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Effect of partial deoxygenated extender on different post thaw incubation periods of physico-morphological and functional attributes during cryopreservation of buffalo spermatozoa

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Abstract

The study was designed to explore the effect of partial deoxygenated extender of different post thaw incubation periods on physico-morphological attributes during cryopreservation of buffalo semen. Semen samples with mass motility of $\geq 4+$ and individual progressive motility ≥ 80 collected from murrh buffalo bulls were utilized. Immediately after collection, a portion of semen was evaluated for progressive motility, hypo-osmotic swelling response (HOS response) and acrosomal intactness and other portion was divided into two equal groups. Group I was kept as control and diluted with Egg yolk-Tris-Glycerol (EYTG) with a maximum of 60×10^6 sperm/mL. Group II was diluted with partially deoxygenated EYTG extender. Both the groups were subjected to equilibration and freezing. Both the group of semen samples were evaluated at pre-freeze and post-thaw stage of progressive motility, viability, HOS response and acrosomal intactness. Post-thaw motility and viability was studied at 0, 15, 30, 45 and 90 minutes of incubation. At pre-freeze and post thaw stage, significantly ($p < 0.05$) higher progressive motility, viability, HOS response and acrosomal intactness was noticed in group II as compared to group I. Post-thaw motility and viability was significantly ($p < 0.05$) higher in group II than that of group I at 0, 15, 30, 45, 60 and 90 minutes of post-thaw incubation.

Keywords: Partial deoxygenation, seminal attributes, post thaw incubation periods, buffalo spermatozoa

1. Introduction

Semen cryopreservation is an important technique for long-term storage of spermatozoa, required for wide application of artificial insemination for genetic improvement of buffalo but its application has been reported on a limited scale in buffalo, because of poor freezability of buffalo spermatozoa when compared to cattle [1] and it remains unpopular because of poor fertility rate [25]. Cryopreservation procedure by itself decreases viability and the number of motile spermatozoa. Buffalo spermatozoa are highly susceptible to changes in osmotic conditions encountered at the time of freezing and thawing process [1].

It is generally accepted that approximately 50% of buffalo spermatozoa are damaged during the process of freezing and thawing which affects spermatozoa motility, viability and acrosomal intactness in comparison to fresh undiluted semen [14]. It is supposed that during freeze-thaw cycle, spermatozoa suffer oxidative stress and the stress is consistent with the hypothesis that reactive oxygen species (ROS) generated during such cycle is detrimental to sperm function. Sperm cells, when frozen and thawed for AI are exposed to oxygen and light radiation which could irreversibly affect sperm functions [28]. Long back in 1943, MacLeod [17] documented the toxicity of oxygen on human spermatozoa. Spermatozoa loses motility more rapidly under 95% oxygen than under nitrogen but could be protected by catalase suggesting that sperm suspensions produce ROS. Removal of O_2 dissolved in the solvent may be done by addition of inert gas such as nitrogen or argon in to the solvent. Partial deoxygenation of the diluter through liquid nitrogen flushing significantly improved the buffalo sperm quality in terms of motility and viability [2]. No reports are available regarding the effect of partial deoxygenated extender on progressive motility and viability at different post-thaw incubation periods.

So keeping in view above facts, the current study was to investigate the effect of partial deoxygenated extender periods on motility and viability at different post thaw incubation in buffalo bull spermatozoa.

2. Materials and methods

2.1 Experimental animals

Three healthy Murrah buffalo bulls (aged between 4- 6 years) with good body condition were maintained under is managerial condition at the Germ Plasm Centre (GPC) of Animal Reproduction Division, ICAR- IVRI, Izatnagar, Bareilly were utilized for the study. All the experimental protocols of the study were carried out as per the Institutional Animal Ethical Committee guidelines. The institute is located at an altitude of 564 feet above the mean sea level at latitude of 28° North and longitude of 79° East. The place has a subtropical climate and experiences both the extremes of hot and cold weather conditions with the relative humidity ranging between 15 to 85% in different months of the year.

2.2. Partial deoxygenation of extender

Extender was partially deoxygenated by mechanical method. In this process, negative pressure was applied at the rate of 550 mm Hg for 10 min to the flask containing extender using a modified vacuum pump (MV8000 Automotive Tune-up kit, Mityvac, Lincoln Helios, India). The process was continued for 10 min and then the flask was sealed air-tight with Parafilm.

2.3. Semen collection and processing

Semen was collected during morning hour using artificial vagina as per standard practice. Immediately after collection, tubes containing the ejaculated semen were placed in a water bath at 37°C and the samples were evaluated initially for various seminal parameters *i.e.*, volume, colour and consistency macroscopically and mass activity and progressive motility microscopically. Only ejaculates with mass motility of +4 and above (on a scale of 0 to +5) and individual motility of 80% or above were selected for further processing.

