

Influence of Filtration or Centrifugation of Semen Diluent on The Viability of Chilled Buffalo-Bull Semen

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

Department of Theriogenology, Faculty of Veterinary Medicine, Zagazig University

ABSTRACT

This study was conducted to investigate influence of filtration or centrifugation of TRIS or milk diluent on the viability and acrosomal integrity of buffalo spermatozoa preserved under refrigeration condition.

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. Semen ejaculates (n=40) were collected twice weekly using artificial vagina. Each time, two successive ejaculates were obtained, pooled and diluted with the following diluents: 1-TRIS (T1 to T4). 2-Milk (M1 to M8), and 3-Combinations of TRIS and milk diluents at different ratios (TM1 to TM11). Some parameters as live percentage, viability indices and acrosomal integrity of spermatozoa were calculated immediately following dilution of semen, then at 3rd, 6th and 9th day of storage at refrigeration temperature (5°C). Data were statistically analysed.

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 indices 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 centrifugated TRIS diluent and one part of centrifugated milk diluent 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.

INTRODUCTION

Artificial insemination was the first biotechnology applied to improve reproduction and genetics of farm animals. It has an enormous impact worldwide in many species, particularly in dairy herds. Artificial insemination remains the main vehicle for the rapid dissemination of valuable genes and the method of choice for dairy farmers world wide to improve the genetic quality of their livestock (1). 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 (2). Egg yolk could contain some deleterious components which are potent to reduce spermatozoa motility that can be removed by centrifugation (3). Also, centrifugated egg yolk milk fructose extender was found to be better than the none treated ones (4), as well as, the supernatant portion of centrifugated TRIS egg yolk for 30 minutes at 24,000 g gave a good protection than the control (5).

Table 3. The different combination ratios of T3 [centrifugated TRIS] and M3 [centrifugated milk] 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 5°C. One hour before semen collection, the diluents warmed in a water bath at 30°C. Following collection, the semen was kept at the same temperature in the same water bath and evaluated before processing. Ejaculates of good acceptable viability (8) 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 the specific diluents) were used for the dilution. Dilution occurred gradually in three steps with 10-15 minutes intervals to avoid dilution shock. The appendorf tubes with their rack and container containing water (at 30°C), was transferred to the refrigerator. The extended semen reached the chilling temperature (5°C) by 90-120 minutes of refrigeration (2).

Assessment of the diluted and preserved semen samples

1-Assessment of live percentage

The percentages of live sperms were assessed 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 (9,14). 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 (15). The scores were calculated to give the viability indices 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) = $\sum a_i t_i = \sum a_1 t_1 + a_2 t_2 + \dots + a_n t_n$; where;
 a_1, a_2, \dots, a_n are the subsequent motility scores.

- t_1, t_2, \dots, t_n are the successive periods at which the corresponding motility scores 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 of one unit corresponding to the 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 motility

3-Estimation of acrosomal picture

The acrosome pictures of spermatozoa in diluted semen were evaluated (16). The acrosome stained with 7.5% Giemsa stock solution [3.8 gm 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 37°C for one week]. Just before staining 3 ml of this solution was diluted with 2 ml of Sorensens M/15 phosphate buffer with 7.0 pH-value [60% Na₂HPO₄+40% KH₂PO₄] and 35 ml of bi-distal water to give 7.5% final concentration of Giemsa mixture. The stains can differentiate the acrosomal picture of spermatozoa according to the following criteria: i-intact acrosomes (purple in color), ii-loose acrosomes (dark lavender in color) and iii-damaged or detached acrosomes (pale lavender or white to pink in color).

Statistical analysis

All data were subjected to two way analysis of variance (ANOVA) (17,18), 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 percentages 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 indices 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).

Table 5. Effect of filtration or centrifugation of TRIS diluents on the percentages of live Buffalo-bull spermatozoa preserved at 5°C (Mean±SE).

Day of preservation	Type of TRIS diluent				
	T1	T2	T3	T4	Total
0 DAY	91.80 ^a ±0.58	92.00 ^a ±0.32	92.20 ^a ±0.38	92.00 ^a ±0.45	92.00 ^A ±0.20
3 rd DAY	70.20 ^d ±0.38	78.60 ^c ±0.60	82.40 ^b ±0.25	82.80 ^b ±0.38	78.50 ^B ±1.18
6 th DAY	51.60 ^g ±0.81	58.80 ^f ±0.49	67.80 ^e ±0.38	67.60 ^e ±0.51	61.45 ^C ±1.56
9 th DAY	23.40 ^k ±0.25	31.40 ^j ±0.25	40.40 ^h ±0.51	38.80 ⁱ ±0.20	33.50 ^D ±1.56
Total	59.25 ^C ±5.73	65.20 ^B ±5.20	70.70 ^A ±4.47	70.30 ^A ±4.60	---

Means with different small superscripts between different treatments are significant at 5% level.

