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Cryo-preservation of buffalo semen in skim milk egg yolk, skim milk, citric acid whey egg yolk and citric acid whey extenders

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Abstract
The present study was conducted on semen of 12 Murrah buffalo bulls kept under identical managemental conditions. The age range of bulls are 3-5 years and weight was 400-600 Kg. The collection of semen from bulls was done in morning hours from 8.00am-9.15am. One collection a week with two consecutive ejaculates were taken from each bull using A.V. technique of Walton. A shorter artificial vagina was used for collection of semen with a temperature ranging from 40-45 ^{0}C depending upon the nature of bull. SMEY (Skim milk 100ml, L-cysteine hydrochloride 0.1g, Benzyl penicillin 1000IU/ml, Streptomycin 0.1mg/ml, Egg yolk 5%), SME(Skim milk 100ml, L-cysteine hydrochloride 0.1g, Benzyl penicillin 1000IU/ml, Streptomycin 0.1mg/ml), CAW(Skim milk 100ml, L-cysteine hydrochloride 0.1g, Benzyl penicillin 1000IU/ml, Streptomycin 0.1mg/ml, Citric acid 0.6gm) and CAWY (Skim milk 100ml, L-cysteine hydrochloride 0.1g, Benzyl penicillin 1000IU/ml, Streptomycin 0.1mg/ml, Citric acid 0.6gm, Egg yolk 5%) were used for the cryo-preservation of semen. SMEY and SME showed the superiority over the CAWY and CAW on the motion analysis of spermatozoa, post-thawing incubation of spermatozoa and HOS, non-eosinophilic count and having less morphological abnormalities of spermatozoa. So it observed that SMEY and SME could be successfully used for cryo-preservation of buffalo semen.

Keywords: Cryo-preservation, extender, semen, murrah buffalo

Introduction
Cryopreservation generates sublethal sperm injury due to chemical, osmotic, thermal and mechanical stresses, which may result in loss of viability, motility, damage of deoxyribonucleic acid (DNA), destruction of acrosomal and plasma membrane [1, 2]. In buffalo natural breeding practice is common in the country compared to Artificial Insemination; low fertility rate with cryopreserved semen is main hindrance in its propagation. Artificial insemination in bull is gaining popularity gradually, but the preservation of buffalo bull semen has been quite a pressing problem for the last several decades. Cryopreservation of buffalo semen increases level of reactive oxygen species molecules that caused lipid peroxidation of biomembrane system by reducing antioxidant potential of cryopreserved semen [3, 4]. Cryopreservation of semen has permitted the rapid expansion of reproductive technology such as artificial insemination. Artificial Insemination and cryopreservation of semen have a significant and good effect on the buffalo population. Several investigators have studied the use of conventional semen extenders for preserving semen of buffalo bull. Buffalo breeders are experiencing the difficulty in preserving the diluted semen at refrigeration temperature as the keeping quality of buffalo semen is much inferior to that of cattle semen with the conventional extenders. Different extenders have been created and enhanced with synthetic compounds to lessen cryodamage or oxidative pressure with changing degrees of accomplishment. Cryodamage during freeze-thawing process to buffalo semen is higher than cattle spermatozoa due to unique physiology of buffalo spermatozoa and higher polyunsaturated phospholipids levels in plasma membrane [5, 6, 7]. Therefore the present study has been undertaken to evaluate the preservability of buffalo semen in milk and a comparison to that of citric acid whey, egg yolk fortified skim milk and citric acid whey.

Materials and methods
The 12 Murrah bulls maintained at the institutional farm where kept under identical managemental condition. The age range of bulls were 3-5 years and weight was 400-600 Kg.

The collection of semen from bulls was done in morning hours from 8.00am-9.15am. One collection a week with two consecutive ejaculates were taken from each bull using A.V. technique of Walton. A shorter artificial vagina was used for collection of semen with a temperature ranging from 40-45 ^{0}C depending upon the nature of bull.

