Original article

Effect of Pellet Volume on Cryopreservation of Barki Ram Semen using Cold Surfaces Made from Cattle Fat

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Abstract

Preservation of semen had a major impact on sheep genetic breeding. The aim of this study was to evaluate the effect of pellet volume on semen freezability after freezing using cold surfaces made from cattle fat. A pool of three to four ejaculates were collected weekly from six rams within a period of ten weeks. Semen was diluted in egg yolk-Tris diluent and processed in 0.1, 0.2, 0.3 ml pellets. Sperm motility was evaluated after dilution, before freezing and post-thawing at 0, 1, 2 and 3-hour incubation. Viability index, acrosome integrity and leakage of intracellular enzymes of Aspartat aminotransferase (AST) and Alkaline phosphatase (AIK) were also evaluated. Spermatozoa exhibited highly significant (P>0.001) percentages of motility at 0, 1, 2 and 3 hours incubation after thawing and viability index in 0.03 ml pellet as compared to 0.2 and 0.1 ml pellets on cattle fat plate, while no significance changes between 0.2 and 0.1 ml. However there were no significant changes in the acrosome integrity and leakage of intracellular enzymes AST and AIK. In conclusion, freezing of Barki ram semen in pellet volume of 0.3 ml on surface made from cattle fat will improve the post-thawing at 0, 1, 2 and 3 hour incubation as well as Viability index.

Keywords: Ram, Semen, Pellet, Cryopreservation

Introduction

Artificial insemination (AI) is undoubtedly the management technique that has most contributed to the genetic improvement programs and ovine breeds conservation, and semen cryopreservation is an established industry used worldwide for performing AI, as it can preserve cells life un definitely (Anel *et al.* 2006).

Cryoprotectants used for freezing of sperm cells provide protection from cold shock and the other damages during freezing. However, the cryopreservation process of mammalian semen results in lower motility and morphological integrity (Watson 2000)

In developing countries, the use of AI was limited by lack of the materials used to cryopreserve spermatozoa such as straws and dry ice. The ability to cryopreserve spermatozoa using relatively inexpensive and readily available materials would permit greater use of spermatozoa from genetically superior animals. Studies using a cold surface made from cattle fat to freeze spermatozoa in pellet from ram, bull and goat semen were generally produced a very good results as using polyvinyl chloride (PVC) straws (Awad 1989; Awad *et al.* 1998; Awad and Graham 2004).

The volume of the pellet and the temperature of the freezing surface are the main factors that regulate the cooling velocity (Mazur *et al.* 1972; Mazur *et al* 1984; Fahrig 2003). Therefore, the purpose of this experiment was to determine the effect of pellet volumes of ram spermatozoa frozen on cold surface made from cattle fat on the post-thaw spermatozoa survival after thawing.

Materials and Methods

Experimental animals

Six rams of local breed (Barki) 2–4 years old were used for semen collection by artificial vagina. Each ram was scheduled for semen collection once weekly for ten weeks. Three to four ejaculates collected with at least 85% initial motility and 3×10^9 sperm cells/ml were pooled to obtain the needed suitable volume used for dilution and processing.

Semen extension

Ram semen samples were diluted in egg yolk-Tris-diluent as described by Salamon and Visser (1972). Semen samples were diluted [(1 part semen: 1 part extender for 0.1 ml pellet), (1 part semen: 2 parts extender for 0.2 ml pellet) and (1 part semen: 3 parts extender for 0.3 ml pellet)], at 30°C with a single stepwise addition of Tris-based extender. The dilution rate was calculated on the basis that each insemination dose contains about 100×10^6 a live motile sperm (Fukui *et al.* 1993). Diluted semen was cooled to 5°C over a period of 1 to 2 hours in a cold handling cabinet and kept at 5°C for another 1 to 2 hours for equilibration (Awad and Graham 2004).

Freezing in pellet

Cattle fat surfaces were prepared immediately prior to use, by melting each of the compounds and filling aluminum foil boxes (13 cm width x l4 cm length x 5 cm depth) to make a layer of 1cm depth (182 ml). The melted fat was left to reach the room temperature before use. Depressions were made on the surface of the plate

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during solidification (Awad and Graham 2004) using a small test tube.

