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Extension of Fresh Bull Semen in Ham's F10 Stored at Different Storage Conditions for 72 Hours

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Abstract: Semen preservation is a critical procedure to guarantee that good quality semen is adequate for advanced assisted reproduction. The present study aimed to evaluate viability of Nguni bull spermatozoa diluted with modified Ham's F10 culture medium stored at different storage conditions (5, 12, 17°C and controlled room temperature 24°C) for 72 h. Following microscopic evaluations, uncontaminated semen samples with progressive motility >70% were pooled before being aliquoted randomly into four test tubes of modified Ham's F10. Diluted samples were distributed unsystematically to each of the four temperature conditions (5, 12, 17 and 24°C) and stored for 72 h. Computer aided sperm analysis was used to evaluate sperm motility, DNA fragmentation and viability. The highest spermatozoa motility rate (86.5%) and viability (26.5%) was observed with 24°C as compared to the other three temperatures 17°C (69.5; 8.0%), 12°C (50.3; 2.5%) and 5°C (35.1; 0.5%), respectively for 72 h. The overall sperm viability rate of the storage conditions 24, 17, 12 and 5°C<30% after 72 h. There was no significant difference in sperm DNA fragmentation amongst the four storage conditions ($p<0.01$). In conclusion, motility and viability rate of Nguni spermatozoa extended with modified Ham's F10 culture medium decreased noticeably regardless of the storage temperature condition.

Key words: Bull semen, Ham's F10, sperm DNA fragmentation, viability, culture medium, sperm motility

INTRODUCTION

In South Africa, livestock like in other developing countries is the most important source of livelihoods for the poor. For example, the poor livestock farmers generates income through sales of their livestock products which in turn they make use of to buy affordable staple food to support their families (Smith *et al*., 2013). Livestock animals namely cattle, goats and sheep in the isolated areas of the Vhembe region of the Limpopo Province in Southern Africa show clear signs of past inbreeding due to lack of selection of phenotypically superior bulls for breeding and poor management practices (Raseona *et al*., 2017). This has resulted in lower reproduction rates which have led to poverty in those areas. Successful application of artificial insemination and other Assisted Reproductive Technologies (ART's) has generally been limited to commercial farmers.

Preserving bull semen in a liquid state stored at refrigerated temperature conditions could be a cheaper alternative than cryopreserving semen in liquid nitrogen, to assist in artificial insemination programs in the isolated rural areas of the Vhembe District. Consequently, production of high quality extended spermatozoa preserved at different temperature conditions for use in AI programs will greatly benefit the rural communities of the Vhembe District living in close proximity.

Artificial insemination is the most frequent assisted reproductive technology used today and still continues to have a remarkable impact on various animal breeding industries (Althouse, 2007). Artificial insemination has many advantages that include the prevention of sexually transmitted diseases, easy transport of genetic material and optimal use of superior males that are not capable of mating properly due to various reasons (Bale, 2003). Through AI a single ejaculate from males with superior genetic materials can be used to breed numerous females to optimize the distribution of mammalian favorable genes. In order to improve herd management before and after the birth of progenies, large groups of females should be synchronized and later be inseminated at the same time. The use of AI also reduces the cost of bulls required for breeding, maintenance expenditures and prevents overuse of males (Heise, 2012).

Bull fertility is the most imperative factor in cattle reproduction as one ejaculate can be used to impregnate numerous cows through artificial insemination. Several scholars reported that male fertility assessment is

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predominantly based on evaluation of semen which includes parameters such as sperm viability and membrane integrity (Christensen *et al*., 2005, 2011), motility (Kathiravan *et al*., 2011; Rodriguez-Martinez, 2013), morphology (Soderquist *et al*., 1991; Nagy *et al*., 2013), acrosome integrity (Grippo *et al*., 1995; Kumaresan *et al*., 2016), DNA integrity (Januskauskas and Zilinskas, 2002) and velocity parameters (Januskauskas *et al*., 2003; Nagy *et al*., 2015).

