http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 5(6): 715-718, 2002

Determination of Glycerol Percentages for Preserving the Black Bengal Buck (*Capra hircus*) Spermatozoa for Long Time

D. Biswas, [1]F.Y. Bari, [1]M. Shamsuddin, [1]M.M. Rahman and [2]M.M. Rahman
Department of Microbiology, Chittagong Government Veterinary College, Pahartali Chittagong-4202, Bangladesh
[1]Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
[2]Department of Surgery and Medicine, Chittagong Government Veterinary College, Pahartali Chittagong-4202, Bangladesh

Abstract: The percentages of glycerol level were determined for long time buck semen preservation and the highest post-thaw motility was observed using fifty-four ejaculates, collected twice in a week from the 3 mature Black Bengal Bucks (18 from each buck). Every 6 ejaculates from each buck were preserved in liquid nitrogen with different percentages of glycerol with tris-glucose-citric acid-egg yolk media by one-step dilution method. The motility was observed in fresh semen, two hours after chilling and 24 hours after freezing. In fresh semen there was no significant variation on sperm motility either between bucks or within the buck. Two hours after chilling the sperm motility was significantly higher ($P<0.01$) in 7% glycerol added diluents than that of 5 and 10% glycerol. Similar results were also observed following 24 hours after freezing ($P<0.01$), where the highest post-thaw motility was 52%. Therefore, 7% glycerol containing media appear to be suitable for preserving the Black Bengal Buck semen for future use.

Key words: Glycerol, post-thaw motility, chilling, black Bengal buck

Introduction

Artificial insemination (AI) is the most powerful tool for livestock improvement ever available to the breeder (Roberts and Foote, 1989). Interest in the AI of goats has increased in recent years largely because of its successful use in cattle breeding programme. It is also enhanced because there has been a decline in the number of goats kept for milk and meat production. Fewer males are available for natural breeding. The male goats are castrated in earlier age (2-11 weeks) to earn more money for their quality meat. As a result availability of mature buck is really a problem for natural service. Selective breeding is necessary to up-grade this species and requires controlled breeding programme. A selective male produces enough spermatozoa, which is used for inseminating thousands of females per year and improve the genetic merits. Increased milk, meat and skin production could be achieved by the development of AI (Roberts and Foote, 1989). Spermatozoa have only a limited survival time outside the reproductive tract at ambient temperature. Researches have been carried out for preserving the buck semen in chilling condition up to 4 days (Shamsuddin and Chanda, 1998). However, it has been noted that buck semen contains phospholipase-A originated from bulbo urthral gland, that can coagulate egg-yolk containing media and hydrolyse lecithin to fatty acid and lysolecithin, the latter is toxic to spermatozoa (Ott and Memon, 1980; Evans and Maxwell, 1987). Possible alternatives to replace egg yolk or reduce it's concentration from the diluents has been used to extend buck semen (Sahni and Tewari, 1992). Even then success has been achieved by inseminating the she goat with chilled buck semen by 2.5% egg yolk (Shamsuddin *et al.*, 2000). For future breeding purpose, the chilling semen could not be used. This could be achieved by methods which reduced or arrested the metabolism of spermatozoa and thereby prolonged their fertile life. Frozen storage of spermatozoa at sub-zero temperature preserves their life for long time. Works have been conducted in several countries for long time preservation of buck semen (Roberts and Foote, 1989). However, in Bangladesh no such work has been performed yet regarding the long time preservation method of Black Bengal Buck semen. Therefore, the present study is designed to determine the suitable glycerol percentage for long time preservation of Black Bengal Buck semen, and to observe the acceptable percentage of post-thaw motility of spermatozoa.

Materials and Methods

Selection of bucks and their management: The experiment was conducted on three Black Bengal bucks at the Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh. The bucks were reared isolated from does. Age, body weight, and scrotal circumference of the bucks were 15 to 25 months, 17 to 21 kg and 20 to 27 cm, respectively.

