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CRYOPRESERVATION OF BUFFALO SPERMATOZOA IN SOY LECITHIN-BASED EXTENDERS

(With 5 Tables)

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مجدي رمضان بدر

لا تزال ممددات السائل المنوي تعتمد في تركيبها على إضافة صفار البيض علي الرغم من الاعتراضات الكثيرة حول استخدامه. وحديثا ظهرت أنواع جديدة من ممددات السائل المنوي خالية من الإضافات الحيوانية (صفار البيض أو اللبن) وبالرغم من ذلك مازالت الممددات التي تحتوى على صفار البيض تستخدم بصورة أساسية لتجميد السائل المنوي الجاموسي. وتهدف الدراسة الحالية إلي دراسة قدرة حيامن الجاموس للتجميد وكذلك قدرتها الإخصابيه معمليا وحقليا عند تجميدها في ممدد البيوسيفوس أو ممدد البيوكسيل وهى ممددات تعتمد في تركيبها علي الصويا ليسيسين بدلا عن استخدام صفار البيض. ولقد أوضحت نتائج الدراسة الحالية أن تجميد السائل المنوى الجاموسى في ممدد البيوكسيل او ممدد البيوسيفوس نتج عنة زيادة معنوية كبيرة في معدل الحركة الأمامية بعدالإسالة (٦١,٦٦ و٦٠,٠٠ % علي التوالي) وكذلك معدل حيوية السائل المنوى (١٣٠,٨٣ و١٢٠,٠٠ على التوالى) وكذلك معدل المحافظة على سلامة غشاء القلنسوة (١١,٠٠و ١١,٣٣ علي التوالى) مقارنة بتلك التي تمديدها في ممدد التريس الذي يحتوى على ٢٠% صفار بيض (٣٨,٣٣% و ٨٨,٣٣ و١٩,٠٠% علي التوالى). كذلك أوضحت نتائج الدراسة الحالية أن تجميد السائل المنوى الجاموسى في ممدد البيوكسيل او ممدد البيوسيفوس نتج عنة انخفاض معنوي كبيرة في معدل أكسده الدهون (٧,٦٤ و٨,٧٣% علي التوالى) مقارنة بتلك التى تمديدها في ممدد التريس الذي يحتوى على ٢٠% صفار بيض(١٢,٩٦%). كما أوضحت نتائج الإخصاب المعملى أن السائل المنوى الجاموسى الذي تم تجميده باستخدام ممدد البيوكسيل أو ممدد البيوسيفوس أدى إلى زيادة معنوية فى معدل إخصاب البويضات (٦٥,٠٨ و٦٣,٢٤% علي التوالى) وكذلك قدرتها علي النمو إلي الطور التوتى (٢٣,٩٤ و٢٢,٨٩% علي التوالى) مقارنة بتلك التى تمديدها في ممدد التريس الذي يحتوى على ٢٠% صفار بيض (٤١,٠٧ و٩,٦٢% على التوالى). كذلك أوضحت نتائج التلقيح الحقلى انه على الرغم من زيادة نسبة الإخصاب للسائل المنوى الذي تم تجميده فى ممددات البيوكسيل أو البيوسيفوس مقارنة بتلك التى تم تمديدها في ممدد التريس الذي يحتوى علي ٢٠% صفار بيض إلا إنها كانت زيادة غير معنوية. ولهذا يمكن أن نستنتج من نتائج هذه الدراسة أن ممددات السائل المنوى التي تحتوى على الصويا ليسيسين يمكن أن تكون الاختيار الأمثل لتجميد السائل المنوى الجاموسى في المستقبل.

