Original Article

In Vitro Comparison of Soybean Lecithin Based-Extender with Commercially Available Extender for Ram Semen Cryopreservation

Mohsen Sharafi, B.Sc.[1], Mohsen Forouzanfar, Ph.D.[2], Sayed Morteza Hosseini, D.V.M.[3], Mehdi Hajian, M.Sc.[3], Somaye Ostadhosseini, D.V.M.[3], Laleh Hosseini, M.Sc.[3], Parvaneh Abedi, B.Sc.[3], Nafiseh Nili, Ph.D.[1], Hamid Reza Rahmani, Ph.D.[1], Ahmad reza Javaheri, B.Sc.[4], Mohammad Hossein Nasr Esfahani, Ph.D.[3]*

1. Animal Sciences Department, College of Agriculture, Isfahan University of Technology, Isfahan, Iran
2. School of Sciences, Islamic Azad University, Marvdasht Branch, Marvdasht, Iran
3. Reproduction and Development Department, Royan Institute for Animal Biotechnology, ACECR, Isfahan, Iran
4. Animal Breeding Center, Karaj, Iran

Abstract

Background: Egg yolk is the main cryoprotectant agent in semen freezing extenders which is used in order to protect spermatozoa against cold shock. However, elimination of animal bioproducts from the cryopreservation protocol is becoming mandatory. Therefore, the aim of this study is to compare a previously studied, homemade soya bean lecithin based extender with a commercially available extender for ram sperm cryopreservation.
Materials and Methods: Samples from three rams were pooled and split into two equal aliquots and diluted (1:20) with i%lecithin - 7%glycerol (L1G7) and Bioxcell®. The effects of L1G7 and Bioxcell® on sperm parameters and the *in vitro* fertilization ability of frozen-thawed ram spermatozoa were assessed.
Results: The results of this study revealed no difference between the two extenders in terms of motility, viability, and capacitation status. The results of *in vitro* fertilization in terms of rate of blastocyst formation were similar for both extenders, but significantly lower than that of freshly processed ram sperm.
Conclusion: We conclude that both extenders are suitable for ram sperm cryopreservation.

Keywords: Cryopreservation, Semen, Extender, *In vitro*, Embryo Development

Introduction

Sperm cryopreservation has become a part of the routine procedure for assisted reproductive techniques (ART) in both humans and animals. Cryoprotectants play a central role in this procedure by resisting sudden temperatures changes, protecting sperm against cold and warm shock as well as preventing ice formation during freezing and dissolution during the thawing process (1-3). Such shocks can alter different compartments of sperm and thereby may alter the integrity and function of the acrosome, nucleus, mitochondria, axoneme and plasma membrane (4-6).
Freezing media consists of one or two cryoprotectants such as glycerol, di methyle sulfoxide (DMSO), egg yolk, milk and lecithin that are added to diluents for the freezing of semen. Diluents usually contain ionic or nonionic substances that sustain osmolarity and have buffering capacity; glucose or fructose as an energy source; and other additives, such as enzymes and antibiotics (7, 8). Egg yolk and skim milk are the most common additives of animal origin that are used for sperm cryopreservation (9-12). Recent studies have raised concerns regarding these two cryoprotectants mainly due to their varied and diverse composition which makes their quality certification difficult (13-15). In addition, they present a potential risk for microbial contamination and production of endotoxins which, in turn, affect the fertilizing capacity of spermatozoa (7, 16). Therefore, the bull stud industry has replaced egg yolks with a combination of phospholipids extracted from lecithin found in plants such as soybean oil (7, 17, 18). Indeed, 10% of soybean lipids are lecithin based which is believed to be the active component of low-density lipoproteins in egg yolks (13, 15, 19).
We previously reported, for the first time, that 1% lecithin can replace egg yolks for ram sperm cryopreservation (20). The objective of this study was to evaluate the potential cryoprotective property of lecithin on ram semen in *in vitro* fertility after undergoing the freezing-thawing process by using 7% glycerol with Bioxcell® (IMV, Aigle, France), a commercially available extender.