Preparation of media: At the day of semen collection, fresh well churned egg yolk (0.250 μl) and glycerol (0.5, 0.7 or 1.0 ml) were added with the stock solution (Table 1) to make 10 ml of complete medium (Table 2). Penicillin and streptomycin sulphate was added at the rate of 1000 IU/ml and 1 mg/ml, respectively.

Table 1: The composition of stock solution for the dilution of goat semen.

Ingredients	Weight
Tris (hydroxy methyl amino methane) (g)	3.786
Glucose (g)	0.625
Citric acid (monohydrate) (g)	2.172
Glass distilled water (ml)	85.00

Table 2: Composition of 10 ml diluent to extend buck semen for freezing

Ingredients	Weight
Egg yolk (μl)	0.250
Glycerol (ml)	0.5
Penicillin (IU)	1000
Streptomycin (mg)	10
Stock solution	To make a total volume of 10 ml

The instruments were washed with tap water and then ringed in alcohol. After that the instruments were again ringed several times in distilled water. The metal and glass-made instruments were autoclaved before use.

Semen collection: The bucks were trained to ejaculate in artificial vagina (AV). The semen was collected twice a week for 7 weeks. Before collection, the prepuce of the buck was wiped clean to prevent semen contamination. During collection, the AV was held in right hand along the buck's flank. The open end of the AV was facing towards the penis and downwards at an angle of 45°. When male mounted, the erected penis was directed into the open end of the AV to permit a vigorous upward and forward

thrust which signify the occurrence of ejaculation. The buck was allowed to withdraw its penis immediately after ejaculation in the AV. The graduated collecting tube was separated from the cone and its mouth was closed with a plastic cap and labeled. Before semen collection, the diluent was prepared and was placed in the water bath at 37°C. After collection, semen was kept at 37°C in water-bath and the prepared media added with it. The individual ejaculate was evaluated for volume, mass activity, motility and sperm concentration.

Semen evaluation:

Volume: The volume of the ejaculate was measured by reading the graduated tube.

Mass activity: To evaluate the mass activity, a drop (25μl) of semen was placed on a prewarmed slide without any cover slip and examined under microscope equipped with phase-contrast optics (100X). The mass activity was scored into 5 scales: 1 = no motion, 2 = free spermatozoa moving without forming any waves, 3 = small, slow moving waves, 4 = vigorous movement with moderately rapid waves and eddies and 5 = dense, very rapidly moving waves and eddies.

Motility: A drop (10μl) of semen diluted at 1:4 ratio with tris was placed on a clean prewarmed slide (37°C) and covered with a cover slip. The motility was determined by eye-estimation of the proportion of spermatozoa moving progressively straight forward at higher magnification (400X).

Concentration: The concentration of spermatozoa (million/ml) was determined by hemocytometer. Semen samples were diluted with water (1:200) to kill the spermatozoa. A drop of diluted semen was placed on the countering chamber from the edge of pipette and spermatozoa were allowed to settle for 5-6 minutes before placing the hemocytometer on the stage of the microscope. The spermatozoa were counted in 5 large squares, each containing 16 small squares. The large squares to be counted are four at corners and one in the middle of 25 large squares. The head of the spermatozoa in the large squares were counted and recorded. The concentration of spermatozoa per ml of semen was calculated by multiplying the total number of spermatozoa in 5 large squares by 10^7 (10 million).

Method of freezing: One-step method was used to freeze the semen in this experiment. Before semen collection, the diluent was prepared and was placed in the water bath at 37°C. After collection, individual ejaculate was diluted with equal amount of diluent. One drop of semen was placed in a worm slide and the sperm motility was recorded. After 5 minutes, the semen was placed at room temperature for 1 hour. At that time another 3 parts of diluents are mixed with the semen. Then the semen was filled into the straws using micropipette. After filling, the straws were sealed by heated forceps. For equilibration the sealed straws were placed in the refrigerator at 5°C for 2 hours. The motility was recorded by using one drop of semen placed on the previously warmed slide under the microscope. The freezing was done in liquid nitrogen vapour in a ice box. The racks were placed in the liquid nitrogen box keeping a gap of 5 cm between the surface of the liquid nitrogen and the straws. After 10 minutes, the straws were transferred into the canister within the liquid nitrogen container at -196°C.

