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STUDIES ON CRYOPRESERVATION OF BUFFALO SPERMATOZOA: II. EFFECTS OF FREEZING RATE, THAWING RATE, GEOMETRY OF SEMEN SAMPLES AND TYPE OF EXTENDER ON THE SURVIVAL OF BUFFALO SPERMATOZOA

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SUMMARY

The effects of four freezing rates (four heights above liquid nitrogen surface; 1, 5, 10 and 15 cm); three thawing rates (water thawing temperature, 22, 37 and 45^{o}C) and geometry of semen sample (size of straw, 0.25 cc and 0.50 cc straw) on the cryosurvival of buffalo spermatozoa were studied. Buffalo semen was diluted at 1:10 (semen : extender) with two extenders (TES-Tris and HEPES-KOH) at 300 mosm and pH 7.0 and contained 20% (v/v) egg yolk and 15% (v/v) succrose solution at the some osmolality, also both extenders contained 3% (v/v) glycerol. Percentage progressive motility were recorded for both unfrozen semen (immediately after dilution, 4 Hr at 5^{o}C before freezing and after 24 hr) and for frozen-thawed semen (immediately post-thawing and 4 Hr post-thawing).

Fast freezing (heights of 5-10 cm above LN_2)yielded higher ($p< 0.05$) post-thaw survival than slower (15 cm above LN_2) or faster freezing one (1 cm above LN_2). Also, faster thawing rate (at 37-45^{o}C) yielded better ($p< 0.05$) post-thawing progressive motility than slower thawing (at 22^{o}C). Half cc straw yielded better ($p<0.05$) post-thaw motility than quarter cc straws.

TES-Tris was superior ($p< 0.05$) to HEPES-KOH in maintaining higher percentage of progressive motility either for unfrozen or frozen-thawed semen. It is concluded that better post-thaw survival of buffalo spermatozoa was obtained with TES-Tris extender and half cc straws and frozen at fast freezing rate (5-10 cm above LN_2) and thawed at faster thawing rate (37-45^{o}C).

Keywords: Buffalo, cryopreservation, spermatozoa, freezing rate, thawing rate, sample size, extender.

INTRODUCTION

Although spermatozoa can tolerate several cooling rates (Entwistle and Martin, 1972; Almquist and Wiggin, 1973; Robbins *et al.*, 1976), there is one freeze rate at which a given type of cell can be successfully frozen. This rate is related to the water permeability of the cell membrane and varies widely among types of cells (Mazur, 1985).

The relationship between glycerol concentrations and cooling velocity was

recognized and studied for bull spermatozoa (Rodriquez *et al.*, 1975; Mortimer *et al.*, 1976 and Robbins *et al.*, 1976).

Shape and volume of the semen packages are limiting factors in achieving exact freezing rates (Larson and Graham, 1973). Graham (1978) and Pace (1984) stated that freezing curves produced by static vapor pressure techniques vary greatly from sample to sample and specially from freeze to freeze which could be due to the amount of liquid nitrogen poured in the freezing chamber and / or the width of the freezing chamber.

The optimal thawing rate depends upon freezing rates, extender type and glycerol concentration (Robbins *et al.*, 1976; Saacke, 1982 and Chandler *et al.*, 1983).

The objectives of this study were to study: a) the effect of four freezing rates in liquid Nitrogen with two different sizes of semen packaging (straws), b) the effect of three thawing rates (temperature of thawing water bath) and c) the effect of two type of extender on the post-thaw survival of buffalo spermatozoa.

MATERIALS AND METHODS.

Semen was collected from three buffalo bulls by artificial vagina and pooled directly into the same collecting tube. Two to three ejaculates were collected from each bull, which was sexually stimulated by allowing one false mounts and 3-5 min. restraint.

Pooled semen was diluted immediately after collection at 1:10 (v/v) semen to extender. Both Tes titrated with Tris and HEPES titrated with KoH were prepared at 300 mosm/kg and 7.0 pH. Diluents contaimed 20% (v/v) Fresh egg yolk and 15% (v/v) sucrose solution at the some osmatic pressure (300 mosm/kg). Diluted semen samples were examined for percent progressive motility before packaging.