The Cattle fat surface plate was cooled by immersing in liquid nitrogen for 15 minutes and then raised at a height of 3cm above the level of the liquid nitrogen. Volumes of 0.1, 0.2 and 0.3 ml were dropped into the depressions on the surface block. After 2 to 3 minutes, the frozen pellets were immersed in liquid nitrogen, transferred into the liquid nitrogen container and stored at -196°C.

Evaluation of frozen semen

Post thawing motility

The pellets were stored for at least 24h before thawing and evaluation were preformed. Samples were incubated in a water bath at 37°C for three hours; the percentage of progressive motile spermatozoa was recorded at 0h, 1h, 2h and 3h.

Viability index

As the semen incubated in water bath for three hours, and the motility was recorded after each hour, the viability index was calculated according to Milovanov (1962).

Acrosome integrity

The integrity of sperm acrosome was assessed by fast green (FCF) stain. Successful staining was accomplished by centrifugation (at 30°C) of 1 m1 thawed semen for three minutes at 3000 rpm, resuspended in 1 ml of a prewarmed (37°C) Tris buffered solution, centrifuged for another three minutes and resuspended again in 1ml Tris buffered solution. Subsequently, one drop of the sperm cell suspension was mixed with one drop of the fast green (FCF) stain for one minute at 37°C and then one drop of this mixture was placed on a clean microscope slide, smeared with a second slide, and air dried on a 37°C (Wells and Awa 1970). Two hundred spermatozoa were examined in each smear under oil immersion of light microscope at l000× magnification.

Leakage of intracellular enzymes

Frozen semen samples were taken immediately after thawing and transferred into a clean dry prewarmed (30°C) 5 ml conical-based glass centrifuge tubes. The thawed semen was centrifuged at 3000 rpm for three minutes and the supernatant fluid was stored at -20°C until determination of aspartate aminotransferase and Alkline phosphatase.

The Aspartate aminotransferase (AST) activity (U/L) in frozen thawed semen was measured spectrophotometerically at a wavelength of 546 nm according to the method of Schmidt and Schmidt (1963) uses a commercially available kit (Pasteur lab. Diagnostic, Giza, Egypt).The Alkaline phosphatase (AIP) activity (U/L) in frozen thawed semen was also measured spectrophotometerically at a wavelength of 495 nm (Belfield and Goldberg 1971) using a commercially available kit (BioDiagnostic, Dokki, Giza, Egypt).

Statistical Analysis

The obtained data were expressed as mean ± SEM using release 11.0.1 of statistic program windows (SPSS 2001). The effect of pellet volumes on the studied parameters was determined by analysis of variance. Differences between means were compared by Least Significance Difference (LSD) procedure.

Results

The effect of pellet volume on different studied parameters of frozen ram semen processed on cold surface made from cattle fat was presented in Table 1. The present study show no significant difference in the mean values of the percentage of sperm motility after dilution (88.50±0.76) and just before freezing (83.50±0.76) in 0.1 ml, 0.2 ml and 0.3 ml pellet. The mean values of the percentage of post-thawing motility at 0, 1, 2 and 3 hours were significantly ($P<0.001$) higher in 0.3 ml pellet size than in 0.2 ml and 0.1 ml pellet size. However, there was no significant difference between 0.1 and 0.2 ml pellet size. A same trend was observed in the viability index. Also, the viability index showed the same trend as post-thawing motility. However, the present study show no significant changes in the Percentage of acrosme integrity as well as the intracellular enzymes of AST and AIP of frozen Barki rams semen in 0.1, 0.2 and 0.3 ml pellets.