Motility is considered to be the most imperative characteristic related to the fertilizing ability of sperm cells and is an articulation of their viability and structural integrity (Kathiravan *et al*., 2011). The traditional evaluation of spermatozoa motility using light microscopes has been proven to be subjective because of the great evaluation variations that existed amongst observers. Utilization of a Computer Aided Sperm Analysis (CASA) system surpasses subjective assessment and also provides an objective and more quicker way to assess spermatozoa motility (Malmgren, 1997; Januskauskas and Zilinskas, 2002). Furthermore, CASA calculates other spermatozoa motion attributes such as Average Path Velocity (VAP), Curvilinear Velocity (VCL), Straight-Lline Velocity (VSL), Straightness (STR), Linearity (LIN), Wobble (WOB) and Beat Cross Frequency (BCF). Computer aided sperm analyzer comprises of an objective microscope connected to a video camera, a video frame grabber card and a computer (Mortimer, 2000; Kathiravan *et al*., 2011).

At ejaculation sperm cells are diluted with seminal plasma from the accessory glands and their motility can only last for couple of hours. In attempts to lengthen their survival in vitro it is important to decrease the metabolic activity by lowering the temperature or chemical inhibitors which also involves dilution (Johnson *et al*., 2000). The correlation between temperature and metabolic rate is frequently conveyed as Q_{10} which estimates the increment rate for each 10°C temperature increase. For instance if the metabolic rate of an animal at 0°C is x then at 10°C the rate would be 2x at 20°C 4x, etc. (Anonymous, 2012). The metabolic rate of a body cell (including spermatozoa) will thus decrease by a factor of 2x for every 10°C drop in temperature. Mammalian spermatozoa react to dilution with a sudden increase in activity, followed by an increase in membrane damage and reduced motility.

Successful liquid preservation of spermatozoa depends on the reversible decrease in metabolic activity and survival of sperm cells. This can be accomplished by provision of effective storage conditions for the chilled semen as well as the utilization of beneficial diluents that sustain DNA integrity, motility and fertilizing capacity of spermatozoa during liquid storage (Garner and Johnson, 1995; Fazeli *et al*., 1997). The present study was designed to examine the effect of different storage conditions (5, 12, 17 and 24°C controlled room temperature) on motility and viability of Nguni bull sperm motility and viability preserved using the modified Ham's F10 culture medium for 3 days and further assessment of the spermatozoa's DNA fragmentation using sperm blue staining protocol.

MATERIALS AND METHODS

Bull semen collection and processing: Semen samples were collected using the Pulsator IV-Auto Adjust™ electro-ejaculator (Lane manufacturing Inc, Denver Colorado, USA) from three Nguni bulls with proven fertility records, aged 2-3 years, kept at the University of Venda experimental farm. Following collection and macroscopic evaluations, uncontaminated semen samples with progressive motility greater than 70% were pooled together to eliminate individual differences and then divided into four aliquots of modified Ham's F10 culture medium at a ratio of 1:4 v/v (semen:extender). Ham's Fl0 was supplemented with 0.025 g/4 mL Maltose monohydrate ($C_{12}H_{22}O_{11}$, H_2O), 10% Bovine Serum Albumin (BSA) and 1% antibiotic cocktail (Penicillin, Streptomycin and Fungizone). The extended samples were randomly stored at 5, 12, 17 and 24°C temperatures for 72 h.

Sperm motility evaluations: Computer aided sperm analyzer was used to evaluate sperm motility at 10x magnification. About 3 μL of semen from each of the four extended samples was placed inside one chamber of the eight chambered Leja® slides (Leja Products B.V, Nieuw-Vennep, Netherlands) on the warm glass stage at 37°C of the CASA microscope for evaluation at 0, 24, 48 and 72 h. For each analysis five fields were captured for all the samples.

