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Effect of Iodixanol Supplementation on Capacitation Like Changes of Bhadawari Buffalo Bull Spermatozoa during Cryopreservation

Sanjay Singh Yadav, Vijay Singh, Atul Saxena, Anuj Kumar, Akhil Patel, Dushyant Yadav, Abhishek Kumar, Pratibha Yadav, Alok Kumar Yadav

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Abstract The present study aims to analyze the Iodixanol supplementation on capacitation like changes of Bhadawari buffalo bull spermatozoa during cryopreservation. 24 Ejaculates from two Bhadawari buffalo bulls were collected using artificial vagina at biweekly interval. Following routine semen evaluation of semen and those which confirmed the standard, Iodixanol was added to the semen sample at 1.25% (v/v) (treatment 1) and 2.5% (v/v) Iodixanol level (treatment 2) and effect of Iodixanol supplementation on capacitation like changes was evaluated. Additional of Iodixanol has reduces the capacitation like changes. The mean percentage of uncapacitated spermatozoa (F pattern) at post thaw stage for Bull B-I were 52.40±1.53, 57.28±1.53, 62.37±1.42 & B-II were 51.02±1.67, 56.24±1.86 and 60.82±1.87 for control and treatment group T_1 and T_2 respectively. The mean percentage of capacitated spermatozoa (B pattern) at post thaw stage for Bull B-I were 43.09±1.74, 39.60±1.56, 35.38±1.49 and B-II was 44.45±1.77, 40.54±1.94 and 36.21±1.87 for control and treatment group T_1 and T_2 respectively. The mean percentage of acrosome reacted spermatozoa (AR pattern) at post thaw stage for Bull B-I were 4.60±0.63, 3.11±0.47, 2.25±0.41 and B-II were 4.60±0.54, 3.22±0.49 and 2.94±0.44 for control and treatment group T_1 and T_2 respectively. Study revealed that capacitation like changes occurs during semen cryopreservation. Addition of Iodixanol reduces capacitation like changes during cryopreservation of spermatozoa in Bhadawari buffalo bull spermatozoa.

Keywords Bull semen, Bhadawari, Iodixanol, Capacitation.

S. S. Yadav, V. Singh, A. Saxena, A. Kumar, A. Patel, D. Yadav, A. Kumar
Department of Veterinary Obstetrics & Gynaecology, College of Veterinary Science & Animal Husbandry, DUVASU, Mathura 281001, UP, India

P. Yadav
Veterinary Officer, Department of Animal Husbandry, UP, India

A. K. Yadav*
PhD Scholar, DCB Division, ICAR-National Dairy Research Institute, Karnal 132001, Haryana, India
e-mail: alokvet3842@gmail.com
*Correspondence

Introduction

Capacitation is an important step in the event of fertilization. *In-vitro* evaluation of the ability of the sperm to undergo capacitation has been used as sperm function test to predict the fertilizing ability of sperm. Cryopreservation procedure induces a series of changes in sperm plasma membrane leading to alteration in membrane permeability. Physiologically, capacitation and acrosome reaction take place within the female reproductive tract stimulated by specific

components of the follicular fluid but can also be induced *in vitro* [1]. Freezing and thawing may alter capacitation time and the frozen thawed sperm undergo capacitation at a faster rate. This finding is in agreement with the report that cryopreservation induced (dilution, cooling, freezing and thawing) structure changes leads to capacitation like changes in bovine, ram and mouse sperm. Several mechanisms have been attributed to the reduced fertility of cryopreserved semen, however, cryopreservation-induced capacitation-like changes in frozen-thawed spermatozoa gained momentum recently [2]. Cryopreservation procedure modify sperm membrane lipid architecture and membrane permeability and reduce the efficiency of enzymes responsible for the efflux of calcium ions [3] leading to elevation of intracellular calcium level, which is a major biochemical changes associated with capacitation.

Demonstration of capacitation or capacitation associated structural modifications through fluorescent labeling of sperm surface antigens has been used to demonstrate capacitation. The evaluation of capacitation by changes in the expression and / or distribution of cell surface molecules by staining with chlortetracycline have been used as method of choice for the semen of bovine. This was first performed with mouse spermatozoa and was subsequently applied to human, monkey and bull spermatozoa. The technique offer the advantage of measuring directly the percentage of non-capacitated, capacitated and acrosome reacted spermatozoa in the same preparation. The assay is based on the ability of the CTC molecules to indicate the localization of membrane Ca^{2+} bind to proteins or glycoproteins. Capacitation changes the distribution of Ca^{2+} in sperm head, plasma membrane causing altered CTC fluorescence patterns in sperm depending in the physiological status of sperm [4].

Variety of additives like anti-oxidants, membrane stabilizers, motility enhancers and chelating agents have been used to protect spermatozoa from deleterious effects of cryopreservation and for improving freezability and fertility of bull semen. Directional cryomicroscopy revealed that the presence of lodixanol alters ice crystal formation into an intricate net of dendrites [5]. Thus, Iodixanol appears to possess cryoprotective properties by helping spermatozoa maintain motility and membrane integrity, possibly through altering ice crystals formation into a more hospitable environment and increasing the glass transition temperature [6].

