EVALUATION OF EGG YOLK-GLYCEROL COMBINATION IN TRIS, CITRATE AND PHOSPHATE EXTENDERS FOR FREEZING OF RAM SEMEN*

S.P. NEMA[1], A.J. DHAMI[2$] AND F.S. KAVANI[3]

Department of Animal Reproduction, Gynaecology and Obstetrics
College of Veterinary Science and Animal Husbandry
Sardarkrushinagar Dantiwada Agricultural University,
Sardarkrushinagar, Gujarat 385 506, India

Received : 23.08.2010 Accepted : 31.01.2011

ABSTRACT

Three buffers, viz., tris fructose citric acid egg yolk glycerol (TFCEG), phosphate buffer with glucose fructose sodium citrate potassium chloride egg yolk glycerol- a synthetic phosphate medium (Phos) and sodium citrate glucose egg yolk glycerol (SCGEG) were tested for optimum egg yolk (6, 12, 20%) - glycerol (2, 4, 6%) combination for straw freezing of Patanwadi ram semen in a 3x3x3 factorial experiment. The motility of ram spermatozoa during pre-freeze and post-thaw stages was preserved well with increasing levels of egg yolk in the medium, however, opposite was true for preservation of normal acrosome. Four per cent glycerol in all the three diluents could provide sufficient protection to sperm motility. However, this level was little detrimental for maintaining the acrosome integrity. The interaction of egg yolk with glycerol revealed that 12 or 20% egg yolk incorporation with 4% glycerol level was good enough for tris and phos buffers, however, for citrate buffer 20 % egg yolk with 4% glycerol level was optimum for freezing ram semen.

Key Words: Dilutors, Egg yolk, Glycerol, Freezability, Post-thaw recovery, Patanwadi ram, Semen.

INTRODUCTION

Vulnerability of ram spermatozoa to dilution and cold shock, sensitivity to the alteration in pH and composition of medium and less tolerance to glycerol pose practical problems in cryopreservation of ram semen leading to lower post-thaw recovery rate (Srivastava *et al.*, 1989). Composition of diluent is a very important criterion for optimum post-thaw survival of spermatozoa (Mathur *et al.*, 1991; Sanchez-Partida *et al.*, 1998). Studies on comparative efficacy of different dilutors and interaction of egg yolk and glycerol levels for straw freezing of ram semen are limited (Gil *et al.*, 2003; Sonmez and Demirci, 2004). Egg yolk and glycerol have been an integral part of dilutor for cryopreservation as they have capability to alter osmolarity of the diluent and have a definite cryoprotective effect (Londhe *et al.*, 2005), however their optimum concentration interplay a decisive role for better recovery at various freezing regimes.

Hence, the present study was attempted to evaluate the best combination of egg yolk and glycerol level in three basic diluents for straw cryopreservation of Patanwadi ram semen.

MATERIALS AND METHODS

This study was conducted on semen of five mature Patanwadi rams maintained at AICRP on sheep breeding, Gujarat Agricultural University, Sardarkrushinagar, North Gujarat. The rams were maintained under uniform nutritional and managerial regime in semi-intensive system. Semen collections were taken at weekly interval from each ram in separate AVs with collection cups. Immediately after collection, the cups were kept at 37°C in a water-bath, samples were evaluated through routine macro-microscopic quality tests and diluted with following extender formulations.

*Part of Ph D thesis of first author, approved by Gujarat Agrl University, Sardarkrushinagar - 385 506.

1. Professor, Department of Gynaecology and Obstetrics, Veterinary College, Mhow, Madhya Pradesh.
2. $Corresponding author: Dr. A.J. Dhami, Prof. & Head, E-mail: dhami_1659@yahoo.com.
3. Retd. Professor and Head, Department Gynaecology and Obstetrics, Veterinary College, Anand 388 001.

