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Effect of curcumin on sperm mucus penetration distance and sperm morphometry in fresh vs frozen-thawed semen of Kankrej bull

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Abstract
The present study was intended to study the cryopreservation of Kankrej bull semen in tris egg yolk fructose dilutor with different concentrations (G I; 0 µM, G II; 25 µM, G III: 50 µM and G IV; 75 µM) of curcumin (CUR) and its effect on cervical mucus sperm penetration of spermatozoa and sperm morphometry using 9 ejaculates artificially collected from 3 mature Kankrej bull. The sperm mucus penetration distance (SMPD) was found to be 14.67±0.78 mm in fresh semen. However, the overall mean penetration distance of cryopreserved bull spermatozoa was found to be 19.11±0.75 mm in G I, 26.11±0.68 mm in G II, 21.89±0.54 mm in G III and 13.78±0.78 mm in G IV Curcumin group, respectively at post-thaw stages of cryopreserved Kankrej bull semen. The SMPD was significantly ($p<$ 0.01) higher in 25 µM CUR as compared to other concentrations. The overall mean head length (HL), head width (HW) and midpiece (ML) length of spermatozoa in fresh semen was found to be 8.77±0.07, 4.49±0.05 and 13.92±0.08 µm, respectively. The overall mean HL, HW and ML using different concentrations of curcumin at post-thawed stage were 8.49±0.06, 4.41±0.05 and 13.74±0.1 in G I; 8.53±0.06, 4.48±0.04 and 13.69±0.08 µm in G II; 8.51±0.05, 4.54±0.08 and 13.88±0.06 µm in G III; 8.65±0.06, 4.49±0.04 and 13.87±0.08 µm in G IV group, respectively. The cryopreservation of semen decreased the head length, but the head width and the mid piece remains unaffected.

Keywords: Cryopreservation, Kankrej bull, curcumin and sperm mucus penetration distance

Introduction
Many decades have passed to the introduction of artificial insemination technology, but the achieved conception rate is still not satisfactory. In India, AI coverage of bovine is about 30 per cent ranging from 71 per cent to even less than 1 per cent for different states having overall conception rate of 35 per cent. This clearly indicates that the benefits of AI can be doubled by improving the quality of AI services. (NDDB). The varieties of factors are responsible for the decreased conception rate of semen following the cryopreservation. More than 50% of spermatozoa get injured during the cryopreservation process (Watson, 1995) [30]. Injuries are due to the solute effect and the formation of intracellular and extracellular ice crystals during cryopreservation (Morrow, 1986) [16] leading to a significant decline in semen quality. The production of reactive oxygen species and the lipid peroxidation is a common reaction resulting in disturbances in the functionality of spermatozoa during cryopreservation of semen (Valko *et al*., 2005) [28].
CUR is an effective ROS scavenger as well as having the ability to counterattack lipid peroxidation. (Bucak *et al*. 2012; Tvrda *et al*. 2016) [6, 27]. There are certain reports/studies available in the literature suggesting strongly that CUR is involved in various energy augmenting and protective properties on male reproductive structures. (Salahshoor *et al*., 2012, Soleimanzadeh and Saberivand, 2013; Tvrda *et al*., 2016) [18, 21, 27]. Use of CUR to prevent the lipid peroxidation in sperm during cryopreservation have been documented (Bucak *et al*., 2010; Bucak *et al*., 2012; Shah *et al*., 2016; Tvrda *et al*., 2016; Tvrda *et al*., 2018 and Abdelnour *et al*., 2020) [7, 6, 19, 27, 26, 1].
The in-vitro sperm mucus penetration test (SMPT) is a sperm function test that measures the ability of sperm in the semen to swim up into a column of cervical mucus or substitute and it is for evaluating semen quality and in the prediction of fertilizing capacity of spermatozoa (Anilkumar *et al*. 2001) [3].

Materials and Methods
Total of 9 ejaculates collected artificially from 3 Kankrej bull were used to study the SMPT and morphometry of fresh and post-thawed spermatozoa cryopreserved in Tris Fructose Egg Yolk dilutor with different concentration (G I, 0 µM; G II, 25 µM; G III, 50 µM and G IV, 75 µM) of CUR.

Sperm Mucus Penetration Test (SMPT)
The Kankrej cow mucus sample having typical fern pattern and free from infection (tested by white side test) was used for the SMPT (Matousek *et al*. 1989) [15] using non-heparinized hematocrit capillary tubes. The distance travelled by the vanguard spermatozoa in bovine mucus was measured in millimetres after 45 minutes of incubation.