SUMMARY

Semen extenders containing egg yolk as a cryoprotectant may pose hygienic risks and are difficult to standardize. Although a new generation of semen extenders free of animal ingredients is available, egg yolk-containing extenders are still widely used for cryopreserving semen. The aim of the present study was to compare the effect of using soy lecithin-based extenders, Biociphos and Bioxcell, and egg yolk-based extender on buffalo spermatozoa freezability and fertilizing potentials. Extension of buffalo bull semen in the Bioxcell and the Biociphos extenders significantly increased ($P<0.01$) the post-thaw sperm motility (61.67 and 60.00%, respectively) and the viability index (130.83 and 120.00, respectively) compared to semen that extended in the TRIS-egg yolk extender (38.33% and 88.33, respectively). Assessment of the post-thaw acrosomal integrity showed significant differences ($P<0.01$) between extenders (11.00, 11.33 and 19.00 %, for Bioxcell, Biociphos and TRIS-egg yolk extenders, respectively). In vitro fertilization results revealed that, extension of buffalo bull semen in the Bioxcell and Biociphos extenders significantly increased ($P<0.05$) the in vitro fertilization rate (65.08 and 63.24%, respectively) and boosted the ability of cleaved oocytes to develop to the morula stage (23.94 and 22.89%, respectively) compared to semen that extended in the TRIS-egg yolk extender (41.07 and 9.62%, respectively). Field trials revealed that, no significant differences ($P>0.05$) were detected between the extenders for the non-return rates. We suggest that consistent with quality standards that should be required for cryoprotectant media, soy lecithin-based diluents might be the best choice as a buffalo semen extender in the future.

Key words: *Cryopreservation, spermatozoa, semen, buffalo.*

INTRODUCTION

The process of cryopreserving semen has profound damage effects on spermatozoa, many of which result in sublethal damage to the sperm cells, and subsequent reduction of fertility. Cryopreservation damage effect includes membrane destabilization due to lateral lipid rearrangement (Quinn, 1985 and De Leeuw *et al.*, 1990), loss of lipids from the membrane (Buhr *et al.*, 1994) and peroxidation of membrane lipids as a result of formation of reactive oxygen species (Flesch and Gadella, 2000). Defining causes of sperm damage during cryopreservation is further complicated because the semen cryopreservation processess is not standardized and there is a wide

variety of freezing diluents in use (Foulkes, 1977 and Watson, 1995). Glycerol is a preferable cryoprotectant for sperm freezing in most mammals. Additionally, complex agents such as egg yolk, skim milk, milk and even serum are used in sperm freezing extenders for different species in order to provide maximal cryoprotection for spermatozoa (Holt, 2000). However, there have been frequent arguments against the use of egg yolk or milk, one of which is the wide variability of composition that make it difficult to analyze the beneficial effects of a particular compound on sperm cryopreservation (Wall and Foote, 1999). Furthermore, egg yolk and milk introduce possible sanitary risks (viruses, bacteria and fungi), with the subsequent production of endotoxins capable of damaging the fertilizing capacity of spermatozoa (Bousseau *et al.,* 1998 and Van Wagtendonk-de Leeuw *et al.,* 2000). Therefore, a chemically defined extender would be helpful to understand the mechanism of both sperm cryodamage and cryoprotection. Recently, new extenders with lecithin-based cryoprotective components were introduced into practice (Gil *et al.,* 2000 and Aires *et al.,* 2003). Previous studies have described how the spermatozoa of a mouse (Storey *et al.,* 1998), goat (Kundu *et al.,* 2000 and Janett *et al.,* 2005) and bovine (Aires *et al.,* 2003) were successfully frozen in a chemically defined extender, but not yet those of buffalo.

In the present study, efforts have been made to develop a method for cryopreservation of the buffalo spermatozoa in a chemically defined extender. Frozen-thawed sperm's function was evaluated by sperm motility and sperm head membrane integrity, in vitro fertilizing potentials and the 56- day non-return rates.

MATERIALS and METHODS

Semen collection and processing:

Semen samples used in this experiment were obtained from six buffalo bulls of a proven fertility, kept at the Animal Reproduction Research Institute farm, Al-Harm. Only semen samples of at least 70% initial motility and $800.00X10^6$ sperm cells/ml were used. Immediately after collection, semen samples were pooled, divided into three equal fractions and diluted at 1:8 ratio at 30°C; the first two fractions were diluted with soy lecithin-based extenders, Biociphos PlusÒ [BP] (IMV Technologies, L'Aigle, French) and Bioxcell [BX] (IMV Technologies, L'Aigle, French). The third fraction was diluted with TRIS-egg yolk extender, which differs from the soy lecithin extenders only in one

component as it contains 20% (w/v) egg yolk instead of soybean lecithin extract. Immediately after dilution, the extended semen was cooled from 37 to 5ºC throughout 60 min in a cold cabinet, filled in polyvinyl chloride (PVC) straws (0.25 ml; IMV, L'Aigle, France) and then frozen for 15 min (–120 °C) above nitrogen gas vapor inside foam box according to Mohammed *et al.* (1998), before being immersed into liquid nitrogen (–196 °C) and stored till used.

