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INFLUENCE OF FILTRATION OR CENTRIFUGATION OF SEMEN DILUENT ON THE VIABILITY OF CHILLED BUFFALO-BULL SEMEN

H. Amer, H. Mansour, A. El-Sayed, A. Swelum

Summary

This study was conducted to investigate influence of filtration or centrifugation of TRIS milk diluent on the viability and acrosomal integrity of buffalo spermatozoa preserved under refrigeration condition. Semen ejaculates (n=40) were collected twice weekly using artificial vagina. Each ejaculate was exposed to the following diluents, TRIS (T1 to T4), Milk (M1 to M8), and combinations of TRIS and milk diluents at different ratios (TM1 to TM11). Some parameters as live percentage, viability indicies and acrosomal integrity of spermatozoa was calculated immediately following dilution of semen, then at 3rd, 6th and 9th day of storage at refrigeration temperature (5ºC). Data were statistically analyzed. The results revealed that centrifugation of TRIS diluent once (T3) or twice (T4), before dilution processes, had a beneficial effect on the examined parameters of diluted semen (live percentage, viability indicies and acrosomal integrity of spermatozoa) during the entire period of storage when compared with control TRIS diluent (T1) or filtrated TRIS diluent (T2). However, T3 was the best of all (T1, T2 or T4). Centrifugation of milk-yolk diluent (M3) was found better than any other treatment for the examined milk diluents. Moreover, M3 was the best of all followed by M4 and M6, then followed by the others. Likewise, a combination of T3 and M3 at a ratio of 50:50 (TM6) was, in many aspects, superior to any other combination (TM1 to TM11) or diluents used in this study for dilution and preservation of buffalo semen at refrigeration temperature. It can be concluded that centrifugation of TRIS or milk diluent once before dilution would be the best treatment. Moreover, combination of one part of centrifuged TRIS diluent and one part of centrifuged milk diluents proved to be the proper diluent of choice for dilution and preservation of buffalo semen. It is recommended to use this combination to dilute and freeze buffalo semen for the artificial insemination program.

Key words: Buffalo bull, semen, filtration, centrifugation, preservation, fertilizing, capacity.

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Introduction

Artificial insemination was the first biotechnology applied to improve reproduction and genetics of farm animals. It has an enormous impact worlwide in many species, particulary in dairy herds. Artificial insemination remains the main vehicle for the rapid dissemination of valuable genes and the method of choice for the dairy farmer worldwide to improve the genetic quality of their livestock (Vishwanath and Shannon, 2000). The breeding value of a buffalo bull can be expanded at least 100 times, as many females can be inseminated artificially at the same time with the same ejaculate. Up to 500 buffaloes can be inseminated if the whole ejaculate was used after proper dilution and preservation. However, the main advantage of using unfrozen semen is that adequate fertility is maintained with lower sperm numbers than with frozen semen (Foote et al., 1960). Egg yolk could contain some deleterious components which are potent to reduce spermatozoa motility that can be removed by centrifugation (Moussa et al., 2002). Also, centrifuged egg yolk milk fructose extender was found to be better than none treated ones (Gil at al., 2000), as well as the supernatant portion of centrifuged TRIS egg yolk for 30 minutes at 24,000 g gave a good protection than the control (Yassen and Foote, 1967). Buffaloes are more prefferable animal for rearing in Egyptian villages due to high resistance to diseases, good quality milk and meat and high conversion rate. In addition, spreading of frozen semen is more difficult and need more effort, cost and experience than liquid semen. For these two reasons, this study aimed to investigate influence of filtration or centrifugation of TRIS semen or milk diluent on the viability and acrosomal integrity of extended buffalo-bull semen preserved under refrigeration condition.

Materials and methods

Semen collection and preliminary assessment

A proven fertile Buffalo-bull aged 5 years was used. It is kept for natural breeding at the experimental farm station belonging to Faculty of Veterinary Medicine, Zagazig University. A total of 40 ejaculates (twice a week) were collected, using artificial vagina (Bearden and Fuquay, 1997) and subjected for evaluation before starting the study. Following approval, two succesive ejaculates with 15 minutes interval were collected, pooled and subjected for dilution and preservation at refrigeration temperature (5ºC)

according to some authors (Zemjanis, 1970; Mansour, 1976; Roberts, 1986).