Immediately after dilution, semen was packaged into. 0.25 ml French straws or 0.50 ml French straws. Straws were seoled by polyvinyl powder and immersed horizontaly in water bath at same temperature ($30^{o}C$) and transferred into the refrigerator at $5^{o}C$, in which they were cooled slowly to $5^{o}C$ over 90 min.

For freezing a special Boat was constructed and developed in order to obtain the same freezing rate from freeze to freeze (Abd Elhakeam, 1988). The height of the straw above the liquid nitrogen (LN_2) was fixed regardless to the amount of LN_2 poured in the freezing chamber.

Straws were mounted on the metal rack which was placed in the boat at one of four different highest (1, 5, 10 and 15 cm) above LN_2 and exposed to LN_2 vapor for 10 min. Straws were plunged directly into LN_2 and stored until evaluation 24 hrs later.

Straws were thawed in water bath at three different temperatures: $22^{o}C$ (tap water), $37\text{-}40^{o}C$ and $45^{o}C$. The time necessary for the temperature of the straw to rise from $-196^{o}C$ to the temperature of the thawing bath was controlled according to the size of the straws. Thirty seconds to one minute for 0.25 ml straws, while it was one to two minutes for 0.50 ml straws in 37-45 and $22^{o}C$ baths, respectively.

Percent progressive motility was recorded for unfrozen semen immediately after dilution (0 hr) after 4 hr: (pre-freezing) and 24 hr. Storage at $5^{o}C$. Progressive motility of frozen- thawed semen was recorded immediately (0 hr) and 4 hr post-thawing storage at room temperature ($23\text{-}28^{o}C$).

A complete randomized block design with a factorial arrangement was used, the data were analyzed using the general linear model (GLM) in SAS programme (Goodnight, 1979). Differences between means were tested by Duncan's multiple

range test (Sall, 1979).

RESULTS AND DISCUSSION

Data showed highly significant ($p< 0.01$) differences in post-thaw (0 hr. and 4 hr. post thawing) progressive motility between freezing rates(FR), thawing rates (THR), size of straws (SZ) and type of extender (EX). Also, there was significant ($p< 0.05$) difference in percent progressive motility of unfrozen semen (after 4 and 24 hrs.) between extenders (Table 3). There was no significant interaction between all factors (FR x THR x SZ x Ex). However, there was a significant ($p< 0.05$) interaction between EX, FR and / orTHR, specially at 0 hrs. post-thawing.

Freezing at 5 cm above LN_2 was the best and yielded the highest post-thaw motility ($p< 0.05$) as shown in Tables (1, 2 and 3). Slow freezing at 15 cm above LN_2 resulted in lower ($p< 0.05$) post-thaw motility compared to faster freezing at 1 cm above LN_2.

Higher thawing temperatures (37 or 45 ^{o}C) yielded higher ($p<0.05$) post-thaw progressive motility (Table 1 and 2) compared to lower thaw temperature ($22^{o}C$) under all freezing rates and type of extenders and also regardless to the other factors studied (Table 3).

Geometry of semen sample affected significantly ($p< 0.05$) the post-thawing survival of buffalo spermatozoa in which 0.5 ml straws yielded the highest progressive motility under all other factor studied (Tables 1 and 2) compared to 0.25 ml straws and also regardless to the other factors (Table 3).

TES-Tris extender was superior to HEPES-KOH in maintaining the highest progressive motility either immediately post-thawing (0 hrs) or 4 hrs. post-thawing regardless to the other factors studied (Table 3). Moreover, unfrozen buffalo spermatozoa maintained higher ($p< 0.05$) progressive motility when diluted with TES-Tris extender rather than dilution with HEPES-KOH, either pre-freezing (4 hrs storage at $5^{0}C$) or after 24 hrs. storage period (Table 3).