The best three basic extenders, viz. (i) Tris fructose citric acid egg yolk glycerol -TFCEG (Kandasamy *et al.*, 1989), (ii) Phosphate buffer with glucose fructose sodium citrate potassium chloride egg yolk glycerol (Phosphate-synthetic medium) - Phos (Watson and Martin, 1975), and (iii) Sodium citrate glucose egg yolk glycerol -SCGEG (Salamon and Lightfoot, 1969), selected based on primary screening of 13 extenders, were used with 6, 12 and 20 per cent egg yolk each in combination with 2, 4 and 6 per cent glycerol level, in a 3x3x3 factorial experiment.

Split-ejaculates after first step dilution (1:3) at 37°C temperature were pooled from five rams and cooled to 5°C over 1.5 hr; in the second step of dilution, equal volume of first step diluted pre-cooled semen and glycerolated buffer (at 3-5°C) was mixed and then equilibrated for 4 hrs in the refrigerator. French medium straws of different colour markings were then filled and sealed with PVA powder. The straws were frozen in liquid nitrogen vapour in a thermocol box using standard freezing protocol, as recommended by Sahni and Mohan (1988). Three freezing trials of each dilutor combination were undertaken to find out the best one. The straws were thawed at 37°C for 15 seconds in a water-bath after 24 hr of freezing. Pre-freeze as well as post-thaw motility and acrosome score (Barth and Oko, 1989) were recorded. The data were analyzed statistically using 3 factors factorial randomized block design.

RESULTS AND DISCUSSION

The results of the study revealed that the pre-freeze and post-thaw motility were maximum when 12 per cent egg yolk was incorporated in TFCEG diluent (47.78 ± 2.78 and 35.00 ± 2.34%, respectively). It was significantly ($P<0.01$) higher than that observed with 6 per cent egg yolk level; however, the motility did not differ significantly between 12 and 20 per cent egg yolk level. The pre-freeze and post-thaw acrosomal scores were significantly ($P<0.01$) lower (14.58 ± 0.33 and 18.15 ± 0.69%) when 6 per cent egg yolk was used as compared to 12 and 20 per cent egg yolk levels

Amongst the three levels of glycerol (2, 4 and 6%) studied in TFCEG diluent, the pre-freeze and post-thaw motility were significantly higher (50.00 ± 2.89 and 36.67 ± 2.20 %, respectively) with 4 per cent glycerol. The acrosomal scores were lower (15.17 ± 0.53 and 18.73 ± 0.75%) with 2 per cent glycerol than with other levels. While studying the interaction of egg yolk and glycerol levels over the seminal attributes, it was found that 4 per cent glycerol in combination of 12 or 20 per cent egg yolk had the maximum and equal pre-freeze and post-thaw motility (55.00 ± 2.89 and 40.00 ± 2.89%, respectively). The motility was significantly ($P<0.05$) higher than the other combinations. The effect of egg yolk-glycerol interaction over the pre-freeze and post-thaw acrosomal score was non-significant

In phosphate buffer, maximum pre-freeze and post-thaw motility (39.44 ± 1.94 and 27.22 ± 1.69%, respectively) were recorded at 20 per cent egg yolk level. Variations in pre-freeze motility between the three egg yolk levels were non-significant. However, the post-thaw motility varied significantly ($P < 0.05$). The pre-freeze acrosomal score was significantly lower with 6 per cent egg yolk level than with 20 per cent egg yolk. The minimum acrosomal score in post-thawed samples was at 12 per cent egg yolk level and it was significantly lower than with 20 per cent egg yolk level .The pre-freeze and post-thaw motility were significantly higher (38.33 ± 1.67 and 26.67 ± 1.86%) at 4 per cent glycerol levels in Phos extender than with 2 per cent glycerol level. The pre-freeze and post-thaw acrosomal scores were minimum (15.73 ± 0.63 and 20.92 ± 0.74%) at 2 per cent glycerol level, however, the post-thaw acrosomal scores did not vary significantly for the three levels of glycerol.