Preparation of semen diluents

1-Tris diluent: TRIS [2-amino-2-(hydroxylmethyl) propane-1,3 diol, BDH Laboratory supply, England] diluent (T) was prepared (Smith et al., 1979; Wall and Foote, 1999). Four treatments were applied for the original TRIS diluent. They included untreated control (T1), filtrated (T2), centrifuged once (T3) and centrifuged twice (T4) as described in Table 1. Filtration was carried out using filter paper Filtrak© no. 388. Medium prosoty Apezialpapier-Filtrak GmbH, Post Barenstein, Germany (Chen et al., 1993; Vishwanath and Shannon, 2000). Centrifugation was carried out at 3310 rpm for 20 min. (Gil et al., 2000).

2-Milk diluent: Milk diluent (M) was prepared (Vishwanath and Shannon, 2000; Paulenz et al., 2003). Eight treatments were applied (M1 to M8) depending upon the step at which egg yolk was added (before or after filtration or centrifugation) as shown in Table 2. Filtration was cerried out using filter paper Filtrak© no. 388. Medium prosoty Apezialpapier-Filtrak GmbH, Post Barenstein, Germany (Chen et al., 1993; Vishwanath and Shannon, 2000). Centrifugation was carried out at 3310 rpm for 20 min. (Gil et al., 2000).

3-Combination of TRIS and Milk diluent: Semen extended with centrifuged TRIS diluent (T3) and centrifuged milk diluent (M3) gave the better viability indicies during the entire course study. Therefore, combination of both diluents was prepared at different ratios (TM1 to TM11) as described in Table 3.

Table 1 – THE DIFFERENT TREATMENTS FOR TRIS DILUENT

Type of treatment	Diluent type			
	T1	T2	T3	T4
None treated (control)	+			
Filtrated		+		
Centrifuged once			+	
Centrifuged twice				+

Table 2 – THE DIFFERENT TREATMENTS FOR MILK DILUENT

Parameter	Diluents type							
	M1	M2	M3	M4	M5	M6	M7	M8
Egg yolk 10%		+	+	+				
Filtration				+			+	+
Centrifugation			+		+	+		
Egg yolk 10%						+		+
Penicilin / Streptomycin	+	+	+	+	+	+	+	+

Table 3 – COMBINATION RATIOS OF T3 AND M3 DILUENTS

No. of TM diluents		1	2	3	4	5	6	7	8	9	10	11
combination ratio	T3	0	10	20	30	40	50	60	70	80	90	100
	M3	100	90	80	70	60	50	40	30	20	10	0

Dilution and preservation processes

The extenders were prepared on the day before semen collection and kept overnight at 5ºC. One hour before semen collection, the diluents warmed in water bath at 30ºC. Following collection, the semen was kept at the same temperature in the same water bath and evalated before processing. Ejaculates of good acceptable viability (Mansour, 1976) were used for extension and preservation. The semen was diluted with the three diluents. The ratio of dilution was 1:10 for the semen to the diluent. Sterile appendorf tubes (previously washed by specific diluents) were used for dilution. Dilution occured gradually ih three steps with 10-15 minutes intervals to avoid dilution shock. The appendorf tubes with their rack and container containing water (at 30ºC) was transferred to the refrigerator. The extended semen reached the chilling temperature (5ºC) by 90-120 minutes of refrigeration (Foote et al., 1960).

Assesment of the diluted and preserved semen samples

1 - Assesment of live percentage

The percentage of live sperms were assesed immediately following dilution (0 day) and after 3rd, 6th and 9th day of preservation. Smears were stained with eosin-negrosin stain prepared to differentiate between live (colorless) and dead (pink) sperm against a blue black ground (Swanson and Bearden, 1951; Roberts, 1986). Percent of live sperms was calculated.

2 – Estimation of sperm motility and viability

The progressive individual motility percentages of the chilled semen were assessed immediately after dilution (0 day) then at the 0, 3rd, 6th and 9th day of refrigeration. The percentages of the progressively motile spermatozoa of the chilled semen were scored in fractions of one unit (Milovanov, 1962). The scores were calculated to give the viability indicies of the bull semen in that specific extender according to the following formula reported by the same author in Table 4.

