Effect of Caffeine, cAMP and Cattle Seminal Plasma on Freezability of Buffalo Bull Semen

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ABSTRACT : An experiment was conducted to investigate the effect of caffeine, cAMP and cattle seminal plasma on preservation of semen at ultra low temperature (-196℃). Each semen sample was divided into four parts equal in volume and sperm concentration; three were treated with caffeine, or cAMP, or cattle seminal plasma (CSP) and the fourth was kept as control. Sperm motility, abnormal spermatozoa, live-dead count and acrosomal damage were studied at different stages of freeze preservation viz.; just after dilution, at 5℃, at glycerolisation, before freezing, just after freezing, 24 hours of storage, and one week of storage. Sperm motility (58.39, 61.33, 52.00 and 50.39 per cent), non-eosinophilic spermatozoa (72.55, 69.98, 63.31 and 67.64 per cent), abnormal spermatozoa (5.71, 4.98, 8.04 and 5.66 per cent) and acrosomal damage (13.28, 13.33, 14.80 and 14.65 per cent) were observed in cAMP, caffeine, cattle seminal plasma and control, respectively, at every stage of freeze preservation. From this study it could be concluded that freezability of buffalo semen can be improved through the addition of caffeine followed by cAMP and cattle seminal plasma. (*Asian-Aus. J. Anim. Sci. 2000. Vol. 13, No. 7 : 901-905*)

Key Words : Buffalo Semen, Sperm Motility, Non-Eosinophilic, Caffeine, cAMP, Cattle Seminal Plasma, Freezability

INTRODUCTION

That buffalo semen is known for its poor quality and freezability is well documented in the literature (Roy et al., 1962; Sengupta, 1963). Semen samples must contain good numbers of forward moving spermatozoa for optimum fertility. Buffalo seminal plasma is believed to have sperm motility inhibitors (Kakar, 1975), and buffalo semen is also deficient in cAMP, a motility promoting factor (Bhatnagar et al., 1979). Effect of addition of caffeine in cattle and buffalo semen has been tried with success by some workers (Haerzettl, 1991; Bhosrekar et al., 1990), but little has been done on the effect of caffeine and cattle seminal plasma on freezability of buffalo semen. Therefore, this study was an endeavour towards the improvement of freezability of buffalo semen by replacing buffalo seminal plasma with cattle seminal plasma, and using cAMP and caffeine as sperm motility promoting factors.

MATERIALS AND METHODS

Selection of bulls

Six healthy Murrah buffalo bulls 3 to 5.5 years of age, maintained under identical nutrition and management conditions at the National Dairy Research Institute, Karnal, India, were taken for the study. These bulls were tested for all diseases and vaccinated against them during the course of the experiment.

Semen collection

The bulls were washed before taking to the site of collection. The semen of the bulls was collected in the early morning by artificial vagina technique (Watson, 1945).

Semen evaluation

Immediately after collection the semen was assessed for the physical attributes viz. volume, mass activity, sperm concentration and sperm motility.

Physical attributes

Volume: Semen volume was measured by taking ejaculate in a graduated tube.

Mass activity: Mass activity was assessed, as described by Tomar et al. (1966) by putting a drop of ejaculated semen spread uniformly over a dry glass slide placed in a microscope on the basis of swirling current. Semen was rated into five categories and given numerical grading; semen with +3 or above grade was used for further processing.

Sperm motility: Sperm motility was observed by taking the average of observations by two persons.

Treatment plan

Every ejaculate was divided into four equal volumes and numbers of sperm before freeze preservation, and subjected to following treatments.

Control: Semen plus extender. Tris buffer (hydroxy methyl amino methane) was used as extender.

Treatment 1. CSP (Cattle seminal plasma): The

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second part of the semen sample was centrifuged at 2000 rpm for 10 min. and buffalo seminal plasma was mixed with an equal volume of cattle seminal plasma.

Treatment 2. (Caffeine): The third part of the semen sample was fortified with 4 mM caffeine (1, 3, 7 trimethyl xanthine anhydrous) immediately after initial dilution of the semen.

Treatment 3. (cAMP): The fourth part of semen sample was treated with 10 μg/ml cAMP (adenosine 3' 5', cyclic monophosphate, Sigma, USA).

Each semen sample was diluted with Tris buffer egg yolk extender @ 30 million sperm/ml after adding the CSP (cattle seminal plasma), cAMP or caffeine.

