Effect Of Propylene Glycol And Some Antibiotics On The Sperm Viability And Bacteriological Quality Of Frozen Buffalo Semen

El-Azab, A. I.[1]; El-Bardisy, M. M.[2] and Tawfik, S. I.[3]

[1] Dept. Theriogen, Fac. Vet. Med., Zagazig Univ. (Benha)

[2] Dept. Bact., Animal Health Res. Instit. Dokki

[3] Abbasia Centre for Frozen Buffalo Semen, Cairo

ABSTRACT

The present study revealed the best viability index for frozen buffalo spermatozoa extended in sodium citrate when compared to those in lactose or Tris buffers. Although glycerol was more effective in cryopreservation of buffalo semen, propylene glycol was successfully used as cryoprotective agent with satisfactory results at concentration of 5% in citrate buffer. The percentage of egg yolk in the extender should not exceed 10% to have better viability index of spermatozoa and minimal count of bacteria in semen. Enerofloxacin or Colistin and Ampicillin could the used effectively as antimicrobial agent in the extender. Thus, the present study suggested an extender composed of citrate 2.9%, fructose 1.2%, propylene glycol 5% and egg yolk 10% with Enerofloxacin 1000 ug/ml or Colistin suphate 250 i.u./ml and Ampicillin base 1000 ug/ml for freezing buffalo semen.

INTRODUCTION

The wide spread application of artificial insemination and realization of its full potential depends largely on producing frozen semen of high quality.In spite of using the best preservation techniques, post-thaw survival of the sperm population in cattle is seldom higher than 50% *(1)*. This percentage is much lower in case of buffalo semen which appears poorer in inital quality and preservability both in liquid and frozen states *(2)*. It has been noticed that about 40% of buffalo ejaculates show static spermatozoa which gain motility on dilution *(3)*. Therefore, many attempts have been made to adopt a profitable extender for freezing bull or buffalo bull semen. Some of known diluents are based on adjusting the buffering system *(4,5)* and the cryoprotective agent *(6,7,8)*. Others depend on justifying concentration of egg yolk as anticooling agent *(9,10)* and type of antimicrobial agents *(11,12, 13,14)*. The present study was designed to assess the quality of buffalo frozen semen using cryoprotective agents and antibiotics other than those routinely used. Thereby, an efficient extender for freezing buffalo semen can be achieved.

MATERIAL AND METHODS

The present study was conducted on two experiments in Frozen Buffalo Semen Centre, Abbasia, during the period from March 1st to June, 30th, 1998. In experiment I, semen was collected from three sexually mature and healthy buffalo bulls with spermatozoa of at least 45% post-thawing motility. The semen was extended in three suggested buffers : 1) 3.03% tris, 1.68% citric acid and 1.20% fructose ; 2) 2.94% citrate and 1.20% fructose and 3) 7.1% lactose and 1.20% fructose. Each buffer was divided into 4 aliquotes to which either 7% glycerol, 5,7 or 9% propylene glycol (El-Nasr Pharm. Chemicals, Cairo) were added as cryprotective agents. The whole extender composed of 80% buffer containing the cryoprotectant and 20% egg yolk. In one portion, semen was extended at 35°C, cooled to 5°C within one hour, packaged in 0.5 ml straws and equilbrated for about 2 hours, then frozen on liquid nitrogen vapour for 10 min followed by dipping and storage in liquid nitrogen for one week. Thenceafter, semen frozen in each of the four buffers with each cryoprotectant was thawed, pooled and incubated in a thermostatically controlled water bath at 40°C for 0, 2 and 4 hours for the viability index [illegible].

