Evaluation of physical and ultra-structural attributes of bulls' semen with variable freezing potential

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Abstract

This experiment was conducted to determine the physical and ultra-structural attributes of fresh bull semen and assess their potential use for freezing and AI. A total of 40 semen samples collected from 4 mature bulls (10 samples per bull) were analyzed. The semen samples were examined for colour, volume, concentration, pH, general and progressive motilities, morphologically normal spermatozoa, acrosome and DNA damage, and lipid peroxidation. Transmission Electron Microscopy (TEM) was also performed to evaluate the ultra-structures of the spermatozoa. Results showed the semen colour varied from bull to bull from creamy-white in bull #1, to milky in bulls #2 and 4, then cloudy in bull #3. Highest sperm concentration, lipid peroxidation and pH were recorded from bull #4. Highest volume, progressive motility, morphology, less acrosome damage and viability were from bull #2. While best values for general motility and DNA damage were obtained from bull #1. TEM revealed 92.5, 90.0 and 82% of intact heads for bulls #1, 2 and 3, respectively, much higher than 62.5% for bull #4 and 32.5, 25.0, 37.5% of total defective spermatozoa for bulls #1, 2 and 3, respectively, much better than 80.0% in bull #4. Conclusively, bulls #1, 2 and 3 were consistently satisfactory in most parameters evaluated and hence their semen can be used for freezing. On the other hand, bull #4 expressed higher ($p<0.05$) sperm concentration but yet was unsatisfactory in most other parameters assessed, including low live:dead ratio and high percentage of abnormalities recorded, manifesting poor potential of freezability.

Key words: Bull, spermatozoa, fresh quality, ultra-structure, Transmission Electron Microscopy

Introduction

Individual cow annual calving is the optimum goal of every dairy and cow-calf operation. Such efficiency depends largely on high quality bull semen used, for successful fertilization and improved herd number. In either artificial insemination (AI) or natural mating processes many females are serviced by few bulls. Individual bulls therefore play an important role by not only being fertile but also by supplying superior quality germ plasma for maintenance of the reproductive performance in their future progeny. Some important physical and chemical parameters are useful in predicting bull's semen quality. Such indices include concentration of spermatozoa, volume of the ejaculate, pH, proportion of dead and abnormal spermatozoa, as well as motility of the spermatozoa. These indices can be influenced by both genetic and environmental factors (Patel and Siddiquee, 2013). Even though a bull could be fertile, its reproductive rate may be low in cases of sub fertility where any combination of the aforementioned parameters are poor, resulting to increased calving intervals, low

herd quality and reproductive rate (Hossain *et al*., 2012). Quality of fresh bull semen is also significantly correlated with the potential fertility of that semen after thawing (Patel and Siddiquee, 2013). If low semen quality is caused by environmental factors such as stress, ambient temperature or humidity, subsequent ejaculates of the same bull could improve by controlling such factors. However, if low semen quality is caused by the bull's genetic factors, the affected bull is usually rejected because they are hardly correctible. In any case, checking individual bull semen quality status is essential. However, it is worth noting that although very useful and often commonly the tradition, researchers argue whether the earlier mentioned andrological tests alone are enough to provide reliable, detailed information on the semen samples. Therefore, acrosome and DNA damage, lipid peroxidation as well as ultra-structural and morphological examination using TEM were also evaluated. Therefore, the current study aimed to determine the physical and ultra-structural attributes of fresh bull semen and assess their potential use for freezing and AI .

Materials and Methods

Animals

Semen samples were collected from four healthy and sexually mature crossbred bulls: Simmental x Brangus (bull #1), Brangus x Hereford (bull #2), Kedah-Kelantan x Brangus (bull #3) and Sahiwal x Friesian (bull #4) at the Universiti Putra Malaysia (UPM) farm (2^{o} 9'18.36" N, 101^{o} 43'49.61" E). The bulls were 3 to 5 years old, with body condition scores (BCS) of 6, 5, 4 and 5 for bulls #1 to #4, respectively, based on a scale of 1 (thin) to 9 (obese), as stated by Eversole *et al*. (2009). All bulls had been used as sires in the past and were maintained under uniform management condition, fed with *Brachiaria decumbens* green chops, supplemented with commercial cattle concentrate and palm kernel cake (PKC) containing approximately 16% crude protein and 2.6% crude fat, given at rate of 3 kg/bull/day. They were also given mineral licks and water *ad libitum*.