Preparation of semen extenders

1. SME: Skim milk 100ml, L-cysteine hydrochloride 0.1g, Benzyl penicillin 1000IU/ml, Streptomycin 0.1mg/ml
2. SMEY: Skim milk 100ml, L-cysteine hydrochloride 0.1g, Benzyl penicillin 1000IU/ml, Streptomycin 0.1mg/ml, Egg yolk 5%
3. CAW: Skim milk 100ml, L-cysteine hydrochloride 0.1g, Benzyl penicillin 1000IU/ml, Streptomycin 0.1mg/ml, Citric acid 0.6g
4. CAWY: Skim milk 100ml, L-cysteine hydrochloride 0.1g, Benzyl penicillin 1000IU/ml, Streptomycin 0.1mg/ml, Citric acid 0.6g, Egg yolk 5%

Assessment of diluted semen

Microscopic assessment of semen was carried out through conventional procedures viz., non-eosinophilic spermatozoa count, morphologically abnormal cell count and computer aided semen analyser (CASA) at various stages of processing and preservation.

Non –eosinophilic spermatozoa count

Eosin-nigrosin stain was prepared by the method described by *Blom* (1950) [8] and *Hancock* (1951) [9]. Fresh stain was prepared every 15 day to avoid artifacts.

Enumeration of morphological abnormal spermatozoa

The slide which was prepared for non-eosinophilic count was also used for enumerating abnormalities. The classification used by *Lagerlof* (1934) [10] was used for the study.

Hypo-osmotic swelling test The procedure used in the present study for the HOS test was similar to the one developed and described by *Jayendran et al.* (1984) [11].

Post-thaw incubation test

The post-thawed semen was taken in to small test tube and kept in the water bath at 37 ^{0}C. The motion parameters of the sample was evaluated using (CASA) at different time intervals.

Statistical Analysis

The statistical analysis was done as per *Harvey* (1975) [12] and DMRT (*Kramer*, 1956) [13].

Results & Discussion

Motion Analysis

The result of motion analysis of spermatozoa in SMEY, SME, CAWY, and CAW at different stages of freezing have been summarized in table 1. At every stage of freezing, there were significant differences (P<0.05) among extenders for %MOT characteristics. SMEY and SME showed better performance then CAWY and CAW. CAWY was comparatively better then CAW throughout experiment. SME showed better VSL then SMEY at pre freezing stages but after freezing they had similar VSL. CAWY and CAW were inferior to skim milk extenders. Curvilinear velocity (VCL) was almost same in SMEY, SME and CAWY before freezing but at post freezing stages CAWY showed inferior result to SME. CAW showed poor VCL through the experiment.

Linearity (LIN) differed significantly ($P< 0.05$) among extenders at initial stage at pre-freezing ($P< 0.01$) and at 7 days post freezing (P< 0.05). Inferiority of CAW over the extenders was indicated at these stages. Similar results was found in ALH. Average path velocity (VAP) was also lesser in CAWY and CAW then skim milk extenders.

Table 1: Least squares analysis of variance for motion characteristics of Murrah bull semen in various extenders at different stages of freezing.

Motion characteristics	Source of variation	On Primary dilution	After glycerolisation	Pre-freezing	Post -freezing		
					0 hr	24 hr	7 days
% MOT	Extender	17.42*	18.40*	255.82**	354.51**	340.93**	422.14**
	error	3.92	4.67	14.59	27.86	36.13	8.57
VSL	Extender	1.41	15.03*	20.09*	20.95	21.98	44.38*
	error	9.67	2.53	5.74	9.70	14.65	7.81
VCL	Extender	492.15*	881.77*	2514.73**	1573.29	1883.91*	2135.28**
	error	88.12	253.00	274.60	741.34	391.96	221.80
LIN	Extender	10.15*	31.71	97.18**	120.69	31.71	24.60*
	error	2.43	9.76	14.42	38.76	10.10	4.31
ALH	Extender	1.63	3.52	5.41*	11.10	4.02	1.18
	error	0.48	1.19	1.24	4.24	1.19	0.37
VAP	Extender	149.78**	256.71*	755.08**	426.31	573.97*	792.95*
	error	25.09	51.45	69.23	203.12	140.11	56.98

**P<0.01 *P<0.05

Degrees of freedom for extender and error are 3 and 12 respectively for each motion characteristic.

