



# Comparative Effect of Different Concentrations of Glycerol and Ethylene Glycol and Temperature on Cryopreservation of Ram Semen

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**Abstract** | To find the suitable cryoprotectants for cryopreservation of ram semen, two cryoprotectants ethylene glycol and glycerol were added to semen at different concentrations and glycerol (5%) was added at different temperatures to Magra ram semen. Ejaculates (n=36) collected from adult Magra rams (n=6) during the breeding season, were subjected to routine evaluation of seminal parameters and were cryopreserved by adding either glycerol (5%) added extender at two temperatures of semen (either 5°C or 30°C) or adding either glycerol or ethylene glycol at different concentrations (2%, 3%, 4%, 5%, 6% or 7%). The post-thaw motility of cryopreserved semen was evaluated 7 days later by thawing at 37°C for 30 seconds. The overall mean sperm concentration, initial individual motility and live percentage of spermatozoa observed were  $2999.33 \pm 3.51$  (million/ml),  $80.05 \pm 0.20$  % and  $85.30 \pm 0.22$  %, respectively. The post-thaw live percentage of spermatozoa, in semen was significantly higher (P 0.01) when the glycerol added extender was mixed at 30°C compared to cooled semen at 5°C. The post-thaw motility of Magra ram semen was highest  $50 \pm 0.74$  % at 6% glycerol concentration whereas it was  $44.18 \pm 1.17$  % in ethylene glycol at 2%. At concentrations of 3% to 7% addition of glycerol resulted in significantly higher post-thaw motility of semen compared to ethylene glycol. It was concluded that it is better to supplement glycerol added (6%) extenders in ram semen at 30°C and ethylene glycol above 2% results in a reduction of post-thaw motility and percent live sperms.

**Keywords** | Magra, Ram, Semen, Glycerol, Ethylene glycol.

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## INTRODUCTION

Artificial insemination (AI) with frozen-thawed semen is not as common in sheep as it is in other domestic species (Kumar and Naqvi, 2014). Many variables do exist in the cryopreservation of ram semen that cryopreserves poorly (Salmon and Maxwell, 2000). There has not been much improvement in the fertility of frozen ram semen that is attributable to the suboptimal development of diluents and cryo-protectants. A wide variety of diluents have been experimented with moderate success (Salamon and Maxwell, 2000). It appears that research effort should be directed at finding new cryoprotective diluents, but directing attention particularly to the preservation of motility

and viability of the spermatozoa (Alvarez et al., 2019). Although addition of glycerol has been suggested as a cryoprotectant for ram semen (Kukovics et al., 2011) the optimum concentration and the temperature of semen at which glycerol should be added to ram semen are sparsely studied, and ethylene glycol addition to ram semen has been poorly reported. The level of glycerol included in diluents for frozen storage of ram semen is ultimately limited by its toxicity (Fahy, 1986), which in turn depends on cooling and freezing rate, diluent composition, and method of addition of glycerol. The present study examined the addition of glycerol (5%) at 5°C or 30°C and different concentrations of glycerol and ethylene glycol on post-thaw motility of ram semen.

**Table 1:** Comparison of post-thaw motility of ram spermatozoa between glycerol and ethylene glycol as cryoprotectant at different percentage levels.

| Cryoprotectant  | Post-thaw motility                         |                            |                            |                            |                           |                           |                            |
|-----------------|--|----------------------------|----------------------------|----------------------------|---------------------------|---------------------------|----------------------------|
|                 | Glycerol and ethylene glycol concentration |                            |                            |                            |                           |                           |                            |
|                 |  | 2 %                        | 3 %                        | 4 %                        | 5 %                       | 6 %                       | 7 %                        |
| Glycerol        | Mean±SE                                    | 44.83 ± 0.628 <sup>A</sup> | 44.76 ± 0.528 <sup>A</sup> | 46.1 ± 1.049 <sup>A</sup>  | 45.36 ± 0.80 <sup>A</sup> | 50.0 ± 0.742 <sup>A</sup> | 46.25 ± 0.966 <sup>A</sup> |
| Ethylene glycol | Mean±SE                                    | 44.183± 0.711 <sup>A</sup> | 41.850± 0.999 <sup>B</sup> | 39.96 ± 0.707 <sup>B</sup> | 38.7 ± 0.646 <sup>B</sup> | 37.3 ± 1.108 <sup>B</sup> | 33.816± 1.037 <sup>B</sup> |

Means with different superscripts differ significantly (P < 0.05).