Semen collection

A total of 40 ejaculates were used, 10 from each bull, were collected by electro ejaculation (Electrojac 6, Lexington,USA) with two ejaculates collected at 4-d interval per bull per week. The samples were kept in a Coleman cooler box containing warm water at 37^{o}C, and later transported to the 'Theriogenology and Cytogenetic' Laboratory of the Faculty of Veterinary Medicine, UPM for evaluation of physical and ultra-structural attributes of fresh semen.

Evaluation of semen

Sperm concentration and motility were determined by Computer Assisted Semen Analyzer (CASA, IVOS Hamilton Thorne Biosciences, version 12.2). The CASA was operated using the following set up; 60 Hz/s frame/second, temperature-37^{o}C, video frequency of 60, magnification factor-1.92, minimum cells size-5 pixel, detection contrast-40 and cell intensity-55. The average path velocity (VAP) was 75µm/s and straightness threshold (STR) was 80%. Ten µl of diluted samples in 0.85% normal saline were placed on Hamilton Thorne research 2X-cell (20 µm) glass slide and loaded on the CASA for analysis (Yimer *et al*., 2011). At least 200 spermatozoa from average of 10 fields were counted per reading (Bucak *et al.*, 2010; Taşdemir *et al*., 2013). The pH was determined using SevenEasy pH meter (Mettler Toledo Ltd., Leicester. England).

Sperm morphology, viability and acrosome damage were determined by eosin-

nigrosin (E & N) stain (Memon *et al*., 2011). A drop of semen sample was mixed with 2 drops of E & N stain. Then, a smear was made, air dried and examined under a phase-contrast microscope, under oil immersion (1000x). A total of 200 spermatozoa were counted. A spermatozoon showing complete normal structures having a smooth-oval head with clearly defined cap (acrosome) joined to the tail by the mid-piece without any visible defect was considered normal. Large/small head sizes, abnormal mid-piece, presence of droplets, broken parts, double head and /or tail were considered abnormalities and therefore, sperms showing any of such characteristics were considered abnormal (Nagy *et al*., 2013). Spermatozoa with clear white heads that did not take up the stain were identified as viable, whereas those that showed partially/completely purple coloured head were non-viable. Percentage acrosome damage was counted according to Khumran *et al*. (2015) in order to assess the acrosome integrity.

DNA damage was assessed through comet assay, a single cell gel electrophoresis (SCGE) for rapid assessment for genotoxicity. The base slides, samples and electrophoresis were prepared and ran in alkaline buffer containing 300 mM NaOH per 1 mM EDTA under pH greater than 13, according to the protocol described by Dhawan *et al*. (2009). The processed sample slides were stained with ethidium bromide (ErBr) stain and viewed under an inverted fluorescent microscope (Nikon Eclipse Ti-s, Japan), equipped with NIS-elements imaging software (BR4.20.00 64-bit) and Nikon Digital Sight camera Ds-Fi2 (K16850). Visible migration of genetic material from spermatozoa was counted as DNA damage. One hundred spermatozoa were counted per slide.

Lipid peroxidation was measured using thiobarbituric acid-reactive substances (TBARS) according to Kaka *et al*. (2015). A 500 µL of the sample were mixed with TBRAS solution and then heated in a water bath at 95°C for 60 min until the development of a pink colour. After cooling, one mL of distilled water and three mL of n-butyl alcohol were added to the extracts and vortexed. The mixtures were centrifuged at 5000 rpm for 10 min. Absorbance of supernatant was read against an appropriate blank at 532 NM using spectrophotometer (Secomam, Domont, France). The TBARS were calculated from a standard curve of 1, 1, 3, 3- tetraethoxypropane and expressed as mg malondialdehyde (MDA) ng/ml sample.