Table 1. Effect of pellet volume on the studied parameters of frozen ram semen processed on cattle fat plate (Means ± SEM)

Semen volume	Sperm motility (%)		Post thawing motility (%)				Viability index	Acrosome integrity (%)	Leakage of intracellular enzymes	
	After dilution	Before freezing	0 hours	1 hours	2 hours	3 hours			AST (U/L)	AIP (U/L)
0.1 ml	88.50 ±0.76^{a}	88.50 ±0.76^{a}	47.50 ±1.54^{a}	45.00 ±1.67^{a}	41.50 ±1.30^{a}	37.00 ±1.11^{a}	147.25 ±4.68^{a}	5.30±0.50 ^{a}	52.30±5.84^{a}	52.70±4.82^{a}
0.2 ml	88.50 ±0.76^{a}	88.50 ±0.76^{a}	50.50 ±0.50^{a}	47.00 ±0.82^{a}	42.00± 0.82^{a}	37.50 ±1.34^{a}	151.75 ±2.89^{a}	5.40±0.33 ^{a}	50.00±4.82^{a}	51.84±4.90^{a}
0.3 ml	88.50± 0.76^{a}	88.50± 0.76^{a}	60.50± 0.90^{b}	56.50± 0.76^{b}	52.50± 1.12^{b}	47.50 ±1.12^{b}	186.75 ±3.01^{b}	5.90 ±0.38 ^{a}	49.00±3.79^{a}	51.42±6.22^{a}

Within columns, mean with different small alphabetical superscripts are significantly different at least at P,0.05.

Discussion

Sperm cryopreservation has become a part of the routine procedure for assisted reproductive techniques 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 (Watson 2000). There appear to be several important factors in the freeze-thaw process that influence the post-thaw quality of the sperm. These include the packaging in which the sperm is frozen, the type of medium used, including the type and concentration of the cryoprotectant, and the freezing and thawing rates that are used (Nordstoga *et al.* 2009).

The lack of effect of pellet volume on sperm motility immediately after dilution and just before freezing (85%) were close to that reported by Khalifa (2001) in Rahmani rams (83.19%) and Badr *et al.* (2004) in Barki rams (83.00%). A slightly low value (80.00%) was reported by Essmail *et al* (2004) in Barki rams. The average values of sperm motility immediately before freezing (82%) were close to 82% reported by Khalifa (2001) in Rahmani rams and (80%) that reported by Gundogan *et al* (2003) in rams. A low value of 73% was reported by Abdel-Malak (1994) in Barki rams. While, a high value of 90% was reported by Upreti *et al*. (1996) and 94.7% reported by Lopez-Saez *et al*. (2000) in rams.

The duration of motility and other sperm characteristics during post-thawing incubation is an indication of the usability of the semen (Saacke and White 1972). The maintenance of higher motility in sperm during incubation reflects a greater possibility to survive in the female genital tract and undergo capacitation and fertilize ova (Fiser *et al.* 1991). Ram spermatozoa are very sensitive to extreme temperature that occur during freezing processes (Salamon and Maxwell 1995) leading to changes in the membrane integrity and ultrastructure of spermatozoa (Watson 1995). With respect to effect of pellet size on the freezability of ram semen, the current study showed that semen frozen in 0.3ml pellet size had significantly higher post-thawing motility after 0, 1, 2 and 3 hours as well as viability index. Lightfoot and Salamon (1969) found that pellets of larger volume resulted in better spermatozoal viability in ram. Similar results reported by Fahrig (2003) who revealed that the mean percentage of motile spermatozoa was low at pellet volumes of 10µl and 50µl, increased to an apparent maximum with pellets of a 100µl volume, and slightly decreased at a pellet volume of 200µl. While, pellet volume did not significantly alter the percentage of sperm motility in boar (Kozumplik 1978), human (Ziegler and Chapitis 1998) and striped trumpeter (Ritar and Campet 2000).

