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Long-Term Storing of Frozen Semen at –196°C does not Affect the Post-Thaw Sperm Quality of Bull Semen

Alfredo Ramírez-Reveco, Jorge Luis Hernández and
Pablo Aros

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Abstract

Today, it is theoretically assumed that frozen storage of semen doses in liquid nitrogen guarantees sperm functionality indefinitely. However, there are few studies that objectively evaluate the effects of long-term storage on sperm quality parameters. In this study, we show a freezability analysis of bull semen stored for 1, 10, 25, 40 and 45 years at –196°C. Sperm viability and full sperm motility were analyzed by CASA system, and acrosome integrity was assessed with Coomassie blue staining. Our results showed that sperm viability and total sperm motility were not affected by long-term cryopreservation at –196°C. Specifically, we did not find any significant differences ($p > 0.05$) associated between different long-time storing analyzed; both parameters showed optimal values of sperm viability and total sperm motility (both over 60%). Additionally, the acrosomal integrity parameter was not affected, showing an optimal range (87±1.6 - 95±0.5%). We conclude that the sperm quality of bovine semen is not affected by long-term storage at –196°C. However, future field trials will be necessary in order to validate that both fertility and embryo viability are maintained for the times analyzed.

Keywords: sperm, cryopreservation, storage, liquid nitrogen, freezability, sperm quality

1. Introduction

Cryopreservation protocols for the bull used in the animal production industry began in the 1950s [1]. Since then, both the packaging type and cryopreservation system were changing on

the time. Primary considerations in the selection of a system for packaging semen were fertility, insemination preference, ease of handling, ease of identification, freedom from contamination, economics of storage and efficiency of ejaculates [2]. Sperm are commonly packaged in one of three ways: (a) glass ampoules, normally containing 0.5–1.2 ml of frozen semen; (b) pellets containing about 0.1 ml; and (c) polyvinyl chloride straws with a volume of 0.25–0.5 ml.

Early field trials showed that the bovine semen frozen to −79°C and packed on dry ice could still yield high fertility [3]. Regarding to time storage factor, studies of sperm motility have indicated a descent in sperm viability after storage [4, 5]. On the other hand, field trials carried out at the Reading Cattle Breeding Centre (Great Britain, 1960) indicated no effect on concep-tion rate when using long-term semen stored in a dry ice alcohol mixture for 4 years [6].

Until the 1970s, it was thought that frozen semen could be indistinctly stored in mechanical freezers at about −25°C, in solid carbon dioxide at −79°C, or in liquid nitrogen at −196°C. However, an inverse relationship between preservation of sperm viability and storage temperature was shown [7]. Briefly, most frozen semen was stored in a mixture of dry ice and alcohol at −79°C, which ultimately decreased fertility [8–10]. Meanwhile, studies of frozen semen stored at −196°C have shown a consistently high non-return rate [11–14].

Furthermore, since the 1970s, there have been mentions that deterioration continues even when sperm are stored in liquid nitrogen; suspecting that aging of spermatozoa may occur if semen is stored for long periods of time, and this may be associated with embryo mortality and delayed return [9, 15].

Studies by Salisbury and Hart [16] suggested that bovine frozen sperm have a low fertility level and promote increased embryonic mortality after 18 months of storage at −196°C, but other studies have been unable to confirm this. In this context, Strom [14] found no evidence of reduced fertility when approximately 60,000 inseminations were performed with semen packaged in pellets, following storage in liquid nitrogen at −196°C for approximately 1–1.5 years. Cassou [17] reported no difference in fertility after 285,551 inseminations with frozen semen in straws were stored at −196°C for up to 4.5 years. Similarly, a field trials of Roettger et al [18], with 100,000 inseminations and using frozen semen stored at −196°C for 5 years, a normal fertility rate was evidenced.

Field trials using frozen semen packaged in ampoules, pellets and straws have indicated that the influence of packaging methods in fertility has been inconsistent [2]. Therefore, according to these authors, if the semen fertility stored in LN is reduced with time, regardless of packaging technique, some factors other than storage are responsible.