Transmission electron microscopy (TEM) was carried out by the following procedure; one milliliter of semen samples were fixed in 2.5% glutaraldehyde for 6 h at 4^{o}C, and then centrifuged at 750 × g for 2 min. The fixative was decanted and then appropriate quantities of animal serum were added to submerge the samples and allowed to clot overnight. The clotted samples were then diced in 1 mm^3 and then fixed again in 2.5% glutaraldehyde for 2 h at 4^{o}C. Following the primary and secondary fixations, samples were then washed in three changes of 0.1 M sodium cacodylate buffer with 10-min interval between each wash. The samples were then post-fixed in 1% osmium tetroxide for 2 h at 4^{o}C. Dehydration in increased concentrations of acetone (35, 50, 75, 95 and 100%) was followed by infiltration of the specimens with gradient acetone: resin mixture; 1:1 for 1 h, 1:3 for 2 h, then 100% resin overnight and repeated 100% resin for 2 h. The samples were then embedded by placing on beam capsules filled with resin, polymerized in oven at 60^{o}C for 48 h. Thick, then ultrathin sectioning and staining of the samples were followed after which the samples were then viewed and examined according to Oliveira *et al*. (2010) under a scanning electron microscope (Leo 1455 VP, Cambridge, UK).

Statistical analysis

Statistical analysis system (SAS V 9.1, SAS Inst. Inc., Cary, NC) was used for data analysis. The means of the semen parameters between different bulls were compared by one way ANOVA. Analyses were conducted at 95% confidence level, and therefore P values less than 0.05 were considered significant.

Results and Discussion

Table 1 shows the sperm characteristics of the 4 bulls. The colour of the semen samples ranged from creamy- white in bull #1 to milky in bulls #2 and 4 and cloudy in bull #3. Average volume of the bulls' ejaculates did not show any significant difference. Mean concentration was lower ($p < 0.05$) in bull #2 than bull #4 but the same as in bulls #1 and 3. Significantly higher general and progressive motilities, as well as viability and better acrosome were achieved in bulls #1 and 2, then bull #3 while lowest scores and high acrosome damage were recorded in bull #4. Morphology was best in bull #2, which was the same with bull #1 and better ($p<0.05$) than bull #3. Bull #4 had the highest abnormal morphology than the others. While the average pH in bull #1 was the same with bull #3 and significantly different from bull #2, the pH was significantly higher in bull #4 when compared to the other 3 bulls in this study.

Table1. Sperm quality characteristics of four bulls of mixed breeds

Parameters	Bulls			
	1	2	3	4
Volume (ml)	8.7±0.6	9.4±0.8	7.0±0.6	7.0±0.4
Concentration (m/ml)	1320.4±163.8^{ab}	939.0±99.6^{b}	1137.0±208.2^{ab}	1624.0±208.5^{a}
General motility (%)	84.6±1.9^{a}	83.1±3.2^{a}	65.8±2.6^{b}	40.2±1.9^{c}
Progressive motility (%)	43.0±3.3^{a}	46.5±3.4^{a}	27.9±2.7^{b}	7.6±0.7^{c}
Morphology (%)	90.7±1.8^{ab}	93.9±2.3^{a}	85.4±1.3^{b}	74.6±2.8^{c*}
Viability (%)	77.3±1.9^{a}	81.8±1.3^{a}	68.4±2.3^{b}	48.5±2.7^{c}
Acrosome damage (%)	8.4±2.1^{c}	8.2±0.7^{c}	12.6±1.5^{b}	18.6±1.0^{a}
pH	6.1±0.1^{c}	6.4±0.0^{b}	6.3±0.1^{bc}	7.1±0.1^{a}

Data are expressed as Mean ± Standard Error (SE) n=10. * abnormal morphology < 20%, Different superscripts within rows denote significant differences ($p<0.05$).

Figure 1 shows percentage DNA damage and MDA production of the 4 bulls. DNA damage was lowest in bull #1 which was the same as bull #2 and significantly better than bull 3, whereas, bull #4 showed the highest DNA damage. Bull #4 had also significantly higher lipid peroxidation when compared to bulls #1, 2 and 3. Except for bull #2 there seemed to be increased percentage DNA damage with increased MDA production among the bulls.