Spermatozoa viability test: Spermatozoa were stained with nigrosin-eosin stain. For staining two microscope slides were pre-warmed at 37°C. About 6 μL of the semen sample and 20 μL of eosin stain was poured on the end of the slide and mixed using a pipette tip on a warm glass stage at 37°C. A drop of 20 μL nigrosin stain was then poured onto the mixture and mixed properly before the second slide also pre-warmed at 37°C could be used to smear across the slide. The smeared slide was immediately placed on a hot slide warmer at 120°C to allow drying. Computer aided sperm analysis viability program was used at 60x magnification to count the number of viable/live and the non-viable/dead spermatozoa. Two-hundred spermatozoa were counted per each stained slide.

Sperm DNA Fragmentation: Sperm DNA fragmentation was assessed using Sperm-halomax kit (Halotech DNA. Madrid, S). The slides were prepared following the Bos-halomax® DNA fragmentation kit protocol. The

processed slides were stained using Sperm-blue and evaluated using the CASA at 60x magnification to count fragmented and non-fragmented spermatozoa. Two hundred spermatozoa were counted per each stained slide.

Statistical analysis: The study was conducted in eight replicates and data was statistically analysed using General Linear Model (GLM) procedures of Minitab program (Mortimer, 2013), using 4×4 factorial in a randomized complete design. Analysis of Variance (ANOVA) was used for analysis of the results. Means of different treatments were compared using Tukey's post hoc test. Significance was set at $p>0.05$.

RESULTS AND DISCUSSION

Spermatozoa samples stored at controlled room temperature 24°C showed no significant decrease in TM, PM and RAP from 0 h until 48 h of storage (Table 1). No significant decrease ($p>0.01$) again in TM and RAP from 24 h until 72 h of storage was observed for 5°C storage temperature. Velocity parameters were also affected by storage temperatures overtime. No significant difference, however, was observed for storage condition 24°C in VCL, VSL, VAP and LIN from 0 h until at 48 h and again on STR velocity overtime. Storage temperature 17and 12°C also didn't show a significant decrease ($p<0.05$) in WOB velocity. Furthermore, samples stored at 5°C did not show any significant difference ($p<0.05$) in VCL, VSL, VAP and LIN.

Table 2 shows viable/live and SDF mean values of Nguni bull spermatozoa after extension with Ham F10 for three days stored at different temperatures. Spermatozoa samples stored at 24°C showed higher viability rate (26.5%) than the other three temperatures 17 (8.0), 12 (2.5) and 5°C (0.5%) and the lowest viability rate obtained with spermatozoa samples at 5°C (0.5%). There was no significant difference ($p>0.05$) between spermatozoa samples stored at 5 and 12°C but they were significantly different to spermatozoa samples at 17 and 24°C ($p<0.05$). Sperm heads with large expanded/dispersed nuclei contain fragmented DNA while heads without expanded/dispersed nuclei are without DNA fragmentation (arrows in Fig. 1a, b). No significant difference in SDF was observed in any of the storage temperatures after 72 h of storage ($p>0.01$).

Results from this study exhibited that spermatozoa motility, velocity parameters and viability decreased drastically over 72 h of preservation regardless of the different storage temperature conditions which is in agreement with the study done by Hara *et al.* (2010) who also observed that spermatozoa quality decreases throughout the refrigeration period regardless of the extender, dilution rate or storage conditions. Spermatozoa samples stored at controlled room temperature 24°C, however, exhibited the highest sperm motility rate as compared to the other three temperatures 5, 12 and 17°C for 72 h. Similarly to this study, Raseona *et al.* (2017) reported that Nguni bull semen extended with modified Ham's F10 survived for 72 h stored at 24°C. Again 24°C

Table 1: Motility rates of extended bull semen in Ham's F10 evaluated at 0, 24, 48 and 72 h stored at 5, 12, 17 and 24°C (n = 3; 8 ejaculates each)