Means with different capital superscripts in the same column or row 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 5°C (Mean±SE)

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

Means with different small superscripts between different treatments are significant at 5% level.

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

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 centrifugated TRIS diluent (T3) and centrifugated 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 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 5°C (Mean±SE).

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

Means with different small superscripts between different treatments are significant at 5% level.

Means with different capital superscripts in the same column or row 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 5°C (Mean ± SE).

riteria	Day of preserv- ation	Type of milk diluent								Total
		M1	M2	M3	M4	M5	M6	M7	M8	
iability indices	mean of 9 days	121.06 ^e ±3.33	138.40 ^{cd} ±3.07	161.86 ^a ±3.42	150.40 ^b ±3.63	134.40 ^d ±2.50	151.73 ^b ±3.68	121.33 ^e ±4.02	141.86 ^c ±4.30	140.13 ⁻⁻⁻ ±5.12
Intact acrosomes	0 DAY	95.00 ^{ab} ±0.37	95.22 ^{ab} ±0.32	95.67 ^a ±0.29	95.33 ^{ab} ±0.24	95.22 ^{ab} ±0.22	95.33 ^{ab} ±0.24	94.44 ^b ±0.50	94.67 ^{ab} ±0.33	95.11 ^A ±0.12
	3 rd DAY	78.78 ^h ±0.50	83.00 ^f ±0.44	86.78 ^c ±0.22	85.44 ^d ±0.22	81.78 ^g ±0.26	84.89 ^{de} ±0.24	81.00 ^g ±0.24	83.89 ^{ef} ±0.29	83.19 ^B ±0.19
	6 th DAY	71.44 ^l ±0.24	75.22 ^j ±0.18	79.33 ^h ±0.44	76.00 ^{ij} ±0.24	73.11 ^k ±0.33	76.44 ⁱ ±0.31	70.56 ^l ±0.37	75.89 ^{ij} ±0.48	74.75 ^C ±0.20
	9 th DAY	32.89 ^r ±0.33	41.11 ^o ±0.67	54.89 ^m ±0.26	49.44 ⁿ ±0.15	38.78 ^p ±0.22	49.78 ⁿ ±0.24	35.44 ^q ±0.24	41.56 ^o ±0.62	42.99 ^D ±0.66
	Total	69.53 ^F ±3.61	73.64 ^C ±3.25	79.17 ^A ±2.57	76.56 ^B ±2.85	72.22 ^D ±3.33	76.61 ^B ±2.82	70.36 ^E ±3.45	74.00 ^C ±3.22	---

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

Table 9. Effect of different ratios of T3 to M3 diluent on the percentages of live Buffalo-bull spermatozoa preserved at 5°C (M±SE).

Type of diluent	Day of preservation				Total
	0 Day	3 rd Day	6 th day	9 th day	
TM1	92.60±0.40 ^a	78.20±0.49 ^f	51.80±0.38 ⁿ	26.40±0.25 ^v	62.25±5.78 ^I
TM2	92.00±0.32 ^a	80.60±0.25 ^{cd}	55.80±0.38 ^m	31.20±0.20 ^u	64.90±5.36 ^H
TM3	92.20±0.20 ^a	80.40±0.25 ^d	62.00±0.45 ^k	36.80±0.38 ^s	67.85±4.78 ^F
TM4	92.40±0.25 ^a	83.60±0.40 ^b	64.80±0.38 ^j	39.60±0.25 ^r	70.10±4.62 ^D
TM5	92.20±0.20 ^a	84.20±0.38 ^b	67.00±0.54 ⁱ	42.60±0.45 ^q	71.50±4.33 ^C
TM6	92.20±0.20 ^a	84.20±0.38 ^b	71.20±0.49 ^g	48.60±0.51 ^o	74.05±3.76 ^A
TM7	92.20±0.20 ^a	84.20±0.38 ^b	69.00±0.54 ^h	45.40±0.40 ^p	72.70±4.07 ^B
TM8	92.20±0.20 ^a	81.60±0.40 ^c	64.80±0.38 ^j	39.60±0.25 ^r	69.55±4.53 ^E
TM9	92.20±0.20 ^a	79.00±0.45 ^{ef}	61.40±0.25 ^k	37.60±0.51 ^s	67.55±4.67 ^F
TM10	92.20±0.20 ^a	79.60±0.51 ^{de}	59.60±0.25 ^l	33.80±0.58 ^t	66.30±5.04 ^G
TM11	92.40±0.25 ^a	79.60±0.51 ^{de}	59.80±0.20 ^l	32.20±0.49 ^u	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 column or row are significant at 5% level.