Table 1. Mean percentage of uncapacitated spermatozoa (F pattern) as observed with CTC assay in the semen of Bhadawari bulls extended in GEYT with Iodixanol supplementation following thawing (mean ± SEM=12).

		Post thaw	
Bull	Control	Treatment 1	Treatment 2
B-I	52.40±1.53^{c} (44.63-62.50)	57.28±1.53^{b} (47.70-66.12)	62.37±1.42^{a} (53.85-72.41)
B-II	51.02±1.67^{b} (44.72-62.50)	56.24±1.86^{a} (48.37-71.81)	60.82±1.87^{a} (54.48-78.74)

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Materials and Methods

The experiment was designed to study the effect of Iodixanol supplementation on capacitation like changes in Bhadawari bull spermatozoa during cryopreservation. For this purpose, 24 ejaculates were collected from two Bhadawari bulls using artificial vagina at biweekly interval. Following evaluation of neat semen and those which confirmed the standard, the semen sample of each bull were split into three equal parts and extended with three different combinations of dilutors i.e. GEYT (control), GEYT with 1.25% (v/v) Iodixanol (T_1) and GEYT with 2.5% (v/v) Iodixanol (T_2). The semen sample which possesses more than 70% progressive motility and above 600 million / ml spermatozoa concentration was subsequently subjected to processing for LN_2 vapor freezing.

Table 2. Mean percentage of capacitated spermatozoa (B pattern) as observed with CTC assay in the semen of Bhadawari bulls extended in GEYT with Iodixanol supplementation following thawing (mean±SEM=12).

Bull	Control	Post thaw Treatment 1	Treatment 2
B-I	43.09±1.74[a]	39.60±1.56[ab]	35.38±1.49[b]
	(31.25-50.85)	(31.40-48.85)	(25.86-43.08)
B-II	44.45±1.77[a]	40.54±1.94[ab]	36.21±1.87[b]
	(31.25-51.55)	(24.54-48.91)	(18.90-42.07)

Table 3. Mean percentage of acrosome reacted spermatozoa (AR pattern) as observed with CTC assay in the semen of Bhadawari bulls extended in GEYT with Iodixanol supplementation following thawing (mean±SEM=12). Unpaired *t* test ; Post thaw ; A vs B ; t =2.564 ($p < 0.05$).

Bull	Control	Post thaw Treatment 1	Treatment 2
B-I	4.60±0.63[a]	3.11±0.47[b]	2.25±0.41[Bb]
	(1.49-9.94)	(0.00-6.76)	(0.00-5.41)
B-II	4.60±0.54[a]	3.22±0.49[ab]	2.94±0.44[Ab]
	(0.50-8.18)	(0.00-6.96)	(0.98-6.52)

After thawing, samples were evaluated for per cent uncapacitated, capacitated and acrosome reacted spermatozoa for assessment of capacitation status and acrosome reaction using chlortetracycline (CTC) staining technique [7]. The technique is based on the principle that Chlortetracycline (CTC) binds with membrane calcium, whose distribution appears to change through capacitation, and is readily visualized by fluorescence microscopy. Thus CTC distribution pattern seems to be related to the capacitation stage. The CTC stock solution containing 750 µM CTC-HCl (Sigma) was prepared daily, wrapped in foil to protect against light, and stored at 4°C until required. 0.5 ml semen (80×10^6 spermatozoa / ml) sample was washed with 5 ml TALP by centrifugation (1000 rpm, 3 minutes). Sperm pellet was obtained after removing the supernatant, again this sperm pellet was resuspended and centrifuge. This procedure was repeated three times. Finally 250 µL of sperm suspension were mixed with 250 µL of CTC solution. Then, this suspension was incubated at 37°C for 20 minutes. After incubation suspension was centrifuge (1000 rpm for 2 minutes) and final sperm suspension was obtained by mixing the sperm pellet with 500 µL TALP. 3 µL of this final stained sperm suspension was taken on a clean grease free slide covered with cover slip and pressed to drain the excess fluid. A total of 200 sperm per slide were observed within 24 hours using a Nikon Eclipse TE 2000-S microscope with phase contrast and epifluorescence optics under blue-violet illumination (excitation at 400–440 nm and emission at 470 nm). Sperm were evaluated according to 1 of 3 CTC staining patterns. Three different forms of CTC pattern observed were: Form I: Even distribution of fluorescence over the entire head (uncapacitated sperm ; pattern F) ; Form II: Fluorescence-free band in the post acrosomal region (capacitated, acrosome intact sperm ; pattern B), fluorescence in anterior portion of the head ; Form III: Fluorescent free head except for a thin bright fluorescent band along the equatorial segment (acrosome-reacted cells, pattern AR).

In all the cases, fluorescence in the middle piece of the flagellum was observed as well. At least 200 spermatozoa per slide were scored.