The effect of volume in the post-thawing motility might be attributed to effect of volume in the velocity of cooling, which has been found to affect sperm survival of ram (Lightfoot and Salamon 1969; Visser and Salamon1974). Cooling rate is a very important variable, and when spermatozoa are frozen as pellets on dry ice, different volumes are likely to cool at different rates. When frozen at various cooling rates, cells of various types exhibit an inverted V-shaped survival curve (Mazur *et al*.; 1972, Mazur *et al.* 1984). Mazur and his colleagues (1984) hypothesized that such a survival curve can be interpreted as resulting from the interaction of two factors oppositely dependent on cooling rate. One-factor results from the changing properties of the extracellular solution as water is removed in the form of ice. The second factor is intracellular ice formation and results from cells becoming increasingly super cooled with decreasing temperature. At high cooling rates since the cells cannot lose water quickly enough to remain in osmotic equilibrium with extracellular solution. The likelihood of intracellular ice formation increases with increasing cooling rate. The results reported herein of the effect of pellet volume, and thus cooling rate, are similar to those reported for spermatozoa of boars, rams, bulls, and humans (Duncan and Watson 1992; Fiser *et al.* 1993; Henry *et al.* 1993; Woelders *et al.* 1997).

In conclusion, the present study concluded that increase the pellet volume up to 0.3 ml will improve the fertility that indicated by increasing in the post-thawing sperm motility at 0, 1, 2, 3 hour and increase of the viability index.

References

Abdel-Malak, M. (1994). Relationship between hormonal and fertility parameters in rams. Ph. D. Thesis, Cairo University.

Anel L, Alvarez M, Martinez-Pastor F, Garcia-Macias V, Anel E and de Paz P (2006). Improvement strategies in ovine artificial insemination. *Reprod .Domast Anim.* 41: 30–42.

Awad MM. and Graham J K. (2004). A new pellet technique for cryopreserving ram and bull spermatozoa using the cold surface of cattle fat. *Anim Reprod Sci. 84*(1): 83-92.

Awad M M, Khalifa RM, El-Alamy MA, El-Keraby F, Holtz W. (1998). Post-thawing characteristics of goat semen frozen in pellets using cooled surface of paraffin wax compared two conventional methods. *Proceedings of the 10th Conference of the Egyptian Society of Animal Production, Assuit University, Egypt.* pp. 13–15.

Awad MM. (1989). Studies on freezing ram semen by two different methods. M. Sc. Thesis. Animal Production Department, Faculty of Agriculture, Ismailia, Egypt.

Badr MR, Abdel Malak MG and Shaker M H. (2004). Influence of some fatty acids and cholesterol addition to semen extender on freezability and in vitro fertilizing potentials of ram spermatozoa. *Assiut Vet Med J.* 50(101): 304-318.

Belfield A. and Goldberg DM. (1971). Revised assay for serum phenyl phosphatase activity using 4-amino-antipyrine. *Enzyme* 12(5): 561-573.

Duncan AE and Watson PF. (1992). Predictive water loss curves for ram spermatozoa during cryopreservation: comparison with experimental observations. *Cryobiology* 29(1): 95-105.

Essmail ME, Badr MR, Samira AE. (2004). Influence of primrose oil and cholesterol-3-sulfate on freezability, ultra-structure changes and in vitro fertilizing potential of ram spermatozoa. *Assiut Vet Med J.* 50(103): 168-187.

Fahrig B M. (2003). Cryopreservation by pellet freezing of epididymal and ejaculated spermatozoa from male dogs. M. Sc. Thesis. Agricultural and Mechanical College, Louisiana State University.

Fiser PS, Fairfull RW, Hansen C, Panich PL, Shrestha JN, Underhill L (1993). The effect of warming velocity on motility and acrosomal integrity of boar sperm as influenced by the rate of freezing and glycerol level. *Mol Reprod Dev*. 34(2): 190 - 195.

Fiser PS, Hansen C, Underhill L and Marcus GJ. (1991). New thermal stress test to assess the viability of cryopreserved boar sperm. *Cryobiology*. 28: 454–459.

Fukui Y, Hirai H, Honda K. and Havashi K. (1993). Lambing rates by fixed-time intrauterine insemination with frozen semen in seasonally anoestrous ewes treated with a progestogen-impregnated sponge or CIDR device. *J Reprod Dev*. 39: 1-5.

Gundogan M, Tekerli M, Ucar M and Turkmenoglu I. (2003). Effect of diluents on motility of ram sperm during storage at 5 degrees C. *Arch Androl*. 49(1): 69-75.