In experiment II, semen was collected from the three aforementioned buffalo bulls. The semen was extended in the buffer containing the best

concentration of propylene glycol with maximal viability index detected in experiment I . To three aliquotes of the buffer, three concentration of egg yolk , 5, 10 or 20% were added. Each aliquote was subdivided into four portions, three of them were treated with antibiotics and the fourth was left as control. Each ml of the extender contained one of the following :1) 1000 ug Enerofloxacin (Baytril, 10%, Bayer, AG, leverkusen) ; 2) 2500 I.U. Colistin sulphate and 1000 ug Ampicillin base (Colampi ; pantex , Vet. Pharm. Prod.) and 3) Combination of 1 and 2 (v/v). After extension , semen was frozen and the thawed - frozen semen was examined as mentioned in experiment (1) . For bacteriological examination the frozen semen was thawed in water bath adjusted at 40°C for 30 sec. In order to isolate aerobic and anoerobic microorganisms, semen was cultured on specific media and incubated by using gas pack gar as needed. All bacterial isolates were identified by cultural, morphological and biochemical characters *(16)*. The totoal bacterial count was carried out by the plate count technique *(17)*. The data were tabulated and sta analysed to estimate the mea and significance between the parameters *(18)*.

RESULTS

The present results (Ta showed a significant (P<0.05) incr the viability index for frozen spermatozoa extended in citrat buffer (19.88± 1.41%) when compa those extended either in Tris yolk ± 1.31%) or lactose yolk (12.09 ± 1 buffers. Regardless of the effe buffer, the viability index of f buffalo spermatozoa appe significantly (P<0.05) higher on glycerol 7% (26.43±1.43%) propylene glycol 5% (18.00±1.29%), (8.23± 0.86%) or 9% (5.17±0.75%) . higher viability index was found w buffalo semen was extended in citr fructose yolk with either 7% glyce (30.40±2.58%) or 5% propylene gly (25.10±2.39%). The difference betw both extenders was not signific (Table 1).

Tabel (1) : The effect of cryoprotectant and buffer on the viability index of frozen buffa spermatozoa after incubation in water bath adjusted at 40° C for 4hs (Mean± S.E.)

Cryoprotectant	Buffer			Overall
	Citrate	Tris	Lactose	Mean
Glycerol 7%	30.40± 2.58^{a1}	22.50±3.04^{a2}	26.40±2.36^{a12}	26.43±1.43^{a}
Propylene glycol:				
5%	25.10±2.39^{a1}	15.50±2.63^{b3}	13.40±2.16^{b2}	18.00±1.29^{b}
7%	13.30±2.56^{b1}	6.20±1.04^{c2}	5.20±1.09^{c2}	8.23±0.86^{c}
9%	10.70±2.06^{b1}	1.70±0.45^{d3}	3.10±0.75^{c2}	5.17±0.75^{d}
Overall mean	19.88±1.41 ^{1}	11.48±1.31^{2}	12.03±1.21^{2}	14.17±0.981

Values with different letters within the same column and different numbers within the same row were significantly different at least at P<0.05.

As shown in table (2). The viability index of frozen buffalo spermatozoa significantly ($P<0.05$) improved after extension of semen in citrate - propylene glycol 5% with egg yolk concentration of 10% (25.00 ± 1.71%) when compared to concentrations of 5% (18.06 ± 1.57%) and 20% (20.56± 1.24%). However, a non - significant difference in the viability index was observed either in absence of antibiotics (20.93 ± 1.69%) or in the presence of Baytril (23.33 ± 1.77%), Colampi (19.81±1.97%) and a combination of both antibiotics (20.74 ± 1.90%). The present results revealed higher viability index for frozen buffalo spermatozoa extended in citrate-propylene glycol - Baytril- egg yolk 5% (24.44± 2.42%) and 10% (26.67±2.00%) as well as citrate - propylene glycol-Colampi - egg yolk 10% (25.56 ± 2.38%) and 20% (21.11 ± 2.47%) .

The obtained results (Table 3) indicated the presence of aerobic and absence of anaerobic bacteria in frozen buffalo semen under investigation . In absence of antibiotics, the total bacterial count for aerobes decreased by increasing egg yolk concentration from 5% (9×10^6) to 10% (7×10^6) and 20% (6×10^6). This count significantly decreased when the extender contained either Baytril or Colampi with egg yolk concentration of 5% (8 and 5×10^6, resp.) 10% (8 and 3×10^6 resp). and 20% (2×10^6) . combination of both Baytril and Colampi did not reveal any total bacterial count with the different egg yolk concentrations (Table 3).