Sperm morphometry
Total of 9 ejaculates (3 from each bull) were studied for the sperm morphometry at the neat and post-freeze stage of cryopreservation. The slides were prepared using the Rose Bengal stain as described by Gupta and Singh (2018) [11] for the morphometric evaluation. A total of 200 spermatozoa from each ejaculates (Neat as well as post thawed semen) were used for sperm biometry estimation using phase contrast microscope (1000x magnification) fitted with a high-resolution digital CCD camera and analyzed with image analysis software (ZEN 2012, Carl Zeiss Microscopy GmbH). The system was first calibrated with images of standard length for known magnifications and measurement accuracy of±0.1 mm.

Statistical analysis
Three factorial CRD (Completely Randomized Design) followed by Duncan New Multiple Range Test (DNMRT) was used for neat and post-thawed semen to determine the difference between different concentrations of CUR and stages using the methodology as described by Snedecor and Cochran (1994) [20]. All statistical analyses of collected data were performed using SPSSver.20 (Statistical Packages for Social Sciences).

Results
The values of sperm mucus penetration distance varied from 13.33 to 16.33 mm with an overall mean value of 14.66±0.78 mm in the neat semen of Kankrej bulls. The mean values of penetration distance were 14.33±1.45, 16.33±1.45 and 13.33±0.88 for the Bull No. K14-76, K15-21 and K15-38, respectively. Statistical analysis revealed that sperm mucus penetration did not differ significantly (p<0.05) among the bulls (Table 1).

Table 1: Neat semen sperm mucus penetration test and sperm morphometry (Mean±S.E.) of Kankrej bulls

Bull no.	SMPT	Sperm morphometry		
	Penetration distance (mm/45 min)	Head length (µm)	Head width (µm)	Mid-piece length (µm)
K14-76 (n = 9)	14.33±1.45	8.67±0.06	4.51±0.04	13.97±0.08
K15-21 (n = 9)	16.33±1.45	8.85±0.07	4.49±0.05	13.84±0.07
K15-38 (n = 9)	13.33±0.88	8.79±0.09	4.48±0.07	13.94±0.08
Overall (n = 27)	14.66±0.78	8.77±0.05	4.49±0.03	13.92±0.04

The detailed findings of penetration distance of vanguard spermatozoa (mm/45 min) of different CUR concentration groups at the post-thaw stage are presented in Table 2.
Significant (p<0.01) difference was observed in Sperm Mucus penetration distance (SMPD) among Groups I, II, III and IV being highest in group II and lowest in group IV. Furthermore, the significant (p<0.01) difference was also found between neat semen (14.67±0.78 mm/45 min) and Post thaw SMPD (20.22±0.83 mm/45 min).

Table 2: Effect of different concentration of curcumin on SMPT (mm/45 min) (Mean±S.E.) of Kankrej bull spermatozoa in neat and post-thawed semen

Neat (n = 9)	Post-thaw			
	Group I (n = 9)	Group II (n = 9)	Group III (n = 9)	Group IV (n = 9)
14.67±0.78^{a}	19.11±0.75^{b}	26.11±0.68^{d}	21.89±0.54^{c}	13.78±0.78^{a}

Means bearing different superscripts in a raw differ significantly (**p<0.01).

Sperm morphometry
The mean values of HL, HW and ML of spermatozoa in fresh semen are depicted in Table.1 showing non-significant difference among the bulls. The findings of sperm morphometry of different treatment groups at the post-thaw stage are presented in Table 3 and 4.

Table 3: Effect of different concentrations of curcumin on sperm morphometry (µm) (Mean±S.E.) of Kankrej bull spermatozoa at post-thaw stage

	Group I	Group II	Group III	Group IV
HL**	8.49±0.06^{a}	8.53±0.06^{a}	8.51±0.05^{a}	8.65±0.06^{b}
HW	4.41±0.05	4.48±0.04	4.54±0.08	4.49±0.04
ML*	13.74±0.11^{ab}	13.69±0.08^{a}	13.88±0.06^{bc}	13.87±0.08^{bc}

Means bearing different superscripts in a raw differ significantly (**p<0.01; *p<0.05)

Table 4: Effect of cryopreservation on Kankrej bull sperm morphometry

	Neat	Post - thaw Overall	P Value
HL	8.77±0.07	8.54±0.01	0.00
HW	4.49±0.05	5.52±0.09	0.66
ML	13.92±0.08	13.80±0.02	0.07