The study (Tables 1,2 and 3) revealed that FR, THR, geometry of semen sample (size of straws, SZ) and EX type affected post-thaw motility of buffalo spermatozoa. Fast to moderate FR (5-10 cm above LN_2) resulted in higher post thaw motility as compared to faster (1 cm above Ln_2) or slower (15 cm above Ln_2) freezing ra. Tolerance by spermatozoa to a wide range of cooling rates has been reported (Almquiat and Wigin, 1973; Robbins *et al.*, 1976). Nagase *et al.* (1964) demonstrated that fast freezing bull sperm on dry ice in pelleted concentrated form in sugar expender survived better in low glycerol levels than at 7.0%. Likewise, higher ($p<0.05$) percentage of motile spermatozoa was recovered in samples frozen at faster rates (1 cm above LN_2) than in those frozen at slower rates (15 cm above LN_2) as it was shown in Table (3). This finding agree with John *et al.* (1969) and Abd ElHakeam *et al.* (1992).

Mazur (1965 and 1985) proposed two mechanisms for freezing damage. One is based on the effects of electrolyte concentration. Slow freezing allows more time to reach the solute precipitation temperature than fast freezing . Thus cells are exposed to the concentrated supercooled solutions for a greater period of time. These solutions can damage the cell by changing the pH, altering tertiary and secondary structures of complex molecules or removing lipids from the cell membrane. The second explanation involves differences in intracellular ice formation. Faster freezing

velocities increases the likehood chance for intracellular ice formation. The resulting crystals are usually small in size, because they did not have enough time to grow. If there crystals remain small, they are often relatively harmless. On the other hand, slow freezing rates minimize the chance of intracellular ice crystal formation. These crystal are usually large when they form, because the lower freezing rate gives them time to grow. Thus, they can cause severe cellular damage. This could explain why very slow freezing rate resulted in lower post-thaw cryosurvival than fast to moderate freezing rates.

Table 1. Effect of freezing rate (FR), thawing rate, geometery of semen sample and extender type on post-thaw progressive motility(%)of buffalo spermatozoa immediately post-thawing.

Factor		Extender type	
		Tes-Tris	Hepes-KOH
FR-1 (1cm)	0.50 cc-22 ^{c}	18.8 ± 1.25 ^{lmnopq}	10.0 ± 0.00 ^{opqr}
	0.25 cc-22 ^{c}	11.3 ± 1.25 ^{nopqr}	06.3 ± 1.25 ^{qr}
	0.50 cc-37 ^{c}	50.0 ± 0.00 ^{bcd}	32.5 ± 7.50 ^{fgjihk}
	0.25 cc-37 ^{c}	45.0 ± 0.00 ^{bcdef}	23.8 ± 3.75 ^{ijklmn}
	0.50 cc-45 ^{c}	52.5 ± 2.50 ^{abc}	33.8 ± 6.25 ^{fghij}
	0.25 cc-45 ^{c}	45.0 ± 5.00 ^{bcdef}	22.5 ± 2.50 ^{ijklmno}
FR-2 (5cm)	0.50 cc-22 ^{c}	26.3 ± 1.25 ^{higkl}	18.8 ± 1.25 ^{lmnopq}
	0.25 cc-22 ^{c}	18.8 ± 1.25 ^{llmnopq}	11.3 ± 1.25 ^{nopqr}
	0.50 cc-37 ^{c}	62.5 ± 2.50 ^{a}	42.5 ± 2.50 ^{cdef}
	0.25 cc-37 ^{c}	47.5 ± 2.50 ^{bcde}	32.5 ± 7.50 ^{fghijk}
	0.50 cc-45 ^{c}	57.5 ± 2.50 ^{ab}	35.0 ± 5.00 ^{efghi}
	0.25 cc-45 ^{c}	45.0 ± 0.00 ^{bcdef}	32.5 ± 7.50 ^{fghjk}
FR-3 (10cm)	0.50 cc-22 ^{c}	20.0 ± 0.00 ^{klmnop}	12.5 ± 2.50 ^{mnopqr}
	0.25 cc-22 ^{c}	12.5 ± 2.50 ^{mnopqr}	07.5 ± 2.50 ^{pqr}
	0.50 cc-37 ^{c}	57.5 ± 2.50 ^{ab}	32.5 ± 2.50 ^{fghijk}
	0.25 cc-37 ^{c}	40.0 ± 0.00 ^{cdef}	27.5 ± 2.50 ^{ghijkl}
	0.50 cc-45 ^{c}	57.5 ± 2.50 ^{ab}	35.0 ± 5.00 ^{efghi}
	0.25 cc-45 ^{c}	37.5 ± 2.50 ^{defgh}	21.3 ± 1.25 ^{jklmno}
FR-4 (15cm)	0.50 cc-22 ^{c}	11.3 ± 1.25 ^{nopqr}	21.3 ± 1.25 ^{gklmno}
	0.25 cc-22 ^{c}	10.0 ± 2.50 ^{opqr}	05.0 ± 0.00 ^{r}
	0.50 cc-37 ^{c}	37.5 ± 2.50 ^{defgh}	25.0 ± 0.00 ^{ghiklm}
	0.25 cc-37 ^{c}	32.5 ± 2.50 ^{fghjk}	23.8 ± 6.25 ^{giklmn}
	0.50 cc-45 ^{c}	40.0 ± 0.00 ^{cdefg}	27.5 ± 2.50 ^{ghijkl}
	0.25 cc-45 ^{c}	27.5 ± 2.50 ^{ghijkl}	22.5 ± 2.50 ^{ijklmno}

