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STUDIES ON CRYOPRESERVATION OF BUFFALO SPERMATOZOA: I. EFFECTS OF TYPE AND LEVEL OF CRYOPROTECTIVE AGENTS AS WELL AS TYPE OF EXTENDER ON THE SURVIVAL OF BUFFALO SPERMATOZOA

A.A. Abd Elhakeam, F.M.R. El-Feel and A.A. Bayoumi.

Deptartment of Animal Production, Faculty of Agriculture, University of Minia, Egypt.

SUMMARY

The effect of three types of cryoprotective agents (Dimethyl sulfoxide, DMSO; Ethylene glycol, EG and Glycerol, GLY) at three levels (0.0 %, 3% and 6% v/v) using two buffers (TES-Tris and HEPES-KoH) prepared at 300 mosm and pH 7.0 on the progressive motility of unfrozen and frozen-thawed buffalo spermatozoa was studied. Extenders contained 20% (v/v) egg yolk and 15% (v/v) sucrose solution (300 mosm). Pooled buffalo semen was diluted at 1:10 (v/v semen: extender) and semen samples were frozen in 0.5 ml straws after 4 hr equilibration period at 5^{o}C post dilution. Frozen straws were stored in liquid Nitrogen for 24 hr before thawing in water at 37^{o}C for 30 sec. Percentages progressive motility were recorded for unfrozen samples stored for 0.0, 4, 24, 48 and 72 hr at 5^{o}C post dilution as well as for frozen- thawed samples at 0.0 and 4 hr post-thawing.

Results showed that freezing of buffalo spermatozoa depended on type and level of cryoprotective agent as well as type of extender. Post-thaw progressive motility was superior ($p< 0.05$) in the presence of Glycerol (54.4%) compared to EG (44.4%) and DMSO (32.9%). The presence of 3% (v/v) or 6% of the agent in the extender resulted in higher ($p< 0.05$) post-thaw motility (46.3% and 41.6%) compared to the absence of the agent (0.0%) which yielded 10.3 % motility.

Meanwhile, TES-Tris extender yielded 41.7% compared to HEPES-koH which yielde 36.7 progressive motility of buffalo spermatozoa post-thawing. The interaction between the three factors (extender*type of agents*level) was not significant. However, the highest post-thaw progressive motility (60.0%) was obtained when buffalo semen was extended in TES-Tris extender containing 3% (v/v) glycerol. It is concluded that buffalo spermatozoa could be successfully frozen in TES-Tris extender containing 3% (v/v) glycerol.

Keywords: Buffalo, spermatozoa, cryoprservation, cryoprotective agents, extender.

INTRODUCTION

Long term and efficient preservation of fertilizing capacity of spermatozoa is a primary goal of research in semen technology. The increase in the efficiency of

artificial insemination is still attracts the attention of A.I specialists in this field throughout the world. In the past, much work has been conducted to determine the optimum conditions for successful freezing of semen. Such investigations are still to be done for buffalo semen. However, many workers have attempted to preserve buffalo spermatozoa in different extenders (Rajamahendran and Dharasen, 1984; Anzar *et al*., 1985; Tuli *et al*., 1985; Ahmed *et al*., 1986 and Singh *et al*., 1987).

The discovery by Polge *et al*. (1949) that glycerol minimized the harmful effects of freezing spermatozoa, opened the era of frozen semen since then , several cryoprotective compounds have been used for preserving spermatozoa of various species. These include Ethylene and propylene glycol (Persidsky and Richard, 1962), Dimethyl sulfoxide (Lovelock and Bishop, 1959). A variety of compounds (141 different compounds) have been screened for their protective effects in freezing of bovine spermatozoa (Jeyendran and Graham, 1980), but no similar studies have been reported for buffalo spermatozoa.

Therefore the objectives of this experiment were to investigate the effect of different types and levels of cryoprotective agents as well as type of extender on the survival of unfrozen and frozen -thawed buffalo spermatozoa.