2.4. Selection of semen ejaculates

A total of 30 ejaculates, ten ejaculates from three bulls each were collected for the experiment on the basis of mass motility as well as individual motility. The mass motility of the semen samples was determined by assessing the motility of the spermatozoa just after semen collection. It was observed under the low power of microscope without cover slip and was graded on the scale of 0 to +5^[23]. The semen samples showing mass motility of +4 or above were utilized for experimental work. The individual progressive motility of spermatozoa (%) in semen samples was determined by using Sperm Quality Analyser (SQAVb) ^[12]. The semen samples having individual motility of 80% or above were selected for further processing.

2.5. Semen freezing and evaluation

French mini straws (0.25 ml) were filled with the extended semen samples, sealed with polyvinyl alcohol powder and kept for 3h at 5°C for equilibration. After equilibration, straws were kept in automatic programmable biological cell freezer (IMV technology, France) until temperature of straws reached -145°C. Then straws were plunged into liquid nitrogen (-196°C) for storage. Semen samples were evaluated at pre-freeze and post thaw stage for progressive motility, viability Hypo-osmotic swelling (HOS) response and acrosomal intactness. A drop of the diluted semen was kept on a clean, grease free, pre-warmed glass slide, cover slip was placed and progressive motility was assessed under high power magnification (Nikon, Eclipse 80i; 400×magnification) of a phase contrast microscope. The live sperm percentage was estimated by differential staining technique using Eosin-Nigrosin stain ^[7] Hypo-osmotic swelling test (HOST) was carried out according to the method described by Jeyendran *et al.*, 1984 ^[11] and acrosomal integrity was assessed using Giemsa stain ^[30].

2.6 Statistical analysis

The data obtained in this study were subjected to statistical analysis by unpaired t-test using SPSS software version 20.0 and results were expressed as mean ± SE.

3. Results and Discussion

Table 1: Mean±SE of effect of partial deoxygenated extender on motility, viability HOS response and acrosomal intactness

	Progressive motility (%)		Livability (%)		Abnormality (%)	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
Gr.I	73.21±0.61 ^B	48.83±0.52 ^b	77.60±0.60 ^B	53.27±0.30 ^b	9.20±0.15	14.10±0.15 ^a
Gr.II	76.14±0.83 ^A	57.66±0.52 ^a	80.90±0.71 ^A	63.43±0.10 ^a	9.06±0.14	11.54±0.32 ^b

Mean showing different superscript in upper case letters (A&B) and lower case letters (a&b) under each seminal attribute differ significantly ($p < 0.05$)

Table 2: Mean±SE of effect of partial deoxygenated extender on HOS response and acrosomal intactness

	HOS response (%)		Acrosomal intactness (%)	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
Gr.I	74.03±0.24 ^B	54.85±0.78 ^b	81.83±0.22 ^B	52.16±0.78 ^b
Gr.II	78.83±0.31 ^A	63.20±0.10 ^a	85.90±0.75 ^A	61.63±1.40 ^a

Mean showing different superscript in upper case letters (A&B) and lower case letters (a&b) under each seminal attribute differ significantly ($p < 0.05$)

The initial progressive motility of the semen sample gives one of the good indicator of the bull's fertility and ability of spermatozoa to withstand the stress during cryopreservation process. In the fresh semen sample Mass motility, progressive motility, livability, abnormality, HOS and acrosomal

intactness recorded were 4.33±0.08, 81.50±0.64%, 88.43±0.57%, 6.80±0.15, 86.03±0.13% and 84.03±0.19%, respectively. The mean mass motility, progressive motility of fresh ejaculates was higher than values of reported by Shukla and mishra (2007) ^[25], Kadirvel *et al.*, (2014) ^[13] Rajoriya *et*

al. (2014) [20] and Sannat *et al.* (2015) [22]. Higher initial progressive motility may be attributed to utilization of only 4+ or more grade semen and twice semen collection in a week. High viability and motility of spermatozoa are important factors for successful artificial insemination (AI) because a significant correlation between post-thawing sperm viability and subsequent conception rate has been reported (Correa *et al.*, 1997) [8] and (Shelke and Dhama, 2001) [24]. Mean percentage of live spermatozoa in fresh ejaculates was $81.50 \pm 0.64\%$, which was similar to that recorded by Bhakat (2015) [5] and Kadirvel *et al.* (2014) [14]. Sperm viability of a semen sample has direct and positive correlations with fertility of a bull. High level of morphological abnormal sperms in semen is associated with low fertility in cattle [29]. Sperm abnormality at fresh stage was correlated with the observation. Average per cent abnormal spermatozoa in the present study were 6.80 ± 0.15 . The mean value in present study was correlated with the observation made by Bhakat (2015) [5] and Javed, (2000) [10]. The hypo-osmotic swelling (HOS) response evaluates the functional integrity of the spermatozoa's plasma membrane and also serves as a useful indicator of fertilizing potential of spermatozoa. HOS positive response of spermatozoa was 84.03 ± 0.19 which was agreement with Rajoriya (2014) [20]; but, higher than that observed by Kumar (2012) [11] and Ramteke (2014) [25]. The higher percentage of HOST positive spermatozoa in this study indicates higher percentage of membrane intact spermatozoa which might be due to the initial selection of excellent quality semen (mass motility +4). Spermatozoa acrosome plays an

important role in fertilization process. The intactness or loss of acrosome has a bearing on the optimal fertility of semen samples. The measurement of intact acrosome is widely accepted parameters for assessing the semen quality. The overall mean value of acrosomal intactness of spermatozoa was 86.03 ± 0.13 . Results in present study was in agreement with [25] and [20] but higher than the finds [11] and [25].