Statistical analysis

Standard statistical procedure was adopted. Means were compared with *t*-test, ANOVA and DMRT.

Results and Discussion

Table 1 reveals that at post thaw stage the mean percentage of uncapacitated spermatozoa in the semen of Bull B-I in control group was found to be 52.40 ± 1.53 where as in treatment group T_1 and T_2 the values were found as 57.28 ± 1.53 and 62.37 ± 1.42. Statistically ANOVA & DMRT), the mean percentage of uncapacitated spermatozoa in the semen of Bull B-I was significantly ($p < 0.05$) higher in treatment groups T_1 and T_2 as compared to control group. Further the treatment group T_1 and T_2 differed significantly ($p < 0.05$). The respective values for Bull B-II were 51.02 ± 1.67, 56.24±1.86 and 60.82±1.87 for control and treatment group T_1 and T_2. Statistically, the mean percentage of uncapacitated spermatozoa in the semen of Bull B-II was significantly ($p < 0.05$) higher in treatment groups T_1 and T_2 as compared to control group. Further, the treatment groups T_1 and T_2 did not differ significantly. Comparison (unpaired *t* test) between

the two bulls for control and treatment groups did not revealed any significant difference.

Table 2 reveals that at post thaw stage the mean percentage of capacitated spermatozoa in the semen of Bull B-I in control group was found to be 43.09±1.74 where as in treatment group T_1 and T_2 the values were found as 39.60 ± 1.56 and 35.38 ± 1.49. Statistically, the mean percentage of capacitated spermatozoa in the semen of Bull B-I was significantly ($p < 0.05$) higher in control group as compared to treatment group T_2 but the treatment group T_1 was neither significant different from treatment group T_2 nor control group. The respective values for Bull B-II were 44.45 ± 1.77, 40.54 ± 1.94 and 36.21 ± 1.87 for control and treatment group T_1 and T_2. Statistically, the mean percentage of capacitated spermatozoa in the semen of Bull B-II was significantly ($p < 0.05$) higher in control group as compared to treatment group T_2 but the treatment group T_1 was neither significant different from treatment group T_2 nor control group. Comparison (unpaired *t* test) between the two bulls for control and treatment groups did not revealed any significant difference.

Table 3 revealed that at post thaw stage the mean percentage of acrosome reacted spermatozoa in the semen of Bull B-I in control group was found to be 4.60 ± 0.63 where as in treatment group T_1 and T_2 the values were found as 3.11 ± 0.47 and 2.25 ± 0.41. Statistically, the mean percentage of acrosome reacted spermatozoa in the semen of Bull B-I was significantly ($p < 0.05$) higher in control group as compared to treatment group T_1 and T_2. Further treatment groups T_1 and T_2 did not differ significantly. The respective values for Bull B-II were 4.60 ± 0.54, 3.22 ± 0.49 and 2.94±0.44 for control and treatment group T_1 and T_2. Statistically , the mean percentage of acrosome reacted spermatozoa in the semen of Bull B-II was significantly ($p < 0.05$) higher in control group as compared to treatment group T_2 but the treatment group T_1 was neither significant different from treatment group T_2 nor control group. Comparison (unpaired *t* test) between the two bulls revealed significant ($t = 2.564$) difference in treatment group T_2 only.

Capacitation is an obligatory maturation process of mammalian spermatozoa that occurs either *in vivo* during transit through female reproductive tract or *in vitro* in a defined media. Several mechanisms have been attributed to the reduced fertility of cryopreserved semen ; however, cryopreservation-induced capacitation-like changes in frozen-thawed spermatozoa gained momentum recently [2]. Earlier studies have demonstrated similarities between the changes associated with capacitation and cryopreservation [8] and named as cryocapacitation [9]. Addition of Iodixanol has improved the various seminal characters of spermatozoa as shown by Saragusty et al. [6] in bovine spermatozoa and [10] in ram semen.

In present study addition of Iodixanol has reduces the capacitation like changes in the semen of Bhadawari bull spermatozoa compared to control group.

We could not found any literature where Iodixanol has been discussed in reference to capacitation. Our study suggests that Iodixanol improves the capacitation like changes, arises during cryopreservation and thawing. For all the parameters consider under this study, Iodixanol used to a level of 2.5% (v/v) (T_2 did not have any toxic effect to the spermatozoa. Hence our result indicate that 2.5% (v/v) of Iodixanol is suitable for freezing buffalo bull spermatozoa in GEYT extender having 20% egg yolk and 7% glycerol with 80 millions / ml concentration of extended semen.

Conclusion

Cryocapacitation is one of the major factors associated with reduced longevity and poor survivability of cryopreserved spermatozoa in female reproductive tract, resulting in reduced fertility of frozen-thawed semen. In present study addition of Iodixanol has reduces the capacitation like changes in the semen of Bhadawari bull spermatozoa compared to control group ; however Iodixanol at 2.5% concentration had shown better result than 1.25%.

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