Today, cryopreservation in liquid nitrogen (−196°C) is a technique that allows for long-term storage of spermatozoa [19]. This is a highly practical method in breeding programs for domestic animals and is used to maintain the establishment and genetic diversity of gene banks [20, 21].

Cryopreservation requires many stages during cooling/freezing and thawing procedures, which interactively affect its success [22, 23]. On the other hand, it is assumed that storage period in deep freezing does not affect sperm viability [24, 25], and there is argument that

spermatozoa retain their fertilizing potential indefinitely when stored at −79°C in dry ice, or at −196°C in liquid nitrogen [26]. However, there is a scarcity of studies designed in order to detect a decrease in reproductive performance of cryopreserved semen as a function of storage time.

In this respect, Mazur [27] proposed that, by accumulative cosmic radiation, more than 3,000 years. Then the question arises, how long cells can be stored in liquid nitrogen without suffering damage? This question is probably irrelevant if the cellular storage temperature is below −120°C, where chemical reactions do not occur in a human timescale. Moreover, at −196°C, the thermally driven reactions only can occur on a geological timescale [28].

There is the possibility of slow accumulation of direct damage from ionizing radiation, but this becomes significant only after centuries of storage [29]. Yet, as previously mentioned, insemination trials with frozen stored semen [16] suggest a far shorter time period of optimal semen storage at the above-mentioned low temperatures, in consideration of fertility rate maintenance. However, other evidence suggests that this could be due to inadequate maintenance of temperatures [26]. There are two studies that strongly reinforce the idea that fertilization potential is maintained in long-term storage in liquid nitrogen. Specifically, the *in vitro* fertilization (FIV) was obtained using frozen spermatozoa, stored in nitrogen liquid during 37 and 27 years, for bovine and human, respectively [30, 31].

In relation to sperm quality parameters, there are few studies that objectively evaluate the effects of long-term sperm storage. Leibo et al. [30] reported a normal bovine sperm motility after 37 years, and Rofeim and Gilbert [32] reported no statistical reduction in human sperm quality after 5 years of follow-up. More recently, although it was not analyzed by computer-assisted sperm analysis (CASA system), Malik et al. [33] showed that the viability and motility of thawed sperm stored in liquid nitrogen during 6 years were lower than 1- to 2-year storage .

The main goal of this study was to assess, through CASA system, the main sperm quality parameters of cryopreserved and stored bull semen in liquid nitrogen for 10, 25, 40 and 45 years.

2. Materials and methodology

2.1. Seminal doses, race of donor and processing

In this study, a total of 75 commercial doses from bulls Friesian breed were used. The cryopreserved germplasm were defined and divided into five groups according to the storage time. For each group, 15 seminal doses from five different donors (three each) were considered. All seminal doses used were collected, processed, packaged, cryopreserved and stored (−196°C) using commercial standard procedures by Center of Artificial Insemination (CIA), belonAustral University Chile (UACh).

The mean storage times or groups of the semen doses analyzed were the following: 45, 40, 25 and 10 years, which were packaged in glass ampoules, pellets, short straws and fine straws,

respectively. As a control, commercial frozen doses cryopreserved in fine straws and stored in liquid nitrogen for 1 year were used. The following thawing protocols were used, in accordance with cryopreservation packaging supports (that is showed in **Figure 1**): Ampoule samples were thawed in thermo-stated water bath at 50°C for 75 s; pellet samples were thawed in a Thermos-stated water bath at 40°C for 55 s; short straw (Mini-Tubes) and fine straw samples were thawed in a thermo-stated water bath at 37°C for 30 s.

Figure 1. Different freezing packaging system used in this study.