Henry MA, Noiles EE, Gao D, Mazur P, Critser JK. (1993). Cryopreservation of human spermatozoa. IV. The effects of cooling rate and warming rate on the maintenance of motility, plasma membrane integrity, and mitochondrial function. *Fertil Steril*. 60(5): 911-8.

Khalifa TAA. (2001). Effect of some antioxidants on viability of preserved buffalo and ram semen. Ph. D. Thesis. Faculty of Veterinary Medicine, Cairo University.

Kozumplik J. (1978). Size of pellets and individual properties of boar semen in relation to the number of motile spermatozoa following defrosting. *Vet Med (Praha)*. 23(8): 457-463.

Lightfoot RJ. and Salamon S. (1969). Freezing of ram semen by the pellet method. III. The effect of pellet volume, composition of the thawing solution, and reconcentration of the thawed semen on survival of spermatozoa. *Australian. J Biol Sci*. 22: 1561–1572.

Lopez-Saez A., Ortiz N, Gallego L and Garde JJ. (2000). Liquids storage (5 degrees C) of ram semen in different diluents. *Arch Androl*. 44(2): 155-64.

Mazur P, Leibo SP. and Chu EHY. (1972). A two-factor hypothesis of freezing injury: evidence from Chinese hamster tissue-culture cells. *Exp Cell Res*. 71: 345-355.

Mazur P, Rall WF.and Leibo SP. (1984). Kinetics of water loss and the likelihood of intracellular freezingn in mouse ova. Influnce of the method of calculating the temperature dependence of water permeability. *Cell Biophys*. 6: 197-213.

Milovanov Vk. (1962). Biology of reproduction and artificial insemination of farm animals. *Seljhozizdat, Moscow* :696.

Nordstoga AB, Soderquist L, Adnoy T and Paulenz H L. (2009). Effect of different packages and freezing/thawing protocols on fertility of ram semen. *Reprod Domest Anim*. 44(3): 527-31.

Ritar AJ and Campet M. (2000). Sperm survival during short-term storage and after cryopreservation of semen from striped trumpeter (Latris lineata). *Theriogenology* 54(3): 467-480.

Saacke RG and White JM. (1972). Semen quality tests and their relationship to fertility. *Proceedings of the 4th NAAB Technology Conference on Artificial Insemination Reproduction*. 18-20 April, Madison, WI, National Association of Animal Breeders, Columbia, MO.

Salamon S and Maxwell WMC. (1995). Frozen storage of ram semen I. Processing, freezing, thawing and fertility after cervical insemination. *Anim Reprod Sci*. 37(3): 185-249.

Salamon S and Visser D. (1972). Effect of composition of Tris-based diluent and of thawing solution on survival of ram spermatozoa frozen by pellet method. *Aust J Biol Sci*. 25: 605-618.

Schmidt E and Schmidt FW. (1963). Determination of serum GOT and GPT. *Enzym Biol Clin*. 3: 1-3.

SPSS (2001). SPSS for Windows, Release 11.0. 1. SPSS Inc.

Upreti GC, Payne SR, Duganzich DM, Oliver J E. and Smith JF. (1996). Enzyme leakage during cryopreservation of ram spermatozoa. *Anim Reprod Sci*. 41: 27-36.

Visser D. and Salamon S. (1974). The effect of pellet volume, dilution rates prefreezing and at thawing and of thawing temperature on the survival and acrosome morphology of frozen ram spermatozoa. *South Afr J Anim Sci*. 4: 147–155.

Watson PF. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *J Reprod Fert.*. 7(4): 871-891.

Watson PF. (2000). The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci*. 60-61: 481-92.

Wells ME and Awa OA. (1970). New technique for assessing acrosomal characteristics of spermatozoa. *J Dairy Sci*. 53(2): 227-232.

Woelders H, Matthijs A and Engel B. (1997). Effects of trehalose and sucrose, osmolality of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. *Cryobiology* 35(2): 93-105.

Ziegler WF. and Chapitis J. (1998). Human motile sperm recovery after cryopreservation: freezing in nitrogen vapor vs. the direct plunge technique. *Primary Care Update for OB/GYNS*. 5(4): 170.