Tabel (2) : The effect of antibiotics and egg yolk concentration on viability index of frozen buffalo spermatozoa incubated at 40°C for 4hr (Mean± S.E.)

Antibiotics	Egg yolk concentration			Overall
	5%	10%	20%	Mean
Without	17.78 ± 2.24^{b2}	21.67 ± 2.33^{a12}	23.33 ± 2.04^{a1}	20.93 ± 1.69^{a}
Baytril	24.44 ± 2.42^{a1}	26.67 ± 2.00^{a1}	18.89 ± 3.09^{a2}	23.33 ± 1.77^{a}
Colampi	12.78 ± 3.13^{b2}	25.56 ± 2.38^{a1}	21.11 ± 2.47^{a1}	19.81 ± 1.97^{a}
Combined	17.22 ± 2.90^{b2}	26.11 ± 2.98^{a1}	18.89 ± 2.32^{a2}	20.74 ± 1.90^{a}
Overall mean	18.06 ± 1.57^{2}	25.00 ± 1.71^{1}	20.56 ± 1.24^{2}	21.20 ± 0.901

Values with different letters within the same column and different numbers within the same row ere significantly different at least at $P<0.05$.

Tabel (3) :The effect of antibiotics and egg yolk concentrations on the total aerobic and anaerobic bacterial count ($x10^6$) in frozen buffalo semen.

Antibioties	Egg yolk concentration			Total	
	5%	10%	20%	Acrobes	Anoerobes
Without	9	7	6	22	0
Baytril	3	3	2	8	0
Colampi	5	0	2	7	0
Combined	0	0	0	0	0
Total	17	10	10	37	0

Table (4) revealed that in absence of antibiotics, Strept. pyogenes and Micrococcus spp. were isolated from semen extended with 5% egg yolk. Staph epidermis and Proteus vulgaris were isolated with 5 and 10% egg yolk. Staph. aureus was isolated with 20 % egg yolk, while E. coli was isolated from semen with 5% , 10% and 20% egg yolk concentrations . In the presence of antibiotics , Micrococcus spp. were isolated from semen extended propylene glycol with egg yolk Colampi. Staph. aureus was with egg yolk 20% and with Colampi. E. coli was isolated semen extended in citrate - p glylcol with either 5% or 10% and Baytril. From the bacteri examination, no anaerobes could be isolated from the frozen semen under conditions of the investigation (Table 4) .

Tabel (4) : Strains isolated from frozen buffalo semen with different types of antibiotics yolk concentrations.

Antibiotics	Without			Baytil			Colampi			Combi	
Egg yolk	5	10	20	5	10	20	5	10	20	5	10
Strept pyogenes	+	-	-	-	-	-	-	-	-	-	-
Micrococcus spp	+	-	-	-	-	-	+	-	-	-	-
E. coli	+	+	+	+	+	-	-	-	-	-	-
Staph epidermis	+	+	-	-	-	-	-	-	-	-	-
Proteus vulgaris	+	+	-	-	-	-	-	-	-	-	-
Staph aureus	-	-	+	-	-	+	-	-	+	-	-
Anaerobes	-	-	-	-	-	-	-	-	-	-	-
Total	5	3	2	1	1	1	1	-	1	-	-

+ : Positive - : Negative

DISCUSSION

One of the main factors influencing freezability of buffalo spermatozoa is the buffering capacity of the extender. Best results where observed with Tris then with either citrate or lactose in freezing buffalo semen *(4)*. . In the present study, contradictory results were noticed where citrate accounted for superior rates of post thaw sperm motility compared to lactose and Tris buffers. In the meantime, conflicting results in the post-thaw sperm viability were reported *(5,19)* indicating the presence of a difficulty to justify the successful buffer suitable for freezing buffalo semen. This difficulty might be attributed to the change in the pH of the buffer system. It has been reported that best pH for freezing buffalo semen milk, Tris or citrate extender was -6.8(20). Highest viability index frozen buffalo spermatozoa was obtained with semen extended in Tris at pH (20) .