Progressive motility, viability, HOS and acrosomal intactness response were significantly higher in group II at pre-freeze ($p < 0.05$) and post thaw ($p < 0.05$) stage than that of group I (Table 1). After subjecting spermatozoa to post-thaw incubation test, there was reduction in progressive motility and viability from 0 min to 90 min. The reduction in motility and viability was more in group I as compared to group II (Fig 1 & 2). Post-thaw motility and viability were significantly ($p < 0.05$) higher in group II than that of group I at 0, 15, 30, 45, 60 and 90 minutes of incubation (Table 2). This was in agreement with findings of Tarapdar *et al.* (2001) [27] who reported significant reduction in motility and viability after 1 hr of incubation. Similarly, Rajoriya (2013) [19] and Lone *et al.* (2016) [16], also reported reduction in post-thaw motility from 0 to 120 min. The retention of higher post-thaw progressive motility and viability by spermatozoa in partial deoxygenated extender treated semen may be due to greater removal of dissolved oxygen from the semen dilutor that resulted in reduction in the sperm metabolism. Presence of more dissolved oxygen in the semen dilutor one of the main factor for production of free radicals [4]. Removal of dissolved oxygen favours the better seminal antioxidant status [3].

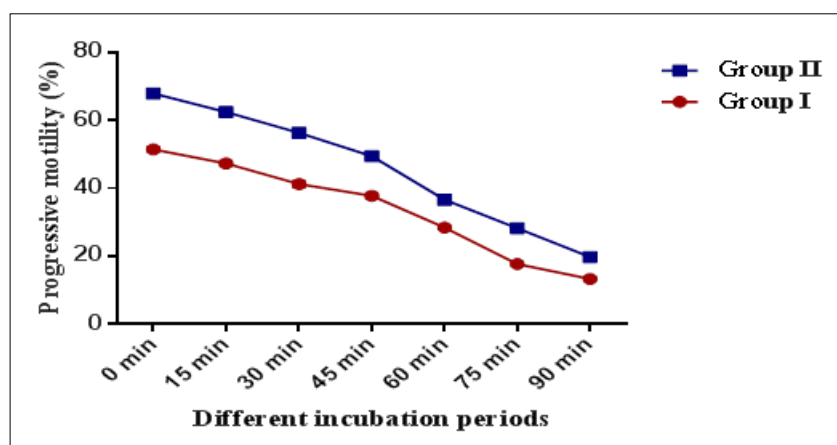


Fig 1: Effect of different post-thaw incubation periods on motility of partial deoxygenated extender buffalo semen.

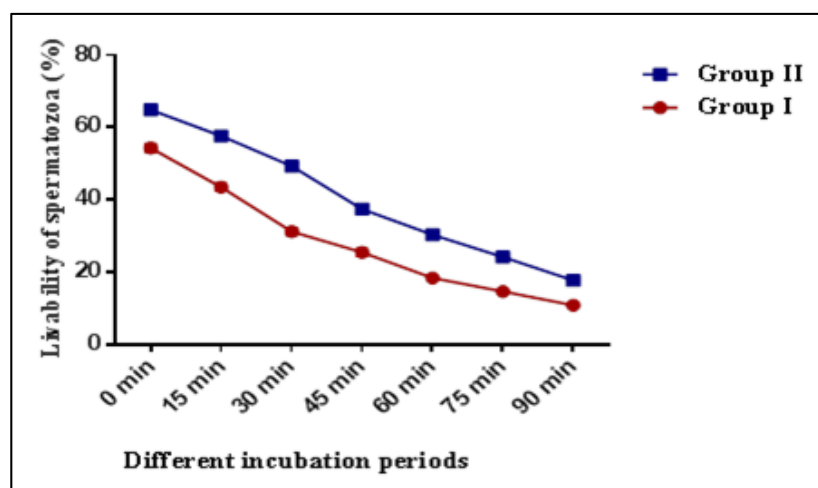


Fig 2: Effect of different post-thaw incubation periods on viability of partial deoxygenated extender buffalo semen.

4. Conclusions

It was found that even after 30 minutes of incubation, partial deoxygenated dilutor treated semen retained around 50% motile and 55% viable spermatozoa. Partial deoxygenated dilutor treated semen, reduction in motility and viability was significantly ($p < 0.05$) less as than that of untreated semen. Thus Partial deoxygenated dilutor treated semen retains post-thaw motility and viability for a prolonged period as compared to semen without any treatment.

5. Conflict of interest

The authors declare that they have no conflict of interest.

6. Acknowledgement

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