: 10.1016/j.rbmo.2010.05.011
- 23. Gürlér H, Malama E, Heppelmann M, Calisici O, Leiding C, Kastelic JP, Bollwein H:** Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm. *Theriogenology*, 86, 562-571, 2016. DOI: 10.1016/j.theriogenology.2016.02.007
- 24. Ballester J, Johannisson A, Saravia F, Håård M, Gustafsson H, Bajramovic D, Rodríguez-Martínez H:** Post-thaw viability of bull Al-doses with low-sperm numbers. *Theriogenology*, 68 (6): 934-943, 2007. DOI: 10.1016/j.theriogenology.2007.07.008
- 25. Ugur MR, Dinh T, Hitit M, Kaya A, Topper E, Didion B, Memili E:** Amino acids of seminal plasma associated with freezability of bull sperm. *Front Cell Dev Biol*, 7:347, 2020. DOI: 10.3389/fcell.2019.00347
- 26. Pace MM, Sullivan JJ, Elliott FI, Graham EF, Coulter GH:** Effects of thawing temperature, number of spermatozoa and spermatozoal quality on fertility of bovine spermatozoa packaged in 5-ml French straws. *J Anim Sci*, 53, 693-701, 1981. DOI: 10.2527/jas1981.533693x
- 27. Garner DL, Johnson LA, Yue ST, Roth BL, Haugland RP:** Dual DNA staining assessment of bovine sperm viability using sybr-14 and propidium iodide. *J Androl*, 15 (6): 620-629, 1994.
- 28. Ibănescu I, Leiding C, Ciornei ŞG, Roşca P, Sfartz I, Drugociu D:** Differences in CASA output according to the chamber type when analyzing frozen-thawed bull sperm. *Anim Reprod Sci*, 166, 72-79, 2016. DOI: 10.1016/j.anireprosci.2016.01.005
- 29. Gadkar S, Shah CA, Sachdeva G, Samant U, Puri CP:** Progesterone receptor as an indicator of sperm function. *Biol Reprod*, 67 (4): 1327-1336, 2002. DOI: 10.1095/biolreprod67.4.1327
- 30. Dogan S, Mason MC, Govindaraju A, Belser L, Kaya A, Stokes J, Rowe D, Memili E:** Interrelationships between apoptosis and fertility in bull sperm. *J Reprod Dev*, 59 (1): 18-26, 2013. DOI: 10.1262/jrd.2012-068
- 31. Roychoudhury S, Sharma R, Sikka S, Agarwal A:** Diagnostic application of total antioxidant capacity in seminal plasma to assess oxidative stress in male factor infertility. *J Assist Reprod Genet*, 33 (5): 627-635, 2016. DOI: 10.1007/s10815-016-0677-5
- 32. Kumar D, Kumar PR, Singh PA, Yadav SP, Sarkar SK, Bharadwaj A, Yadav PS:** Characteristics of frozen thawed semen in predicting the fertility of buffalo bulls. *Indian J Anim Sci*, 84, 389-392, 2014.
- 33. Karunakaran M, Devanathan TG:** Evaluation of bull semen for fertility-associated protein, in vitro characters and fertility. *J Appl Anim Res*, 45 (1): 136-144, 2017. DOI: 10.1080/09712119.2015.1129343
- 34. Jiang XP, Zhou WM, Wang SQ, Wang W, Tang JY, Xu Z, Zhang ZX, Qin C, Wang ZJ, Zhang W:** Multivariate model for predicting semen cryopreservation outcomes in a human sperm bank. *Asian J Androl*, 19 (4): 404-408, 2017. DOI: 10.4103/1008-682X.178488
- 35. Singh P, Kumar D, Kumar P, Singh I, Yadav PS:** Cryopreservation and quality assessment of buffalo bull semen collected from farmer's doorstep. *Agric Res*, 2 (2): 148-152, 2013. DOI: 10.1007/s40003-013-0056-8
- 36. Nagy Á, Polichronopoulos T, Gáspárdy A, Solti L, Cseh S:** Correlation between bull fertility and sperm cell velocity parameters generated by computer-assisted semen analysis. *Acta Vet Hung*, 63 (3): 370-381, 2015. DOI: 10.1556/004.2015.035
- 37. Bollwein H, Bittner L:** Impacts of oxidative stress on bovine sperm function and subsequent in vitro embryo development. *Anim Reprod*, 15 (Suppl. 1): 703-710, 2018. DOI: 10.21451/1984-3143-AR2018-0041
- 38. Morrell JM, Valeanu AS, Lundeheim N, Johannisson A:** Sperm quality in frozen beef and dairy bull semen. *Acta Vet Scand*, 60:41, 2018. DOI: 10.1186/s13028-018-0396-2
