Changes in adenosine 5'-triphosphate, adenylate energy charge and adenosine 3'5'-cyclic monophosphate during the freezing of buffalo semen

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Summary. In freshly ejaculated buffalo semen ($N = 4$) there were 24.61 ± 5.28 nmol ATP and 40.39 ± 5.94 nmol total adenylate/ 10^8 spermatozoa, and 97.75 ± 7.06 pmol cAMP/ 10^9 spermatozoa. The semen was frozen in 4 steps (I, dilution; II, cooling; III, glycerolization and equilibration; IV, freezing and thawing). Motility, ATP, total adenylate and cAMP were significantly lower after Step IV than after Step I.

Motility and ATP concentration were significantly correlated in egg-yolk-Tris ($r = 0.530$, $P < 0.05$), skim milk-egg yolk ($r = 0.754$, $P < 0.01$), egg yolk-citrate-glucose ($r = 0.784$, $P < 0.01$) and citric acid-whey ($r = 0.551$, $P < 0.05$). Cyclic AMP and motility in egg yolk-Tris were also correlated ($r = 0.714$, $P < 0.01$). The adenylate energy charge was stable in all 4 freezing steps.

Introduction

Preservation of semen of a number of domestic animals either at 4°C or under frozen conditions is required for large scale breeding through the use of artificial insemination. The quality of semen at each step of preservation can be judged by evaluation of motility which is the most widely used assessment technique in lieu of actual fertility trials (Linford, Glover, Bishop & Stewart, 1976). Motility, however, is known to be dependent upon a number of intrinsic and extrinsic factors which include pH, temperature, prolonged incubations and cyclic AMP content.

During the freezing of semen, the spermatozoa are subjected to various stresses such as dilution of the semen, cooling, glycerolization and equilibration, and freezing and thawing. The decrease in the propagation of the flagellar wave may be related to effects on the adenosine triphosphate content or the adenylate energy charge ($\text{ATP} + \frac{1}{2}\text{ADP}/(\text{ATP} + \text{ADP} + \text{AMP})$) which has been proposed as a linear measure of the energy stored in the adenine nucleotide pool of living cells (Atkinson & Walton, 1967). In addition, the decreased motility may also be due to a reduction in adenosine 3'5'-cyclic monophosphate (Tash & Mann, 1973; Chaudhry & Anand, 1975). The present study was designed to observe the changes of ATP, adenylate energy charge and cyclic AMP at various steps during the freezing of buffalo semen.

Materials and Methods

Source of semen

Semen was collected from 4 breeding Murrah buffalo bulls once weekly by artificial vagina. Only ejaculates showing high initial motility with a score of 3.5 to 4.5 (zero, no motility; 5, the best motility) were used without pooling. Sperm counts were made with a haemocytometer and

sperm motility in extended semen at various steps during the freezing process was assessed by examining a uniform drop of semen under a coverslip on a warm stage at 37°C; the assessments were made independently by two experienced workers using two scales of 0 to 5 and 0 to 10. The percentage of live/dead spermatozoa was ascertained by the method of Hancock (1951) using nigrosin-eosin stain. Seminal plasma free of spermatozoa was prepared by centrifuging fresh semen at 12 000 g for 30 min at 4°C.

Preparation of diluents

Egg yolk-citrate-glucose (EYCG). This diluent contained 1.4 g sodium citrate, 1.2 g glucose, 0.9 g glycine and 0.125 g cysteine-HCl dissolved in 100 ml glass-distilled water; the pH was adjusted to 6.8. Egg yolk was then added to a final concentration of 20%.

Egg yolk-Tris (EY-Tris). This diluent contained 2.65 g Tris, 1.26 g citric acid, 0.625 g glucose and 0.125 g cysteine-HCl dissolved in 100 ml glass-distilled water; the pH was adjusted to 6.8. Egg yolk was added to a final concentration of 20%.

Skim milk-egg yolk (SM-EY). Cow skim milk powder (9.25 g) was reconstituted in 100 ml distilled water and heated at 92°C for 10 min. The milk was filtered and then 0.625 g glucose was added. The pH was adjusted to 6.8. Egg yolk was then added to a final concentration of 10%.

Citric acid-whey (CAW). Fresh cow skim milk was heated to 92°C for 10 min in a water bath and brought to room temperature. The milk was curdled with citric acid to a final concentration of 6%. The precipitate was separated by filtration and the pH of the supernatant adjusted to 6.8.