Evaluation of semen freezability:

Frozen semen samples were thawed in a water bath at 40ºC for 30 second. Sperm motility was assessed subjectively, post-thawing and after 1, 2 and 3 hours of thawing. Post-thawing viability indices were recorded according to Milovanov (1962). Post-thaw acrosomal defects were recorded in smears stained by Fast Green (FCF) according to Wells and Awa (1970).

Measurement of membrane lipid peroxidation:

Membrane lipid peroxidation was estimated by the end point generation of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) test according to Golal *et al.* (1998).

Evaluation of in vitro fertilizing potential of the treated semen:

Frozen semen extended in different extenders was used to evaluate the fertilizing potentials of the treated semen in vitro.

In vitro oocyte maturation:

Ovaries were obtained from buffaloes at a local slaughterhouse and transported to the laboratory within two hours in 0.9% NaCl containing 75 µg/ml potassium penicillin G and 50 µg/ml streptomycin sulfate maintained at 30°C. Oocytes were aspirated from medium-sized follicles (2-8mm in diameter) with an 18-gauge needle fixed to a 10-ml disposable syringe. Oocytes surrounded by a compact cumulus mass and having evenly granulated cytoplasm were washed 3 times in maturation medium, and 10-15 oocytes were transferred into each drop of pre-equilibrated maturation medium previously covered with warm mineral oil and cultured at 39°C in humidified atmosphere of 5% CO_2 in air for 24 hours according to Totey *et al.* (1992).

Sperm preparation and oocyte insemination in vitro:

Three straws from each treatment were thawed in a water bath at 40ºC for 30 sec. Immediately after thawing, the most motile spermatozoa were separated by swim up technique in sperm-TALP medium containing 6 mg/ml bovine serum albumin, for 1 hour (Parrish *et al.*, 1988). The uppermost layer of the medium containing the most spermatozoa was collected. The selected spermatozoa were washed

twice by centrifugation at 2000 rpm for 10 min. The sperm pellet was resuspended in the fertilization TALP medium containing 10 µg/ml heparin, for in vitro sperm capacitation. After appropriate dilution, 2 µl of sperm suspension was added to the fertilization drops, containing matured oocytes, at a final concentration 2 $X10^6$ sperm cell/ml. Gametes were co-incubated in the fertilization drops under sterile mineral oil for 18 hour at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity. At the end of gametes co-incubation, some of the inseminated oocytes were fixed in acetic acid-ethanol (1:3) and stained with 1% aceto-orcein stain and examined under phase-contrast microscope (X 400) for evaluating the in vitro fertilization rate. The presence of the second polar body, swollen or decondensing sperm head or even a detached sperm tail in the ooplasm was regarded as an evidence of sperm penetration. Penetrated oocytes with a male pronucleus or male and female pronuclei were regarded as an evidence of fertilization.

In vitro culture:

The inseminated oocytes were freed from cumulus cells and the attached spermatozoa by gentle pipetting and cultured in TCM-199 medium with Hepes modification for 7- days at 39°C in an atmosphere of 5% CO_2 in air with maximum humidity. The proportional of cleaved oocytes was recorded 48 hour after insemination and those developed to the morula and blastocyst stages were recorded at 5-7 day post-insemination under a stereomicroscope according to Totey *et al.* (1992).

Fertility Study:

As a practical test of cryoprotectant efficacy, a preliminary fertility trial was performed using sperm frozen in the conventional TRIS-egg yolk extender and the chemically defined soy lecithin extenders. Buffaloes were randomly assigned to one of three treatment groups: group 1 (35 buffaloes) was inseminated using semen extended in TRIS-egg yolk extender; group 2 (42 buffaloes) was inseminated using semen extended in Biociphos extender and group 3 (53 buffaloes) was inseminated using semen extended in Bioxcell extender. Pregnancy diagnosis was performed 45 days post-insemination by transrectal palpation and ultrasound.

Statistical analysis:

All data were analyzed by using Costat Computer Program, Version 3.03 copyright (1986), and were compared by the least significant difference least (LSD) at 1% and 5% levels of probability. The results were expressed as means ± S.E.M. In vitro fertilization rate,

embryo development and total conception rate were analyzed by chi-square analysis (X^2).

