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Research Article

Effect of Low Temperature Thawing on the Motility and Fertility of Cryopreserved Water Buffalo and Zebu Bull Semen

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Abstract: The study was conducted to evaluate the quality of cryopreserved buffalo and zebu semen thawed and held at low temperature. Progressive motility of frozen semen thawed in a 37 °C water bath for 45 s (control) was compared with that of semen held in ice water (3-5 °C) for 180 min. Semen collected from 3 buffalo and 2 zebu bulls was used for this purpose over 3 weeks (replicates). Fertility was compared after performing 28 inseminations with buffalo semen and 100 inseminations with zebu semen either after control thawing or thawing straws in ice water for 30-60 min. Progressive motility of buffalo and zebu semen thawed and held in ice water for 30 and 90 min, respectively, was not different from that of semen thawed at 37 °C. The conception rate of buffaloes and zebu cows (69.2% and 60.0%) inseminated after control thawing was higher than that of animals inseminated with semen kept in ice water for 30-60 min (53.3% and 47.7%). However, the difference between conception rates after insemination with the 2 thawing methods was not significant in either case. It is concluded that there was a trend for decreased fertility for both buffalo and zebu semen when inseminations were performed after thawing and holding semen for 30-60 min in ice water.

Key Words: Water buffalo, zebu cattle, semen, freezing, thawing, fertility

Introduction

Cryopreserved bovine semen processed in egg yolk-citrate extender is recommended to be thawed in a 33-35 °C water bath for 45 s (1). However, in some cold locations, semen thawing is practised in the air or in the pocket as user-friendly alternatives (2). Another user-friendly method being practised with cryopreserved water buffalo and zebu cattle semen is using ice water to thaw and carry semen from the artificial insemination (AI) centre to the insemination site. It has been reported that thawing bovine semen at 35 or 37 °C resulted in greater survival of spermatozoa in terms of motility and acrosome integrity as compared to thawing at 5 °C for 1-4 min (3-5). Bovine spermatozoa thawed in 37 °C water had higher fertility than those thawed in ice water (6). A limited scale artificial insemination programme is in progress in water buffaloes and zebu cattle in Pakistan, where semen is processed in Tris-citric acid-yolk extender. The inseminators are advised to thaw semen at 35-37 °C for 45-60 s; however, a practice of thawing and carrying semen in ice water has been observed in the field.

Although the effect of different thawing temperatures on the post-thaw quality of bovine semen has been extensively studied, repetitions are as important as the original to confirm the findings in different species/breeds and under different environmental conditions. The present study was designed to evaluate the effect of thawing and holding cryopreserved buffalo and zebu semen in ice water (3-5 °C) on progressive motility of spermatozoa over a 3-h period as compared to thawing at 37 °C for 45 s. The effect on fertility was also assessed after holding frozen semen in ice water for 30 to 60 min before insemination.

Materials and Methods

The work was conducted at the National Agricultural Research Centre, Islamabad, Pakistan (Lat. 33.7°N; Long. 73.1°E; Alt. 508 m) during 2003.

Semen collection and processing

Three bulls of the Nili-Ravi breed of water buffalo and 2 bulls of Sahiwal zebu cattle were used for semen donation. The bulls were 8 to 10 years old and were trained to donate semen in an artificial vagina (AV) at the start of puberty. Two ejaculates from each bull were collected in the AV at 10 min intervals weekly for

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3 weeks (replicates) and evaluated for volume, percentage of progressively motile spermatozoa (at ×400 by phase contrast microscope; ×400; Leica, LeitzWetzlar, Germany), and sperm concentration (by a digital photometer; Dr. Lange LP 300 SDM, Minitub, Germany). Semen samples used for the study had >60% progressive motility and >0.8 billion sperm per ml. After a holding time of 15 min at 37 °C in a water bath, the semen was processed in an extender containing Tris (hydroxymethyl)-aminomethane (3.03%; w/v), citric acid (1.66%; w/v), egg yolk (20%; v/v), fructose (0.2%; w/v), glycerol (6%; v/v), penicillin (1000 IU per ml), and streptomycin (1000 mg per ml). The pH of Tris-citric acid buffer was 6.8. The extended semen that contained approximately 60 million progressively motile spermatozoa per ml of extender was cooled to 4 °C over 2 h and then equilibrated for 4 h. The 2 extended ejaculates from the same bull were pooled after equilibration before filling in 0.5 ml French straws. Semen was frozen in a programmable cell freezer (KRYO 10 series III, UK) at the rate of 3 °C per min from 4 to -15 °C and then at the rate of 10 °C per min from -15 to -80 °C. The straws were plunged into liquid nitrogen (-196 °C) after holding for 1 min at -80 °C.