The effect of interaction of egg yolk and glycerol over the pre-freeze and post-thaw motility were non-significant. However, the maximum values were with 20 per cent egg yolk in combination with 6 per cent glycerol. The effect of egg yolk-glycerol interaction over the pre-freeze acrosomal score was highly significant with the minimum score (14.72 %) at 6 per cent egg yolk and 2 per cent glycerol combination, whereas the post-thaw acrosomal score did not vary significantly between the different combinations. Effects of different egg yolk and glycerol levels in citrate buffer over the mean seminal attributes revealed that the maximum pre-freeze and post-thaw motility (28.89 ± 2.32 and 18.33 ± 2.64 %, respectively) were obtained when the egg yolk level was 20 per cent, whereas acrosomal scores were minimum (16.94 ± 0.56 and 21.51 ± 0.74 %) when the egg yolk level was 6 per cent. The acrosomal score at pre-freeze and post-thaw stage varied significantly ($P < 0.05$). Similarly, the pre-freeze and post-thaw motility were maximum (30.56 ± 1.94 and 20.00 ± 2.04%) when 4 per cent glycerol was incorporated in citrate buffer. The minimum acrosomal score at the corresponding stages was 17.59 ± 0.54 and 22.08 ± 0.75 per cent when the level of glycerol was 6 per cent. The effect of interaction of egg yolk and glycerol over all the seminal attributes

was non-significant in citrate buffer diluent. However, apparently the maximum pre-freeze and post-thaw motility were at 20 per cent egg yolk in combination with 4 per cent glycerol.

Dispersion of egg yolk in the buffer incorporates motility-preserving factor, but this also has some substance, which disrupts acrosome (Waston and Martin, 1975; Graham 1978). Thus, higher motility with higher egg yolk level in the dilutor is justified. The finding corroborates with the concept of Gil *et al.,* (2003) that high level of lipoprotein provides protection to spermatozoa.

Role of cryoprotectants, such as glycerol in the dilutor has been emphasized by Polge *et al.,* (1949). However, Watson and Martin (1975) suggested that the concentration of glycerol in diluent depends on several factors, such as composition of dilutor, freezing process and packaging system. Results of present study indicate that for straw cryopreservation of ram spermatozoa, 4 per cent glycerol v/v in all the three diluents was capable of providing sufficient protection of spermatozoa in terms of motility; however, it was slightly detrimental for maintaining the acrosomal integrity as compared to 2 per cent glycerol incorporation. Thus, findings of the present study are in close agreement with the reports of Sahni and Roy (1972) and Sonmez and Demirci (2004) for the different dilutors as they also found that 3-4 per cent glycerol levels were beneficial for cryopreservation of ram spermatozoa. However, Londhe *et al.,* (2005) observed comparatively higher (5-7 % v/v) levels of glycerol incorporation in the diluent to be beneficial.

Saroff and Mixner (1955) established a relationship of egg yolk and glycerol for optimum semen freezing protocol. They suggested that the egg yolk in some manner is a tied up part of the glycerol and as the levels of egg yolk component increases in the dilutor the requirement of glycerol also increases. Several other researchers (Srivastava *et al.,* 1989; Sanchez-Partida *et al.,* 1998) also reported that the protective effect of glycerol on sperm during freezing was dependent on the level of egg yolk inclusion in the diluent. Gil *et al.,* (2003) observed no beneficial effect of increasing egg yolk level above 10% in milk-based diuent and 6.4% glycerol in commercial diluent Bioexcell (IMV, France) in protecting the acrosomal integrity of ram spermatozoa during freezing.

In present study, the interaction of egg yolk with glycerol for cryopreservation of Patanwadi ram semen in straws for tris and phosphate buffers revealed that 12 or 20 per cent egg yolk incorporation with 4 per cent glycerol gave better post-thaw motility. However, for citrate buffer 20 per cent egg yolk with 4 per cent glycerol combination was preferable for post-thaw motility of spermatozoa. Salamon and Visser (1972) and Sonmez and Demirci (2004) recommended 3-5 % glycerol in tris buffer, while Londhe *et al.,* (2005) found 4-6 % glycerol as optimum for sodium citrate buffer.