Table 10. Effect of different ratios of T3 to M3 diluent on the viability and percentages of intact acrosomes of Buffalo-bull spermatozoa preserved at 5°C (Mean±SE).

Type of diluent	Viability indices	Intact acrosomes				
	mean of 9 days	0 Day	3 rd Day	6 th day	9 th day	Total
TM1	134.40 ^f ±4.60	95.80 ^a ±0.38	84.20 ^f ±0.73	74.20 ^k ±0.58	42.60 ^t ±0.60	74.20 ^H ±4.51
TM2	142.56 ^{ef} ±5.23	96.20 ^a ±0.49	87.20 ^{cde} ±0.20	75.80 ^j ±0.58	48.20 ^s ±0.66	76.85 ^F ±4.13
TM3	153.60 ^{cd} ±4.15	96.00 ^a ±0.54	87.60 ^{cd} ±0.40	76.00 ^{ij} ±0.54	52.40 ^q ±0.40	78.00 ^E ±3.76
TM4	159.84 ^{ab} ±4.05	96.40 ^a ±0.51	88.40 ^c ±0.40	77.40 ^{hi} ±0.60	55.60 ^p ±0.40	79.45 ^D ±3.51
TM5	164.16 ^{ab} ±4.33	96.00 ^a ±0.32	90.00 ^b ±0.63	80.40 ^j ±0.51	58.80 ^o ±0.58	81.30 ^C ±3.22
TM6	171.36 ^a ±3.83	96.20 ^a ±0.38	91.00 ^b ±0.00	86.00 ^e ±0.71	64.40 ^m ±0.40	84.40 ^A ±2.78
TM7	169.44 ^a ±3.60	96.00 ^a ±0.32	90.80 ^b ±0.38	83.20 ^f ±0.96	61.20 ⁿ ±0.38	82.80 ^B ±3.04
TM8	158.88 ^{ab} ±6.36	96.20 ^a ±0.38	88.20 ^c ±0.80	78.00 ^h ±0.54	56.20 ^p ±0.58	79.65 ^D ±3.42
TM9	154.08 ^{cd} ±4.63	96.20 ^a ±0.38	86.40 ^{de} ±0.51	76.60 ^{hij} ±0.51	53.20 ^q ±0.58	78.10 ^E ±3.64
TM10	148.08 ^{de} ±4.75	96.00 ^a ±0.32	86.20 ^{de} ±0.58	72.40 ^l ±0.51	50.00 ^r ±0.54	76.15 ^{F^G} ±3.96
TM11	147.84 ^{de} ±4.19	96.00 ^a ±0.00	85.80 ^e ±0.80	72.40 ^l ±0.75	48.20 ^s ±0.38	75.60 ^G ±4.09
Total	154.90 ±3.42	96.09 ^A ±0.11	87.8 ^B ±0.32	77.49 ^C ±0.58	53.71 ^D ±0.84	---

Means with different small superscripts between different treatments are significant at 5% level.