2.2. Sperm quality analysis of seminal doses

2.2.1. Plasma membrane integrity (viability assessment)

Plasma membrane integrity was determined using the acridine orange (AO)/propidium iodide (PI) double-staining technique, according to Córdova et al. [34], with modifications. Briefly, post-thawing samples (3 μL) were mixed (1:1) with a staining aqueous solution composed of 20 μM AO and 10 μM PI in a tempered microscope slide. Stained samples were analyzed using the CASA System (viability module of the Sperm Class Analyser®, Microptic, Spain) coupled to an epifluorescence microscope (Nikon E200, Japan) with a high-velocity camera (Basler AG, Germany). Viability percentages were established from a minimum of 1000 spermatozoa for each sample.

2.2.2. Sperm motility assessment

Sperm motility was assessed using the CASA system (Motility module of the Sperm Class Analyser®, Microptic, Spain), according to Ramírez et al. [35], with modifications. A total of 6 μL aliquots of samples was then placed on a prewarmed (37°C) slide and covered with a 24 mm² coverslip. The motility analysis by CASA system was based on the analysis of 25 consecutive, digitalized photographic images taken over a time lapse of 1 s, obtained from a single field using a negative objective (10× magnification) and a phase contrast microscope (Nikon E200, Japan), coupled to a high-velocity camera (Basler AG, Germany, scA780 54tc). Four or seven separate fields were taken for each sample (at less 500 spermatozoa analyzed). Sperm motility parameters were as follows: curvilinear velocity (VCL); linear velocity (VSL); mean velocity (VAP); linearity coefficient (LIN): (VSL/VCL) × 100(%). Straightness coefficient

(STR): (VSL/VAP) × 100(%). Wobble coefficient (WOB): (VAP/VCL) × 100(%). Mean amplitude of lateral head displacement (ALH); frequency of head displacement (BCF). Bovine configuration of CASA system used was as follows—capture: 25 frames/s; particle area range: 5–70 µm², classification according to velocity (VAP): static < 10 µm/s < slow < 25 µm/s < medium < 50 µm/s < rapid. The progressive motility was defined as the percentage of spermatozoa showing an STR above 70%.

2.2.3. Sperm acrosomal integrity assessment

The structural status of sperm acrosomes was assessed using Coomassie G-250 staining, according to Larson et al. [36]. Briefly, sperm aliquots were washed in TBS, fixed and permeabilized for at least 30 min at 4°C in 100% methanol. Permeabilized spermatozoa dried onto slides were then covered with a droplet of staining solution (0.22% W/V Coomassie blue G-250; 50% methanol and 10% glacial acetic). The samples were washed with excess of bidistilled water, dried and observed under 100 × oil immersion lens. Percentage of stained cells was determined by counting of at less 300 spermatozoa.

2.3. Statistical analysis

Statistical analyses were performed using one-way ANOVA with post hoc Bonferroni multiple comparison tests. For the analysis, we used GraphPAD (Prism 6) software and differences were considered significant and highly significant for p values of <0.05 and <0.01, respectively.

3. Results and discussion

Our results showed that integrity of plasma membrane is not altered by long-term storage at −196°CC (**Figures 2A** and **3A**). Specifically, we did not find any significant differences associated with the different storage times analyzed (>10 years) or in relation to control (p < 0.05), with a post-thaw sperm viability percentage that oscillated between 60 ± 1.8 and 68 ± 2.1. A similar situation was observed in the acrosomal integrity analysis, wherein the only significant difference observed associated with storage time was specifically between 45 and 40 years (p < 0.01) (**Figures 2B** and **3C**). Despite these differences, the percentage of acrosomal integrity after thawing ranged between 87 ± 1.6 and 95 ± 0.5, and ultimately acceptable enough even for fresh semen. On the other hand, comparative analysis of total sperm motility did not show significant differences between times storage analyzed. Specifically, all values shown are above 60%, ranging between 60 ± 2.4 and 66 ± 3.2, similar to viability results (**Figure 2A** and **2C**).