$^{a-r}$=Means follawed by the same letter verticaly or herizontally are not significantly different (P>0.05)

The detrimental effect of slow freezing rates on the survival of spermatozoza in general, and those protected by low level of glycerol in particular, may be attributed to the solution effect" rather than intracellular ice formation. It has been reported that

intracellular ice formation was not a prime cause of cell death in mouse and human semen (Sherman, 1962). The same was true in fowl semen frozen at slow rates (Ravie and lake, 1982)

Table 2 .Effect of freezing rate (FR), thawing rate , geometery of semen sample and extender type on post-thaw progressive motility (%) of buffalo spermatozoa 4 Hrs post-thawing.

Factor		Extender type	
		Tes-Tris	Hepes-KOH
FR-1 (1cm)	0.50 cc-22c	03.8 ± 1.25 hijk	00.0 ± 0.00 k
	0.25 cc-22c	01.3 ± 1.25 jk	00.0 ± 0.00 k
	0.50 cc-37c	17.5 ± 2.50 abcd	11.3 ± 1.30 ehdgcf
	0.25 cc-37c	17.5 ± 2.50 abcd	02.5 ± 2.50 kji
	0.50 cc-45c	15.0 ± 5.00 abcdef	11.1 + 3.75 ehdgcf
	0.25 cc-45c	11.3 ± 6.25 cdefjh	02.5 ± 2.50 kji
FR-2 (5cm)	0.50 cc-22c	05.0 ± 0.00 ghijk	02.5 ± 2.50 ijk
	0.25 cc-22c	06.3 ± 8.80 ghijk	00.0 ± 0.00 k
	0.50 cc-37c	22.5 ± 2.50 k	17.5 ± 2.50 bdac
	0.25 cc-37c	18.8 ± 1.25 abc	12.5 ± 2.50 ebdgcf
	0.50 cc-45c	18.8 ± 3.80 abc	15.0 ± 5.00 ebdacf
	0.25 cc-45c	16.3 ± 1.30 abcde	10.0 ± 0.00 ehdgif
FR-3 (10cm)	0.50 cc-22c	00.0 ± 0.00 k	00.0 ± 0.00 k
	0.25 cc-22c	00.0 ± 0.00 k	00.0 ± 0.00 k
	0.50 cc-37c	21.3 ± 1.25 a	07.5 ± 0.00 fghijk
	0.25 cc-37c	08.8 ± 1.25 efghij	06.3 ± 1.30 ghijk
	0.50 cc-45c	20.0 ± 0.00 ab	07.5 ± 2.50 fghijk
	0.25 cc-45c	10.0 ± 0.00 defghi	02.5 ± 2.50 ijk
FR-4 (15cm)	0.50 cc-22c	00.0 ± 0.00 k	00.0 ± 0.00 k
	0.25 cc-22c	00.0 ± 0.00 k	00.0 ± 0.00 k
	0.50 cc-37c	10.0 ± 2.50 defghi	02.5 ± 2.50 ijk
	0.25 cc-37c	07.5 ± 2.50 fghijk	05.0 ± 5.00 ghijk
	0.50 cc-45c	12.5 ± 2.50 k	07.5 ± 0.00 fghijk
	0.25 cc-45c	06.3 ± 1.25 ghijk	06.3 ± 1.25 ghijk

a-k Means follawed by the same letter verticaly or herizontally are not significantly different (P>0.05).