- Absolute index of viability (Sa) = $\sum a_1t_1 + a_2t_2 + ... + a_nt_n$, where:

a_1, a_2, ..., a_n are the susequent motility scores;
t_1, t_2, ..., t_n are the successive periods at which the corresponding motility scores are interpreted;
t – value (time index) was computed by the formula: $t = T_{n+1}-T_{n-1}/2$, where:
T_{n+1} = Time from beginning of the trial till examination next to the given;
T_{n-1} = Time from beginning of the trial till examination previous to the given.
The formula of first observation was $L_1 = T_2/2$;
The formula of last observation was $t_x = T_x-T_{x-1}/2$

Table 4 – SCORES IN FRACTIONS CORRESPONDING TO PERCENTAGE VALUE OF PROGRESSIVE MOTILITY

Motility score	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	N
Corresponding value in percent	95	85	75	65	55	45	35	25	15	5	0
	to										
	100	94	84	74	64	54	44	34	24	14	

N – No of motility

3 – Estimation of acrosomal status

The acrosome status of spermatozoa in diluted semen were evaluated (Kovacs and Foote, 1992). The acrosome stained with 7.5% Giemsa stock solution [3.8 Giemsa stain was ground with 375 ml absolute methanol in a pestle and mortar, then 125 ml of glycerol was added and stain mixture was stored at 37ºC for one week]. Just before staining 3 ml of this solution was diluted with 2 ml of Sorenses M/15 phosphate buffer with 7.0 pH-value [60% Na_2HPO_4 + 40% KH_2PO_4] and 35 ml of bi-distal water to give 7.5% final concentration of Giemsa mixture. The stains can differentiate the acrosomal status of spermatozoa according to the following criteria: i - intact acrosomes (purple in color), ii - loose acrosomes (dark laveneder in color) and iii - damaged of detached acrosomes (pale lavander or white to pink in color).

Statistical analysis

All data were subjected to two way analysis of variance (ANOVA) (Little et al., 1991; Almor et al., 2006) using computerized statistical analysis SPSS/PCT 2001 (11.0 production facility). The analytic design was factorial (general linear model) to clarify the effect of different diluents in different days on the individual motility, the livability index and the acrosome integrity. Treated means were compared by the least significant difference test (LSD) at 5% level of probability.

Results

The percentage of live spermatozoa (Table 5) and those with intact acrosome (Table 6) of TRIS (T1 to T4) diluted buffalo semen during the entire period of storage showed significant differences. However, T3 diluted semen exhibited the highest values, followed by T4 diluted samples. Moreover, the viability indicies recorded for T3 and T4 diluted semen samples were significantly higher ($P<0.05$-0.01) than those calculated for T1 or T2 diluted ones (Table 6).

The percentage of live spermatozoa (Table 7) and those with intact acrosome (Table 8) of milk diluted (M1 to M8) semen samples during the entire course of the study showed significant differences. However, the highest values were recorded for M3 extended semen. Therefore, eleven diluents were prepared by combination of centrifuged TRIS diluent (T3) and centrifuged milk diluent (M3) at various ratios (TM1 to TM11). It was found that semen extended in TM6 diluent (Table 9, 10) gave better overall values when compared with TRIS or milk diluent. However, results with TM6 (one part T3 : one part M3) were, in many aspects, superior to any other combinations (TM1 to TM11) or diluents used in this study as shown in Fig. 1.

Table 5 – EFFECT OF FILTRATION OR CENTRIFUGATION OF TRIS DILUENTS ON THE PERCENTAGES OF LIVE BUFFALO-BULL SPERMATOZOA PRESERVED AT 5ºC (MEAN±SE)

Day of preservation	Type of TRIS diluents				
	T1	T2	T3	T4	Total
0 DAY	91.80±0.58	92.00±0.32	92.20±0.38	92.00±0.45	92.00±0.20 A
3rd DAY	70.20±0.38	78.60±0.60	82.40±0.25	82.80±0.38	78.50±1.18 B
6th DAY	51.360±0.81	58.80±0.49	67.80±0.38	67.60±0.51	61.45±1.56 C
9th DAY	23.40±0.25	31.40±0.25	40.40±0.51	38.80±0.20	33.50±1.56 D
Total	59.25±5.73 C	65.20±5.20 B	70.70±4.47 A	70.30±4.60 A	- - -

Means with different small superscripts between different treatments are significant at 5% level.
Means with different capital superscripts in the same row or column are significant at 5% level.