In respect to progressive motility, a higher significant value was found in samples from frozen semen stored by 40 years (p < 0.01) (**Figures 2D** and **3B**). Additionally, a similar behaviour in other cinematic parameters (VSL, VAP, LIN and WOB) was observed in these samples, showing higher values (p < 0.001) (**Table 1**). Consistently, lowest values of hyperactivity, ALH and BCF were observed in the frozen semen stored for 40 years (p < 0.001). This great differences in progressive motility and other cinematic parameters in samples of seminal doses storage by

40 years may have been influenced by the thawing solution used in the pellets tube, particularly with the presence of sodium bicarbonate (30.9 mM) [37].
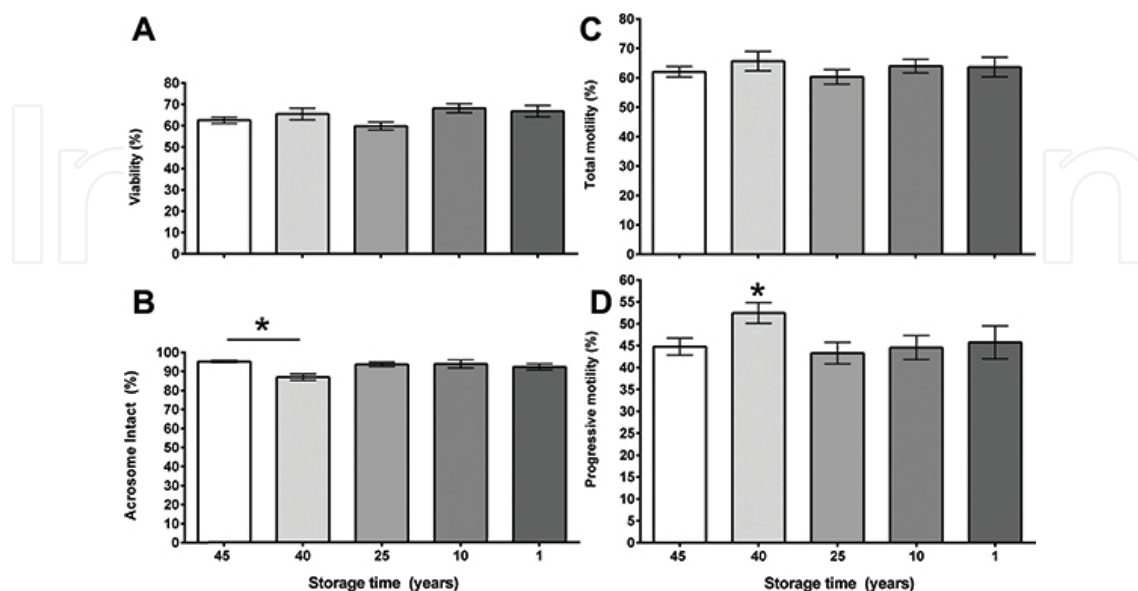

Figure 2. Freezability evaluation of frozen-thawed bovine sperm. A) Plasma membrane integrity or viability (%) analysis by CASA system using double stain (propidium iodide and acridine orange). A minimum of 1000 spermatozoa were counted for each assay. B) Acrosomal integrity (%) analysis by Coomassie G-250 staining. A minimum of 200 spermatozoa were counted for each assay. C) Total sperm motility (%) analysis by CASA System, VCL > 10 μm/s. A minimum of 500 spermatozoa were counted for each assay. D) Progressive sperm motility (%) analysis by CASA System, STR > 70%. A minimum of 500 spermatozoa were counted for each assay. Each bar (storage time) represents the mean ± SEM of a total of 15 doses were analyzed (from five bulls, three doses for each time analyzed). Significant differences among time storing are shown: one-way ANOVA/Bonferroni post-test (p < 0.001 or by different letters).

The results shown differ from those obtained by Malik et al. [33], who reported a significant decrease in both the viability and motility associated with prolonged storage (6 years versus 1–2 years). It is highly probable that these discrepancies, in the case of both viability and motility, are due to differences in the sensitivity of the technique used, nigrosin/eosin staining and bright field microscopy versus acridine orange/propidium iodine and epifluorescent microscopy in our case. This could also be due to the evident differences associated with the use of analysis of subjective sperm motility analysis versus our use of CASA system.