Treatment	ST (h)	TM (%)	PM (%)	RAP (%)	VCL (μm/sec)	VSL (μm/sec)	VAP (μm/sec)	LIN (%)	STR (%)	WOB (%)
24°C	0	96.0^{a}	45.3^{a}	39.5^{a}	105.8^{ab}	60.0^{a}	81.0^{a}	57.0^{ab}	74.3^{a}	76.5^{a}
	24	90.8^{a}	39.0^{ab}	45.0^{a}	120.5^{a}	56.3^{a}	77.0^{a}	46.3^{abcd}	72.8^{ab}	64.0^{ab}
	48	87.3^{ab}	33.8^{bc}	39.8^{a}	116.0^{a}	49.8^{ab}	70.0^{ab}	42.8^{bcde}	71.0^{abc}	60.5^{bc}
	72	72.0^{bc}	22.8^{cde}	21.3^{bc}	78.8^{cd}	33.0^{c}	45.0^{cd}	41.0^{cde}	72.3^{ab}	56.3^{bcd}
17°C	0	94.5^{a}	43.5^{ab}	38.8^{a}	108.5^{a}	62.8^{a}	83.3^{a}	57.8^{a}	75.5^{a}	76.5^{a}
	24	82.0^{ab}	25.5^{cd}	33.8^{ab}	106.0^{ab}	36.5^{bc}	54.8^{abc}	34.0^{def}	66.3^{abcd}	51.5^{bcde}
	48	60.25^{c}	13.5^{ef}	12.8^{cd}	80.5^{bcd}	25.3^{cd}	39.0^{cd}	31.3^{ef}	64.8^{abcd}	48.5^{cdef}
	72	42.5^{d}	6.0^{fg}	2.5^{d}	56.5^{de}	21.3^{cde}	30.5^{de}	36.8^{def}	68.0^{abcd}	53.3^{bcde}
12°C	0	96.3^{a}	42.0^{ab}	37.3^{a}	107.8^{a}	59.5^{a}	81.3^{a}	55.3^{abc}	73.0^{a}	75.5^{a}
	24	57.5^{cd}	15.3^{def}	11.0^{cd}	77.5^{d}	27.8^{c}	39.5^{cd}	36.0^{def}	70.0^{abc}	51.3^{bcde}
	48	26.5^{e}	1.8^{g}	0.8^{d}	50.8^{e}	11.8^{def}	20.8^{ef}	22.3^{fg}	54.0^{cde}	41.0^{ef}
	72	21.0^{ef}	0.0^{g}	-0.0^{d}	32.3^{ef}	7.8^{ef}	13.5^{ef}	23.5^{fg}	55.5^{bcd}	42.5^{ef}
5°C	0	97.0^{a}	42.8^{ab}	37.8^{a}	103.5^{abc}	56.3^{a}	78.0^{a}	54.5^{abc}	72.5^{ab}	75.5^{a}
	24	25.3^{e}	1.0^{g}	0.8^{d}	45.8^{ef}	12.0^{def}	20.0^{ef}	25.8^{fg}	58.8^{abcd}	43.8^{def}
	48	6.5^{f}	0.0^{g}	0.0^{d}	31.5^{ef}	4.5^{f}	11.8^{f}	13.8^{g}	37.0^{e}	37.5^{f}
	72	11.8^{ef}	0.0^{g}	0.0^{d}	31.0^{f}	5.5^{f}	9.8^{f}	23.0^{fg}	51.5^{de}	43.5^{def}
SEM		3.0	2.2	2.5	5	3.1	3.5	2.9	3.4	2.5
Temp. means										
24°C		86.5^{a}	34.2^{a}	36.4^{a}	105.3^{a}	49.8^{a}	68.3^{a}	46.8^{a}	72.6^{a}	64.3^{a}
17°C		69.5^{b}	22.1^{b}	21.9^{b}	87.9^{b}	36.4^{b}	51.9^{b}	39.9^{b}	68.6^{ab}	57.4^{b}
12°C		50.3^{c}	14.8^{c}	12.3^{c}	67.1^{c}	26.7^{c}	38.8^{c}	34.3^{c}	63.1^{b}	52.6^{c}
5°C		35.1^{d}	10.9^{c}	9.6^{c}	50.4^{d}	19.6^{d}	29.9^{d}	29.3^{d}	54.9^{c}	50.1^{c}
SEM		1.5	1.1	1.3	2.5	1.5	1.8	1.4	1.7	1.3
Temperature (T)		**	**	**	**	**	**	**	**	**