Table 6 – EFFECT OF FILTRATION OR CENTRIFUGATION OF TRIS DILUENTS ON THE PERCENTAGES OF INTACT ACROSOMES OF BUFFALO-BULL SPERMATOZOA PRESERVED AT 5ºC (MEAN±SE)

Criteria	Day of preservation	Type of TRIS diluents				
		T1	T2	T3	T4	Total
Viability indicies	mean of 9 days	126.24±5.33	143.04±5.40	159.82±4.33	159.36±4.46	147.15±7.98
Intact acrosome	0 DAY	97.20±0.38	97.80±0.38	97.80±0.38	97.80±0.38	97.65±0.18 A
	3rd DAY	87.40±0.40	91.60±0.51	94.20±0.38	94.00±0.32	91.80±0.64 B
	6th DAY	71.40±0.60	78.20±0.80	86.00±0.71	85.60±0.40	80.30±1.40 C
	9th DAY	38.40±0.68	48.40±0.92	59.80±0.86	59.80±0.20	51.60±2.07 d
	Total	73.60±5.09 C	77.00±4.36 B	84.45±3.40 A	84.30±3.38 A	- - -

Means with different small superscripts between different treatments are significant at 5% level.
Means with different capital superscripts in the same row or column are significant at 5% level.

Table 7 – EFFECT OF FILTRATION, CENTRIFUGATION AS WELL AS SEQUENCE OF YOLK ADDITION TO MILK DILUENTS ON THE PERCENTAGES OF LIVE BUFFALO-BULL SPERMATOZOA PRESERVED AT 5ºC (MEAN±SE)

Type of milk diluent	Day of preservation				
	0 DAY	3rd DAY	6th DAY	9th DAY	Total
M1	90.76±0.37	74.78±0.29	48.00±0.33	16.78±0.00	57.56±4.78 G
M2	90.76±0.37	80.78±0.29	58.00±.0.33	29.00±0.17	64.61±4.01 D
M3	90.78±0.28	84.22±0.22	69.11±0.31	43.89±0.26	72.00±3.05 A
M4	90.78±0.28	83.44±0.18	62.78±0.28	34.89±0.11	67.97±3.67 B
M5	90.89±0.26	78.44±0.29	52.00±0.41	24.11±0.32	61.36±4.34 E
M6	90.89±0.26	82.33±0.24	63.00±0.41	35.00±0.11	67.81±3.64 B
M7	90.89±0.31	76.44±0.29	50.11±0.39	18.78±0.00	59.06±4.69 F
M8	90.78±0.22	82.33±0.22	60.22±0.36	28.89±0.20	65.56±4.04 C
Total	90.79±0.10 A	80.35±0.40 B	57.90±0.81 C	28.92±1.03 D	- - -

Means with different small superscripts between different treatments are significant at 5% level.
Means with different capital superscripts in the same row or column are significant at 5% level.

Table 8 – EFFECT OF FILTRATION, CENTRIFUGATION AS WELL AS SEQUENCE OF YOLK ADDITION TO MILK DILUENTS ON THE PERCENTAGES OF INTACT ACROSOMES OF BUFFALO-BULL SPERMATOZOA PRESERVED AT 5ºC (MEAN±SE)

Type of diluents	Viability indicies	Day of preservation				
		0 DAY	3rd DAY	6th DAY	9th DAY	Total
M1	121.06±3.33	95.00±0.37	78.78±0.50	71.44±0.24	32.89±0.33	69.53±3.61 F
M2	138.40±3.07	95.22±0.32	83.00±0.44	75.22±0.18	41.11±0.67	73.64±3.25 C
M3	161.86±3.42	95.67±0.29	86.78±0.22	79.33±0.44	54.89±0.26	79.17±2.57 A
M4	150.40±3.63	95.33±0.24	85.44±0.22	76.00±0.24	49.44±0.15	76.56±2.85 B
M5	134.40±2.50	95.22±0.22	81.78±0.26	73.11±0.33	38.78±0.22	72.22±3.33 D
M6	151.73±3.68	95.33±0.24	84.89±0.24	76.44±0.31	49.78±0.24	76.61±2.82 B
M7	121.33±4.02	94.44±0.50	81.00±0.24	70.56±0.37	35.44±0.24	70.36±3.45 E
M8	141.86±4.30	94.67±0.33	83.89±0.29	75.89±0.48	41.56±0.62	74.00±3.22 C
Total	140.13±5.12	95.11±0.12 A	83.19±0.19 B	74.75±0.20 C	42.99±0.66 D	- - -

Means with different small superscripts between different treatments are significant at 5% level.
Means with different capital superscripts in the same row or column are significant at 5% level.