Freezing protocol

The semen was frozen as per the method described by Jindal (1994). Tris (hydroxy methyl amino methane) buffer was used with ten per cent egg yolk to prepare the extender. The extension rate was fixed as per the sperm concentration per insemination dose. Total extender was divided into two equal parts, A and B.

A: Non glycerolated: semen was half diluted with extender

B: Glycerolated: Extender plus 12 per cent glycerol was so adjusted that the final glycerol concentration become 6 per cent.

Cooling: Both A and B parts were kept in a water bath at 30℃ and then both in the water bath were kept in a cold cabinet and allowed to cool to 5℃ in a period of about 2.5 h.

Glycerolisation: Both A and B parts were mixed at 5℃.

Equilibration: After mixing, semen remained at 5℃ for 3-4 hrs.

Filling and sealing of straws: Mini French (0.25 ml) straws were used for semen packaging. Before filling and keeping in cold handling cabinet the straws were kept under UV rays for 15 min. The filling and sealing of the straws was done with the IMV automatic filling and sealing machine.

Freezing: After filling and sealing, the straws were exposed to liquid N_2 vapour for 13 min. The straws finally were plunged into the liquid N_2 container for storage.

Thawing: The thawing of semen straws was done at 40℃ for 30 seconds.

Morphological attributes

Live-dead count: Live dead count was enumerated according to procedure described by Blom (1950); Eosin-Nigrosin stain was used to prepare the slides. The spermatozoa which took the stain were considered to be dead and the rest were taken to be live.

Abnormal spermatozoa: The abnormal spermatozoa were counted in the same slides which were prepared for live-dead count using oil immersion; a total 150 sperm in different fields of the slide were examined. Typical abnormalities were head, tail, and mid-piece defects.

Acrosomal damage: Giemsa's stain was used to prepare the slide for examination, as for abnormals, of 150 sperm, using oil immersion. The acrosomal cap of a sperm that took the stain was considered to be intact, but otherwise was considered to be damaged.

All the physical and morphological attributes viz, sperm motility, live-dead count, abnormal sperms and acrosomal damage were recorded after dilution, at 5℃, after glycerolisation, before freezing, immediately after freezing, 24 hrs. after freezing and one week after freezing.

Statistical analysis

The data analysis was done by analysis of variance to study the effect of treatments on different physical and morphological attributes of semen during the stages of freeze preservation. The statistical model used for ANOVA as described by Snedecor and Cochran, 1967, was as follows :

$Y_{ijk} = u+A_i+B_j+E_{ijk}$

u = Overall mean,

A_i = is the effect of i th treatment (i=1, 2, 3),

B_j = is the effect of j^{th} h of storage (j=1, 2, 3,···, 9),

E_{ijk} = is random error.

RESULTS

Sperm motility

The average sperm motility after dilution, post thawing and one week after freezing was 75.33, 45.82 and 36.55 per cent, respectively (table 1). The rate of decrease in sperm motility was maximum (16.40%) when semen was exposed to liquid N_2 vapours and no significant difference in sperm motility was found between post freezing and up to one week of storage (table 1). Average sperm motility per cent was significantly ($p<0.01$) higher in the samples fortified with caffeine (61.33%) and cAMP (58.39%) than in those with cattle seminal plasma (52.00%) and control (50.33%) (table 1). Both cAMP and caffeine fortification resulted in better post-thaw sperm motility as compared to cattle seminal plasma and control, whereas no significant difference was found between cattle seminal plasma and control.

Non-eosinophilic spermatozoa

The mean percentages of non-eosinophilic spermatozoa under various treatments and stages of freezing are presented in table 2. The maximum (16.52%) decrease in liveability of spermatozoa was after plunging into liquid N_2 and minimum (2.12%)

Table 1. Sperm motility (%) at various stages of cryopreservation of buffalo semen with different additives