There is an argument that spermatozoa store at −79°C (in dry ice) or at −196°C (in liquid nitrogen) retain their fertilizing potential indefinitely [26]; however, the storage time results that studies are controversial. Effectively, although Mazur [29] proposed that several centuries are required, of liquid nitrogen storage, for that ionizing cosmic radiation alters or damages the DNA of the cell. However, there are studies that raise a discrepancy respect that cryopreservation, for several years, completely stops the processes of sperm biochemistry, but whose storage times do not exceed 6 years.

Fourteen five years ago, Salisbury and Hart [16] proposed that bovine frozen sperm have a low fertility level and promote increased embryonic mortality after 1.5 years of storage at −196°C. More recently, Haugan et al. [19] based on results of field trials indicated that the

likelihood of conception decreased only a little more than one percentage after 5.5 years of storage, but that level of decline seems to be so important because the calving rate predicted by multiple logistic regression was 59.2%, optimal value according to commercial standard for frozen semen. Contrary, field trial results of Strom [14] found no evidence of reduced fertility when was used frozen semen storage by 1–1.5 years. Additionally, Cassou [17] and Roettger et al [18] reported no difference in fertility when were used frozen semen stored at −196°C for up to 4.5 and 5 years, respectively. Unfortunately, there are no field trials in that and both pregnancy and calving rates have been analyzed; to rule out or confirm effects of prolonged storage on embryo mortality, this would be the only one way to resolve the question.
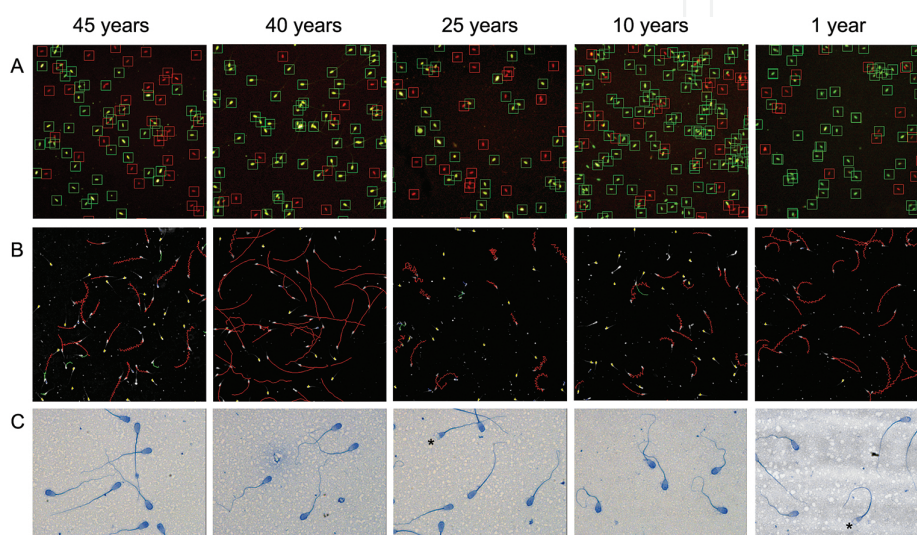

Figure 3. Representative's field captures for sperm quality parameters analyzed. (A) Plasma membrane integrity analysis, green or red fluorescents marks correspond to sperm recognized as live or dead, respectively. Images obtained with epifluorescence microscope, objective: 10×. (B) Sperm motility analysis, the tracking in red, green, blue and yellow, correspond to sperm sorted according velocity: rapid, medium, slow and static, respectively. Images obtained with phase contrast microscope, objective: 10×-negative. (C) Acrosomal integrity analysis, Coomassie blue G-250 stained acrosome-intact sperm or those with acrosome reacted or damaged (asterisks). Images obtained with bright field microscope, objective: 40×.