MATERIALS AND METHODS

The stock solutions of both TES-Tris and HEPES-KoH buffers were prepared at 300 mosm/kg and pH 7.0. Each extender contained 15% (v/v) sucrose solution at the same osmotic pressure (300 mosm) and 20% (v/v) fresh egg yolk (Bayoumi, 1995). Three cryoprotective agents were used namely: Glycerol (Gly), dimethyl sulfoxide (DMSO) and ethylene glycerol (EG), each at two levels 3% or 6% (v/v) in both extenders. Extenders without cryoprotective agents were used as controls. All extenders were centrifuged at 5000 r.p.m for 10 min till clear and supernatant were decanted and used for semen dilution.

Semen was collected from three mature buffalo bulls by means of an artificial vagina. Buffalo bulls were sexually stimulated and one to three ejaculates were collected and pooled in the same collecting tube. Pooled semen was examined for percentage of progressive motility and concentration. Semen samples with less than 60% progressive motility were excluded.

Pooled semen was mixed thoroughly before dilution at the rate of 1:10 (v/v) semen to diluent. Semen was diluted at room temperature with the fourteen extenders in test tubes. Diluted semen samples were packaged into 0.5ml French plastic straws, which were sealed using polyvinyl powder. Three straws per each treatment were prepared, labeled and coded. Straws were placed horizontally in water bath at the same temperature, and transferred into the cold room (refrigerator) to be cooled slowly to $5^{o}C$ within one and half hour and left for four hours equilibration period before freezing.

For freezing, cold straws were mounted horizontally on metal rack which was placed in freezing Floating boat at 5-10 cm above LN_2 surface. Straws were exposed to LN_2 vapor for 10 min, then plunged directly into the LN_2 and stored for 24 hr before thawing. Frozen straws were thawed in water bath at $37-40^{o}C$ for 30 sec.

Percentages of progressive motility were recorded for unfrozen semen samples immediately (0.0 hr) after dilution , before freezing (4 hr at $5^{o}C$), after 24 , 48 and 72 hr storage at $5^{o}C$. Frozen-thawed semen samples were evaluated for motility

immediately (0.0 hr) post-thawing and after 4 hr storage at room temperature post-thawing.

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

Concerning post-thaw progressive motility, it was observed that type of extender did not affect ($p > 0.05$) the motility either immediately (0.0 hr) or 4 hr post-thawing. However, TES-Tris extender yielded higher motility than HEPES-KOH as shown in Table (1), in which the values were 41.7, 17.0% for TES-Tris compared with 36.7% and 14.4% for HEPES-KOH at 0.0 and 4 hr post-thawing, respectively. Meanwhile, there were no significant interaction between type of extender and the other factors studied (type of cryoprotective agents and levels).

Table 1. Overall effects of type of extender as well as type and levels of cryoprotective agents on post - thawing Progressive motility(%) of buffalo spermatozoa .

Factors	Progressive motility ± S.E	
	0.0 Hrs Post-thawing	4 Hrs Post-thawing
EXTENDER		
TES TRIS	41.7±4.9 a	17.0±4.3 a
HEPES KOH	36.7±6.4 a	14.4±3.4 a
TYPE OF CRYOPROTECTIVE		
Glycerol	54.4±5.7 a	26.3±6.4 a
Dimethyl-slufoxide	32.9±6.3 b	10.8±2.4 c
Ethylene-glycol	44.4±6.6 a	16.3±4.0 b
LEVELS (V/V)		
0%	10.3±2.3 b	3.1±1.5 c
3%	46.3±6.7 a	22.6±5.5 a
6%	41.6±6.3 a	13.0±2.9 b

a-c Means in the same column, within the same factor, followed by the same letters are not significantly different (P> 0.05).

Type of cryoprotective agents had a significant ($p < 0.01$) effect on post-thaw motility regardless to the other factors studied. Glycerol was superior in maintaining higher (54.4%) progressive motility post-thawing compared with DMSO (32.9%) or EG (44.4%) as shown in Table (1) and the same trend was observed at 4 hr post-thawing.

The absence of cryoprotective agents in the extenders resulted in a significant ($p<0.05$) lower percentage of post-thaw progressive motility (10.3 and 3.1 % compared to the presence of the agent at 3% (46.3 and 22.6%) or 6% (41.6 and 13.0%) in the extender at 0.0 and 4 hr post-thawing, respectively. Meanwhile, the level of 3% maintained sperm motility better ($p<0.05$) than 6%, especially at 4 hr post-thawing as shown in Table (1).