Table 9 – EFFECT OF DIFFERENT RATIOS OF T3 AND M3 DILUENTS ON THE PERCENTAGES OF LIVE BUFFALO-BULL SPERMATOZOA PRESERVED AT 5ºC (MEAN±SE)

Type of diluents	Day of preservation				
	0 DAY	3rd DAY	6th DAY	9th DAY	Total
TM1	92.60±0.40	78.20±0.49	51.80±0.38	26.40±0.25	62.25±5.78 I
TM2	92.00±0.32	80.60±0.25	55.80±0.38	31.20±0.20	64.90±5.36 H
TM3	92.20±0.20	80.40±0.25	62.00±0.45	36.80±0.38	67.85±4.78 F
TM4	92.40±0.25	83.60±0.40	64.80±0.38	39.60±0.25	70.10±4.62 D
TM5	92.20±0.20	84.20±0.38	67.00±0.54	42.60±0.45	71.50±4.33 C
TM6	92.20±0.20	84.20±0.38	71.20±0.49	48.60±0.51	74.05±3.76 A
TM7	92.20±0.20	84.20±0.38	69.00±0.54	45.40±0.40	72.70±4.07 B
TM8	92.20±0.20	81.60±0.40	64.80±0.38	39.60±0.25	69.55±4.53 E
TM9	92.20±0.20	79.00±0.45	61.40±0.25	37.60±0.51	67.55±4.67 F
TM10	92.20±0.20	79.60±0.51	59.60±0.25	33.80±0.58	66.30±5.04 G
TM11	92.40±0.25	79.60±0.51	59.60±0.25	32.20±0.49	66.00±5.18 G
Total	92.25±0.07 A	81.38±0.32 B	62.47±0.76 C	37.62±0.85 D	- - -

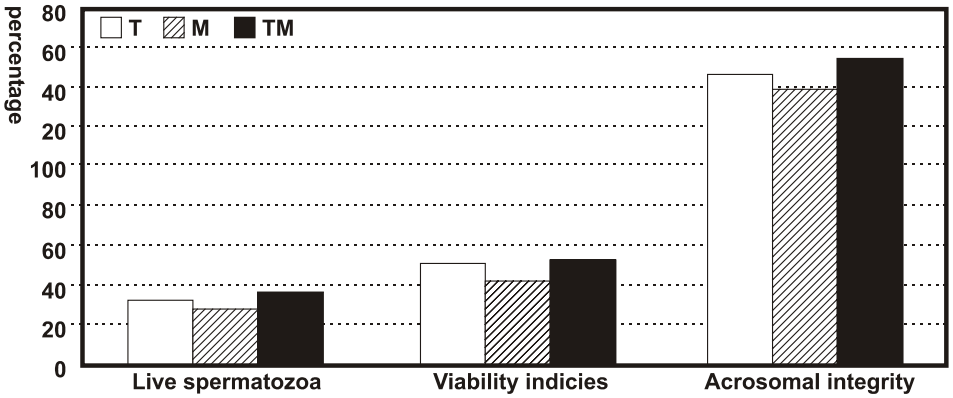
Means with different small superscripts between different treatments are significant at 5% level.
Means with different capital superscripts in the same row or column are significant at 5% level.

Table 10 – EFFECT OF DIFFERENT RATIOS OF T3 TO M3 DILUENTS ON THE VIABILITY AND PERCENTAGES OF INTACT ACROSOMES OF LIVE BUFFALO-BULL SPERMATOZOA PRESERVED AT 5ºC (MEAN±SE)

Type of diluents	Viability indicies	Intact acrosomes				
	mean od 9 days	0 DAY	3rd DAY	6th DAY	9th DAY	Total
TM1	134.40±4.60	95.80±0.38	84.20±0.73	74.20±0.58	42.60±0.60	74.20±4.51 H
TM2	142.56±5.23	96.20±0.49	87.20±0.20	75.80±0.58	48.20±0.66	76.85±4.13 F
TM3	153.60±4.15	96.00±0.54	87.60±0.40	76.00±0.54	52.40±0.40	78.00±3.76 E
TM4	159.84±4.05	96.40±0.51	88.40±0.40	77.40±0.60	55.60±0.40	79.45±3.51 D
TM5	164.16±4.33	96.00±0.32	90.00±0.63	80.40±0.51	58.80±0.58	81.30±3.22 C
TM6	171.36±3.83	96.20±0.38	91.00±0.00	86.00±0.71	64.40±0.40	84.40±2.78 A
TM7	169.44±3.60	96.00±0.32	90.80±0.38	83.20±0.96	61.20±0.38	82.80±3.04 B
TM8	158.88±6.36	96.20±0.38	88.20±0.80	78.00±0.54	56.20±0.58	79.65±3.42 D
TM9	154.08±4.63	96.20±0.38	86.40±0.51	76.60±0.51	53.20±0.58	78.10±3.64 E
TM10	148.08±4.75	96.00±0.32	86.20±0.58	72.40±0.51	50.00±0.54	76.15±3.96 F
TM11	147.84±4.19	96.00±0.00	85.80±0.80	72.40±0.75	48.20±0.38	75.60±4.09 G
Total	154.90±3.42	96.09±0.11 A	87.80±0.32 B	77.49±0.58 C	53.71±0.84 D	- - -