Storage time at −196°C	VCL (μ/s)	VSL (μ/s)	VAP (μ/s)	LIN (%)	SRT (%)	WOB (%)	ALH (μ)	BCF (Hz)
45 years	68.23 ± 1.8[a]	34.88 ± 1.5[a]	43.94 ± 1.6[a]	47.8 ± 1.3[a]	71.5 ± 1.3[a]	63.1 ± 0.9[a]	2.57 ± 0.0[a]	9.38 ± 0.29[a]
40 years	77.27 ± 2.5[a]	52.63 ± 2.1[b]	65.47 ± 2.9[b]	60.2 ± 1.6[b]	72.5 ± 1.4[a]	78.4 ± 1.8[b]	1.97 ± 0.1[b]	7.07 ± 0.6[b]
25 years	75.67 ± 2.1[a]	32.46 ± 1.4[a]	48.26 ± 1.9[a]	42.5 ± 1.3[a]	63.9 ± 1.3[b]	63.0 ± 1.2[a]	2.81 ± 0.0[a]	9.46 ± 0.3[a]
10 years	78.64 ± 2.8[a]	34.03 ± 2.1[a]	50.23 ± 2.2[a]	41.7 ± 2.1[a]	63.0 ± 2.0[b]	62.2 ± 1.6[a]	2.94 ± 0.1[a]	8.87 ± 0.2[a]
1 year	69.08 ± 5.0[a]	32.42 ± 2.3[a]	45.02 ± 3.2[a]	45.0 ± 2.1[a]	66.9 ± 2.2[a,b]	63.5 ± 1.3[a]	2.59 ± 0.1[a]	9.19 ± 0.4[a]

Values are expressed as mean ± SEM. Different superscript letters (a and b) indicate significant differences among storage times (one-way ANOVA, p < 0.001).

Table 1. Effect of storage time at −196°C on bull sperm kinetic parameters.

Our result showed that the more important parameters of sperm quality non-present changes associated to storage times analyzed (1–10–25–45 years). Considering that the plasma and acrosomal membrane integrity are two irreversible parameters of sperm quality, and that the motility is commonly believed to be one of the most important characteristics associated with the fertilizing ability of semen [38]. Our freezability data, analyzed as a whole, suggest that fertilizing potential of the seminal dose is commercially analyzed, independent of storage time, and it is high. In this respect, Budworth et al. [39, 40] observed significant correlation of the sperm motility and sperm velocity with the competitive fertility index. Moreover, Amann [41] reported a high level of correlation between competitive fertility index and sperm motility, VCL, VSL parameters, with 0.80, 0.68 and 0.70, respectively.

We conclude, and categorically, that the basic parameters of sperm quality of bovine semen are not affected by long-term storage at −196°C. Complementary analysis, including other aspects as to mitochondrial metabolism, reactive oxygen species (ROS) levels, DNA fragmentation and chromatin integrity, could shed light on possible and potential changes induced for prolonged storage.

Future studies of embryo production by *in vitro* fecundation (IVF) and field trials are needed, in order to confirm effects associated to long-term sperm storing at −196°C on fertility, embryonic viability and calving rate.

Acknowledgements

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References

[1]   Walters EM, Benson JD, Woods EJ & Critser JK. The history of sperm cryopreservation. In: Pacey, A.A., & Tomlinson, M.J., editors. Sperm banking: theory and practice. Cambridge: Cambridge University Press; 2009; p. 1–17.

[2]   Berndtson WE & Pickett BW. Techniques for the cryopreservation and field handling of bovine spermatozoa. In: The integrity of frozen spermatozoa. Proceeding of a Round-table Conference held on 6–7 April 1976. National Academy of Sciences Washington, DC. 1976; p. 53–77.

[3]   Bratton RW, Foote RH & Cruthers JC. Preliminary fertility results with frozen bovine spermatozoa. Journal of Dairy Science. 1955; 38 (1): 40–46.

[4]   Dunn HO & Hafs HD. Extenders and techniques for freezing bovine spermatozoa. Journal of Dairy Science. 1953; 36 (6): 577–577.