The mean values of HL, HW and ML were 8.49±0.06, 4.41±0.05 and 13.74±0.11 in G I; 8.53±0.06, 4.48±0.04 and 13.69±0.08 in G II, 8.51±0.05, 4.54±0.08 and 13.88±0.06 in G III and 8.65±0.06, 4.49±0.04 and 13.87±0.08 in G IV, respectively. The statistical analysis established a highly significant difference (p<0.01) in HL among G I, G II and G III and G IV. Highly significant (p<0.01) difference of HL was found between neat and overall mean of post-thawed spermatozoa of all the groups. However, HW did not differ significantly between neat and post-thaw spermatozoa. ML differed significantly (p<0.05) between the different concentration of CUR groups being smallest in G II (Table 3).

Discussion
To observe the vanguard spermatozoa different types of capillary tubes [(Round; David *et al*. 1979; Kremer (1965); Verberckmoes *et al*., (2002); flat capillary tubes; Alexander *et al*. (1981); Gillan *et al*., 2008; Katz *et al*., 1980)] [8, 14, 29, 2, 9, 13] have been used. The round capillary tubes was used in the present study by considering the advantage i.e. they are easier to fill with cervical mucus than flat tubes. The observed SMPD in the present study was more in post thawed spermatozoa compared to the neat semen sperm. This might be due to the thawing-induced cryo-capacitation-like changes to the spermatozoa, due to which spermatozoa become hyperactive (Thomas *et al*., 2006, Talukdar *et al*., 2015) [25, 22]. Sperm freeze-thawing induces capacitation, and sudden

occurrence of acrosome reaction-like changes in mammalian spermatozoa (Bailey *et al*., 2003) [4]. However, the SMPD of spermatozoa cryopreserves with 75 µm of CUR showed a drastic reduction in the SMPD. Similar observations have also been recorded in Hariana bull semen by Gupta *et al*. (2022) [12]. They recorded significant difference among different groups of CUR concentrations at post thawed vanguard distance, as 30.49±0.55 mm in 10 µM CUR group, 28.00±0.48 mm in 25 µM CUR group, 23.09±0.43 mm in 50 µM CUR group and 16.05±0.38 mm in CUR group following 60 minutes of incubation. So, it could be concluded that higher concentration (75 µM) of CUR in semen extender is disastrous for the post thawed motility of sperm. However, further studies with different parameters are obligatory to know the use of higher concentrations of CUR in the cryopreservation of bovine spermatozoa. Further, differences in observations by various workers may be due to sperm abnormalities, sperm antibodies, age and quality of cervical mucus (Tang *et al*., 1999) [23] and the material used as well as time allowed for incubation for the test.

Fertilizing capacity of spermatozoa has been shown to be strongly related to sperm mucus penetration test (SMPT) in bull (Tas *et al*., 2007) [24]. In the present study, at post-thaw stage, maximum penetration distance was found in G II having 25 µM CUR compared to other treatment groups including the control. The higher distance covered in G II compared to other groups suggested that curcumin at lower concentration in dilutor act as antioxidant and avoid the lipid peroxidation of spermatozoa while cryopreservation of semen.

It was noticed that HL of spermatozoa decreased significantly due to cryopreservation of semen, but the HW and ML remained unaffected. Various authors studied morphometric of spermatozoa of various species. Gravance *et al*. (1998) [10] found no significant differences in the means between the extended and cryopreserved samples in bull which do not corroborate with the present findings. However, Rana *et al*. (2020) [17] found significant decline of sperm head length following the cryopreservation of Murrah buffalo bull semen, which support the present findings. However, they have also recorded the significant reduction in head width, midpiece length, tail length, total length and head area between neat and post thawed spermatozoa.

Changes in sperm chromatin structure may explain the differences in morphometric dimensions found between fresh and cryopreserved spermatozoa. The probable explanation for the alteration in sperm morphometric dimensions was put forth by Blottner *et al*. (2001) [5]. They opined that after cryopreservation and thawing, the surface area of sperm heads tended to decrease, which could be due to changes in sperm chromatin structure. Further, they hypothesized that sperm chromatin condensation occurred due to cryopreservation. Multiple types of injuries, like heat stress, colling and freezing as well as thawing can tempt changes in sperm chromatin structure, resulting in acid-induced denaturation.

Conclusion

Addition of 25 µM CUR as an additive in dilutor for cryopreservation of bovine semen resulted in improving the quality of frozen-thawed semen in respect to sperm mucus penetration. The cryopreservation decreased the head length of bovine spermatozoa but the head width and midpiece length remains unaffected.

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