Means with different small superscripts between different treatments are significant at 5% level.
Means with different capital superscripts in the same row or column are significant at 5% level.

Figure 1 – THE OVERALL LIVE PERCENT, VIABILITY INDICIES AND ACROSOMAL INTEGRITY OF BUFFALO SPERMATOZOA (MEAN OF 9 DAYS) RECORDED FOR TRIS (T), MILK (M) AND TRIS-MILK (TM) DILUENTS. THE MEANS ARE SIGNIFICANT AT 5% LEVEL



Discussion

There was no significant difference between the percentage of motile spermatozoa in all extenders of each experiment before and after dilution, which may be attributed to good preparation of the diluents and gradual dilution of semen at the same temperature minimizing the intra cellular ions reduction by out fluxing following initial extension (Yassen and Foote, 1966) and the diluents offered the main requirements for sperm cells as mentioned previously (Bearden and Fuquay, 1997). The percentage of spermatozoa parameters were significantly higher in T3 (centrifuged TRIS-yolk) and M3 (centrifuged milk-yolk) diluents than the non treated or filtrated ones. These results may be attributed to the removal of some deleterious components present in the egg-yolk or maintaining the highest protective ability of low density fraction (LDF) lipoprotein. Our results were in agreement with the previous results (Gil et al., 2000) which reported that the centrifuged egg yolk milk fructose extender was found to be better than none treated ones and the centrifuged TRIS-egg yolk for 30 minutes at 24,000 g (Yassen and Foote, 1967). The previuos authors found that the supernatant portion gave a good protection than the control and the sperm motility in the washed egg yolk precipitate was significantly reduced by removing some protective substances or altered the residual precipitate in an undesirable way. Furthermore, egg yolk could contain some deleterious components which are potent to reduce semen motility that can be removed by centrifugation (Moussa et al., 2002). The last authors added that adsorption and gelation of LDF-components around the spermatozoa

membrane could form a protective film against ice-crystals generated during freezing. Moreover, the increase of LDF-apoproteins concentration in the diluent above 10% leads to a decrease in sperm performance after freeze-thaw which may be related to the osmotic pressure of the diluents that declined when the concentration of LDF-apoprotein increases in the diluents. Our results were supported by some authors (Pace and Graham, 1974; Watson and Martin, 1975), who found that egg yolk may have harmful substances which inhibit respiration of spermatozoa and reduce their motility. Recent study showed that the LDF-lipoprotein has a very high capacity for BSP-protein binding which may prevent their detrimental effect on sperm membrane which is important for sperm storage and represents the major mechanism of sperm protection by egg yolk (Manjunath et al., 2002). This binding is rapid, specific, saturable and stable. Our results were also supported by previous findings (Amirat et al., 2004) which reported that after cryopreservation in LDL extender, a higher percentage of motile bull spermatozoa (>50%) and a higher cleavage rate than that obtained when using Optydill. As well as the improvement may be attributed to the disperzing of the fat globules by centrifugation, which interfere with microscopic examination of the extended semen (Vishwanath and Shannon, 2000). Moreover, better results in terms of motility and movement characteristics when replacing whole egg yolk by 8% (w/v) LDF-lipoprotein (Moussa et al., 2002).