There were no significant interactions between the three factors studied. However,

the highest post-thaw progressive motility (60.0%) was obtained with TES-Tris- 3% glycerol (Table 2) followed by HEPES-KoH-3% glycerol which yielded 54.4% post-thaw motility.

Table 2. Post-thawing progressive motility (%) of frozen buffalo spermatozoa as affected by type of extender as well as type and level of cryoprotective agents.

Extenders		Progressive motility + S.E	
		0Hrs Post-thawing	4Hrs Post-thawing
TES TRIS (*)			
Control	0 %	10.6±3.4 ^{e}	00.6±0.0 ^{e}
Glycerol	3 %	60.0±7.9 ^{a}	34.4±9.7 ^{a}
Glycerol	6 %	48.9±2.2 ^{abc}	20.6±5.5 $^{a\ bcd}$
(DMSO)	3 %	36.1±4.3 ^{bcd}	19.4±3.9 ^{bcd}
(DMSO)	6 %	31.1±4.4 ^{cd}	12.8±3.4 ^{cde}
E.G.	3 %	6.7±3.9 ^{ab}	18.9±4.4 ^{bcd}
E.G.	6 %	48.3±8.5 ^{abc}	12.8±2.4 ^{cde}
HEPES KOH			5.5±2.4 ^{de}
Control	0 %	11.1±1.1 ^{e}	31.7±6.7 ^{ab}
Glycerol	3 %	54.4±4.0 ^{ab}	18.3±3.5 ^{bcd}
Glycerol	6 %	54.4±8.7 ^{ab}	5.60±0.6 ^{de}
(DMSO)	3 %	31.7±11.1 ^{dc}	6.10±1.5 ^{de}
(DMSO)	6 %	26.1±6.3 ^{de}	25.6±7.8 ^{bac}
E.G.	3 %	38.9±6.2 ^{bd}	7.80±1.5 ^{de}
E.G.	6 %	40.6±7.8 ^{bdac}	

$^{a-e}$ Means in the same column, undern the same storge period, followed by the same letters are not significantly different ($P > 0.05$).
*Control = without cryoprotective agents, DMSO = dimthyl slufoxide, E.G = ethylene glycol.

Unfrozen semen, did not show any significant effect due to type of extender, type and level of cryoprotective agents on progressive motility either immediately after dilution (0.0 hr) or before freezing (4 hr at 5°C). The same trend was observed for type and level of cryoprotective agents till 72 hr storage period at 5°C.However, type of extender affected ($p< 0.05$) motility of unfrozen buffalo especially with longer storage period at 5°C (48 and 72 hr), in which TES-Tris was superior to HEPES-KoH (Table, 3).

Also, there were no significant interactions between the three factors studied. However, the highest value (60.0%) at 72 hrs storage period for progressive motility was obtained with TES-Tris extender contaning 6% ethylene glycol (Table, 4).

The results of this study revealed that the absence of cryoprotective agents in the extender (TES-Tris or HEPES-KoH) used for liquid preservation of buffalo spermatozoa at 5°C (Tables 3 and 4) did not affect ($p> 0.05$) the progressive motility at any storage period (up to 72 hr). However, the recovery after freezing was significantly ($p< 0.05$) very low with this absence compared to its presence in the extender (Tables 1 and 2). Meanwhile, the type of the cryoprotective agent used is very important. In this study, it was observed that glycerol was the main choice followed by EG and the DMSO was the last to protect buffalo spermatozoa during freezing and thawing process as judged by percentage of progressive motility. At the

same time, it was observed that level of cryoprotective agents to some extend is important, in which levels above 3% is not beneficial for cryopreservation of buffalo spermatozoa. The highest post-thaw progressive motility was obtained with 3% level compared to the 6% or 0.0% level. Also, the results of this experiment indicated that buffalo spermatozoa could be successfully frozen in the presence of 3% glycerol in the extender. These findings are in agreement with those reported in the literature. Jeyendran and Graham (1980) concluded that of the low-molecular-weight compounds tested (DMSO, EG,.. etc) glycerol yielded the highest recovery of spermatozoa as evaluated by post-thaw motility and GOT retained in the spermatozoa. Also, they reported that DMSO, Diethylene glycol and trimethylene glycol with TES-Tris yolk extender appeared not to be different in their protective effect in freezing bovine spermatozoa. El-Azab *et al.*(1984) studied the influence of buffer, glycerol level, and cooling rate on the survival of buffalo spermatozoa frozen in straws. They reported that the best glycerol level ranged 3-7% whereas, the equilibration time varied between 3 and 6 hr. Also, Flukirger *et al.* (1976) found that the addition of 3.2% glycerol to Tris diluent was the best for buffalo semen resulting in an average sperm motility of 47% after freezing.