- 39. Ismail NH, Osman K, Mohd Yusof FZ, Syed Mohamad SF, Jaafar FHF, Ibrahim SF:** Improvement of post-thaw sperm kinematics and dna integrity of cross-bred bovine sperm by incorporating dgc as selection method prior to cryopreservation. *J Agric Sci*, 9 (13): 24-31, 2017. DOI: 10.5539/jas.v9n13p24
- 40. Sieme H, Oldenhof H, Wolkers WF:** Sperm membrane behaviour during cooling and cryopreservation. *Reprod Domest Anim*, 50, 20-26, 2015. DOI: 10.1111/rda.12594
- 41. Kasimanickam R, Kasimanickam V, Thatcher CD, Nebel RL, Cassell BG:** Relationships among lipid peroxidation, glutathione peroxidase, superoxide dismutase, sperm parameters, and competitive index in dairy bulls. *Theriogenology*, 67 (5): 1004-1012, 2007. DOI: 10.1016/j.theriogenology.2006.11.013
- 42. Ryu DY, Song WH, Pang WK, Yoon SJ, Rahman MS, Pang MG:** Freezability biomarkers in bull epididymal spermatozoa. *Sci Rep*, 9:12797, 2019. DOI: 10.1038/s41598-019-49378-5
- 43. Nijis M, Creemers E, Cox A, Janssen M, Vanheusden E, Castro-Sanchez Y, Thijs H, Ombelet W:** Influence of freeze-thawing on hyaluronic acid binding of human spermatozoa. *Reprod Biomed*, 19, 202-206, 2009. DOI: 10.1016/S1472-6483(10)60073-9
- 44. Castro LS, Hamilton TRS, Mendes CM, Nichi M, Barnabe VH, Visintin JA, Assumpção MEOA:** 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, 2016. DOI: 10.1186/s40104-016-0076-x

- 45. El-Regalaty H:** Effects of cryopreservation of buffalo and bovine spermatozoa on sperm dna damage and early embryonic development. *J Anim Poult Prod*, 8 (7): 167-172, 2017. DOI: 10.21608/jappmu.2017.45843
- 46. Kumaresan A, Gupta MD, Datta TK, Morrell JM:** Sperm DNA integrity and male fertility in farm animals: A review. *Front Vet Sci*, 7:321, 2020. DOI: 10.3389/fvets.2020.00321
- 47. Enciso M, Cisale H, Johnston SD, Sarasa J, Fernández JL, Gosálvez J:** Major morphological sperm abnormalities in the bull are related to sperm DNA damage. *Theriogenology*, 76 (1): 23-32, 2011. DOI: 10.1016/j.theriogenology.2010.12.034
- 48. Karoui S, Díaz C, González-Marín C, Amenabar ME, Serrano M, Ugarte E, Gosálvez J, Roy R, López-Fernández C, Carabaño MJ:** Is sperm DNA fragmentation a good marker for field AI bull fertility?. *J Anim Sci*, 90 (8): 2437-2449, 2012. DOI: 10.2527/jas.2011-4492
- 49. Li MW, Lloyd KCK:** DNA fragmentation index (DFI) as a measure of sperm quality and fertility in mice. *Sci Rep*. 10:3833, 2020. DOI: 10.1038/s41598-020-60876-9
- 50. Hitit M, Ugur MR, Dihn TT, Sajeev D, Kaya A, Topper E, Tan W, Memili E:** Cellular and functional physiopathology of bull sperm with altered sperm freezability. *Front Vet Sci*, 7:581137, 2020. DOI: 10.3389/fvets.2020.581137
- 51. Lewis SEM, Sterling ESL, Young IS, Thompson W:** Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. *Fertil Steril*, 67 (1): 142-147, 1997. DOI: 10.1016/s0015-0282(97)81871-7
- 52. Smith R, Vantman D, Ponce J, Escobar J, Lissi E:** Andrology: Total antioxidant capacity of human seminal plasma. *Hum Reprod*, 11 (8): 1655-1660, 1996. DOI: 10.1093/oxfordjournals.humrep.a019465
- 53. Siciliano L, Tarantino P, Longobardi F, Rago V, De Stefano C, Carpino A:** Impaired seminal antioxidant capacity in human semen with hyperviscosity or oligoasthenozoospermia. *J Androl*, 22 (5): 798-803, 2001.
- 54. Bucak MN, Tuncer PB, Sariözkan S, Baspınar N, Taspınar M, Çoyan K, Bilgili A, Peker Akalın P, Büyükleblebici S, Aydos S, Ilgaz S, Sunguroğlu A, Öztuna D:** Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: Antioxidants protect DNA integrity against cryodamage. *Cryobiology*, 61, 248-253, 2010. DOI: 10.1016/j.cryobiol.2010.09.001