Dilution and freezing of semen

Before dilution (Step I), each diluent was fortified with penicillin G (1000 i.u./ml) and streptomycin sulphate (1 mg/ml). One volume of semen was diluted with 4 volumes of the diluent containing 3% glycerol. The diluted semen was cooled to 5°C in a refrigerator over a period of 1½ h (Step II). The semen was further diluted with an equal volume of the diluent containing 11% glycerol in four aliquots at intervals of 15 min and then left for equilibration for 6 h (Step III). The equilibrated semen was frozen in polyvinyl straws and the sperm density was 50×10^6 (Bhosrekar, 1974). The straws were stored in liquid nitrogen at -196°C for 24 h before thawing at 37°C for 1 min (Step IV). The conditions used at the four freezing steps were rigorously maintained for the four diluents used and for the different samples of semen.

Extraction and assay of adenine nucleotides

Undiluted semen, seminal plasma or semen (0.5 ml) at the four steps of freezing (diluted, cooled, glycerolized and equilibrated, frozen and thawed) was mixed with 0.2 ml 20% ice-cold trichloroacetic acid. The precipitate was removed by centrifugation at 13 000 g for 15 min at 4°C and the supernatant was neutralized with NaOH for assay of adenine nucleotides. ATP was assayed by the luciferase reaction using liquid scintillation spectrometry as described by George, Randles & Brand (1975). ATP + ADP and ATP + ADP + AMP were similarly assayed after the appropriate enzymic conversion by the procedure of Adam (1963). The adenylate energy charge was calculated by using the equation: $(\text{ATP} + \frac{1}{2} \text{ADP})/(\text{ATP} + \text{ADP} + \text{AMP})$.

Extraction and assay of cyclic AMP

The extraction of cyclic AMP by boiling the semen for 3 min in water bath or by homogenizing it in 6% trichloroacetic acid and its estimation by protein binding assay were carried out as described by Chaudhry & Anand (1975).

Statistical analysis

The data were analysed for statistical significance by Student's *t* test (Snedecor & Cochran, 1967).

Results

ATP concentration of buffalo spermatozoa

The ATP content of buffalo spermatozoa processed immediately after ejaculation (within 3 min) was estimated to be 9.22–38.07 nmol/10⁸ spermatozoa (24.61 ± 5.28 (s.e.m.) nmol of 5 determinations). The total adenylate pool was 40.39 ± 5.94 nmol/10⁸ spermatozoa and the ratio of ATP/ADP was 1.77. The amount of adenine nucleotides estimated was solely due to sperm cells because in seminal plasma none of the adenine nucleotides was detected. The latter observation is in agreement with those reported on human and bovine seminal plasma (Chulavatnatol & Haesungcharen, 1977; Foulkes & MacDonald, 1979).

Changes during freezing

The motility of the spermatozoa decreased non-significantly between the dilution and equilibration steps and there were no differences between the four diluents. However, at the last step of freezing and thawing motility declined significantly ($P < 0.01$) in all the 4 diluents although the decline was variable depending upon the diluent used (Table 1). The maximum loss in motility occurred in CAW (60%), followed by SM-EY (40%), EYCG (39%) and EY-Tris (35%). Analysis of variance showed that differences in motility after thawing were not significant for the diluents EY-Tris, SM-EY and EYCG but the change in CAW was significant ($P < 0.01$). The percentages of eosinophilic spermatozoa changed similarly in all 4 diluents (Table 1); there was little change between Steps I and II and Steps II and III, but the increase between Steps III and IV was significant ($P < 0.01$).

Table 1. Percentages of motile and eosinophilic spermatozoa in buffalo semen at four steps of the freezing process (I, dilution; II, cooling to 5°C; III, glycerolization and equilibration; IV, freezing and thawing)

Freezing steps	Egg yolk–Tris		Skim milk–egg yolk		Egg yolk–citrate–glucose		Citric acid–whey	
	% Motile	% Eosino-philic	% Motile	% Eosino-philic	% Motile	% Eosino-philic	% Motile	% Eosino-philic
I	67.0 $\pm$ 1.0	26.7 $\pm$ 4.2	67.5 $\pm$ 1.1	30.7 $\pm$ 6.1	67.0 $\pm$ 1.0	25.2 $\pm$ 4.0	67.3 $\pm$ 0.9	28.0 $\pm$ 5.4
II	62.2 $\pm$ 1.4	33.0 $\pm$ 2.2	63.5 $\pm$ 3.7	38.7 $\pm$ 3.7	65.0 $\pm$ 2.3	31.0 $\pm$ 4.1	58.0 $\pm$ 5.8	35.0 $\pm$ 2.6
III	64.5 $\pm$ 1.1	33.2 $\pm$ 4.2	62.0 $\pm$ 3.1	43.0 $\pm$ 4.0	63.2 $\pm$ 1.8	39.0 $\pm$ 5.5	57.0 $\pm$ 5.0	32.5 $\pm$ 2.0
IV	43.5 $\pm$ 3.3	64.7 $\pm$ 2.0	40.2 $\pm$ 1.8	70.7 $\pm$ 11.2	40.8 $\pm$ 4.5	73.2 $\pm$ 5.9	27.2 $\pm$ 5.2	67.7 $\pm$ 4.3

Values are mean $\pm$ s.e.m. for 5 samples.