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

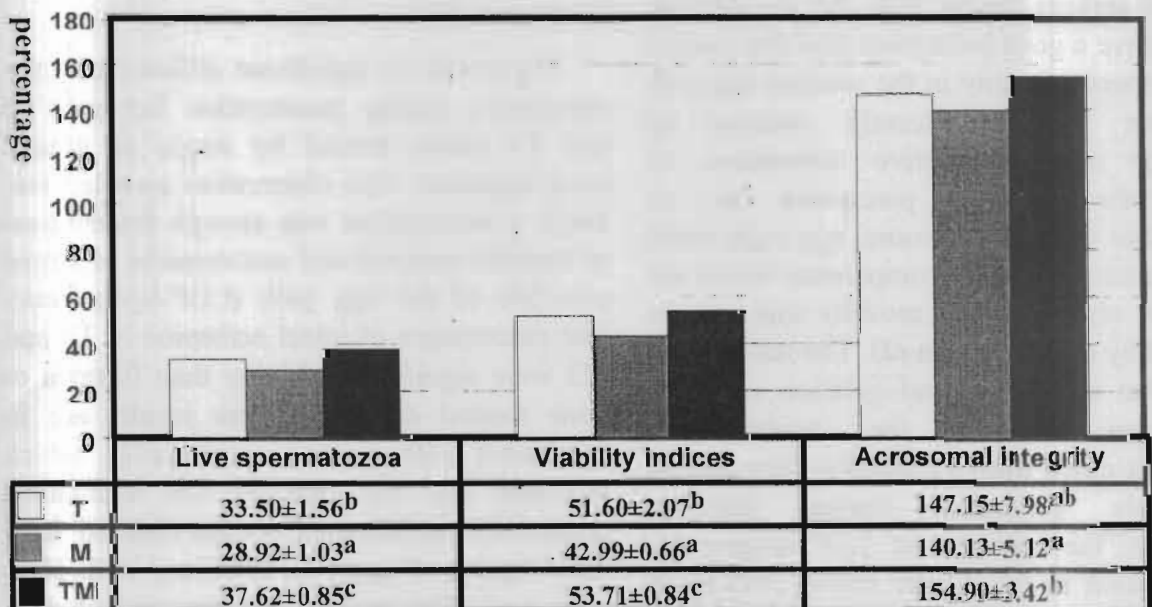


Figure 1: The overall live percent, viability indices and acrosomal integrity of buffalo spermatozoa (mean of 9 days) recorded for TRIS (T), milk (M) and TRIS-milk (TM) diluents.

Means with different small superscripts in the same column 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 extention (19), and the diluents offered the main requirements for sperm cells as mentioned previously (6). The percentage of spermatozoal 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 (4), which reported that the centrifugated egg yolk milk fructose extender was found to be better than the none treated ones, and the centrifugated TRIS-egg yolk for 30 minutes at 24,000 g (5). The previous 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 deletrious components which are potent to reduce semen motility that can be removed by centrifugation (3). The last authors added that adsorption and gelation of LDF-apoproteins around the spermatozoal 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 diluent that declined

when the concentration of LDF-apoprotein increases in the diluent. Our results were supported by some authors (20,21), 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 (22). This binding is rapid, specific, saturable and stable. Our results were also supported by previous findings (23), which reproted that after cryopreservation in LDL extender, a higher percentage of motile bull spermatozoa (>50%) and a higher cleavage rate than that obtained when using Optidyll. As well as the improvement may be attributed to the dispersing of the fat globules by centrifugation, which interfere with microscopic examination of the extended semen (1). Moreover, better results in terms of motility and movement characteristics when replacing whole egg yolk by 8% (w/v) LDF-lipoprotein (3).

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 (11), which indicated that high fertility was maintained when only the supernatant that resulted from centrifugtion 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 another findings (24) 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 continuous 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 (25). 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 (22). 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 5°C 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 5°C is, however, a very cheap and effective way of establishing an AI program for cattle (2).

As represented in this study, the sperm viability and intact acrosomes with filtrated milk egg yolk (M4) was significantly higher during preservation than filtrated milk plus egg yolk (M8). This result was nearly similar to previous results (26), which showed that sperm survival in extenders containing high concentrations of egg yolk salts was significantly low. The percentage of motile sperms as well as intact acrosomes were 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 (22). 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 (27). Milk can be filtrated after heating or poured slowly from boiler leaving the surface scum albumin on the side of boiler (9). Recently, it has been recorded (1,12) 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 (ultrafiltrate, microfiltrate and alpha-lactalbumin fraction) decreased spermatozoal survival (28). 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 spermatozoal preservation. Heating increases the diameter of casein micelles, partially denatured whey proteins and enzymes, release -SH groups and decrease the concentration of minerals because of adsorption of ions onto the casein micelles (28). 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 have permitted efficient spermatozoa protection. At 4°C, 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 become 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 (29), 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 previously tried (30). Boiled milk mixed with citrate buffer to preserved bovine semen. In 1972, some authors (31) used a diluent 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 5-143 days. More recent studies (32) 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 a new diluents containing the two main beneficial actions of both milk and TRIS (33,34). 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 centrifugated 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 centrifugated 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 (35-37). The later authors as well as another (38) reported that lactalbumin enhances sperm viability (assessed as the hypoosmotic response) and sperm motility when added to other extenders.