[5]   Buch NC, Smith VR & Tyler WJ. Bull and line differences in the survival of spermatozoa after freezing and thawing. Journal of Dairy Science. 1956; 39: 1712–1716.

[6]   Melrose DR. Ultra-low temperature storage of sperm. In: Maule, JP., editors. The semen of animals and artificial insemination. 1st ed. Bucks, England: Commonwealth Agricultural Bureaux; 1962; p. 109–132.

[7]   Gomes WR. Artificial insemination. In: Cole, HH., & Cupps, PT., editors. Reproduction in domestic animals. 3rd ed. London: Academic Press Inc; 1977; p. 257–284.

[8]   Mixner JP & Wiggin SH. Stored frozen semen fertility. AI Digest. 1960; 8 (7): 19.

[9]   Salisbury GW & Flerchinger FH. Aging phenomena in spermatozoa. I. Fertility and prenatal losses with use of liquid semen. Journal of Dairy Science. 1967; 50 (10): 1675–1678.

[10] Stewart DL. Observation on the fertility of frozen semen stored at −79°C and −196°C. In: In Proceedings of International Congress on Animal Reproduction and Artificial Insemination; 1964; Vol. 4. p. 617.

[11] Clegg EO & Pickett BW. Effect of storage at −196°C on fertility. AI Digest. 1966; 14 (3): 12–13.

[12] Foote RH. Aging of spermatozoa during storage in liquid nitrogen. In: In Proceedings of the Fourth NAAB Technical Conference on Artificial Insemination and Reproduction. 1972; Vol. 28.

[13] Lindström U. Pellets: experiences and results of storage over 3 years. In: VII International Congress on Animal Reproduction and Artificial Insemination, Munich. 1972; p. 1428–1435.

[14] Strom B. Influence upon fertility of bull semen of storage time in liquid nitrogen. In: International Congress on Animal Reproduction and Artificial Insemination [Proceedings]. 1968; p. 1171–1174.

[15] Salisbury GW. Effect of season and storage at −79°C to -88°C on fertility and prenatal losses. Journal of Dairy Science. 1967; 50 (10): 1683–1689.

[16] Salisbury GW & Hart RG. Gamete aging and its consequences. Biology of Reproduction. 1970; (2): 1–13.

[17] Cassou R. A technique for semen preservation and its application to breed improvement programmes. AI Co-op Centre. 1972; 61-L'Aigle, France. Personal publication (Cited by Berndtson WE & Pickett BW, 1976) [2].

[18] Roettger LW, Salisbury GW, Lee AJ, Boyd LJ & Ingalls W. *In vitro* aging of frozen bull semen. Journal of Dairy Science. 1975; 58 (5): 767–768.

[19] Haugan T, Gröhn YT, Kommisrud E, Ropstad E & Reksen O. Effects of sperm concentration at semen collection and storage period of frozen semen on dairy cow conception. Animal Reproduction Science. 2007; 97 (1): 1–11.

[20] Saint JM, Lecoq R, Seigneurin F, Blesbois E & Plouzeau E. Cryopreservation of semen from endangered pheasants: the first step towards a cryobank for endangered avian species. Theriogenology. 2003; 59 (3): 875–888.

[21] Shivaji S, Kholkute SD, Verma SK, Gaur A, Umapathy G, Singh A, Sontakke S, Shailaja H, Reddy A, Monika S, Sivaram V, Jyotsna B, Bala S, Ahmed SM, Bala A, Chandrashekar BVN, Gupta S, Prakash S & Singh L. Conservation of wild animals by assisted reproduction and molecular marker technology. Indian Journal of Experimental Biology. 2003; 41: 710–723.

[22] Medeiros CMO, Forell F, Oliveira ATD & Rodrigues JL. Current status of sperm cryopreservation: why isn't it better? Theriogenology. 2002; 57 (1): 327–344.

[23] Neild DM, Gadella BM, Chaves MG, Miragaya MH, Colenbrander B & Agüero A. Membrane changes during different stages of a freeze–thaw protocol for equine semen cryopreservation. Theriogenology. 2003; 59 (8): 1693–1705.