In vitro semen evaluation

Post-thaw progressive motility of semen was evaluated after storage of at least 24 h in liquid nitrogen. Semen was either thawed in a 37 °C water bath for 45 s (control) or thawed and held in ice water (3-5 °C) for 180 min (3 straws of semen used for each thawing method). Semen from straws thawed at 37 °C was pooled in a 5 ml plastic tube and its progressive motility was assessed within 2 min post-thawing. The semen from the 3 straws thawed in ice water was also pooled in a 5 ml plastic tube and its progressive motility was assessed from 30 min to 180 min at 30 min intervals. The temperature of ice water was maintained at 3-5 °C during this period by adding more ice cubes if needed. Progressive motility was evaluated subjectively (at ×400) by placing a drop of semen with the help of a Pasteur pipette on a slide. The drop was covered by a cover slip and the slide was placed on the stage of a phase contrast microscope attached to a closed circuit television. The pipettes, slides, cover slips, and microscope stage were pre-warmed to 37 °C.

In vivo semen evaluation

Twenty-eight inseminations were made in healthy water buffaloes maintained by farmers around the town of Daultala near Islamabad (during November and December, 2003) with 1 of the buffalo bull's semen, and 100 inseminations were performed in healthy zebu cows (from March to May 2003) with semen from 1 of the Sahiwal bulls. The inseminations were performed either after thawing semen at 37 °C for 45 s or after thawing semen straws in ice water at 3-5 °C. Based on the progressive motility data (Table 1), semen thawed and held in ice water for 30-60 min was used for the inseminations.

The thaw method was alternated for every 2 inseminations. All the inseminations were performed by the same technician 12-24 h after the appearance of oestrus symptoms. For insemination, a sterile AI gun covered with a disposable plastic sheath and loaded with thawed semen straw (0.5 ml) was inserted into the vagina and then guided into the cervix by means of a gloved hand in the rectum. The inseminating gun was passed through the folds of the cervix to deposit the semen in the body of the uterus.

The animals used were in their 2^{nd} to 5^{th} lactation and had calved at least 60 days before insemination. Females were equally spaced in terms of lactation number between the control and ice water groups. The semen used in both cases was from one of the replicates tested for post-thaw progressive motility evaluation. Animals were palpated for pregnancy 60-90 days post-insemination. In the case of cows, 84 animals were tested for pregnancy out of the 100 inseminated.

The livestock farmers in the area are either landless or own ≤ 2 ha land each. They maintain 2-3 buffaloes/cows each. The animals are fed on seasonal green fodder (oats, maize, or wheat), hay (maize and millet), wheat straw, and cotton seed cake, along with some grazing on the banks of water channels during the rainy season. Milk production averages 1800 and 800 l per lactation, respectively, in buffaloes and zebu cows. Oestrus detection is based on observations like mucus discharge and decrease in milk production in buffaloes, and bellowing, restlessness, and jumping in cows. About 10% buffaloes and zebu cows are served by AI in the area.

Statistical analyses

Progressive motility of semen thawed at 37 °C and of that held in ice water over various time intervals was expressed as mean ± SD. The values were compared by analysis of variance, and $P < 0.05$ was considered statistically significant. Significant differences between

means were determined by Tukey's test. Conception rate with control semen and the semen held in ice water was compared by chi-square test. Analyses were performed using Minitab statistical package 12.22.

Results

Progressive motility of buffalo and zebu spermatozoa thawed at 37 °C for 45 s and those held in ice water is presented in Table 1. A 30 min holding time in ice water did not affect the progressive motility of buffalo semen ($P > 0.05$) as compared with the control sample, but the difference became significant at 60 min. The decline in progressive motility of zebu semen was lower as it did not differ between control samples and those held in ice water for up to 90 min.

The conception rate in buffaloes and zebu cows after insemination with semen thawed at 37 °C for 45 s (control) or semen thawed and held in ice water for 30-60 min is given in Table 2. Although the conception rate was lower in animals inseminated with semen held in ice water both in buffaloes and zebu cows, the differences between control-thawing and ice water method were not significant. The P values for the chi-square test were 0.39 and 0.26, respectively. The P value was 0.16 for pooled data (buffalo + zebu).