There was no significant difference in the parameters during preservation between T3 and T4 (those treated by single or double centrifugation). This observation revealed that single centrifugation was enough for removal of harmful material and maintenance of active principle of the egg yolk (LDF-lipoprotein). The percentages of intact acrosome in T3 and M3 were significantly higher than filtrated or none treated diluents. These results are in agreement with previous study (Wall and Foote, 1999), which indicated that high fertility was maintained when only the supernatant that resulted from centruifugation of egg yolk combined with TRIS was used. Our results may be attributed to concentration of LDF-lipoprotein that reacts with detrimental factor (BSP-protein) in seminal plasma. These observations were supported by other findings (Vishwanath et al., 1992) which suggested that the egg yolk lipoproteins compete with detrimental seminal plasma cations peptides in binding to the sperm membrane and thus guarding them from this harmful effect. Also, the continous exposure of sperm to seminal plasma that contains BSP-protein is detrimental to the sperm membrane and this may render the membrane very sensitive to sperm storage in liquid or frozen state (Therien et al., 1999). Therefore, BSP-proteins act as both beneficial and detrimental

factors to sperm depending on the concentration of the seminal plasma and exposure time. The mechanism by which the lipoprotein protects sperm acrosome during preservation was clarified (Manjunath et al., 2002). The last authors cited that BSP-proteins interact with the LDF (major component of egg yolk extender), which represents the major mechanism of sperm protection by egg yolk. The fertility of bovine semen stored at 5ºC in most diluents remains acceptable for 2-4 days. The decline in fertility that occurs after this time is initially due to decreased motility and survival in the female genitalia rather than to sperm death. Short-term storage of semen by chilling to 5ºC is, however, a very cheap and effective way of establishing an AI program for cattle (Foote et al., 1960).

As represented in this study, the sperm viabililty and intact acrosomes with filtrated milk egg yolk (M4) was signicifantly higher during preservation than filtrated milk plus egg yolk (M8). This result was nearly similar to previous results (Garcia and Graham, 1987), which showed that sperm survival in extenders containing high concentrations of egg yolk salts was significantly low. The percentage of motile sperma as well as intact acrosomes was significantly higher in treated (M3, M4, M6 and M8) milk egg yolk diluents. This result may be attributed to presence of LDF-lipoprotein in egg yolk milk diluents in higher level than in milk diluents alone improving its protective effect on sperm cell membrane and acrosome. Our results are similar to previous results (Manjunath et al., 2002). Milk contains lipoprotein and phospholipids and these milk components interact with the BSP-protein and prevent their detrimental effect on sperm membrane. Also, milk proteins effectively reduce the damage of cold shock and the triglycerides were ineffective (Choong and Wales, 1962). Milk can be filtrated after heating or poured slowly from boiler leaving the surface scum albumin on the side of boiler (Roberts, 1986). Recently, it has been recorded (Chen et al., 1993; Vishwanath and Shannon, 2000) that whole homogenized milk satisfies the requirements of good semen diluter if it is obtained from a reliable source, heat-treated, then cooled and filtered. In addition, some milk fractions (ultra filtrate, micro filtrate and alpha-lactalbumin fraction) decreased spermatozoa survival (Batellier et al., 1997). Others (beta-lactoglobulin and negative phosphocaseinate) were protective. Additionally, the filtration not affects lactose concentration as well as pH, but reduce osmolarity and nitrogen content. This may be one of the causes improving spermatozoa preservation. Heating increases the diameter of casein micelles, partially denatured whey proteins and enzymes, release –SH gropus and decrease the concentration of minerals because of adsorption of ions onto the casein micelles (Batellier et al.,

1997). Enzymes denaturation seems to be the most important modification of milk to explain the difference of sperm survival between skim milk and ultra-high temperature treated skim milk. This was confirmed by the fact that heating affected only fractions containing soluble proteins. However, optimization of milk soluble proteins has permitted efficient spermatozoa protection. At 4ºC, the optimal whey protein concentration is 20 g/L (3 times the milk concentration), and the optimal beta-lactoglobulin concentration is 40 g/L (15 times the milk concentration). It is important to note that beta-lactoglobulin is the major soluble protein in the milk (and in the whey protein). The percentage of live intact acrosome decreased by days in all diluents, but the rate becomes higher after 6 days. This may be attributed to aging of sperm as well as chilling. This explanation was nearly similar to that previously cited (Bedford, 1972), which obtained disintegration of the acrosome of the spermatozoa as a result of chilling, therefore, this might be expected to affect its fertilizing capacity.