[24] Hammerstedt RH, Graham JK & Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive. Journal of Andrology. 1990; 11 (1): 73–88.

[25] Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reproduction, Fertility and Development. 1995; 7 (4): 871–891.

[26] Vishwanath R & Shannon P. Storage of bovine semen in liquid and frozen state. Animal Reproduction Science. 2000; 62 (1): 23–53.

[27] Mazur P. Fundamental aspects of the freezing of cells with emphasis on mammalian ova and embryos. In: Proceedings of 9th International Congress on Animal Reproduction. A.I. Madrid, USA. 1980; p. 99–114.

[28] Gao D, Mazur P & Critser JK. Fundamental cryobiology of mammalian spermatozoa: scientific principles. In: Karow, A., & Critser, J., editors. Reproductive tissue banking. 1st ed. USA: Academic Press; 1997; p. 263–328.

[29] Mazur P. Freezing of living cells: mechanisms and implications. American Journal of Physiology-Cell Physiology. 1984; 247 (3): C125–C142.

[30] Leibo SP, Semple ME & Kroetsch TG. *In vitro* fertilization of oocytes by 37-year-old cryopreserved bovine spermatozoa. Theriogenology. 1994; 42 (8): 1257–1262.

[31] Horne G, Atkinson AD, Pease EHE, Logue JP, Brison DR & Lieberman BA. Live birth with sperm cryopreserved for 21 years prior to cancer treatment: case report. Human Reproduction. 2004; 19 (6): 1448–1449.

[32] Rofeim O & Gilbert BR. Long-term effects of cryopreservation on human spermatozoa. Fertility and Sterility. 2005; 84 (2): 536–537.

[33] Malik A, Laily M & Zakir MI. Effects of long term storage of semen in liquid nitrogen on the viability, motility and abnormality of frozen thawed Frisian Holstein bull spermatozoa. Asian Pacific Journal of Reproduction. 2015; 4 (1): 22–25.

[34] Córdova A, Strobel P, Vallejo A, Valenzuela P, Ulloa O, Burgos RA, Menarim B, Rodriguez-Gil JE, Ratto MH & Ramírez-Reveco A. Use of hypometabolic TRIS extenders and high cooling rate refrigeration for cryopreservation of stallion sperm: presence and sensitivity of 5′AMP-activated protein kinase (AMPK). Cryobiology. 2014; 69 (3): 473–481.

[35] Ramírez AR, Castro MA, Angulo C, Ramió L, Rivera MM, Torres M, Rigau T, Rodriguez-Gil JE & Concha II. The presence and function of dopamine type 2 receptors in boar sperm: a possible role for dopamine in viability, capacitation, and modulation of sperm motility. Biology of Reproduction. 2009; 80 (4): 753–761.

[36] Larson JL & Miller DJ. Simple histochemical stain for acrosomes on sperm from several species. Molecular Reproduction and Development. 1999; 52 (4): 445–449.

[37] Grove D. Andrological field diagnosis on cattle in the tropics. 1st ed. *Deutsche Gesellschaft fur Technische Zusammenarbeit GmbH*. (GTZ). 1977; p. 286.

[38] Kathiravan P, Kalatharan J, Karthikeya G, Rengarajan K & Kadirvel G. Objective sperm motion analysis to assess dairy bull fertility using computer-aided system—A review. Reproduction in Domestic Animals. 2011; 46 (1): 165–172.

[39] Budworth PR, Amann RP & Hammerstedt RH. A microcomputer-photographic method for evaluation of motility and velocity of bull sperm. Journal of Dairy Science. 1987; 70 (9): 1927–1936.

[40] Budworth PR, Amann RP & Chapman PL. Relationships between computerized measurements of motion of frozen-thawed bull spermatozoa and fertility. Journal of Andrology. 1988; 9 (1): 41–54.

[41] Amann RP. Can the fertility potential of a seminal sample be predicted accurately? Journal of Andrology. 1989; 10: 89–98.