After 24 hours, thawing was done by plunging the frozen semen straws in water bath at 37°C for 12 seconds. A drop of thawed semen was placed on a previously warmed slide and sperm motility was estimated as before.

The ANOVA test was performed to obtain the difference in sperm motility after the addition of different percentages of glycerol in fresh condition, 2 hours after chilling and 24 hours after freezing within the bucks. Similar test was used to test the difference in sperm motility for significance between bucks.

Results

Fresh semen evaluation: The mean volume, mass activity and concentration of semen collected from buck I, II & III are presented in Table 3. The volume of individual buck ejaculates varied from 200.00 to 700.00μl. The volume of semen was highest in Buck II (433.0 ± 49.0); however, the differences between bucks were not significant. The mass activity varied from ±3 to ±4. There was no significant variation in mass between bucks. The sperm concentration varied from 182 to 300 X10^7/ml. Sperm concentration did not differ between Bucks.

Table 3 : Characteristics of fresh buck semen (Mean ±SE)

Buck No.	Volume (μl/ejaculate)	Mass activity (±)	Concentration (X10^7/ml)
I	372 ± 42^a	3.7 ± 0.2^a	249.5 ± 8.4^a
II	433 ± 49^a	3.8 ± 0.2^a	265.4 ± 9.7^a
III	355 ± 45^a	3.8 ± 0.2^a	239.3 ± 10.4^a

Figures within the same column having the same superscripts are not significantly different at P>0.05

Table 4: Effects of glycerol concentration on sperm motility (Mean ±SE) at different times when glycerol was added

Buck No.	Parameters	Glycerol (%) 5 (Mean ±SE)	7 (Mean ±SE)	10 (Mean ±SE)
I	Fresh semen	^{a}93.0 ± 2^x	^{a}90.0 ± 1^x	^{a}91.0 ± 2^x
II		^{a}93.0 ± 1^x	^{a}92.0 ± 1^x	^{a}93.0 ± 1^x
III		^{a}93.0 ± 1^x	^{a}91.0 ± 2^x	^{a}94.0 ± 1^x
I	2 h after chilling	^{b}78.0 ± 1^y	^{a}83.0 ± 1^y	^{b}75.0 ± 2^y
II		^{b}78.0 ± 2^y	^{a}83.0 ± 2^y	^{b}74.0 ± 2^y
III		^{b}73.0 ± 1^y	^{a}82.0 ± 2^y	^{b}74.0 ± 2^y
I	24 h after freezing	^{b}4.0 ± 1^z	^{a}51.0 ± 2^z	^{b}5.0 ± 1^z
II		^{b}4.0 ± 1^z	^{a}51.0 ± 2^z	^{b}6.0 ± 1^z
III		^{b}6.0 ± 2^z	^{a}52.0 ± 1^z	^{b}6.0 ± 1^z

Number of observations in each cell = 6, a,b = Mean ±SE in the same row and x,y,z = Mean ±SE in the same column with different superscripts differs significantly from each other (P<0.05).

Table 5: Sperm motility after treatment with different concentrations of glycerol for various period of time (data polled over buck)

Glycerol level (%)	Sperm motility (%) Fresh semen	2 hours after chilling	24 hours after freezing
5	92±0.3	76±1.00^b	5±1.00^b
7	88±1.0	83±1.00^a	51±1.00^a
10	93±1.0	74±1.00^b	7±1.00^b

Number of observations in each cell = 18, a,b = Mean ±SE in the same column with different superscript letters differ significantly from each other (P<0.01).