[a-d] Mean values followed by different superscript letters in the same column are significantly different at ($p<0.01$); SEM = Standard Error Mean; ST = Storage Time; RAP = Rapid Motility; PM = Progressive Motility; TM = Total Motility; VAP = Average Path Velocity; VSL = Straight Line Velocity; VCL = Curvi Linear Velocity; WOB = Wobble; STR = Straightness; LIN = Linearity ** = Highly significant

Table 2: Bull spermatozoa viability (live/dead) and sperm DNA fragmentation percentages in Ham's F10 after 72 h stored at 5, 12, 17 and 24°C (n = 3; 8 ejaculates each)

Treatment	Viability (%)	SDF (%)
24°C	26.5[a]	29.3[a]
17°C	8.0[b]	29.8[a]
12°C	2.5[c]	29.5[a]
5°C	0.5[c]	29.5[a]
SEM	0.8	1.1
Temperature (T)	**	Ns

[a-c] Mean values followed by different superscript letters in the same column are significantly different at ($p<0.01$); SEM = Standard Error Mean; SDF = Sperm DNA Fragmentation; ** = Highly significant; ns = Not significant

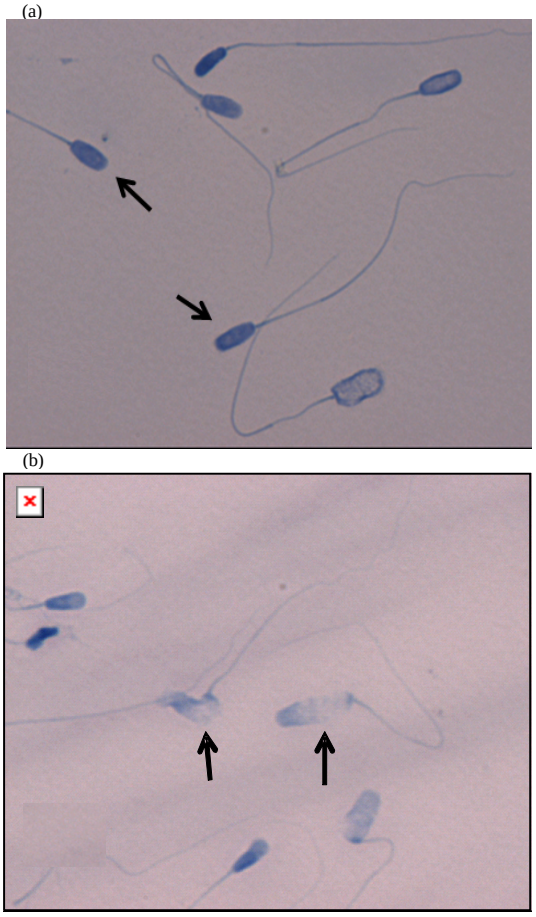

Fig. 1: Images obtained when sperm-blue staining was combined with the Sperm-Halomax protocol indicating Sperm DNA Fragmentation (SDF); a) Arrows indicate sperm heads without DNA fragmentation and b) Arrows indicate heads with fragmented DNA

storage temperature condition showed no significant decrease in TM, PM and RAP from 0 h until 48 h of storage. Spermatozoa velocity was also influenced by the decrease in temperature and more radical decrease was observed at 12 and 5°C. At 5°C VCL was <50% after 24 h and at 72 h it was 31%. The VCL is always the highest compared to the three velocity values (Mortimer, 2002) which is what was observed in this study.