Table 3.Overall effects of type of extender as well as type and levels of cryoprotective agents on Progressive motility(%) of unfrozen buffalo spermatozoa stored at 5°C.

	Progressive motility ± S.E				
FACTORS	0.0 Hrs	4 Hrs	24 Hrs	48 Hrs	72 Hrs
EXTENDER					
TES TRIS	76.7±2.0 ^{a}	71.4±4.8 ^{a}	63.1±3.7 ^{a}	57.6±3.6 ^{a}	54.5±4.5 ^{a}
HEPES KOH	79.1±0.9 ^{a}	68.3±3.5 ^{a}	60.7±2.9 ^{a}	48.8±2.5 ^{b}	42.0±3.7 ^{b}
TYPEL OF CRYOPROTECTIVE					
Glycerol	76.3±2.4 ^{a}	70.0±3.9 ^{a}	58.3±2.5 ^{b}	48.3±2.0 ^{ab}	43.8±2.7 ^{a}
Dimethyl-slufoxide	79.6±0.4 ^{a}	72.5±3.9 ^{a}	65.0±3.2 ^{ab}	54.2±2.2 ^{ab}	50.0±3.8 ^{a}
Ethylene-glycol	79.2±0.9 ^{a}	67.5±4.5 ^{a}	60.4±4.8 ^{ab}	55.4±5.8 ^{a}	50.0±6.9 ^{a}
LEVELS (V/V)					
0 %	75.0±3.3 ^{a}	69.2±5.0 ^{a}	65.8±3.9 ^{a}	56.7±1.6 ^{a}	53.3±1.6 ^{a}
3 %	77.8±1.7 ^{a}	68.3±5.1 ^{a}	58.9±4.0 ^{ab}	54.2±5.1 ^{a}	47.5±7.0 ^{a}
6 %	78.9±0.8 ^{a}	71.7±3.2 ^{a}	63.6±2.4 ^{ab}	51.1±1.6 ^{a}	48.3±2.1 ^{a}

$^{a-b}$ Means in the same column, within the same factor, followed by the same letters are not significantly different ($P > 0.05$) .

Rapatz (1966) and Foote (1984) suggested that the addition of cryoprotective agent such as glycerol to a cell suspension reduces the exposure of the cells to high concentrations of solutes since the glycerol does not crystalize, but aids in keeping concentration of solutes from reaching toxic levels during freezing " salt buffering". Also, since glycerol dehydrates the cells, so, formation of intracellular ice is also, prevented (Berndston and Foote, 1969 and Farrant, 1980). This may explain the

importance of the presence of such cryoprotective agents in the extender as compared to its absence for successful freezing of buffalo spermatozoa.

Table 4. Progressive motility (%) of unfrozen buffalo spermatozoa stored at 5°C as affected by type of extender as well as type and level of cryoprotective agents.