Discussion

A high percentage of cryopreserved bovine semen available commercially to producers is processed in egg yolk-based extenders, which generally should be thawed by plunging straws in a 33 to 35 °C water bath for a minimum of 40 s. However, semen thawing is practised in the air, in the pocket, or in ice water as user-friendly alternatives. The present study evaluated the progressive motility of buffalo and zebu semen cryopreserved in Tris-citric acid-yolk extender and held for 3 h in ice water. Fertility was assessed by inseminating semen thawed and held in ice water for 30-60 min after removal from liquid nitrogen.

The progressive motility of cryopreserved buffalo semen in ice water for 30 min did not differ significantly

Table 1. Progressive motility of semen thawed at 37 °C or thawed and held in ice water (3-5 °C) over 3 h.

Thawing method	Post-thaw progressive motility of spermatozoa (%) (mean ± SD)	
	Buffalo semen	Zebu semen
37 °C (45 s)	48.3 ± 5.0[a]	46.7 ± 6.8[a]
Ice water (30 min)	42.2 ± 5.6[ab]	42.2 ± 6.8[ab]
Ice water (60 min)	38.3 ± 5.0[bc]	40.0 ± 5.5[ab]
Ice water (90 min)	36.7 ± 5.6[bc]	36.7 ± 6.8[abc]
Ice water (120 min)	31.1 ± 4.9[cd]	30.8 ± 5.8[bc]
Ice water (150 min)	26.2 ± 7.4[de]	30.0 ± 8.4[bc]
Ice water (180 min)	22.7 ± 6.0[e]	25.8 ± 8.6[c]

Semen was processed in Tris-citric-yolk extender and packaged in 0.5 ml French straws.
[a,b,c] Values with different superscript in the same column differ ($P < 0.05$).

Table 2. Conception rate with semen thawed at 37 °C or in ice water (3-5 °C).

Thawing method	Buffalo semen		Zebu semen	
	No. Inseminated	No. Pregnant (%)	No. Inseminated	No. Pregnant (%)
37 °C (45 s)	13	9 (69.2)	40	24 (60.0)
Ice water (30-60 min)	15	8 (53.3)	44	21 (47.7)

from that of semen thawed at 37 °C for 45 s. However, progressive motility declined gradually and significantly from 60 min onward as compared to the control. The decline in the progressive motility of zebu semen was slower as the motility of semen did not differ significantly from the control up to 90 min holding time in ice water. The visual estimation of percentage of progressively motile spermatozoa is likely the most common type of semen analysis conducted in the laboratory, but it is not consistently highly correlated with fertility (7). It has been stated that although post-thaw thermal insults might not reduce sperm motility it should not be concluded that the fertility of semen handled under such conditions was equal to that of the controls (2). The progressive motility, acrosomal integrity, and plasma membrane integrity of bovine semen have been reported to be better for semen thawed at 37 °C than that thawed at 5-7 °C (5). However, the results of the present study are closer to Kaproth et al.'s (8) observations, who noted that sperm motility of semen processed in milk was unchanged for post-thaw intervals ranging between 5 and 20 min for semen thawed and held at 6 or 35 °C.

The conception rate of buffaloes (69.2%) inseminated with semen thawed at 37 °C was higher than that of buffaloes inseminated with semen thawed and held in ice water for 30-60 min (53.3%). Similarly, the conception rate of cows inseminated with zebu semen thawed at 37 °C (60.0%) was higher than that of cows inseminated with semen thawed in ice water (47.7%). However, the conception rate did not differ significantly between control and low temperature thawing methods in either case. Kaproth et al. (8) showed that thawing and holding bovine semen for 20 min at 35 °C did not affect the conception rate in cows. However, Pace et al. (6) observed that cattle spermatozoa thawed in 37 °C water had higher fertility than those thawed in ice water. The conception rate with ice water thawed semen was lower in both buffalo and zebu semen in the present study; however, a statistical difference was not observed between the 2 thawing methods, and this might have been due to the low number of inseminations. It has been suggested that an average pregnancy rate calculated from <100 observations might be ≥12 percentage units above or below the true value (9). Due to the limited use of AI in Pakistan, a higher number of animals could not be included in the trial.

It is concluded that the progressive motility of cryopreserved buffalo or zebu semen thawed and held in ice water for 30 and 90 min, respectively, was not different from that of semen thawed at 37 °C for 45 s. There was a trend for decreased fertility in both buffalo and zebu semen when inseminations were performed after thawing and holding semen for 30-60 min in ice water. Further fertility trials based on higher numbers of inseminations are required to confirm the findings of this study.

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