The ATP content of buffalo spermatozoa fell gradually during the 4 freezing steps, from 16–18 to 5–7 nmol ATP/10⁸ spermatozoa ($P < 0.01$) and there were no differences between the 4 diluents at each step (Table 2). The greatest loss occurred between Steps II and IV; significant ($P < 0.01$) change occurred between Steps II and III in diluents SM-EY and CAW and between Steps III and IV in EY-Tris and EYCG.

The reduction in the total adenylate pool followed a similar pattern, falling from 27–31 nmol/10⁸ spermatozoa at Step I to 10–14 nmol/10⁸ spermatozoa at Step IV. The adenylate energy charge of the spermatozoa during all steps was stable; the values ranged between 0.65 and 0.79 (Table 2).

Table 2. Mean $\pm$ s.e.m. ($n = 4$) values for adenine nucleotides, total adenylate pool and energy charge of spermatozoa at four steps of the freezing process of buffalo semen

Diluent	Freezing steps*	Adenine nucleotides (nmol/ 10^8 spermatozoa)			Total adenylate pool (nmol/ 10^8 spermatozoa)	Energy charge
		ATP	ADP	AMP		
Egg yolk-Tris	I	17.36 $\pm$ 2.73	6.65 $\pm$ 2.79	5.22 $\pm$ 4.67	29.22 $\pm$ 4.53	0.75 $\pm$ 0.12
	II	12.73 $\pm$ 2.12	7.74 $\pm$ 2.63	4.64 $\pm$ 2.63	25.12 $\pm$ 2.11	0.65 $\pm$ 0.05
	III	11.39 $\pm$ 2.43	8.96 $\pm$ 2.44	1.52 $\pm$ 0.95	21.88 $\pm$ 1.76	0.72 $\pm$ 0.04
	IV	7.17 $\pm$ 1.08	6.17 $\pm$ 3.88	1.36 $\pm$ 0.57	14.70 $\pm$ 3.29	0.72 $\pm$ 0.04
Skim milk-egg yolk	I	15.78 $\pm$ 1.88	8.35 $\pm$ 3.68	2.66 $\pm$ 1.35	26.80 $\pm$ 3.03	0.77 $\pm$ 0.08
	II	12.44 $\pm$ 2.48	11.59 $\pm$ 4.33	3.97 $\pm$ 2.45	27.99 $\pm$ 3.17	0.68 $\pm$ 0.06
	III	7.04 $\pm$ 2.10	7.79 $\pm$ 2.90	1.40 $\pm$ 0.77	16.23 $\pm$ 4.32	0.65 $\pm$ 0.05
	IV	6.00 $\pm$ 1.78	3.69 $\pm$ 2.43	0.55	10.24 $\pm$ 2.77	0.76 $\pm$ 0.05
Egg yolk-citrate-glucose	I	16.52 $\pm$ 0.87	9.64 $\pm$ 2.93	5.09 $\pm$ 3.52	31.24 $\pm$ 5.18	0.72 $\pm$ 0.08
	II	15.99 $\pm$ 2.55	5.90 $\pm$ 2.02	6.05 $\pm$ 1.05	27.94 $\pm$ 3.14	0.68 $\pm$ 0.03
	III	12.49 $\pm$ 2.91	7.93 $\pm$ 1.51	2.59 $\pm$ 1.05	23.01 $\pm$ 3.20	0.71 $\pm$ 0.04
	IV	5.41 $\pm$ 0.99	3.06 $\pm$ 1.68	2.08 $\pm$ 1.26	10.55 $\pm$ 2.27	0.66 $\pm$ 0.04
Citric acid-whey	I	18.78 $\pm$ 1.61	8.59 $\pm$ 2.98	3.33 $\pm$ 1.86	30.75 $\pm$ 4.61	0.76 $\pm$ 0.04
	II	17.30 $\pm$ 3.06	7.31 $\pm$ 2.44	1.76 $\pm$ 0.96	26.37 $\pm$ 2.97	0.79 $\pm$ 0.06
	III	11.87 $\pm$ 2.48	8.24 $\pm$ 3.24	1.35 $\pm$ 0.97	21.50 $\pm$ 2.53	0.74 $\pm$ 0.06
	IV	7.14 $\pm$ 0.37	4.54 $\pm$ 2.90	1.99 $\pm$ 1.24	13.66 $\pm$ 2.49	0.69 $\pm$ 0.06

* I, Dilution; II, cooling to 5°C; III, glycerolization and equilibration; IV, freezing and thawing.