Extender		Progressive motility + S.E				
		0.0 Hrs	4 Hrs	24 Hrs	48 Hrs	72 Hrs
TES TRIS						
Control*	0%	73.3±3.3 ^{a}	73.3±3.3 ^{a}	63.3±3.3 ^{a}	60.0±0.0 ^{ab}	56.7± 3.3 ^{ab}
Glycerol	3%	73.3±3.3 ^{a}	73.3±3.3 ^{a}	53.3±3.3 ^{bc}	51.7±1.7 ^{abc}	48.3±4.4 ^{abc}
Glycerol 6%		75.0±2.9 ^{a}	70.0±5.8 ^{a}	60.0±0.0 ^{abc}	55.0±2.9 ^{abc}	50.0±0.0 ^{abc}
(DMSO)	3%	78.3±1.7 ^{a}	70.0±5.8 ^{a}	70.0±5.8 ^{a}	56.7±8.8 ^{ab}	56.7± 8.8 ^{ab}
(DMSO)	6%	80.0±0.0 ^{a}	73.3±3.3 ^{a}	66.7±3.2 ^{a}	60.0±0.0 ^{ab}	56.7± 3.3 ^{ab}
E.G.	3%	78.3±1.7 ^{a}	66.7±8.8 ^{a}	60.0±5.8 ^{abc}	56.7±8.8 ^{ab}	53.3±12.0 ^{ab}
E.G.	6%	78.3±1.7 ^{a}	73.3±3.3 ^{a}	68.3±4.4 ^{a}	63.3±3.3 ^{a}	60.0± 0.0 ^{a}
HEPES KOH						
Control	0%	76.7±3.3 ^{a}	69.0±7.6 ^{a}	68.3±4.4 ^{a}	53.3±3.3 ^{abc}	50.0±0.0 ^{abc}
Glycerol	3%	76.7±3.3 ^{a}	66.7±3.3 ^{a}	56.7±3.3 ^{abc}	46.7±3.3 ^{bc}	43.3±3.3 ^{abc}
Glycerol	6%	80.0±0.0 ^{a}	70.0±3.3 ^{a}	63.3±3.3 ^{ab}	40.0±5.8 ^{c}	33.3± 3.3 ^{c}
(DMSO)	3%	80.0±0.0 ^{a}	73.3±3.3 ^{a}	63.0±3.3 ^{ab}	50.0±0.0 ^{abc}	43.3±3.3 ^{abc}
(DMSO) 6%		80.0±0.0 ^{a}	73.3±3.3 ^{a}	60.0±0.0 ^{abc}	50.5±0.0 ^{abc}	40.0± 0.0 ^{bc}
E.G.	3%	80.0±0.0 ^{a}	60.0±5.8 ^{a}	50.0±5.8 ^{c}	45.0±7.6 ^{bc}	40.0±10.0 ^{bc}
E.G.	6%	80.0±0.0 ^{a}	70.0±0.0 ^{a}	63.3±3.3 ^{ab}	56.7±3.3 ^{ab}	50.0±5.8 ^{abc}

$^{a-c}$Means in the same column, undern the same storge period ,followed by the same letters are not significantly different (P > 0.05) .
* Control = without cryoprotective agents., DMSO = dimthyl slufoxide., E.G= ethylene glycol.

It has been reported (Rapatz, 1966) that high glycerol concentrations were detrimental to sperm during freezing and /or thawing and this is may explain the superiority of 3% level of glycerol to the higher level (6%) of the cryoprotective agent in yielding higher post-thaw motility.

It is well recognized, that as the total solids and electrolytes contents of an extender decrease the optimal glycerol concentration required for successful freezing is decreased. Bull, boar and ram semen have been successfully frozen in Zwitterionic buffer (non salt buffer) based extenders with egg yolk as the only cryoprotectant (Gibson and Graham, 1969; Crabo *et al.*, 1971; Abd Elhekeam *et al.*, 1991).

It is may be worth mentioning that the one step dilution of buffalo semen with the extender contaning the cryoprotective agent at room temperature (30°C) did not impair the survival rate either pre or post-freezing in this study. Jainudeen and Santhana (1982); Bhandari *et al.* (1983) and Fathel- Bab *et al.*(1985) indicated that no significant difference in post-thaw motility was observed whether glycerol was added initially at 35°C or after cooling in Tris-yolk diluent.

Type of diluent used appear to be important for successful freezing of buffalo spermatozoa. The present study revealed that TES-Tris diluent has a protective action higher than HEPES- KoH as it is judged by higher post-thaw progressive motility. This finding is in agreement with those reported by Crabo and Jeyendran

(1979). Also, similar results were observed by Anzar *et al.* (1985), when three extenders were compared (Lactose, Lactose-Fructose and Tris-Tes-Fructose) for post-thaw motility of frozen buffalo spermatozoa. They reported that the best results were obtained with Tris-Tes-Fructose extender, which was significantly ($p< 0.01$) better than the other two extenders.