Received: 6 Jun 2009, Accepted: 21 Sep 2009
*** Corresponding Address: Reproduction and Development Department, Royan Institute for Animal Biotechnology, ACECR, Isfahan, Iran**
Email: mh.nasr-esfahani@royaninstitute.org

Royan Institue
International Journal of Fertility and Sterility
Vol 3, No 3, Nov-Dec 2009, Pages: 149-152

Materials and Methods

All chemical reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise indicated.

Extender preparation

The basic extender used in this study was prepared according to our previous publication (20). Briefly, 1% (w/v) soybean lecithin and 7% (v/v) glycerol was added to a basic extender and named L1G7.

Semen collection and processing

The present study was conducted in the Department of Reproduction and Development at Royan Institute for Animal Biotechnology (Isfahan, Iran). Semen samples were obtained from three mature Bakhtiari rams (3 and 4 years of age) with proven fertility by using an artificial vagina. The range of semen parameters for the samples were: volume 0.75-2 ml, sperm concentration ≥ 3×10^9 spermatozoa/ml, sperm motility ≥ 70%, and frequencies of total morphological abnormalities≤ 10%. Samples used for cryopreservation were pooled from the three rams. Samples were split in two equal aliquots and diluted (1: 20) with L1G7 and Bioxcell® (IMV, L'Aigle, France). For gradual cooling, the diluted semen samples were refrigerated at 4°C for 2-3 hours. Subsequently, 0.25 ml French straws were filled with diluted semen and placed 4 cm above liquid nitrogen (LN2) for 12 minutes before plunging into LN2 and stored until used for assessment of sperm parameters and *in vitro* fertilization.

Semen evaluation

Semen parameters, the hypo-osmotic swelling test (HOST) and chlortetracycline (CTC) assay were carried out according to Forouzanfar et al. (20), Revell and Marode (21) and Perez et al. (22), respectively.

In vitro fertilization (IVF)

Oocyte recovery and in vitro maturation

This procedure was carried out according to Forouzanfar et al. (20). Briefly, aspirated cumulus-oocyte complexes (COCs) were cultured in hepes-buffered TCM 199, penicillin- streptomycin 100 IU/ml and 10% (v/v) fetal calf serum (FCS) for 24 hours in 5% CO_2 at 39°C and maximum humidity. The conditions for maturation were a slightly modified version of those described by Shirazi et al. (23).

Sperm preparation and IVF

Matured COCs were washed four times in the fertilization medium before being transferred into final fertilization medium fertilization medium (Fert-TALP) (24). Oocytes were transferred in groups of 40-50 into 200 µl drops of fertilization medium. For insemination, three straws of frozen sperm representing one treatment, were thawed (37°C, 40 seconds) and pooled. Motile spermatozoa were obtained by centrifugation at 700 rpm, on Pure Sperm (Nidacon; Gothenburg, Sweden) for 10 minutes at room temperature. A total 2×10^6 spermatozoa/ml was added to each droplet of fertilization medium containing matured COCs and incubated for 24 hours in 5% CO_2 in humidified air at 39°C.

In vitro culture

After *in vitro* fertilization, cumulus cells were removed from presumptive zygotes by vortexing in 2 ml phosphate buffered saline solution (PBS) for 1 minute.

In vitro embryo culture was carried out in modified SOF medium (25) containing FCS (10% v/v) under mineral oil in a humidified atmosphere of 5% CO_2 and 90% N_2 at 39°C. The cleavage rate and number of embryos that reached the blastocyst stage were recorded at 3 and 7-8 days post insemination, respectively (day 0 = day of insemination).

Statistical analysis

After freezing - throwing, we had two strategies one for sperm parameters and one for assessment of fertilization and embryo development. Six and three replicates were done for assessment of sperm parameters and fertilization / embryo development respectively.