The results indicated that post thaw progressive motility of buffalo spermatozoa frozen in the presence of 3% glycerol was increased as the thawing temperature increased. Thawing at 37-45°C was superior to thawing at lower temperature (22°C). These findings are in agreement with those reported by Pace *et al.* (1981) and Motwani *et al.* (1986). Motwani *et al.* (1986) obtained a post-thaw motility of 23.1, 26.4, 29.1, 41.0, 44.3, 47.6, 50.2 50.6 and 51.7 % on thawing at 20°C for 30 sec.,

20°C for 5 min., 20°C for 2 min. 34°C for 30 sec., 42°C for 30 sec., 34°C for 5 min. 34°C for 2 min. respectively. Robbins *et al.* (1976), Saacke (1982), Chandler *et al.* (1983) and Abd Elhakeem *et al.* (1992) emphasized that different optimal thawing rates depend upon freezing rates, extender type and glycerol concentration. Semen thawed more rapidly than it is frozen is desirable (Mazur, 1977; Foote, 1984). This minimizes recrystallization or ice crystal growth which could occur during slow rewarming.

Table 3. Overall effects of type of extender, freezing rate, thawing rate and geometry of semen sample on progressive motility(%) of unfrozen as well as frozen baffulo spermatozoa.

Factors	Unfrozen at 5 °C			Frozen-thawed	
	0 Hrs	4 Hrs	24 Hrs	0 Hrs	4 Hrs
Extender					
Tes -Tris	75.0±0.0 a	72.5±0.0 a	65.0±0.0 a	36.0±1.7 a	10.4±2.0 a
Hepes KOH	75.0±0.0 a	65.0±0.0 b	55.0±0.0 b	23.0±3.1 b	05.4±1.6 b
Freezing rate (Height above LN ,cm)					
1 cm		29.3±2.6 b		07.8±2.4 b	
5 cm		35.8±2.9 a		12.1±2.5 a	
10 cm		30.1±2.2 b		07.0±0.7 b	
15 cm		22.7±1.9 b		04.8±1.5 c	
Thawing rate (Thawing temperature)					
22°C		13.1±1.3 b		01.2±0.9 b	
37°C		38.3±2.8 a		11.8±2.1 a	
45°C		37.0±3.2 a		10.8±2.3 a	
Size of straw					
0.50cc straw		33.6±0.9 a		09.5±1.8 a	
0.25 cc straw		25.4±1.9 b		06.3±1.7 b	

a-c Means in the same column within the same factor followed by the same letter are significantly different (P>0.05).

Thawing temperature is one of the most critical factors that influence the cryosurvival and the integrity of frozen-thawed buffalo spermatozoa. Mazur (1985) reported that the warming phase of the freeze-thaw process is as important to cell survival as the cooling phase.

Geometry of semen sample (size of straw) had a significant effect on post thaw progressive motility of buffalo spermatozoa. Buffalo semen frozen in 0.5 ml straws yielded higher post-thaw progressive motility than semen frozen in 0.25 ml straws. This finding disagree with those reported in the literature and this conflict may be attributed mainly to differences in experimental conditions such as species differences, type of extender used, glyecrol concentration, freezing technique and thawing time and method.

Generally, it is agreed that freezing semen in pellets and smaller straws allows much faster freezing and thawing rates due to a larger surface to volume ratio than semen packaged in ampoules or longer size of straws. The longer surface area results in a rapid removal of the latent heat of fusion. In contrast, the smaller surface area in relation to larger volume results in slower removal of the latent heat of fusion

and produce a lengthy plateau at the freezing point. Pursel and Park (1985) and Park and Pursel (1985) found a significant interaction between cooling rate and plateau. The duration of plateau significantly affected the percentage of normal acrosome. The percentage of boar sperm with loose acrosomal caps were lower for sperm frozen with a 0.0 min. plateau than for sperm frozen with a 5 min. plateau. They concluded that minimizing of the freezing point plateau and prevention of supercooling reduced sperm damage in the 0.0 to -10.0°C phase of the freezing process. Therefore, it was reported that smaller semen volume permit faster freezing and thawing rates and results in higher cryosurvival of spermatozoa (Pursal and Park, 1985; Abd Elhakeam *et al.*, 1992).