**Table 2:** Comparison of post-thaw live percentage of ram spermatozoa between glycerol and ethylene glycol as cryoprotectant at different percentage level

| Cryo-protectant | Post-thaw live percentage of spermatozoa   |                            |                            |                           |                            |                            |                            |
|-----------------|--|----------------------------|----------------------------|---------------------------|----------------------------|----------------------------|----------------------------|
|                 | Glycerol and ethylene glycol concentration |                            |                            |                           |                            |                            |                            |
|                 |  | 2 %                        | 3 %                        | 4 %                       | 5 %                        | 6 %                        | 7 %                        |
| Glycerol        | Mean±SE                                    | 55.166± 0.344 <sup>A</sup> | 55.083± 0.680 <sup>A</sup> | 56.73 ± 1.00 <sup>A</sup> | 57.833± 1.258 <sup>A</sup> | 61.0 ± 0.979 <sup>A</sup>  | 57.183± 0.377 <sup>A</sup> |
| Ethylene glycol | Mean±SE                                    | 56.266± 1.47 <sup>A</sup>  | 52.816± 1.307 <sup>B</sup> | 51.6 ± 0.744 <sup>B</sup> | 50.150± 0.670 <sup>B</sup> | 48.716± 0.890 <sup>B</sup> | 47.133± 0.751 <sup>B</sup> |

Means with different superscripts differ significantly (P < 0.05).

## MATERIALS AND METHODS

### SEMEN COLLECTION AND DILUTION

Semen was collected from Magra rams (n=6) managed under similar management conditions at the Department of Livestock Production and Management of our Institute during the months of August-September. Six ejaculates were collected from each ram by allowing the ram to mount on an estrus ewe and using the artificial vagina described previously (Kukovics et al., 2011). Immediately after collection the ejaculates were submitted to the laboratory and kept in warm water maintained at 37°C. A part of the ejaculates was taken for the fresh semen evaluation parameters including sperm concentration, individual sperm motility and percent live sperms (Purohit, 2001; Kukovics et al., 2011) and the remaining part of ejaculates were immediately put to dilution and processing. The minimum criteria were fixed as average concentration 2000 to 3,000 million/ml, >70 percent initial motility and < 20 percent dead spermatozoa. If the ejaculates did not meet the above criterion the sample was discarded and collected again on a subsequent day. The ejaculates were diluted with Tris-glucose diluents as suggested previously (Kukovics et al., 2011).

Using split sample ejaculate technique, the ejaculates were diluted with three types of semen extenders. One for evaluation of semen when glycerol added (5%) diluents were mixed at 30°C and 5°C, in the other two aliquots either glycerol or ethylene glycol was added at concentrations of 2%, 3%, 4%, 5%, 6% or 7% at 5°C (Saxena and Tripathi, 1984). The dilution of the ejaculates was done in such a way so as to maintain the level of sperm concentration of

50 million sperms per ml.

### SEMEN CRYOPRESERVATION

Semen cryopreservation was done as per methods described previously (Kukovics et al., 2011). Briefly, diluted semen was cooled in the cold handling cabinet at the rate of 1°C per 3 minutes from ambient temperature to 5°C within 45 to 60 minutes. Cooled semen was kept at this temperature for 4 hours for equilibration. The Filling of straws was done by the manual method.