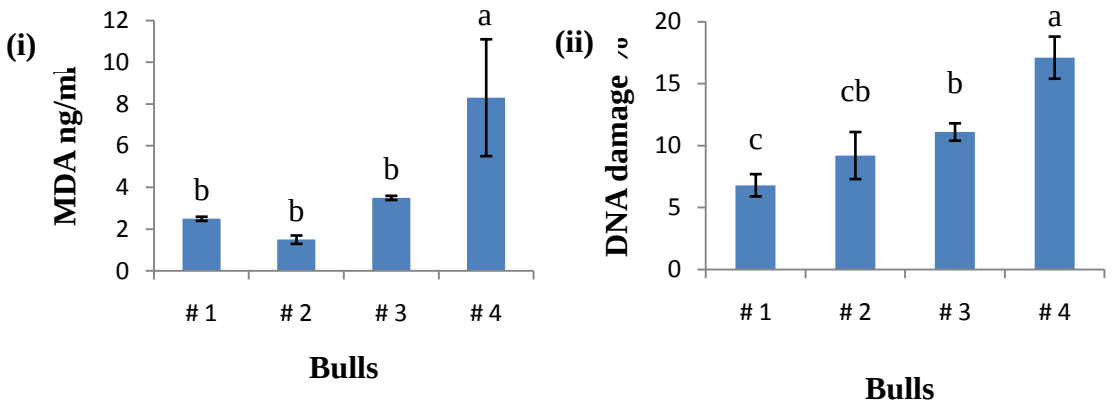

Figure 1. (i) MDA production (ng/ml) of the 4 bulls and (ii) Mean DNA damage (%) Different letters [a,b] denote significant difference ($p < 0.05$)

Table 2 shows results of the fine structures of the spermatozoa as assessed through TEM. The TEM results revealed 92.5, 90 and 82.5% intact heads from bulls #1, 2 and 3 respectively, with the lowest percentage from bull #4 (62.5%). Similar pattern was repeated in the total (non-sperm head) damage. The lowest (best) to the highest (worst) percentage damage were bull #1 (32.5%), #2 (25%) and #3(37.5%) with bull #4 of 80% total damage amounting to more than double of each of the rest of the bulls. Figure 2 shows various micrographs (A to G) illustrating both intact and defective ultra-structures taken from the TEM.

Table 2. Characteristics of the bulls' sperm ultra-structures assessed by TEM

Fine structures of the sperm	Bulls			
	1	2	3	4
Intact heads (%)	92.5	90.0	82.5	62.5
Acrosome reacted (%)	5.0	7.5	12.5	17.5
Acrosome loss (%)	2.5	0.0	2.5	17.5
Defective nuclear material (%)	0.0	0.0	2.5	2.5
Bent spermatozoa (%)	0.0	2.5	5.0	7.5
Inflamed plasma membrane (%)	10.0	2.5	5.0	10.0
Inflamed mitochondria (%)	15.0	12.5	10.0	25.0
Total damaged (%)	32.5	25.0	37.5	80.0

Data are expressed as Mean, n= 2.