Stages of cryo-preservation	cAMP	Caffeine	CSP	Control	Av.
Immediately after dilution	75.33 ± 1.67^{a}	75.33 ± 1.67^{a}	75.33 ± 1.67^{a}	75.33 ± 1.67^{a}	75.33
At 5℃	69.50 ± 1.09^{cb}	73.00 ± 1.51^{c}	67.60 ± 2.75^{b}	63.80 ± 2.89^{a}	68.41
At glycerolisation	62.50 ± 2.14^{b}	63.33 ± 3.80^{b}	58.50 ± 2.16^{a}	55.60 ± 4.62^{a}	59.98
Before freezing	61.00 ± 3.04^{ab}	68.00 ± 1.71^{c}	58.00 ± 3.61^{a}	62.16 ± 2.40^{b}	62.29
Immediately after freezing	48.00 ± 4.51^{c}	50.50 ± 4.04^{c}	40.30 ± 3.70^{a}	44.50 ± 3.70^{b}	45.82
24 hrs after freezing	44.30 ± 5.66^{c}	52.16 ± 3.12^{d}	34.50 ± 4.22^{b}	30.60 ± 6.66^{a}	40.39
One week after freezing	48.10 ± 3.32^{c}	47.00 ± 3.15^{c}	30.30 ± 4.15^{b}	20.80 ± 4.90^{a}	36.55
Av.	58.39	61.33	52.00	50.39	

The values with similar superscripts are not significantly different from each other.

Table 2. Non eosinophilic spermatozoa (%) at various steps of processing for cryopreservation in buffalo semen with various additives

Stages of cryopreservation	cAMP	Caffeine	CSP	Control	Av.
Immediately after dilution	88.16 ± 1.33	88.16 ± 1.33	88.16 ± 1.33	88.16 ± 1.33	88.16
At 5℃	76.33 ± 2.38	81.83 ± 2.09	75.60 ± 3.79	81.16 ± 2.72	78.73
After glycerolisation	81.10 ± 2.40	79.66 ± 1.33	68.00 ± 3.14	76.00 ± 1.92	76.20
Before freezing	75.16 ± 3.44	73.83 ± 3.96	63.80 ± 3.37	76.16 ± 2.02	72.23
Immediately after freezing	57.80 ± 5.40	57.30 ± 4.70	49.16 ± 4.25	59.00 ± 3.04	55.81
24 hrs after freezing	61.80 ± 3.70	50.80 ± 3.97	53.16 ± 3.38	49.00 ± 6.60	53.59
One week after freezing	67.50 ± 4.24	58.30 ± 5.29	45.33 ± 2.40	44.00 ± 6.80	53.78
Av.	72.55	69.98	63.31	67.64	

Live sperm percentage did not vary significantly between treatments.

Table 3. Abnormal spermatozoa (%) at various stages of cryopreservation buffalo semen with various additives

Stages of cryopreservation	cAMP	Caffeine	CSP	Control	Av.
Immediately after dilution	3.50 ± 0.67^{a}	3.50 ± 0.67^{a}	3.50 ± 0.67^{a}	3.50 ± 0.67^{a}	3.50
At 5℃	6.66 ± 2.70^{b}	5.33 ± 1.08^{a}	7.66 ± 1.50^{b}	5.00 ± 0.93^{a}	6.16
After glycerolisation	5.00 ± 1.29^{a}	5.60 ± 0.55^{ab}	10.60 ± 1.20^{c}	6.50 ± 1.09^{b}	6.92
Before freezing	6.66 ± 0.66^{b}	5.33 ± 1.02^{a}	8.60 ± 1.78^{c}	9.00 ± 1.59^{d}	7.39
Immediately after freezing	7.66 ± 1.08^{cb}	4.83 ± 0.91^{a}	8.33 ± 1.68^{c}	6.83 ± 1.22^{b}	6.91
24 hrs after freezing	5.50 ± 0.76^{a}	6.16 ± 0.98^{a}	9.50 ± 0.96^{b}	5.50 ± 1.18^{a}	6.66
One week after freezing	5.00 ± 1.06^{b}	4.16 ± 0.94^{ab}	8.16 ± 1.49^{c}	3.33 ± 0.33^{a}	5.16
Av.	5.71	4.98	8.04	5.66	

The values with similar superscripts are not significantly different from each other.

was after 24 h of storage. The overall percentage of live spermatozoa immediately after dilution (88.16%) decreased by 34.38 per cent after one week of freezing (53.78%) and the difference was significant between the stages of freezing.

Spermatozoal abnormality

The average per cent value of abnormal spermatozoa with respect to four treatments viz. cAMP, caffeine, cattle seminal plasma and control during the freezing process are presented in table 3. The maximum (3.89%) increase in sperm abnormality was after plunging into liquid N_2 and minimum (0.25%) was after 24 h of storage. The overall sperm abnormality varied from 7.39 to 3.50 per cent across the stages of freezing process. The significant ($p<0.01$) effect of various stages of the freezing process was observed on the sperm quality. The quality differed significantly ($p<0.01$) between the treatments.