In conclusion, centrifugation of TRIS or milk diluents once before dilution would be the best treatment. However, combination of one part of centrifugated TRIS diluent and one part of centrifugated milk diluent proved to be the proper diluent of choice for dilution and preservation of buffalo semen. We recommend to follow 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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الملخص العربي

تأثير عمليتي الترشيح والطرود المركزي للمخففات على حيوية الحيامن أثناء تبريد السائل المنوي المخفف لطلانق الجاموس

حسين عامر - حسن منصور - علي عبدالرحيم - أيمن سويلم
قسم التوليد والتاسل والتلقيح الاصطناعي - كلية الطب البيطري - جامعة الزقازيق

استهدفت هذه الدراسة إلقاء الضوء على تأثير كل من عمليتي الترشيح والطرود المركزي لمخففات الترس واللين على حيوية وقلنسوة الحيامن في السائل المنوي المخفف لطلانق الجاموس والمخزن في الثلجة عند درجة حرارة ٥ درجات مئوية. تم استخدام طلوقة جاموسى متميزة من مزرعة كلية الطب البيطري بجامعة الزقازيق يتراوح عمرها حوالي الخمس سنوات وتتصف بخصوبة عالية حيث تم الاحتفاظ به للتوثيب الطبيعي. تم تجميع السائل المنوي بمعدل مرتين أسبوعيا (٤٠ قذفة) باستخدام المهبل الصناعي وفي كل مرة تم تجميع قذفتان متتاليتان بينهما ١٥ دقيقة. تم تخفيف السائل المنوي المجمع بإحدى المخففات التالية: ١- مخففات الترس (مخفف الترس-١ الى -٤)؛ ٢- مخففات اللين (مخفف اللين-١ الى -٨)، ثم ٣- مخففات الترس+اللين (وهو عبارة عن مزج من أفضل مخففات الترس واللين وتشتمل على مخفف الترس+اللين-١ وحتى -١١). تم حساب النسبة المئوية للحيامن الحية، حيوية وحركة الحيامن وكذلك النسبة المئوية للقلنسوة السليمة للحيامن وذلك مباشرة بعد التخفيف ثم عند اليوم الثالث، السادس والتاسع من التخزين في درجة حرارة الثلجة (٥ درجات مئوية). تم إجراء التحليلات الإحصائية للنتائج.

أظهرت النتائج أن الطرد المركزي لمخفف الترس مرة واحدة (مخفف الترس-٣) أو مرتين (مخفف الترس-٤) قبل عملية تخفيف السائل المنوي له تأثير إيجابي على كل المعايير التي تم قياسها على السائل المنوي المخفف (النسبة المئوية للحيامن الحية، حيوية وحركة الحيامن وكذلك النسبة المئوية للقلنسوة السليمة للحيامن) بالمقارنة بالمخفف الضابط (مخفف الترس-١) ومخفف الترس المرشح (مخفف الترس-٢) وذلك طوال فترة التجربة (مباشرة بعد التخفيف ثم عند اليوم الثالث، السادس والتاسع من التخزين). على أية حال، كان مخفف الترس المطرود مركزيا مرة واحدة (مخفف الترس-٣) هو أفضل المخففات بالمقارنة بمخففات الترس-١، الترس-٢ و الترس-٤. أظهر مخفف اللين المطرود مركزيا لمرة واحدة والمحتوى على مح البيض (مخفف اللين-٣) نتائج أفضل (النسبة المئوية للحيامن الحية، حيوية وحركة الحيامن وكذلك النسبة المئوية للقلنسوة السليمة للحيامن) بالمقارنة بالمعالجات الأخرى لمخفف اللين (مخففات اللين-١ إلى اللين-٨). على أية حال، كان مخفف اللين-٣ الأفضل تبع بمخفف اللين-٤، ٦- ثم المخففات الأخرى وذلك عند اليوم الثالث، السادس والتاسع من التخزين في درجة حرارة الثلجة. بالمثل، كان المخفف المركب من الترس واللين بنسبة ٥٠:٥٠% (مخفف الترس+اللين-٦) هو أفضل الأنواع حيث أعطى أعلى نسب من الحيامن الحية، حيوية وحركة الحيامن وكذلك القلنسوة السليمة للحيامن بالمقارنة بالأنواع الأخرى من المخففات المركبة من الترس واللين (مخففات الترس+اللين-١ وحتى -١١)، وذلك أثناء فترة تخزين حيامن الجاموس في درجة حرارة الثلجة (اليوم الثالث، السادس والتاسع من التخزين).

يستخلص من هذه الدراسة؛ أن الطرد المركزي لمخففات السائل المنوي (الترس أو اللين) لمرة واحدة قبل التخفيف هو أفضل المعالجات لتخزين السائل المنوي للجاموس. علاوة على ذلك، إضافة جزء من مخفف الترس المطرود مركزيا لمرة واحدة إلى جزء مماثل من مخفف اللين المطرود مركزيا لمرة واحدة أثبت أنه أفضل الأنواع لتخفيف وتخزين السائل المنوي للجاموس عند درجة حرارة الثلجة. لذلك ينصح باستخدام هذا المخفف المركب لتجميد السائل المنوي للجاموس أثناء برامج التلقيح الاصطناعي.