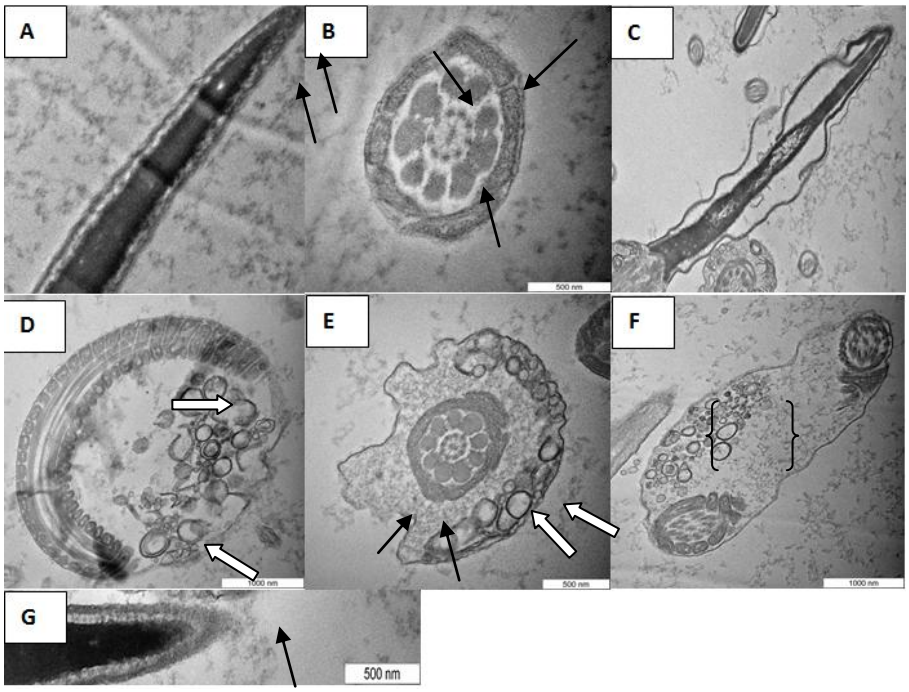

Figure 2. Electron micrographs showing TEM sections of the bulls' spermatozoa

(A) Sagittal section of a spermatozoon head presenting intact structures, acrosomal membrane (black arrows) completely surrounding the acrosomal ground substance. **(B)** Cross section of normal midpiece showing 9 mitochondria (black arrows). **(C)** Spermatozoon head with complete loss of acrosome, inflamed plasma membrane, condensed nuclear material. **(D)** Bend spermatozoon with inflamed plasma membrane, displaced organelles (white arrows). (E) Midpiece containing displaced and inflamed mitochondria (white arrows) with ruptured plasma membrane (black arrows). **(F)** Broken tail with displaced and inflamed mitochondria (within brackets). **(G)** Sperm head presenting 'Physiological' acrosome reaction (black arrow).

Cow infertility is often the focus in most herd fertility management instances, whereas bulls influence is as much critical. Studies show that more than 20% of unselected bulls could be sub-fertile (Penny, 2014). When cow management is good and infectious diseases are controlled, the other factor that could affect fertility in a herd is quality of the semen used, which is well determined by the individual source bulls. In natural mating farms where bulls are rotated around breeding cows during mating periods, presence of sub fertile bulls may be masked but it consequently results to fertility inefficiencies. Accurate evaluation and timely intervention is essential for optimum reproductive efficiency. In this study, ejaculates of 4 bulls were evaluated and their sperm subtle structures were also characterized by TEM in order to obtain more detailed information about the potential use of the bulls' semen for AI/freezing.

General and progressive motilities, morphology, viability and acrosome damage

Bulls #1, 2 and 3 showed highly significant values in general and progressive

motilities, as well as morphology and viability when compared to bull #4 (Table 1). Bull #4 was lower in these parameters which were indications of poor semen quality. Sperm motility is evidence of viability and the two parameters (motility and viability) are the most closely related parameters to fertility Davis and Williams (1939).

Abnormal sperm morphology of less than 30% poses a setback to fertility (Menon *et al*., 2011) by affecting its motility. Semen with 30% and above in abnormalities are therefore, usually not accepted for samples intended for reproduction. While morphologically normal spermatozoa in the present study agree with previous reports, the percentage of normal morphology in bull #4 of this study was not satisfactory. Intact acrosome is important in the fertilization process. The acrosomal cap undergoes biochemical changes in composition and ultra-structure during fertilization. Acrosomal enzymes are released during this process, which play a key role in sperm penetration through the zona pellucida. Damage or loss of acrosome therefore hinders the ability of spermatozoon to fertilize an oocyte (Patel and Siddiquee, 2013). The mean value of damaged acrosome in the present study is in close agreement with Ray and Ghosh (2013).

Concentration

Significant differences could exist from bull to bull in terms of ejaculates' sperm concentration. The result in the present study is in agreement with recent studies (Hossain *et al*., 2012; Patel and Siddiquee, 2013) which were undertaken in various breeding bulls, mixed breeds and Kankrej bulls, respectively. Sperm concentration has been shown to have positive correlation with motility thereby considered as an initial indicator for semen quality (Shelke and Dhami, 2001). However, in the present study we observed that this had not been the case with bull #4, while the bull showed highest average concentration it expressed significantly poor values in the other parameters when compared to bull #1, 2 and 3, which might probably result from the magnitude of spermatozoal damage and or high lipid peroxidation recorded from the bull.

Volume

The average volume of ejaculates in this study was higher than some previous studies (Hossain *et al*., 2012; Patel and Siddiquee, 2013). However, the mean ejaculate volume in this study is within acceptable range of standard bovine ejaculate volume (5 to 15 ml). The difference here may be from the method of collection. Electro Ejaculator was used in the present study, which tends to result in higher volume of ejaculates (due to excessive accessory gland secretion) than artificial vagina that was used in the previously mentioned studies.