The analysis of variance (ANOVA) was used for comparisons of means. When the ANOVA test showed statistical differences, the mean of the treatments were compared using Duncan's multiple range test (DMRT) and a confidence level of $p<0.05$ was considered to be significant. Statistical evaluations were carried out using the Statistical Package for Social Studies Software (SPSS, 16.0). Information on CTC staining was analyzed using the GENMOD procedure of SAS (version 9) to fit a generalized linear model with a logit link function.

Results

The effects of L1G7 and Bioxcell® on sperm parameters and the *in vitro* fertilization ability of frozen-thawed ram spermatozoa are shown in Tables 1 and 2, respectively. Comparison between these two extenders showed no significant difference in terms of sperm motility or viability between the two groups (Table 1).

Table 1: Mean percentages of motility, viability and acrosome status of ram spermatozoa frozen with L1G7 and Bioxcell® extenders.

	Extenders	
Sperm Parameters	**L1G7**	**Bioxcell®**
Total Motility	52.6 ± 3.9	50.1 ± 3.7
Viability	49.1 ± 3.4	47.4 ± 2.8
Uncapacitated (F)	10.5 ± 2.7	8.4 ± 3.1
Capacitated (B)	61.7 ± 3.2	59.8 ± 2.9
Acrosome-reacted (AR)	27.8 ± 1.9	31.8 ± 2.4

No significant differences were observed between the two extenders at p≤0.05

Fig 1 shows the pattern of staining for uncapacitated, capacitated, and acrosome reacted sperm. The percentage of each pattern was compared between the two extenders and the results showed no difference between the two groups (Table 1 and Fig 1).

Table 2: Number of in vitro matured oocytes, as well as their cleavage and blastocyst rates, that have been inseminated with fresh or thawed spermatozoa using L1G7 and Bioxcell®. Blastocyst rate is expressed in terms of the number of inseminated oocytes or cleaved embryos.

		Proportion (%) of embryos developed to		
Semen type	**Oocytes (n)**	**Cleavage**	**Blastocyst/ oocytes**	**Blastocyst/ cleaved embryos**
Fresh	220	90.6 [a]	34.3	40.2
Frozen/ thawed (Bioxcell ®)	255	74.6 [b]	31.4	42.7
Frozen/ thawed (L1G7)	240	73.2 [b]	32.6	41.9

Different letters within the same column show significant differences among the groups (p≤0.05).

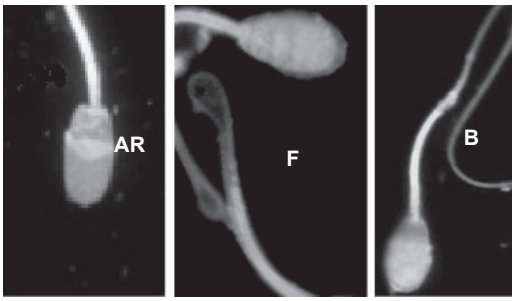

Fig 1: Fluorescent patterns of spermatozoa stained with CTC staining. Uniform fluorescence head or non-capacitated sperm (F pattern), post-acrosomal region without fluorescence or capacitated sperm (B pattern), and fluorescent freehead or a thin fluorescent band on the equatorial segment or acrosome reacted sperm (AR pattern).

Results for the cleavage rate and blastocyst development per oocytes and per cleaved embryos for fresh and frozen sperm, using L1G7 and Bioxcell®, are presented in Table 2. The cleavage rate was significantly higher in oocytes fertilized with fresh semen as compared to those fertilized with L1G7and Bioxcell® ($p < 0.05$). The blastocyst rates in terms of inseminated oocytes were 32.6, 34.3 and 31.4% in L1G7, fresh, and Bioxcell® groups, respectively. The differences between the three groups were not significant. The rates of blastocyst formation in terms of cleaved oocytes were also not significant between the three groups.