An analysis of the 16 samples (from 4 bulls) and the 4 diluents showed that there was a significant correlation of ATP concentration with observed motility (Table 3). At each step the regression was linear.

Table 3. Correlation between ATP content and observed motility of buffalo spermatozoa in different diluents during freezing of the semen

Diluents	% Motile spermatozoa	ATP (nmol/ 10^8 spermatozoa)	Linear regression		Correlation coefficient (r)	P
			Slope	Intercept		
Egg yolk-Tris	63.2 $\pm$ 2.5	12.16 $\pm$ 1.35	0.986	51.26	0.530	<0.05
Skim milk-egg yolk	55.3 $\pm$ 5.9	10.31 $\pm$ 1.38	3.218	22.12	0.754	<0.01
Egg yolk-citrate-glucose	64.2 $\pm$ 2.2	12.72 $\pm$ 1.47	1.230	64.18	0.784	<0.01
Citric acid-whey	54.3 $\pm$ 5.2	13.72 $\pm$ 1.52	1.873	28.64	0.551	<0.05

Values are mean $\pm$ s.e.m. for 16 samples (4 bulls, 4 freezing steps).

The cyclic AMP (cAMP) content of freshly ejaculated buffalo spermatozoa was estimated to be 97.75 ± 7.06 pmol/ 10^9 spermatozoa (mean $\pm$ s.e.m. for 4 determinations). Dilution with EY-Tris increased cAMP by 35% (132.59 ± 5.55 pmol/ 10^9 spermatozoa); values at Steps II and III changed little (134.16 ± 9.26 and 116.22 ± 15.42 pmol/ 10^9 spermatozoa, respectively) but decreased significantly ($P < 0.05$) at Step IV (43.14 ± 7.37 pmol/ 10^9 spermatozoa). There was a significant positive correlation ($r = 0.714$, $P < 0.01$) and linear regression (slope 1.842) between cyclic AMP content and motility of buffalo spermatozoa at all steps of the freezing process.

Discussion

The freezing of semen subjects the spermatozoa to the combined effects of dilution, glycerolization and changing temperatures, and the freezing of buffalo semen led to a decline in

motility and ATP concentration. Anand, Kaur & Chaudhry (1978) reported that the decline in motility at the time of dilution of semen was minimal but enzyme release was maximum, indicating cellular injury to the spermatozoa. This was supported by the present results with buffalo semen: the increase in cyclic AMP content during Steps I, II and III was not due to the egg yolk because no cyclic AMP could be detected in egg yolk by protein-binding assay. The change was also unrelated to temperature because Hammerstedt & Hay (1980) observed no change in cyclic AMP content when bovine spermatozoa were incubated between 10 and 40°C. The considerable decrease of AMP content at Step IV was presumably due to sperm cell damage. The positive correlation between cyclic AMP and observed motility at all the freezing steps is consistent with the postulated relationship of cAMP with sperm motility. ATP concentration was also positively correlated with sperm motility (Table 3). Similar observations relating motility to the ATP content of bovine spermatozoa during the freezing process have been made (Von Prinzen, 1978; Foulkes & MacDonald, 1979). The significant positive correlations between motility and the two nucleotides, ATP and cyclic AMP, during the freezing of buffalo semen obtained in the present study suggest that an objective assessment of semen quality may be obtained through the estimation of ATP/cyclic AMP.

The adenylate energy charge of a normal physiological cell ranges between 0.80 and 0.95 and when subjected to metabolic stress it may fall but not below 0.50 (Atkinson, 1971). This was found true for buffalo spermatozoa during all steps of the freezing process (Table 2). In spite of the decrease observed in ATP content and the total adenylate pool during the freezing process, the stabilization of adenylate energy charge in the buffalo sperm cell would indicate ATP conservation through metabolic regulation. The loss in ATP content occurring during the freezing process could be because of a slow rate of metabolism at low temperatures and cell damage/death which ensue as a result of freezing and thawing. Glucose utilization by buffalo spermatozoa at 5°C where it was kept for 6 h for equilibration before freezing was found to be 25% of that observed at 30°C before cooling. The rate increased 2-fold after freezing and thawing (data not shown). That cell damage/death could be a major contributory factor is supported by the appreciable increase in the percentages of eosinophilic spermatozoa (Table 1) as well as the reduced cyclic AMP and ATP contents. Jones & Stewart (1979) have reported that freezing of bull semen not only affects the plasma membrane and underlying acrosomal membrane but also damages the mitochondria and plasma membrane of the middle piece. We are now looking at the ultrastructure of buffalo spermatozoa to study the damage caused by freezing the semen.

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