Colour

All the colours maintained by the bulls' semen in this study were normal and usually varied from bull to bull, similar colours had been observed in previous studies (Patel and Siddiquee, 2013). These colours are thought to be influenced by lipochrome pigment, which is derived from the epithelium of ampulla during seminal secretion and therefore considered harmless to spermatozoa and have no effect on bull fertility (Patel and Siddiquee, 2013). The samples used were also free from physical impurities, semen coagulation, blood tinged and soiled.

pH

pH is the measure of acidity (lower values) or alkalinity (higher values) of a given sample. According to Roberts (1986), optimum pH common to most domestic species (including bulls) within which spermatozoa performed at its best and has the potential to neutralize vaginal acid ranges between 6.4 to 7.4. pH outside this range tends to lower spermatozoal motility and a low pH reported to immobilize spermatozoa (Acott and Carr, 1984). Although the average pH of the bulls under investigation was within the range suggested by Robert (1986), bull #4 showed average pH significantly higher than the rest of the bulls even though it (bull #4) had the highest concentration and lowest motility (Table 1). This seems to agree with the finding reported by Davis and Williams (1939) who studied the correlation between pH, volume, motility and concentration of semen and stated that the higher the pH value the lower the volume and the motility, and the greater the concentration.

DNA damage and lipid peroxidation

The primary objective of fertilization is successful delivery of intact DNA to the ovum. Damaged DNA affects survival of spermatozoa during freeze–thaw procedures (Januskauskas *et al.*, 2001). It is also reported to be associated with sub fertility and infertility in a number of studies (D'Occhio *et al*., 2013). However, values obtained in this study (Table 1) were higher than those obtained by Januskauskas *et al*. (2001). Conditions of sub fertility and infertility have been blamed on lipid peroxidation owing to the exposure of bull spermatozoa to inappropriate production of reactive oxygen species (Brouwers and Gadella, 2003). Reactive oxygen species (ROS) are normally produced by living cells during respiration and low levels are beneficial for processes that lead to fertilization. Increased ROS production also results from dead, dying and phagocytic cells. Endogenous antioxidants are usually released within the semen plasma in order to strike a balance with the ROS production. When the amount of ROS exceeds its beneficial limit in the semen, then oxidative degeneration of the sperm sets in (Bansal and Bilaspuri, 2010). Sperm membrane lipid peroxidation may alter the membrane permeability which leads to adenosine triphosphate (ATP) depletion (Lemma, 2011), reduced sperm motility (Bansal and Bilaspuri, 2010), damage DNA and interfere with sperm-ovum binding process (Brouwers and Gadella, 2003). From the above explanation we may conclude that the significantly high lipid peroxidation characterized by increased MDA production recorded in bull #4 may partly result from dead and phagocytic cells activities that are consequently manifested by low viability and motility result of the bull's sperm. The high MDA production in the same bull (#4) may also be associated with its high DNA damage.

TEM

Subtle anomalies present in the ultra-structures were only assessed by TEM. The TEM result revealed more detailed structural and physiologic state of the spermatozoa (Table 2). Physiological state as well as plasma membrane and mitochondria integrities are of much significance in providing information for assessment of the bulls' spermatozoal anticipated fertility. In the present study, the TEM showed increased sperm defects than obtained from morphological evaluation of each bull, with most of the highly concentrated spermatozoa of bull #4 been defective. This may not be unconnected with the high MDA production in the bull's semen.

Conclusion

The current study revealed that bulls #1, 2 and 3 were consistently satisfactory in most parameters evaluated and hence can be used for freezing. Whereas, bull #4 expressed significantly higher sperm concentration but yet, unsatisfactory in most other tests conducted. An indication of poor freezing potential which may be attributed to the low live:dead ratio, high MDA (LPO) and high abnormalities recorded from both morphological and TEM evaluation of sperm and may therefore not be a good candidate for cryopreservation/AI. The ultra-structural evaluation has complemented the traditional variables being checked. It revealed intactness and otherwise information about genetic materials and extent of other deformities of the sperm such as those of mitochondria, plasma membrane and acrosome, which may not have been accessed except by TEM. Lack of such details may affect the quality of decision about a bull's semen potential. While bulls whose semen samples first fail the traditional andrological evaluations may be retained (not quickly rejected) for prolonged period of repeated checks with some environmental-factors adjustment, in the hope that the semen quality may improve when the stressors are corrected. The TEM test exposes deformities that could be connected to genetic factors of the bull which are hardly correctible, thereby saving time, resources and protecting the next generation offspring from inheriting subfertility. Furthermore, freeze-thaw circle tends to reduce the overall quality of semen, therefore recruiting samples with pre-freezing low quality for the procedure amounts only to waste.

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