Thus, it could be inferred that the motility of ram spermatozoa during pre-freeze (chilling and equilibration) and even at post-thaw stages (freeze thaw process) could be preserved better with increasing levels of egg yolk in the medium. However, opposite was true for preservation of acrosome.

ACKNOWLEDGEMENT

We thank the Officer-in-charge of AICRP on sheep, Livestock Research Station, GAU, Sardarkrushinagar for permitting us to utilize their animals and facilities, and the Dean of the College for financial support.

REFERENCES

Barth, A.D. and Oko, R.J. (1989). Preparation of semen for morphological examination. In: Abnormal Morphology of Bovine Spermatozoa. Iowa State Univ. Press, Ames, Iowa, pp.18.

Gil, J., Lundeheim, N., Soderquist, L. and Rodriguez, M.H. (2003). Influence of extenders, temperatures and addition of glycerol on post-thaw sperm parameters in ram semen. *Theriogenology,* **59:** 1241-55.

Graham, E.F. (1978). "Fundamentals of the preservation of spermatozoa". The integrity of frozen spermatozoa. National Academy of Science, Washinton, D.C., pp. 4-44.

Kandasamy, N., Iyue, M. and Ulaganathan, V. (1989). Post-thaw motility and fertility of ram spermatozoa frozen in tris diluent. *Indian J. Anim. Sci.,* **59:** 972.

Londhe, P.M., Takarkhede, R.C,, Sahatpure, S.K., Karalkar, S.V. and Kolte, A.Y. (2005). Effect of different glycerol levels in egg yolk-citrate diluent on freezability of ram semen. *Indian Vet. J.,* **82:** 634-36.

Mathur, A.K., Srivastava, R.S., Joshi, A. and Kalra, D.B. (1991). Cryopreservation of ram semen in egg yolk lactose raffinose citrate glycerol extender. *Int. J. Anim. Sci.,* **6:** 79.

Polge, C., Smith, A.U. and Parkes, A.S. (1949). Survival of spermatozoa after vitrification and dehydration at low temperature. *Nature,* **164:** 666.

Sahni, K.L. and Mohan, G. (1988). A modified method of freezing bovine semen for screening of bulls under field conditions. *Indian J. Anim. Sci.*, **58:** 1046.

Sahni, K.L. and Roy, A. (1972). A study on the effect of deep freezing (-79°C) on post-thawing revival of sheep and goat spermatozoa. *Indian J. Anim. Sci.*, **42:** 102.

Salamon, S. and Lightfoot, R.J. (1969). Freezing ram spermatozoa by the pellet method. I. Effect of diluent composition in survival of spermatozoa. *Aust. J. Biol. Sci.*, **22:** 1527.

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

Sanchez-Partida, L.G., Setcheli, B.P. and Maxwell, W.M.C. (1998). Effect of compatible solutes and diluents composition on the post-thaw motility of ram spermatozoa. *Reprod. Fert. Develop.*, **10:** 347-57.

Saroff, J. and Mixner, J.P. (1955). The relationship of egg yolk and glycerol content of dilutors and glycerol equilibration time to survival of bull spermatozoa after low temperature freezing. *J. Dairy Sci.*, **38:** 292.

Sonmez, M. and Demirci, E. (2004). The effect of ascorbic acid on the freezability of ram semen with extender containing different proportions of glycerol. *Turk Veterinerlik ve Hayyancilik Dergisi* 28: 893-99 (cited from *Anim. Breed.Abstr.*, **73:** 2887).

Srivastava, R.S., Mathur, A.K., Kalra, D.B. and Rawat, P.S. (1989). Cryopreservation of ram semen in straws. *Indian J. Anim. Sci.*, **59:** 970.

Watson, P.F. and Martin, I.C.A. (1975). Effect of egg yolk, glycerol and the freezing rate on the viability and acrosomal structures of frozen ram spermatozoa. *Aust. J. Biol. Sci.*, **28:** 153.