Another point that necessary in freezing of semen is implementation of the cryoprotective agent . It has been assumed that the requirements for effective cryoprotectant agent are low molecular weight, an ability to penetrate living cells, a high solubility in aqueous electrolyte solutions and non toxic. In this respect , glycerol has been

emphasized as the most effective cryoprotective agent for low temperature preservation of semen. However, conflicting results have been obtained on using glycerol as cryoprotective agent for buffalo spermatozoa *(9,10,23,24)*. Trials have been adopted to compare effects of other cryoprotective agents on freezability of bovine spermatozoa, e.g. polyethylene glycol *(8,25)*, polyvinyl pyrolidone *(7)* and butylated hyroxytoluene *(19)*. Compounds containing hydroxyl groups (e.g. glycerol, ethylene glycol) were relatively less effective cryoprotective agents, particularly for rabbit spermatozoa *(22)*, than those containing amide (e.g. formamide, lactomide, acetamide, medmide) or methyl (e.g dimethyl formaide, dimethyl sulphoxide) groups. On the other hand, successful cryopreservation of in- vitro - bovine blastocyst was obtained by vitrification in a mixture of glycerol and propylene glycol **(26)**. Recently, propylene glycol has been used as neutral solvent for the natural oil of Nigela sativa. The latter could be infused intra uterine in treatment of bovine endometritis with encouraging results on rate of conception **(27)**. Hereby, the present study intended to justify implementation of propylene glycol in cryopreservation of buffalo semen. From the data obtained (Table 1), it has been concluded that although glycerol is more effective in cryopreservation of buffalo spermatozoa, propylene glycol could be successfully used as a cryoprotective agent with satisfactory results at a concentration of 5% in citrate buffer.

Another point of interest is to choose the most suitable concentration of egg yolk as one of the main requirements of an extender. It has been evident that the percentage of egg yolk with Tris-glycerol buffer can be reduced to 5% for buffalo semen (9), a finding which was confirmed in the present study even with citrate propylene glycol buffer. In the meantime, the percentage of post thaw sperm viability index increased by using 10% egg yolk when compared to 5% and 20% concentration (Table 2). This finding partially agreed with that observed previously *(10)* where the post thawing motility of buffalo spermatozoa was depressed by increasing egg yolk concentration over 5% for milk, [illegible] and citrate extenders.

It is important to [illegible] dilution of semen [illegible] achieving the best [illegible] of spermatozoa [illegible] result in high rate of conception [illegible] microbial growths in semen are controlled. Previous reports recommended different systems for controlling the microbial content of semen *(12, 13, 14)*. As a point for establishing a new extender in freezing buffalo semen, the present study suggested using Enerofloxacin or Colistin sulphate and Ampicillin as effective antimicrobial agents (Tables 3,4). These agents were added to citrate 2.9%- Fructose 1.2% - Propylene glycol 5% - egg yolk 10% to have reasonable post thaw viability index for frozen buffalo spermatozoa. However, further study is necessary to evaluate the effect of the suggested extender adopted in the present study on the conception rate of frozen buffalo semen.

ACKNOWLEDGEMENT

The authors express their gratitudes to *Dr. Moghazi,* [illegible] Researcher, NRC, Cairo for providing antibiotics used in the study.

REFERENCES

1-Ollero, M. ; Bescós, O.; Cebrián-Pariz, J. A. and [illegible] *- Blanco, T. (1998)* : Loss of plasma membrane proteins of bull spermatozoa through the freezing - [illegible] process [illegible] [illegible]-[illegible].

2-El-Sheltawi, M. A. (19[illegible]): Evaluation of locally processed frozen semen Ph. D. Vet. Thesis, Cairo Univ., pp. 67-73.