Motility immediately after dilution: The average sperm motility immediately after addition of different percentages of glycerol (5, 7 and 10%) two hours chilling and 24 hours after freezing in Bucks is shown in Table 4. The sperm motility varied from 80 to 95%. The sperm motility was not significant (P>0.05) either between bucks or between concentrations of glycerol added.

The mean sperm motility two hours after chilling in glycerol-containing diluent is 73-83%. The highest sperm motility was observed with the addition of 7% of glycerol (82±2 to 83±2%). The difference (P>0.01) in sperm motility between bucks after addition of equal concentration of glycerol was non significant. However, individual bucks differed when these were treated with different concentrations of glycerol (P<0.05).

The average sperm motility of frozen-thawed semen produced from different bucks varied from 4 to 52%. The highest sperm motility was observed when semen was treated with 7% glycerol (51 to 52%). Similar to chilled semen, the differences between bucks were non significant when treated with equal concentrations of glycerol. However, significant variation

($P<0.01$) in sperm motility was observed between different proportions of glycerol used for semen dilution. The 7% glycerol produce the highest motility compared with 5 and 10%.

When the data was pooled over bucks (Table 5) between different percentages of glycerol, the result was similar as within the same buck where 7% glycerol produced the higher sperm motility (83 ± 1 and 51 ± 1%) compared with 5 and 10% glycerol ($P<0.01$).

Discussion

When good quality semen is preserved from high quality animals this makes it possible to preserve semen for a prolonged period, thereby conserving genes for future use and providing insurance against loss of a particular sire. However, transportation of semen, both locally and internationally, is also facilitated, and semen can be collected and stored outside the normal breeding period. Consequently, much more widespread use of sires is achieved when semen is frozen-stored. In fresh semen, the average sperm motility after addition of diluent varied from 90 to 94%. Similar results were published elsewhere (Madrid Bury *et al.*, 1995; Gundogan, 1999). The sperm motility did not vary depending on the percentages of glycerol added in the study. This indicates that glycerol at the level of 5, 7 and 10% does not affect the spermatozoa during a short-time exposure.

In this experiment the diluent was added with the semen at room temperature and filling and sealing was also done at room temperature. Two hours after equilibration the motility of spermatozoa at pre-freezing stage declined from that of fresh semen. This result is agreeable with some other works where similar equilibrium time was used (Salamon and Ritter, 1982; Evans and Maxwell, 1987; Jansen, 1992; Mareco and Arosteguy, 1995; Shao Guezhi *et al.*, 1996 and Singh and Purbey, 1996). The drop of sperm motility before freezing might be due to the processing when spermatozoa may lack the adjustment potential at falling temperature. However, Dutta *et al.* (1996) found that 4 hours equilibrium time gave the best post thaw sperm motility in tris diluent. Therefore, it seems that a 2 - 4 hours equilibrium time is suitable for freezing good quality semen.

In this experiment the freezing time was 10 minutes in liquid nitrogen vapour, where the temperature varied from −80 to -100°C (Evans and Maxwell, 1987). The sperm motility varied from 51 to 52%. Watson (1990) recommended 50% sperm motility as a lower bench mark in the preserved semen of bull and other ruminants. Evans and Maxwell (1987) and Jennrong *et al.* (1999) also observed the similar percentage of sperm motility 24 hours after preservation. They used the similar time in liquid nitrogen vapour as we did. Sperm motility did not vary between bucks where a certain percentage of glycerol was added. This result is in agreement with Dutta *et al.* (1996).

In the present experiment, 7% glycerol produced higher post-thaw sperm motility than did 5 and 10%. The result is in agreement with some other reports published on bucks (First *et al.*, 1961a; Das and Pajkonwar, 1995). These authors also found better sperm motility with 6-8% glycerol than that with 4 and 10% glycerol. However, the present observation differ from that of Fiser *et al.* (1981), Perez-Llano and Matoes Rex (1995), where they found better sperm motility with 4% glycerol. This difference could be due to the difference in egg yolk percentage and equilibration time. Higher percentage of egg yolk is detrimental to goat spermatozoa, because goat semen contains an enzyme phospholipase-A originated from bulbo-urethral glands. When medium containing egg yolk is used for dilution of semen, the enzyme breaks down the lecithin of the egg yolk to toxic product which is detriment to spermatozoa (Evans and Maxwell, 1987).