Acrosomal damage

The average percentages of acrosomal abnormalities with respect to various treatments viz.: cAMP, caffeine, cattle seminal plasma and control during the various stages of freezing process are presented in table 4. The overall percentage of spermatozoa with

Table 4. Acrosomal damaged spermatozoa (%) at various stages of cryopreservation of buffalo semen with additives

Stages of cryopreservation	cAMP	Caffeine	CSP	Control	Av.
Immediately after dilution	6.00 ± 1.55^{a}	6.00 ± 1.55^{a}	6.00 ± 1.55^{a}	6.00 ± 1.55^{a}	6.00
At 5℃	9.40 ± 1.59^{b}	7.16 ± 1.16^{a}	10.00 ± 1.20^{b}	10.33 ± 1.26^{b}	9.22
After glycerolisation	8.25 ± 0.21^{b}	6.00 ± 1.27^{a}	9.75 ± 1.63^{b}	5.75 ± 2.43^{a}	7.43
Before freezing	13.50 ± 1.39^{b}	13.33 ± 1.28^{b}	12.83 ± 0.92^{b}	10.50 ± 1.07^{a}	12.54
Immediately after freezing	15.50 ± 1.24^{a}	21.00 ± 1.33^{b}	21.83 ± 0.79^{b}	21.33 ± 1.45^{b}	19.19
24 hrs after freezing	16.83 ± 1.07^{a}	20.50 ± 1.15^{b}	20.33 ± 2.01^{b}	19.16 ± 2.25^{b}	19.00
One week after freezing	21.33 ± 1.91^{a}	23.00 ± 1.33^{b}	23.16 ± 1.57^{b}	28.33 ± 0.55^{c}	23.55
Av.	13.28	13.33	14.80	14.65	

Values with similar superscripts are not significantly different from each other.

damaged acrosome varied from 6.00 to 23.55 per cent from dilution stage to one week of storage. The maximum (7.37%) damage in the acrosome occured between the pre-freezing and plunging into liquid N_2 stages. A significant ($p<0.01$) difference for acrosomal damage between the treatments was observed.

DISCUSSION

The earlier studies in our laboratory on buffaloes (Pawan Singh and Raina, 1998) and studies on other species (Burge, 1973; Joseph et al., 1977; Robert et al., 1978) revealed that 4 mM concentration of caffeine is an ideal concentration for fortification of semen to improve the preservability. Caffeine is believed to have a stimulatory effect on kinetic activity and respiration of spermatozoa (Drevius, 1973), as this is an inhibitor of certain enzymes involved in spermatozoal glycolysis (Hardman et al., 1971). Cyclic nucleotides and certain inhibitors of cyclic nucleotide phosphodiesterase have been shown to induce and promote the motility of spermatozoa (Gehlaut et al., 1987). A number of methyl compounds have been used for enhancing sperm motility by several workers with encouraging results (Haesungcharrern and Chula-vatnatol, 1973; Turner et al., 1978). It has been demonstrated that caffeine increases intracellular cAMP which is essential for regulation of spermatozoal motility (Hokins et al., 1975). cAMP brings about the conversion of the inactive form of glycogen phosphorylase into its active form through breaking down glycogen to simple sugar (Lehninger, 1987).

The role of caffeine as an inhibitor of adenylate phosphodiesterase helps in increasing cAMP level, which might be responsible for the longer preservation of buffalo semen with caffeine fortification shown in the present study. Keeping in view the hypothesis that caffeine increases cAMP in the cell, caffeine and cAMP fortification should be tried for preservation of buffalo semen at ultra low temperature (-196℃). Buffalo seminal plasma is deficient in cAMP and believed to have poteneous inhibitors of spermatozoal motility. Its removal improves the keeping quality of buffalo semen (Kakar, 1975), and the subsequent addition of cattle seminal plasma has been found in the present study to enhance the preservability of spermatozoa. The superiority of all the three treatments over the control (table 1) leads to the conclusion that buffalo seminal plasma lacks motility supporting factors and might even have motility inhibitors (Kaker and Ganguli, 1979). The present study also found that cAMP, whether added directly in the semen or its enhancement through addition of caffeine, has a important role in maintenance and sustaining of quality of frozen buffalo spermatozoa.

The above findings revealed that the fortification of buffalo semen with these compounds not only helps preservability but also helps maintain integrity of spermatozoa during the process of freeze preservation. This has been further substantiated by acrosomal membrane integrity, which gave the similar trends of results with the various treatments.

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