RESULTS

Effect of freezing extenders on sperm motility, viability, and acrosomal integrity:

Data presented in table 1 revealed that, extension of buffalo semen in Bioxcell and Biociphos extenders enhanced sperm freezability and increased significantly (P<0.01) the post-thawing motility (61.67 and 60.00%, respectively), viability index (130.83 and 120.00, respectively) and maintained the acrosomal integrity (11.00 and 11.33%, respectively) compared to that extended in egg yolk-based extender (38.33%, 88.34 and 19.00%, respectively).

Table1: Comparison between egg yolk and soy lecithin-based diluents on buffalo spermatoza motility, viability, and acrosomal integrity.

Different treatments	Extension motility (%)	Post-thawing motility (%)	Viability index	Acrosomal defect (%)
Control	75.00±2.89 [a]	38.33±1.64 [b]	88.34±10.94 [b]	19.00±2.65 [a]
Biociphos	78.33±1.66 [a]	60.00±5.78 [a]	120.00±10.42[a]	11.33±0.87 [b]
Bioxcell	78.33±1.67 [a]	61.67±6.02 [a]	130.83±2.21 [a]	11.00±2.08 [b]
Over all mean	77.22±1.48	53.33±5.49	113.06±7.89	13.77±2.01

Values with different letters in the same columns are significantly different at least (P<0.05).

Effect of freezing extenders on lipid peroxidation and antioxidant activity of buffalo spermatozoa:

Data presented in table 2 showed that, extension of buffalo semen in soy lecithin extenders before freezing could delete the harmful effect of the oxidative stress during cryopreservation. Extension of buffalo semen in chemically defined extender; Bioxcell and Biociphos extenders decreased significantly (P<0.01) membrane lipid peroxidation (7.64 and 8.73%, respectively) compared to that extended in egg yolk-based extender (12.96%) and increased significantly (P<0.01) the activity of pyruvate kinase enzyme (5.43 and 4.53%, respectively) compared to that extended in egg yolk-based extender (3.39%).

Table 2: Comparison between egg yolk and soy lecithin-based diluents on lipid peroxidation and antioxidant activity of buffalo spermatozoa.

Different treatments	Lipid peroxidation (%)	Pyruvate kinase (%)	Total antioxidant (%)
Control	12.96±1.19[a]	3.39±0.16 [b]	0.22±0.10 [b]
Biociphos	8.73± 1.17 [b]	4.53±0.13 [a]	0.46±0.09 [ab]
Bioxcell	7.64±1.21 [b]	5.43±0.39[a]	0.75±0.05 a
Over all mean	9.77±1.23	4.45±0.39	0.48±0.11

Values with different letters in the same columns are significantly different at least (P<0.05).

Effect of cryoprotectants on the in vitro fertilization and embryo development rate:

Data presented in tables 3 and 4 demonstrated that, extension of buffalo semen in chemically defined extender, Bioxcell and Biociphos, increased significantly (P<0.01) the in vitro fertilization rate (65.08 and 63.24%, respectively), the cleavage rate (53.52 and 51.85%, respectively) and the morula stage development (23.94 and 22.89%, respectively) compared to that extended in egg yolk containing extender (41.07, 34.62 and 9.62%, respectively). The current results showed higher apparent embryo development to the blastocyst stage in the soy lecithin-based extenders compared to egg yolk-based extender. However, these distinctions were not statistically significant (P > 0.05).

Table 3: Comparison between egg yolk and soy lecithin-based diluents on buffalo spermatozoa in vitro fertilizing potentials**.**

Different treatments	No. of oocytes	Penetration rate (%)	Fertilization Rate (%)
Control	56	32 (57.14) [a]	23 (41.07) [a]
Bioxcell	63	44 (69.84) [a]	41 (65.08) [b]
Biociphos	68	46 (67.65) [a]	43 (63.24) [b]

values with different letters in the same columns are significantly different at least (P<0.05)

Table 4: Comparison between egg yolk and soy lecithin based diluents on buffalo spermatozoa in vitro embryo development.

Different treatments	No. of oocytes	Cleavage rate NO. (%)	Morula NO. (%)	Blastocyst NO. (%)
Control	52	18 (34.62) [b]	5 (9.62) [a]	3 (5.77) [a]
Bioxcell	71	38 (53.52) [a]	17 (23.94) [b]	9 (12.68) [a]
Biocephos	83	43 (51.81) [a]	19 (22.89) [b]	10 (12.05) [a]

Values with different letters in the same columns are significantly different at least (P<0.05)

Effect of cryoprotectants on the 56-day non return rate:

The results showed that the soy lecithin-based extenders and egg yolk-based extender perform equally well clinically. The current results showed higher apparent conception rate in the Biociphos and Bioxcell extenders (61.90 and 62.26%, respectively) compared to egg yolk-based extender (54.29%). However, these distinctions were not statistically significant ($P > 0.05$).