Discussion

The use of animal free culture medium also known as chemically defined medium is becoming mandatory in assisted reproductive technology (19, 26). Egg yolks are the effective agent in semen extenders (13, 19, 27). Considering the aforementioned fact, in addition to inconsistency in the individual quality differences that are inherent in egg yolks (7, 14, 26), it is apparent that substitution of egg yolk with animal free products would have several advantages including enhancing consistency in the components of semen extenders and exclusion of hygienic risks (7, 14, 16-20). The results of our previous study revealed that the substitution of egg yolk with 1% lecithin resulted in development of a suitable extender for cryopreservation of ram semen (20). In this study we compared the L1G7 extender with a commercially available product, Bioxcell®. The results of this study revealed that both extenders are efficient for cryopreservation of ram semen. We observed no significant difference in terms of sperm motility, viability and capacitation status between the two extenders. In addition, no significant difference was observed in terms of cleavage rate and blastocyst formation between the two extenders. However, the cleavage rates of both extenders were lower than fresh sperm, indicating that additional investigation for optimization of ram cryopreservation is required. Therefore, from the results of this study it can be concluded that both extenders are suitable for ram semen cryopreservation.

The results of this study only reveal that these two extenders are suitable for ram semen cryopreservation for *in vitro* use. In order to evaluate the use of the extenders for in vivo use, further studies are necessary. Indeed soybean lecithin based extender has been used for cryopreservation of bovine semen and resulted in higher motility, viability and non-return rate after artificial insemination compared with egg yolk based extender. To our knowledge, the only report available on ram semen cryopreservation using Bioxcell® has resulted in a pregnancy rate of 35.9% (NRR-21), which was far below the rate reported for

other soybean based-extenders applied in bovines. Moreover, there is a report on using a commercial soybean lecithin based extender (AndroMed®) in sheep which resulted in a 56.7% pregnancy rate after artificial insemination (26).

Conclusion

Considering that these products have GM, therefore we recommend the use of commercially available products for field work. Therefore, from our study there is a need to investigate the efficiency of our homemade extender to be used for artificial insemination.

Acknowledgements

This study was funded by a grant from Royan Institute, Islamic Republic of Iran. The authors would like to gratefully thank Dr. Hamid Gourabi and Dr. Ahmad Vosough for their full support. There is no conflict of interest in this article.