In this study semen was collected on every third day during the non breeding and early breeding season. Most of the times volume of semen per ejaculate was between 300-500μl. However, in two occasions the volume was 200μl and in other two occasions the volume was 700μl. The mean volume was in accord with the result of Shamsuddin *et al.* (2000) within the same breed and Karatzas *et al.* (1997) in different breeds. The volume of semen in present study was lower than that of some other published works (Ibrahim, 1997; El-Ashry *et al.*, 2000). This difference could be due to seasonal variation. Semen volume of buck is larger during the breeding seasons because the sexual glands are more active during that time (Karatzas *et al.*, 1997; Karagiannidis *et al.*, 2000).

The good mass activity of fresh semen is the key factor to have the good motility of preserved semen. In this study the average mass activity varied from 3.7 to 3.8. This result is consistent with the results of other published work within the same breed and in different breed (Shamsuddin and Chanda, 1998; Bhuskat *et al.*, 2000; Shamsuddin *et al.*, 2000).

The mass activity observed in this study was lower than that reported by some other works (Karatzas *et al.*, 1997). Mass activity is the outcome of collective movement of the spermatozoa. This means both sperm concentration and motility contribute to the mass activity. It is likely that, buck ejaculate more concentrated semen during the breeding seasons.

In present study the average sperm concentration varied from 250 to 265×10^7/ml. This result is similar to that of Evans and Maxwell (1987) in goat and Debas *et al.* (1997) in ram. However, different sperm concentrations have been reported elsewhere (Karatazas *et al.*, 1997; Ibrahim, 1997; Shamsuddin *et al.*, 2000). This variation in sperm concentration could have resulted from differences in season when semen was collected.

References

Bhuskat, S.N., R.C. Takarkhede, N.S. Mangle and S.Z. Ali, 2000. Comparative study of some semen attributes of different breeds of bucks. Indian Vet. J., 77: 963-965.

Das, K.K. and C.K. Rajkonwar, 1995. Effects on the motility of buck semen during freezing with lactose-egg yolk-glycerol extender. Int. J. Anim. Sci., 10: 127-128.

Debas, S.D., B.N. Suthar and F.S. Kavani, 1997. Seasonal variation in seminal characteristics of Patanwadi ram. Ind. J. Anim. Reprod., 18: 70-72.

Dutta, S., B.B. Ghosh, S.K. Bondyopadhyay, R. Roy Choudhury, S. Basu and R. Dutta Gupta, 1996. Effect of different extenders, glycerol levels and equilibration times in deep-freezing of buck semen. Ind. J. Anim. Hlth., 35: 35-38.

El Ashry, M.A., H.M. Saleh, H.A. El-Fouly and S.O. Amin, 2000. The effect of different levels of caged layer droppings in male lambs diets on growth performance and semen characteristics. Egyptian J. Nutrition and Feeds, 3: 1-14.

Evans, G. and W.M.C. Maxwell, 1987. Salamon's artificial insemination of sheep and goats. Bulterworths, Sydney, Boston, London, Durtan, pp: 107-141.

First, N.L., H.A. Henneman, E.T. Magee and J.A. Williams, 1961a. The frozen storage of ram semen. J. Anim. Sci., 20: 74-78.

Fiser, P.S., L. Ainsworth and G. Langford, 1981. Effect of osmolarity of skim-milk diluents and thawing rate on cryosurvival of ram spermatozoa. Cryobiology, 18: 399-403.

Gundogan, M., 1999. Correlation of testes measurements with semen characteristics and blood serum testosterone levels of rams. Hayvancilik Arastirma Dergisi, 9: 49-52.