Type of extender influenced the motility of both unfrozen and frozen thawed buffalo spermatozoa frozen in the presence of 3% glycerol. TES-Tris extender significantly maintained ($p< 0.05$) higher percentage of progressive motility compared with HEPES-KOH extender. These results confirm our previous results in this series of studies.

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دراسات على تجميد الحيوانات المنوية للجاموس: ٢- تأثير معدل التجميد ومعدل الإسالة وحجم عينة السائل المنوى وكذلك نوع المخفف المستخدم على حيوية الحيوانات المنوية للجاموس.

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درس تأثير أربع معدلات تجميد (أربع ارتفاعات فوق سطح النيتروجين السائل) ١، ٥، ١٠، ١٥سم) وثلاثة معدلات تسييح (درجة حرارة ماء التسييح ٢٢، ٣٧، ٤٥ °م) وكذلك حجم عينة السائل المنوى (حجم الأسترو ز - القصيبات - ٢٥ومل، ٥٠ومل) بالإضافة إلى دراسة تأثير نوع المخفف المستخدم على حيوية الحيوانات المنوية للجاموس بعد التجميد والتسييح.

تم تخفيف السائل المنوى للجاموس بمعدل ١ :١٠ (سائل منوى : مخفف) باستخدام مخففين (تيس- ترس (TES - Tris) وهيبيز أيدروكسيد البوتاسيوم (HEPES - KOH) عند ضغط أسموزى مقداره ٣٠٠ ملى أوزمول ودرجة حموضة ٧ بالإضافة إلى ٢٠٪ (حجم / حجم) صفار بيض طازج و ١٥٪ (حجم / حجم) محلول سكر سكروز (٣٠٠ ملى أوزمول) - كلا المخففين أضيف إليه جليسرول بنسبة ٣٪ (حجم / حجم). تم تسجيل النسبة المئوية للحركة التقدمية للحيوانات المنوية لكل من السائل المنوى الغير مجمد مباشرة بعد التخفيف وكذلك بعد ٤ ساعات على درجة ٥ °م قبل التجميد وبعد ٢٤ ساعة تخزين على درجة ٥ °م - وكذلك بالنسبة للسائل المنوى المجمد بعد تسييحه مباشرة ومرة أخرى بعد ٤ ساعات من التسييح.

أظهرت النتائج أن معدل التجميد السريع (٥ - ١٠ سم فوق سطح النيتروجين السائل) أنتج نسبة حيوية أعلى ($P < 0.05$) من معدل التجميد البطيء (١٥ سم فوق سطح النيتروجين السائل) وكذلك من معدل التجميد الأسرع (١سم فوق سطح النيتروجين السائل). أيضا معدل التسييح السريع (درجة حرارة ٣٧ - ٤٥ °م) أنتج نسبة حيوية للحيوانات المنوية أعلى ($P < 0.05$) مقارنة بمعدل التسييح البطيء (على٢٢°م) . الأسترو ز (القصيبات) حجم ٥٠و مل أنتجت أعلى نسبة حيوية بعد التسييح مقارنة بالأسترو ز ٢٥و مل. مخفف التيس-ترس (TES - Tris) حافظ على نسبة حيوية عالية سواء على درجة ٥ °م أو بعد التجميد والتسييح مقارنة بمخفف الهيبيز -أيدروكسيد البوتاسيوم (HEPES - KOH)

وخلص البحث إلى أن أحسن نسبة حيوية للحيوانات المنوية للجاموس بعد التجميد والتسييح يمكن الحصول عليها باستخدام مخفف التيس-ترس (TES - Tris) مع ٥٠و مل أسترو ز وتجميده بمعدل تجميد سريع (٥ - ١٠ سم فوق سطح النيتروجين السائل ثم تسييحه على معدل تسييح سريع (٣٧ - ٤٥ °م).