In a thermo-cool box, the semen straw racks were placed and liquid nitrogen was added after placing the equilibrated straws at 5°C in a horizontal position inside the straw rack 4 to 5 cm above the level of liquid nitrogen. The straws were kept for 10 minutes in the liquid nitrogen and then plunged into the goblet of a liquid nitrogen jar.

### EVALUATION OF POST-THAW MOTILITY

After 7 days of frozen storage, the frozen semen straws were thawed in a water bath at 37°C for 30 seconds and the post-thaw motility was evaluated under a microscope.

### STATISTICAL ANALYSIS

The obtained data were analyzed statistically using standard statistical procedures mentioned previously (Snedecor and Cochran, 1994).

## RESULTS

The overall mean sperm concentration, initial individual motility and live percentage of spermatozoa observed were 2999.33 ± 3.51 (million/ml), 80.05 ± 0.20 % and 85.30 ±

0.22 %, respectively.

### EFFECT OF DIFFERENT TEMPERATURE FOR ADDING GLYCEROL

The addition of glycerol (5%) at 30°C resulted in a significantly higher ( $P < 0.05$ ) mean post-thaw motility of  $48.72 \pm 0.43\%$  compared to  $30.76 \pm 0.26\%$  when glycerol was added at 5°C. Similarly, the post-thaw live percent of sperms was significantly higher ( $P < 0.05$ ) in thawed semen to which glycerol was added at 30°C ( $53.01 \pm 0.23\%$ ) compared to glycerol added at 5°C ( $38.66 \pm 0.54\%$ ).

### EFFECT OF DIFFERENT CONCENTRATIONS OF GLYCEROL AND ETHYLENE GLYCOL

The addition of 2%, 3%, 4%, 5%, 6% or 7% of glycerol at 5°C as a cryoprotectant had little effect on the post-thaw motility of cryopreserved ram semen, however; 6% glycerol resulted in the optimum post-thaw motility (Table 1). In comparison to glycerol, ethylene glycol resulted in significant ( $P < 0.05$ ) decrease in post-thaw motility of sperms beyond the 2% level of addition. At all concentrations tested except 2% glycerol resulted in significantly higher post-thaw motility of cryopreserved ram spermatozoa.

Similarly, addition of glycerol at 2%, 3%, 4%, 5%, 6% and 7% resulted in non-significantly higher increase in post-thaw percent of live sperms up to 6% beyond which the post-thaw live sperm percent decreased (at 7%) (Table 2). Ethylene glycol addition resulted in highest post-thaw percent live sperms at 2% only beyond which the post-thaw live sperm percent decreased sequentially with increasing concentrations of ethylene glycol. Glycerol addition resulted in significantly higher post-thaw live sperm percent compared to ethylene glycol at all concentrations tested.

## DISCUSSION

In the present investigation, the overall mean sperm concentrations observed were  $2999.33 \pm 3.51$  million/ml and were similar to previous reports (Bhosrekar et al., 1994; Ibrahim, 1997; Azawi and Ismaeel, 2012). The sperm concentration was observed to be affected by a variety of factors like season, method of collection (Tiwari and Sahni, 1975; Dabas et al., 1997). The overall mean  $\pm$  SE values of individual sperm motility observed in the present study were similar to previous reports on ram semen (Tiwari et al., 1972; Tejaswi et al., 2016). However, the findings of Srivastava et al. (1973) revealed a higher percentage of motility of spermatozoa (86.6 %) in Marwari sheep. In the present investigation the mean  $\pm$  SE values of live percentage of spermatozoa observed were  $85.30 \pm 0.22$  per cent lying within the range  $82 \pm 0.37$  percent as per reported previously (Soltanpour et al., 2014; Tejaswi et al., 2016).

However, other workers reported a slightly lower live percentage of live spermatozoa, 81.33 percent (Srivastava et al., 1973) and 81.39 per cent, (Tiwari et al. 1972).