References

1. Graham EF, Carbo BF, Brown KI. Effects of some zwitter ion buffers on the freezing and storage of spermatozoa bull. J Dairy Sci. 1972; 55: 372-378.
2. Larson JM, Mckinney KA, Mixon BA, Burry KA, Wolf DP. An intrauterine insemination-ready cryopreservation method compared with sperm recovery after conventional freezing and post-thaw processing. Fertil Steril. 1997; 143-148.
3. Watson PF. Cooling of spermatozoa and fertility capacity. Reprod Dom Anim. 1999; 31(1): 135-40.
4. Parks JE, Graham JK. Effects of cryopreservation procedures on sperm membranes. Theriogenology. 1992; 38(2): 209-222.
5. Quinn PJ, White IG, Cleland RW. Chemical and ultrastructural change in ram spermatozoa after washing, cold shock and freezing. J Reprod Fertil. 1965; 37: 185-549.
6. Nath J. Correlative biochemical and ultrastructural studies on the mechanism of freezing damage to ram semen. Cryobiology. 1972; 9(4): 240-246.
7. Aires VA, Hinsch KD, Mueller-Schloesser F, Bogner K, Mueller-Schloesser S, Hinsch E. In vitro and in vivo comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. Theriogenology. 2003; 60: 269-279.
8. Vishwanath R, Shannon P. Storage of bovine semen in liquid and frozen state. Anim Reprod Sci. 2000; 62: 23-53.
9. Andersen K, Aamdal J, Fougner JA. Intra-uterine and deep cervical insemination with frozen semen in sheep. Zuchthygiene. 1973; 8: 113-8.
10. Olafsson T. Insemination of sheep with frozen semen. Result obtained in a field trial in Norway. Zuchthygiene. 1980; 15: 50-9.
11. Marco-Jimenez F, Puchades S, Moce E, Viudes-de-Carto MP, Vicente Js, Rodrigues M. Use of powdered egg yolk vs fresh egg yolk for the cryopreservation of ovine semen. Reprod Domest Anim. 2004; 39(6): 438-441.
12. Sodrequist L, Lundeheim N, Nilson B. Assessment of fertility after using different procedure to thaw ram spermatozoa frozen in mini-straw. Reprod Dom Anim. 1999; 34: 61-6.
13. Moussa M, Matinet V, Trimeche A, Tainturier D, Anton M. Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. Theriogenology. 2002; 57: 1695-1706.
14. Amirat L, Tainturier D, Jeanneau L, Thorin C, Gerard O, Courtens JL, Anton M. Bull semen in vitro fertility after cryopreservation using egg yolk LDL: a comparison with Optidyl®, a commercial egg yolk extender. Theriogenology. 2004; 61: 895-907.
15. Gil J, Rodiguez-Irazoqui M, Lundeheim N, Soderquist L, Rodriguez-Martinez H. Fertility of ram semen frozen in Bioxcell®® and used for cervical artificial insemination. Theriogenology 2003; 59: 1157-1170.
16. Bousseau S, Brillard JP, Guienne M, Guerin B, Camus A, Lechat M. Comparison of Bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of Bovine semen frozen in egg yolk or lecithin-based diluents. Theriogenology. 1998; 50(5): 699-706.
17. van Wagtendonk-de Leeuw AM, Haring RM, Kaal-Lansbergen LM, den Daas JH. Fertility results using bovine semen cryopreserved with extenders based on egg yolk and soybean extract. Theriogenology. 2000; 54: 57-67.
18. Thun R, Hurtado H, Janett F. Comparison of Biociphos-plus and Tris-egg yolk extender for cryopreservation of bull semen. Theriogenology. 2002; 57: 1087-94.
19. Jeyendran R, Acosta V, Land S, Coulam C. Cryopreservation of human sperm in a lecithin-supplemented freezing medium. Fertil Steril. 2008; 90(4): 1263-1265.
20. Forouzanfar M, Sharafi M, Hosseini SM, Ostadhosseini S, Hajian M, Hosseini L, et al. In vitro Comparison of Egg Yolk and Soybean Lecithin-based Extenders for Cryopreservation of Ram Semen. Theriogenology. A Head of Press.
21. Revell G, Marode RA. An osmotic resistance test for bovine semen. Anim Reprod Sci. 1994; 36: 77-86.
22. Perez LJ, Valcarcel A, de Las Heras MA, Moses DF, Baldassarre H. In vitro capacitation and induction of acrosomal exocytosis in ram spermatozoa as assessed by the chlortetracycline assay. Theriogenology. 1996; 45(5): 1037-1046.
23. Shirazi A. Ostad-hosseini S, Ahmadi E, Heidari B, Shams-Esfandabadi N. In vitro developmental competence of ICSI-derived activated ovine embryo. Theriogenology. 2009; 71(2): 342-348.
24. Parish JJ, Susko-parish, JL, First NL. Effect of heparin and chondroitin sulfate on the acrosome reaction and fertility of bovine sperm in vitro. Theriogenology. 1985; 24(5): 537-549.
25. Hosseini SM, Moulavi F, Hajian M, Abedi P, Forouzanfar M, Ostad-Hosseini S, et al. Highly efficient in vitro production of bovine blastocyst in cell-free sequential synthetic oviductal fluid vs. TCM 199 Vero cell co-culture system. IJFS. 2008; 2: 66-73.
26. Fukui Y, Kohno H, Togari T, Hiwasa M, Okabe K. Fertility after artificial inseminatin using a soybean-based extenders in sheep. J Reprod Dev. 2008; 54: 286-289.
27. Waston PF. The role of lipid and protein in the protection of ram spermatozoa at 5 c by egg yolk lipoprotein. J Reprod Fertil. 1981; 2: 337-40.