Post–thaw incubation test

The result of post-thaw incubation test using SMEY, SME, CAWY and CAW have been summarized in table 2. The overall motility (PTM 71.25±2.43%) was 52.25±2.01%, 49.75±0.63% and 41.75±0.85% respectively at 1, 2 and 3 hrs. of incubation at 37 °C in SMY extenders. The corresponding values for SME (PTM 69.00±2.08%) were 54.25±3.33%, 51.00±1.83% and 44.25±0.85% and for CAWY (PTM 47.00±9.53), these values were 41.00±6.12%, 41.75±3.9% and 42.50% respectively. In CAW (PTM 40.00±1.83%) the corresponding values were 43.50±2.63%, 26.00±0.40% and 22.75±0.63%, respectively in SMEY, SME, CAWY and CAW extenders. The % MOT was significant at every stages of incubation and of SMEY and SME over CAWY and CAW was revealed at all these stages. In VSL, VCL, LIN, ALH and VAP motion characteristics, similar trends were observed.

Table 2: Least squares analysis of variance for motion characteristics of Murrah bull semen in various extenders at 37 ^{0}C incubation.

Motion characteristics	Source of variation	Incubation stages (hrs.) 0 1 2 3			
% MOT	Extender	340.93**	119.55*	189.53**	155.44**
	error	36.13	20.54	6.77	0.79
VSL	Extender	21.98	39.23*	23.78	18.11**
	error	14.65	10.36	9.02	2.83
VCL	Extender	1883.91*	235.55	1804.97**	837.71
	error	391.96	333.05	165.34	402.54
LIN	Extender	31.72	8.54	63.28*	28.03
	error	10.10	14.70	13.08	26.70
ALH	Extender	4.02	1.42	2.62	2.15
	error	1.19	1.52	1.28	1.95
VAP	Extender	573.97*	61.61	350.94**	186.96
	error	140.11	102.67	42.92	82.74

**P<0.01 *P<0.05 Degrees of freedom for extender and error are 3 and 12 respectively for each motion characteristic.

HOS

The average values of percentage of HOS-reactivity of spermatozoa in SMEY, SME, CAWY and CAW at pre and post freezing stages have been presented in table 3. The differences in values were statistically significant at both pre and post freezing stages. The SMEY and SME showed better HOS reactivity then CAWY and CAW at both the stages.

Non-eosinophilic count

The result of non-eosinophilic count are summarized in table 3. Here also same trend was observed similar that to HOS-test. The SMEY was better than other extenders at pre-freezing stage. At post-freezing stage SMEY and SME were superior over CAWY and CAW.

Morphological abnormalities

The percentage of abnormal spermatozoa using SMEY, SME, CAWY and CAW were almost same at both pre and post freezing stages. The results of the morphological abnormalities of spermatozoa in the above extenders have been presented in table 3. Statistically there were non significant differences among extenders at both the stages.

Table 3: Least square analysis of variance for seminal characteristics of Murrah bull spermatozoa in various extenders (pre and post freezing).

Source of variation	Degrees of freedom	Characteristics					
		HOS		Non-eosinophilic		Morphological abnormalities	
		A	B	A	B	A	B
Extender error	3	33.79**	400.32**	37.90**	386.68**	0.63	1.20
	12	4.21	40.60	4.57	43.41	0.97	1.09

*P < 0.01 A= Pre freezing, B= Post freezing

From the aforesaid findings, it is observed that SMEY and SME are better than CAWY and CAW for buffalo semen extender. The CAWY and CAW could not show good motility, membrane integrity and livability of buffalo spermatozoa. On the other hand, SMEY and SME both performed satisfactorily as revealed by various characteristics studied in study. The above results were in agreement to findings by *Patil et al*. (1981) [14], *Tuli et al*. (1981a) [15], *Matharoo* and *Singh* (1980) [16], and *Dudeja* (1981) [17]. The reason for inferior results shown by CAWY and CAW might be due to absence of casein, lowered level of lactose and phospholipids.

Conclusion

SMEY (SKIM MILK EGG YOLK) and SME (SKIM MILK EXTENDER) showed the superiority over the CAWY (CITRIC ACID WHEY EGG YOLK) and CAW (CITRIC ACID WHEY EXTENDERS) on the motion analysis of spermatozoa, post-thawing incubation of spermatozoa and HOS (HYPO-OSMOTIC SWELLING), non-eosinophilic count and having less morphological abnormalities of spermatozoa. So it observed that SMEY (SKIM MILK EGG YOLK) and SME (SKIM MILK EXTENDER) could be successfully used for cryo-preservation of buffalo semen.

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