3-Nainar, A. M. ; Easwaran, B. M. and Ulaganathan, V. (1990) : Studies on non- motile spermatozoa (static semen) in buffalo bull semen . Ind. Vet. J. 67 (2) : 133-135.

4-Dhami, A.J. and Sahni, K. L. (1994) : Role of different extenders and additives in improving certain biologicial indices of frozen bull and buffalo semen . Ind. Vet. J. 71 (7) : 670-676.

5-Kumar, S. ; Sahni, K. L. ; Mohan, G. and Bisht, G. S. (1994) : Effect of certain cryoprotectants on freezing of buffalo semen in milk dilutors. Ind. J. Anim. Sci.,64 (11) : 1161-1169.

6-Ak, K. and Ileri , I. K. (1991) : The effect of different levels of glycerol added to milk powder - egg yolk extender on the deep freezing of bull semen in turkey Vet. Fakul. Dergisi. Istanboul, 17 (1) : 39-44.

7-Leeuw , F. E. ; Leeuw, A. M. ; Daas, J. H. ; Colenbronder, B. and Verkleij , A. J. (1993) : Effects of various cryoprotective agents and membrane stabilizing compounds in bull sperm membrane integrity after cooling and freezing. Cryobiology , 30 (1) : 32-44.

8-Chestimedjieva , S. B. ; Vaisberg, C. N. and Kolev, S. I. (1996) : On the lipid composition of buffalo spermatozoa frozen in different cryoprotective media . Bulg . J. Agric. Sci., 2 (1) : 23-26.

9-Sahni, K. L. and Mohan, G. (1990) : Yolk as a cryoprotectant in deep - freezing of bovine semen . Ind. J. Anim. Sci. 60 (1) : 828-829.

10-Kumar, S. ; Sahni, K. L. and Mohan, G. (1992) : Freezing of buffalo semen in milk Tris and sodium citrate dilutors with different levels of yolk and glycerol in relation to pH of dilutors . Ind. J. Anim. Sci., 63 (5) : 499-509.

11-Ahmed , K. (1987): Antibiotics in bull semen. Effects of new antibio. on post thaw survival and fertility frozen bull spermatozoa . Diss. Ab Inter., - B, Sci., & Eng., 47 (7) N 2689.

12-Sullivan , J. J. ; Bean, B. Kellgren , H. D. ; Lorton, S. P. Koproth, M. and Marshall, C. I (1988) : Effect of gentamicin, tylo and linco-spectin on fertility of froze bovine spermatozoa extended in eg yok - citrate or heated . whole mil extenders . 11th Int. Congr. Anim. Reprod. and A. I. Dublin. Ireland Vol. 3, Paper No. 301.

13-Hassan , H. M. (1993) : Effect certain antibiotics on some bacter isolated from contaminated buffal semen: M. V. Sc. Thesis. Zagazi Univ., pp. 51-92.

14-Gerard , O. ; Visser, J. P. an Jansen, H. D. (1995) : Influence two extension procedures the ubiquitous microflora an the biological quality of froze bovine semen . Reprod-In-Dom 30 (5) : 289 - 294.

15-Milovanov, V. K. (1962) : and reporduction and insemination of farm anim (title). Monograph selkhoz Moscow.

16-Daily, W. R. and Scott, (1978) : Diagnostic m 5th Ed., The C. V. Mosby Co., Louis, pp. 109-110.

17-Dilielle, L. E. ; Fuber, L. E. (1978) : antibiotics on vibrio bovine spermatozoa Am. J. V 18 , 86-92.

18-Denenberg , V. H. (1
Statistics and experimental for behavioral and biol researchers . An introduction Wiley and Sons, New Yo 101-102.

19-Killian , G. ; Hanadel, T. ; McNutt, T. ; Henault , M. ; Wegner, C. and Dunlop, D. (1989) : Evaluation of butylacted hydroxytoluene as a cryopreservative added to whole or skin milk diluent for bull semen. J. Dairy Sci., 72 (2) : 1291-1295.