Ibrahim, SA., 1997. Seasonal variations in semen quality of local and crossbred ram raised in the United Arab Emirates. Anim. Reprod. Sci., 49: 161-167.

Jansen, H.B., 1992. The effect of the packaging temperature on bovine semen quality and fertility results. 12th International Congress on Animal Reproduction. The HAGUE, The Netherlands. August 23 rd to August 27th. Congress proceedings, pp: 1563-1565.

Jennrong, Y., H. Janchi, H. Mingchiang, J.R. Young, J.C. Huang and M.C. Hsieh, 1999. Effects of dilution and freezing process on survival rate and motility in frozen-thawed goat semen. J. Taiwan Livestock Res., 32: 267-277.

Karagiannidis, A., S. Varsakeli and G. Karatzas, 2000. Characteristics and seasonal variations in the semen of Alpine, Saanen and Damascs goat bucks born and raised in Greece. Theriogenology, 53: 1285-1293.

Karatzas, G., A. Karagiannidis, S. Varsaleli and P. Brikas, 1997. Fertility of fresh and frozen thawed goat semen during the nonbreeding season. Theriogenology, 48: 1049-1059.

Madrid Bury, N., J. Aranguren Mendez, C. Gonzalez Stagnare, R. Lopez, M. Rodriguez, J. Goicochea and G. Alfani, 1995. Semen characters in crossbred West African Dwarf rams in Venezuela during the hot season. Revista Argentina de Produccion Anim., 15: 969-970.

Mareco, G. and R.P. Arosteguy, 1995. Successful freezing of goat semen in straw. Veterinaria Argentine, 12: 306-309.

Ott, R.S. and M.A. Memon, 1980. Sheep and Goat manual. Society for Theriogenology, pp: 24-33.

Perez-Llano, B. and E. Mateos-Rex, 1995. Effect of external cryoprotectant and the percentage of glycerol on the *in vitro* quality of frozen goat semen. Investigacion-Agraria-prosuccion-Y-Sanidad-Animales, 10: 211-221.

Roberts, R.J. and R.H. Foote, 1989. Artificial insemination. In: Veterinary Obstetrics and Genital Diseases (Theriogenology). Roberts R.J. (ed.), Published by Woodstock, Vernont 05091, pp: 894-923.

Sahni, K.L. and S.B. Tewari, 1992. Caprine semen evaluation, processing and artificial insemination in India. In: Research on Goats: Indian Experiences, Bhattacharyya, N.K. and Singh, K. (eds.), Mathura (UP), Central Institute for Research on Goats, pp: 94-107.

Salamon, S. and A.J. Ritar, 1982. Deep freezing of Angora goat semen: Effects of diluent composition and method and rate of dilution on survival of spermatozoa. Australian J. Biol. Sci., 35: 295-303.

Shamsuddin, M. and P.K. Chanda, 1998. Effects of diluents and dilution rates on the preserved buck semen. 4th Asian Symposium on Animal Biotechnology (Kamiina, Japan) October 7-9, 1998 pp: 177-181.

Shamsuddin, M., Y. Amiri and M.M.U. Bhuiyan, 2000. Characteristics of buck semen with regard to ejaculate numbers, collection intervals, dilution and preservation periods. Reprod. Domestic Anim., 35: 53-57.

Shao Guezhi, Huang Meiyu, Sun Zhiran and Kong Weitangn Li Wenge, 1996. Study on the technology of deep freezing semen of down bearing goats. Chinese J. Anim. Sci., 32: 34-35.

Singh, L.P. and L.N. Purbey, 1996. Preservability of goat spermatozoa in tris and citrate extenders at −196°C and 5°C. Ind. J. Anim. Sci., 66: 1139-1141.

Watson, P.F., 1990. Artificial insemination and the preservation of semen. In: Marshall's physiology of reproduction. Vol. 2. Reproduction in the male. Lammong G.E. (ed.) 4th edition. Churchill Livingstone. Edinburgh, London, Melbourne and New York, pp: 747-869.