The post-thaw motility and live sperm percentage of ram semen was observed higher when glycerol was mixed at 30°C compare to 5°C which was also observed by other workers (Salamon, 1976; Kukovics et al., 2011). Salmon (1976) and Evans and Maxwell (1987) suggested that the addition of glycerol at 30°C is a practical and widely used method for dilution of ram semen for frozen storage. The findings of above investigations are well in accordance with the present study. However, other workers suggest that addition of glycerolated diluent at 4-5°C was more suitable than 30°C (Colas, 1975; Graham et al., 1978; Neubert and Menger, 1981; Fiser and Fairfull, 1989), while some other workers believed that there is no difference to addition of glycerolated diluent either at 32°C or 3°C or 30°C or 5°C (Mattos et al., 1982).

Temperature change induces stress on spermatozoa membranes. It is probable that these are related to phase change in lipids and altered functional state of membranes. Cold shock is then seen merely as the extreme state of a continuum of stress, influenced by the rate of onset of the phenomenon. Such stresses on the membrane may be continued below 0°C since phase changes are not complete at 0°C (Watson, 2000). However, it is well known that a major phase change occurs in the vicinity of 5-15°C (Drobnis et al., 1993), and this may well be the prime temperature range for temperature dependent injury.

The post-thaw motility and live percentage of ram semen were observed to be highest when glycerol was mixed at 6 % concentration level which was also observed by other workers (Farshad et al., 2009; Silva et al., 2012). Graham et al. (1978) have previously reported that glycerol levels above 6 % were detrimental to post-thaw revival of spermatozoa. The addition and removal of cryoprotectant in molar proportions result in a substantial but transient osmotic stress to the plasma membrane of spermatozoa, depending upon the relative permeability of the cryoprotectant (Gao et al., 1993).

While glycerol offers cryoprotection to spermatozoa, it may also cause structural damage during pre-freeze processing. Consequently, it was suggested that glycerol should be added no more than 20-30 min. before freezing. Effective cryoprotection after short contact (5-10 s) with glycerol has been demonstrated for bull and boar and also for ram semen (0-5 min.), which support the view that penetration of glycerol into the cell is not essential for protection. Removal of glycerol from thawed semen by centrifugation or by dialysis did not affect on lambing, (Salamon and Maxwell, 2000). The level of glycerol included in diluent for

frozen storage of ram semen is ultimately limited by its toxicity (Fahy, 1986), which in turn depends upon cooling and freezing rate, diluent composition and method of addition of glycerol.

Contrary to the above findings some workers also get successes in obtaining fertility with ram semen frozen without glycerol have been reported by Lopatko (1976) and Abdelhakeam et al. (1991) who claimed 26% and 52% lambing rates respectively.

In the present investigation the maximum post-thaw motility and live percentage of ram sperms were observed when ethylene glycol was mixed at 2% concentration level as also observed in a previous study (Silva et al., 2012). The action of glycerol on ram and bull spermatozoa seems to be different, as it more easily penetrates ram spermatozoa (Nauk et al., 1970) which was also support to the present findings that show higher survival of post-thaw spermatozoa with 5 % glycerol compared to 5 % ethylene glycol.

## CONCLUSION

It was concluded that it is better to supplement glycerol added (6%) extenders in ram semen at 30°C and ethylene glycol above 2% results in reduction of post-thaw motility and percent live sperms.

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## CONFLICT OF INTEREST

The authors have no conflicts of interest.

## AUTHORS CONTRIBUTION

The first author conducted the research for his MVSc under the guidance of second (P K Pareek) and fourth author and the third author (Amit Kumar) prepared this manuscript.