Our experiment to mix milk with buffer containing diluents (TRIS) was perviously tried (Lyer, 1952). Boiled milk mixed with citrate buffer to preserved bovine semen. In 1972, Pavithran et al. used a diluents composed of skim milk, lactose, citrate and egg yolk that produced a fertility rate of 77% from buffalo bull semen stored in liquid nitrogen for 4-143 days. More recent studies (Karabinus et al., 1991) improved post-thaw quality of bull semen by combining equal fractions of heated whole milk and 20% egg yolk citrate. The useful effect of mixing milk and TRIS was to create new diluents containing the two main beneficial actions of both milk and TRIS (Melrose, 1956; Foote, 1975). In this study, it was observed that the mixing of T3 with M3 diluent with a ratio of 50:50 (TM6) gave the better results in the percentages of live spermatozoa and those with intact acrosomes during preservation than the other different ratios. We can conclude that by synergistic action and increase amount of LDF-lipoprotein, offering fructose, reducing fructolysis and preserving sperm energy in centrifuged TRIS yolk (T3) diluent and a good protective effect of milk proteins as lactalbumin which enhances sperm viability and sperm motility, as well as offering of lactose and another nutritive materials to the sperm in centrifuged milk yolk (M3) diluent. In addition, the explanation of improving the efficacy of mixing milk to TRIS diluents may be attributed to the presence of milk proteins those have a good protective effect on spermatozoa (Salamon and Maxwell, 1995a,b; Watson, 1995). The later authors as well as anothers (38) reported that lactalbumin enhances sperm viability (assessed as the hypoosmotic response) and sperm motility when added to other extenders.

Conclusion

Centrifugation of TRIS or milk diluents once before dilution would be the best treatment. However, combination of one part of centrifuged TRIS diluent and one part of centrifuged milk diluent proved to be the proper diluent of choice for dilution and preservation of buffalo bull semen. We recommend following up these trials with another aim to freeze and increase the fertilizing capacity of buffalo-bull spermatozoa using TRIS-milk diluent.

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UTJECAJ FILTRIRANJA I CENTRIFUGIRANJA RAZRJEĐIVAČA SPERME NA VIJABILNOST OHLAĐENE SPERME BIKA BUFALA

Sažetak

Cilj ovoga rada bio je istražiti utjecaj filtriranja i centrifugiranja razrjeđivača TRIS i mliječnog razrjeđivača na vijabilnost i akrosomalni integritet sperme bufala čuvane u uvjetima hlađenja. Ejakulati sperme (n=40) sakupljani su dvaput tjedno primjenom umjetne vagine. Svaki ejakulat izložen je sljedećim razrjeđivačima: TRIS (T1 do T4), mlijeko (M1 do M8) i kombinacija razrjeđivača TRIS i mliječnog razrjeđivača (TM1 do TM11). Neki su parametri, kao živi postotak, vijabilnost i akrosomalni integritet sperme, izračunati odmah nakon rezrjeđivanja sperme, zatim 3., 6. i 9. dan čuvanja na temperaturi hlađenja (5°C). Podaci su statistički analizirani. Rezultati su pokazali da centrifugiranje razrjeđivačem TRIS jedanput (T3) ili dvaput (T4) prije procesa razrjeđivanja ima povoljan utjecaj na ispitivane parametre razrjeđivanja sperme (živi postotak, vijabilnost i akrosomalni integritet sperme) u cijelom razdoblju čuvanja, u usporedbi s kontrolnim razrjeđivačem TRIS (T1) ili filtriranim razrjeđivačem (T2). Međutim, T3 je bio najbolji od svih (T1, T2 ili T4). Centrifugiranje razrjeđivača mlijeko-žumanjak (M3) pokazalo se boljim od bilo kojeg tretiranja ispitivanih mliječnih razrjeđivača. Štoviše, M3 je bio najbolji, slijede M4 i M6, a zatim ostali. Isto tako, kombinacija T3 i M3 u omjeru 50:50 (TM6) bila je u mnogo pogleda bolja od bilo koje druge kombinacije (TM1 do TM11) razrjeđivača upotrijebljenih u ovom istraživanju. Može se zaključiti da bi centrifugiranje TRIS ili mliječnim razrjeđivačem jedanput prije razrjeđivanja bio najbolji tretman za očuvanje sperme bufala na temperaturi hlađenja. Osim toga, kombinacija jednog dijela centrifugiranog razrjeđivača TRIS i jednog dijela centrifugiranih mliječnih razrjeđivača pokazala se pravim izborom za razrjeđivanje i očuvanje sperme bufala. Preporuča se upotreba ove kombinacije za razrjeđivanje i zamrzavanje sperme bufala za program umjetne oplodnje.

Ključne riječi: bik bufalo, sperme, filtriranje, centrifugiranje, čuvanje, oplodnja, kapacitet

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