Table 5: Comparison between egg yolk and soy lecithin-based diluents on buffalo spermatozoa conception rate.

Different treatments	No. of inseminated buffaloes	56- non-return rate	Total conception rate %
Control	35	19	54.29 [a]
Biociphos	42	26	61.90 [a]
Bioxcell	53	33	62.26 [a]

Values with different letters in the same columns are significantly different at least ($P<0.05$)

DISCUSSION

A loss of mobile spermatozoa (close to 50% loss) is common after freezing of bull semen. The results of the present study have clearly demonstrated that the soy lecithin-based extenders increased the freezability and the fertilizing potentials of buffalo spermatozoa compared with TRIS- egg yolk extender. These results are in accordance with those obtained by Gil *et al.* (2000) , Moussa *et al.* (2002), Amirat *et al.* (2004) and Ricker *et al.* (2006) and are in opposition to those obtained by Van Wagtendonk-de Leuw *et al.* (2000). The beneficial effect of soy lecithin extender on the sperm function may be attributed to the ability of the lecithin to maintain the integrity of cell membranes, facilitating the movement of fluids inside and outside the cell and without lecithin, cell membranes would harden and would no longer stay semi-permeable (Zeisel, 2000). Moreover, lecithin act as a sink for the cholesterol from the sperm plasma membrane (Dobiasova and Frohlich 1999). By bringing down cholesterol levels, lecithin can protect the spermatozoa from cryoinjury via enhanced membrane permeability. Membrane cholesterol efflux induces an enhanced membrane fluidity and permeability (Purdy *et al.,* 2005 and Badr and Abd el-Malak 2007). By increasing membrane fluidity and permeability the amount of intracellular ice formation, which is lethal to the cell in cryopreservation, will be decreased (Mazur, 1985 and Essmail *et al.,*

2004). Additionlly, the removal of sperm plasma membrane cholesterol content could be useful to protect sperm during cryopreservation by improving viability without promoting premature capacitation (Galantino-Homer *et al.,* 2006). Furthermore, the beneficial effect of soy lecithin extender on semen cyropreservation may be attributed to the high concentration of linoleic acid (LA) in its constituent. Once LA in lecithin granules enters the body, it is converted to γ-linoleic acid, which is the precursor of prostaglandin E. Addition of prostaglandin E to the semen extender increassed the life span of the spermatozoa and the cleavage rate of the inseminated oocytes (Kolev and Dimov 1998). Moreover, the current data revealed that soy lecithin decreased significantly membrane lipid peroxidation, throughout reduction of malondialdehyde (MDA) production in the frozen-thawed spermatozoa. It is well established that cryopreservation induces lipid peroxidation in mammalian spermatozoa (Alvarez and Storey, 1992, Aitken, 1995, Golal *et al.,* 1998 and Chatterjee and Gagnon, 2001). Therefore, the improved buffalo semen freezability and in vitro fertilizing potentials that extended in soy lecithin extenders may be attributed to the ability of the lecithin to protect the spermatozoa from the destructive effects of oxidation, a naturally occurring process in the cryopreservation due to the presence of oxygen.

On the other hand, the present study demonstrated that TRIS-egg yolk extender appeared to protect the cryopreserved buffalo spermatozoa less effectively than soy lecithin extenders. These results are in accordance with those obtained by Amirat *et al.* (2005)**.** This effect of egg yolk-based extender may be attributed to; a high viscosity of the extender limits the diffusion of small molecules and ions from the eutectic phase emerging around growing ice crystals, towards the liquid phase (Pace and Graham, 1974 and Courtens and Réty, 2001). The present results showed that extension of buffalo semen in Tris-based extender increased significantly the acrosomal abnormalites. These acrosomal destructions could result from intrusion of Ca_2+, which is present in high concentrations in egg yolk and rapidly enters the cells when the temperature is below 30°C (Courtens *et al.,* 1989).

In conclusion, the present study suggest that soy lecithin extenders are viable alternative to conventional egg yolk-based freezing extenders for cryopreserving buffalo spermatozoa. Therefore, soy lecithin extender could largely replace egg yolk in extenders for buffalo bull semen freezing.

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