## REFERENCES

- Abdelhakeam AA, Graham EF, Vazquez IA (1991). Studies on the presence and absence of glycerol in unfrozen and frozen semen: Fertility trials and the effect of dilution method of freezing ram semen in the absence of glycerol. *Cryobiol.* 28:36-42. [https://doi.org/10.1016/0011-2240\(91\)90005-9](https://doi.org/10.1016/0011-2240(91)90005-9)
- Alvarez M, Anel-Lopez L, Boixo JL, Chamorro C, Neila-Montero M, Montes-Garrido R, dePaz P, Anel L (2019). Challenges in sheep artificial insemination: A particular

insight. *Reprod. Domest. Anim.* 54 (Suppl 4):32-40. <https://doi.org/10.1111/rda.13523>

- Azawi OI, Ismaeel MA (2012). Effects of seasons on some semen parameters and bacterial contamination of awassi ram semen. *Reprod. Dom. Anim.* 47(3):403. <https://doi.org/10.1111/j.1439-0531.2011.01888.x>
- Bhosrekar MR, Ghalsassi PM, Purohit JR, Mangurkar BR (1994). A note in deep freezing of ram semen. *Indian. J. Agr. Res.* 15(2):157-158.
- Colas G (1975). Effect of initial freezing temperature, addition of glycerol and dilution on the survival and fertilizing ability of deep-frozen ram semen. *J. Reprod. Fertil.* 42:277-285. <https://doi.org/10.1530/jrf.0.0420277>
- Dabas SD, Suthar BN, Kavani FS (1997). Seasonal variations in seminal characteristics of patanwadi ram. *Indian. J. Agr. Res.* 18(1):70-72.
- Drobnis EZ, Crowe LM, Berger T, Anchordoguy T, Overstreet JW, Crowe JH (1993). Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. *J. Exp.* 2001. 265: 432-437. <https://doi.org/10.1002/jez.1402650413>
- Evans G, Maxwell WMC (1987). *Salamon's Artificial Insemination of Sheep and Goats.* Butterworths, Sydney, pp 1-208.
- Fahy GM (1986). The relevance of cryoprotectant toxicity to cryobiology. *Cryobiol.* 23:1-13. [https://doi.org/10.1016/0011-2240\(86\)90013-1](https://doi.org/10.1016/0011-2240(86)90013-1)
- Farshad A, Khalili B, Fazeli P (2009). The Effect of Different Concentrations of Glycerol and DMSO on Viability of Markhoz Goat Spermatozoa During Different Freezing Temperatures Steps. *Pak. J. Biol. Sci.* 12: 239-245. <https://doi.org/10.3923/pjbs.2009.239.245>
- Fiser PS, Fairfull RW (1989). The effect of glycerol-related osmotic changes on post-thaw motility and acrosomal integrity of ram spermatozoa. *Cryobiol.* 26:64-69. [https://doi.org/10.1016/0011-2240\(89\)90033-3](https://doi.org/10.1016/0011-2240(89)90033-3)
- Gao GY, Ash Worth E, Watson PF, Kleinhans FW, Mazur P, Critser JK (1993). Hyperosmotic tolerance of human spermatozoa. Separate effect of glycerol, sodium chloride and sucrose on spermolysis. *Biol. Reprod.* 49:112-123. <https://doi.org/10.1095/biolreprod49.1.112>
- Graham EF, Crabo BG, Pace MM (1978). Current status of semen preservation in the ram, boar and stallion. *J. Anim. Sci.* 47 (Suppl. 2):80-119.
- Ibrahim SA (1997). Seasonal variations in semen quality of local and crossbred rams raised in the United Arab Emirates. *Anim. Reprod. Sci.* 49:161-167. [https://doi.org/10.1016/S0378-4320\(97\)00063-8](https://doi.org/10.1016/S0378-4320(97)00063-8)
- Kukovics S, Gyoker E, Nemeth T, Gergatz E (2011). Artificial insemination of sheep-possibilities, realities and techniques at the farm level. In: Milad Minafi (Ed) *Artificial insemination in farm animals.* In Tech Open. <https://doi.org/10.5772/16642>
- Kumar D, Naqvi SMK (2014). Effect of time and depth of insemination on fertility of Bharat Merino sheep inseminated trans-cervical with frozen-thawed semen. *J. Anim. Sci. Technol.* 56(8):1-8. <https://doi.org/10.1186/2055-0391-56-8>
- Lopatko MI (1976). A method of freezing animal semen in media without glycerol and egg yolk. *Proc. 8<sup>th</sup> Int. Congr. Anim. Reprod. A.I., Crakow* 4: 830-833.
- Mattos R, Günzel AR, Neves J (1982). Einfluss verschiedener kryobiologischer Faktoren auf die Qualität and