Viability was less than 30% for all the four storage temperatures conditions 24 (26.5), 17 (8.0), 12 (2.5) and 5°C (0.5%) and this indicated a drastic decrease in sperm viability due to the fact that the modified Ham's F10 culture medium used for semen extension did not contain any form of composition to protect spermatozoa against damage from cold shock. Progressive and rapid motility were less than 2% at 24 h and 0% at 72 h for both 17 and 5°C storage temperatures. Despite the fact that the culture medium Ham's F10 was supplemented with maltose and BSA, motility and viability still decreased greatly. According to Rizal *et al.* (2003), polysaccharide, disaccharide and monosaccharide sugars are the sources of energy for spermatozoa and can be used as extracellular cryo-protectants to protect spermatozoa during cryopreservation process. Sariozkan *et al.* (2012) in their study found that di-(trehalose) and tri-(raffinose) saccharides had higher cryoprotective effect than monosaccharides (fructose). Additionally, a study by Wattimena *et al.* (2009) revealed that maltose disaccharide sugar protected spermatozoa against cold shock and also served as the substrate for energy source during preservation at 5°C for 5 days. Our results showed that both maltose and BSA did not protect the sperm cells from cold shock damage but only acted as energy source substrates for spermatozoa during preservation.

The present study also evaluated SDF because the transfer of heritable information to the forthcoming generations relies on sperm DNA integrity. Sperm DNA integrity is becoming to be a critical indicator of sperm fertility. In addition, Love *et al.* (2005) reported that the integrity of sperm DNA is a more target indicator of sperm functionality rather than sperm motility. Gandini *et al.* (2006) reported that not only did cold shock trigger sperm DNA damages but also deteriorated the sperm functional parameters. In the present study, however, differences in storage temperatures had no effect on sperm DNA fragmentation as no significant difference amongst all the four different storage temperatures was observed. The loss in sperm functionality thus could have resulted from the in vitro manipulations which spermatozoa are highly susceptible to (Nakatsukasa *et al.*, 2001).

Studies had demonstrated that the release of Reactive Oxygen Species (ROS) by dead cells into their surrounding environment is a technique of digesting dead cells. Because of the large quantity of unsaturated fatty acids present, the discharged ROS will then accumulate and increase in amount (Lenzi *et al.*, 1996 and 2002).

Furthermore, ROS from different sources including electron discharge from the sperm mitochondria would also aggravate this issue. Additionally, thou seminal plasma has a number of antioxidant enzymes that controls the level of ROS, diluting semen may enable oxidative stress to occur (Sawyer *et al*., 2003). The drastic decrease in viability in this study could have also resulted from the continuous accumulation of ROS because the modified Ham's F10 extender contained no antioxidants to supplement the limited antioxidant defense of the extended spermatozoa over a 3 days period.

Oxidative pressure is the main factor controlling vitality and spermatozoa function (Aitken and Curry, 2011). Past investigations have additionally demonstrated that the exorbitant endogenous oxidative pressure could prompt remarkable sperm DNA destruction. Since, the phosphodiester backbones and deoxyribonucleic acid bases are connected by double bonds, these molecules turn to be highly vulnerable to peroxidation (Teebor *et al*., 1988). Sperm DNA fragmentation has been associated with the reduction in sperm viability (Uysal and Bucak, 2007) and this would almost certainly be what was found in this study. Our results indicated that viability was greatly reduced in all the four storage temperatures which could have also attributed to the increase in SDF.

CONCLUSION

Motility and viability rate of bull spermatozoa extended using the modified Ham's F10 culture medium decreased noticeably regardless of the storage temperature condition. The controlled room temperature 24°C, however, exhibited the highest sperm motility rate as compared to the other three storage temperatures 5, 12 and 17°C. Furthermore, differences in storage temperature condition did not have any effect on SDF as no significant difference was observed between the four temperatures. Studies on antioxidant and buffer addition to protect sperm cells from ROS and the effect of cold shock needs to be further explored.

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