Therefore, it could be concluded that, although the presence of cryoprotective agent in the extender is not beneficial to short-term liquid preservation of buffalo spermatozoa at $5^{o}C$, it is very essential for successful coryopreservation. Glycerol with 3% (v/v) level in TES-Tris extender provided the maximum protection for buffalo spermatozoa during freezing and thawing followed by ethylene glycol which provided more protection during freezing of buffalo spermatozoa than Dimethyl sulfoxide.

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دراسات على تجميد الحيوانات المنوية للجاموس.١. تأثير نوع ومستوى مساعدات التجميد وكذلك نوع المخفف على حيوية الحيوانات المنوية للجاموس.

عبدالهادى عبدالحكيم - فوزى محمود رحيم الفيل - أحمد عبدالجليل بيومى

قسم الإنتاج الحيوانى - كلية الزراعة - جامعة المنيا.

درس تأثير ثلاثة أنواع من مساعدات التجميد (داى ميثايل سلفوكسيد ، ايثيلين جليكول والجليسرول) كلا بثلاثة مستويات (صفر، ٣، ٦ ٪ حجم / حجم من المخفف) باستخدام مخففى (التيس-ترس (TES - Tris) وهيبيز أيدروكسيد البوتاسيوم (HEPES - KOH) والتى تم تحضيرها على ضغط أسموزى ٣٠٠ ملى أوزمول ودرجة حموضة ٧ - محتوية على ٢٠٪ (حجم / حجم) صفار بيض طازج وكذلك ١٥٪ (حجم / حجم) محلول سكر سكروز (٣٠٠ ملى أوزمول).

تم تخفيف السائل المنوى للجاموس بمعدل ١ :١٠ (حجم / حجم) سائل منوى إلى مخفف وعينات السائل المنوى المخففة تم تجميدها باستخدام أستروز (قصيبات) حجمها ٠,٥٠ مل - بعد ٤ ساعات فترة توازن فى الثلاجة على درجة ٥ °م من التخفيف. بعد ٢٤ ساعة تخزين فى النيتروجين السائل تم تسييح الأستروز فى حمام مائى درجة حرارته ٣٧ °م لمدة ٣٠ ثانية والنسبة المنوية لحركة الحيوانات المنوية التقدمية سجلت للسائل المنوى الغير مجمد والمخزن لمدد صفر، ٤، ٢٤، ٤٨، ٧٢ ساعة على درجة ٥ °م وكذلك سجلت بعد صفر، ٤ ساعات من التسييح.

أظهرت النتائج أن نجاح تجميد السائل المنوى للجاموس يعتمد على نوع ومستوى مساعد التجميد وكذلك نوع المخفف المستخدم. النسبة المئوية للحركة التقدمية للحيوانات المنوية بعد التجميد والتسييح كانت أحسن ٥٤،٤٪ (P < 0.05) فى وجود الجليسرول مقارنة بالايثيلين جليكول ٤٤،٤٪ والذى ميثايل سلفوكسيد ٣٢،٩٪ . احتواء المخفف على ٣٪ أو ٦٪ (حجم/حجم) من مادة التجميد أدت إلى رفع نسبة الحيوية بعد التسييح (٤٦،٣٪ و ٤١،٦٪) مقارنة بعدم وجوده (صفر٪) والتى أنتجت ١٠،٣٪ نسبة حيوية.

فوق ذلك لوحظ أن مخفف التيس-ترس (TES - Tris) أنتج ٤١،٧٪ نسبة حيوية مقارنة بمخفف الهيبيز أيدروكسيد البوتاسيوم(HEPES - KOH) والذى أنتج ٣٦،٧٪.

لم يكن هناك أى تداخل معنوى بين العوامل تحت الدراسة ولكن لوحظ أن أعلى نسبة حيوية بعد التجميد والتسييح وكانت ٦٠،٠٠٪ تم الحصول عليها عند تخفيف السائل المنوى الجاموسى بمخفف التيس-ترس TES - Tris والمحتوى على ٣٪ جليسرول (حجم/حجم).

وخلص البحث إلى أنه يمكن تجميد وحفظ السائل المنوى الجاموسى بنجاح باستخدام مخفف التيس-ترس TES - Tris فى وجود ٣٪ (حجم/حجم) جليسرول.