- Befruchtungsfähigkeit von tiefgefrorenem Schafesperma. In: Physiologie und Pathologie der Fortpflanzung. Verhandlungsbericht VII. Veterinär-Humanmedizinische Tagung, Gissen. Wissenschaftlicher Dienst Alete, Munich, pp. 179-182.
- Nauk VA, Lansberg ES, Sherikin VN (1970). Cryogenic changes in ram spermatozoa. *Ovtsevodstvo*. 10:25-27. (In Russian).
  - Neubert L Menger H (1981). Problems der Tieftemperaturkonservierung von Schafbockspermien. I. Mitteilung : Der Einfluss verschiedener Kryoprotektiva auf die Spermien. *Arch. Exp. Veterinaermed.* 35:51-56.
  - Purohit GN (2001). In: Assisted Reproductive Technologies in Farm Animals – A Manual. Kanta Publisher, Bikaner, India, Pp. 10-44.
  - Salamon S, Maxwell WMC (2000). Storage of ram semen. *Anim. Reprod. Sci.* 62:77-111. [https://doi.org/10.1016/S0378-4320\(00\)00155-X](https://doi.org/10.1016/S0378-4320(00)00155-X)
  - Salamon S (1976). Fertility of ram and boar semen after long-term storage. *Proc. 8<sup>th</sup> Int. Cong. Anim. Reprod. A.I.*, 23-27 August 1976, Cracaw, pp. 1069-1070.
  - Saxena VB, Tripathi SS (1984). Preservation of ram semen at 3<sup>o</sup> - 5<sup>o</sup> C. *Indian J. Anim. Sci.* 54(8): 813-815.
  - Silva ECB, Cajueiro FDP, Silva S, Vidal H, Guerra MMP (2012). In vitro evaluation of ram sperm frozen with glycerol, ethylene glycol or acetamide. *Anim. Reprod. Sci.* 132(3-4):155-158. <https://doi.org/10.1016/j.anireprosci.2012.05.014>
  - Soltanpour F, Moghaddam G, Asadpour R, Abbas RS (2014). Effect of antioxidant combinations on sperm quality of cross breed rams during liquid storage. *International J. Adv. Biol. Biomed. Res.* 2(3):732-740.
  - Snedecor and Cochran (1994). *Statistical methods*. The Iowa state University press, Ames, Iowa, USA.
  - Srivastava AK, Tiwari SB, Sahni KL (1973). Studies on biochemical changes in ram semen during storage and preservation. *Annual Report (C.S.W.R.I., Avikanagar, Rajasthan)* pp. 49-54.
  - Tejaswi V, Narayanaswamy M and Govardhana Sagar N (2016). Studies on some fresh semen characteristics in rams of Indian twinning breed – The Nari suvarna during winter months (Short Photoperiod). *International J. Sci. Environ. Technol.* 5(3): 1697-1706.
  - Tiwari SB, Sahni KL (1975). Breed difference in semen quality during different seasons. *Annual Report (C.S.W.R.I., Avikanagar, Rajasthan)* pp. 64-66.
  - Tiwari SB, Sahni KL, Rai AK (1972). Studies on ram semen with special reference to physical, biochemical and storage problem. *Annual Report (C.S.W.R.I., Avikanagar, Rajasthan)* Pp. 85-86.
  - Watson PF (2000). The causes of reduced fertility with cryopreserved semen. *Anim. Reprod. Sci.* 60-61:481-492. [https://doi.org/10.1016/S0378-4320\(00\)00099-3](https://doi.org/10.1016/S0378-4320(00)00099-3)