20-Mary G. Abdel-Malak : Abdel -Malkak G.; Abdel - Aziz , A. ; Farahat, A. A. and Abdou , M. S. S. (1993) : Viability of frozen buffalo semen in Tris- yolk - fructose extender . Egypt Soc. Anim. Reprod. Fert., 5th Ann. Congr. Cairo, pp. 171-177.

21-Lovelock, J. E. (1957): Biophysical aspects of the freezing of living cells. In "The preservation and transplantation of normal tissues. Eds. G. E. W. Wolstenholme and Cameron, Churchilk, Edinburgh, pp. 131 - 138.

22-Hanada , A. and Nagase, H. (1980) : Cryoprotective effects of some amides on rabbit spermatozoa . J. Reprod. Fert., 60 : 247-252.

23-Maha S. Ziada; Abdel -Malak, G. and Abdou , M. S. S. (1992) : Effect of glycerol concentration and thawing regimes on the viability of frozen buffalo spermatozoa. Egypt. Soc. Anim. Reprod. Fert., 4th Ann. Congr., Cairo, pp. 133-143.

24-Ramakrishanan, P. and Ariff, M. O. (1994) : Effect of glycerol level and cooling rate on post - thaw semen quality of malaysian swamp buffalo . Proc. 4th world buffalo congr., Sao paulo. Brazil, 3 : 540-542.

25-Kalev, S. I. (1996) : Effect of different cryoprotective media on the acrosomal integrity of buffalo bull spermatozoa after deep freezing . Bulg. J. Agric. Sci. 2 (1) : 27-30.

26-Kuwayama , M. ; Hamano, S. and Nagai, T. (1992): Vitrification of bovine blastocysts obtained by in vitro culture of oocytes matured and fertilized in vitro . J. Reprod. Fert. 96 : 187-193.

27-Maarof, A. A. (1998) : Trials for treatment of repeat breeding in cattle . Ph. D. Thesis, Zagazig Univ. (Benha) , pp. 48-92.

الملخص العربى

تأثير البروبيلين جليكول وبعض المضادات الحيوية على حيوية الحيامن والكفاءة البكتريولوجية للسائل المنوى المجمد للجاموس

د. عبدالسلام إبراهيم العزب [١] ، د. محمد مكين البرديسى [٢] ، ط.ب. شفيق إبراهيم توفيق [٣]

١-قسم التوليد والتناسل والتلقيح الإصطناعى بكلية الطب البيطرى بمشتهر جامعة الزقازيق - فرع بنها

٢- قسم البكتريولوجى - معهد بحوث صحة الحيوان بالدقى

٣- مركز السائل المنوى المجمد بالعباسية

أظهرت هذه الدراسة أن أفضل دليل لحيوية حيامن الجاموس وجد عند تخفيفها فى مخفف سترات الصو... بالمقارنة بتلك التى تم تخفيفها بإستخدام مخففات اللاكتوز والتريس . كما وجد عند تجميد السائل المنوى للجا... أنه بالرغم من أن الجليسرول كان أفضل كفاءة فإن البروبيلين جليكول قد استخدم بنجاح وأعطى نتائج معقولة... إضافته بنسبة ٥٪ لمخفف السترات . ولقد أظهرت النتائج أنه لكى يتم الحصول على دليل حيوية جيد للحيامين... عدد من البكتريا فى السائل المنوى لابد أن لاتزيد نسبة صفار البيض عن ١٠٪ . وقد أمكن إستخ... لإنروفلوكساسين ، الكولستين ، لأميسلين كفاءة كعوامل مضادة للبكتريا فى المخفف . لذلك فإن هذه الدراسة ... مخفف لسائل منوى مجاموس يتكون من سترات صوديوم (٢ر٩٪) ، فراكتوز (٢ر١٪) ، بروبيلين جل... (٥٪) ، صفار بيض (١٠٪) . بالاضافة إنروفلوكساسين (١٠٠٠ ميكروجرام لكل ملى) ، سلفات كولستين (... وحدة لكل مللى) وأميسلين (١٠٠٠ ميكروجرام لكل ملى) .