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EFFECT OF GLYCEROL LEVEL, PACKAGING METHOD AND THAWING RATE ON POST-THAWING MOTILITY AND ACROSOMAL INTEGRITY OF BARKI BUCK SEMEN

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SUMMARY

Thirty-six ejaculates were collected from 12 Barki bucks and diluted in a Tris-based extender at three different glycerol levels: 5, 7.5 and 10 %. Diluted semen was divided into two portions: one portion was filled in straws at room temperature (20° C), then equilibrated at 4°C for 6 hrs. The other portion equilibrated at 4° C before filling in straws. Straws were frozen at –80° C for 10 minutes. Two thawing rates were used: one of which at 40° C for 20 seconds and the other one at 65° C for 5 seconds. Thawed samples incubated at 37° C for 6 hours. Percent motility and acrosomal integrity were evaluated at 0, 2, 4 and 6 hours of incubation. Glycerol level significantly ($P<0.01$) affected post-thawing motility and acrosomal integrity at 0, 2, 4 or 6 hrs. of incubation. Five percent of glycerol was superior in improving post-thawing percent intact acrosomes, however, 5 and 7.5 % glycerol levels similarly improved post-thawing motility as compared with 10 % glycerol level. Filling straws after equilibration significantly ($P<0.01$) improved post-thawing motility and acrosomal integrity. Thawing at 65°C for 5 seconds was superior in maintaining post-thawing motility and acrosomal integrity. Significant ($P<0.01$) interaction of glycerol level and packaging method was observed. Filling straws after equilibration of semen diluted at 7.5 % glycerol level improved post-thawing acrosomal integrity and progressive motility. However, for semen diluted at 5% glycerol level, packaging before equilibration improved the two measured criteria. For cryopreservation of Barki buck's spermatozoa diluted in a Tris-based extender, it can be advised to use a glycerol level of 7.5 %, filling straws after equilibration at 4° C for 6 hours and using a thawing rate of 65° C for 5 seconds to get optimal post-thawing motility and acrosomal integrity.

Keywords: Buck, spermatozoa, glycerol; packaging, freezing, thawing, motility, acrosome

INTRODUCTION

Genetic improvement of local breeds of goats can be achieved by a continuous selection program within the local flocks in addition to crossing with foreign breeds to improve productivity of local ones. Successful freezing of buck semen and applying of AI technique help in accurate and rapid genetic improvement through selection and/or crossing programs.

It was reported that local breeds of goats have better sexual activity during autumn (Ashmawy, 1979; El-Sayed *et al.*, 1983 and Abdel-Fatah, 1990) as well as better seminal quality either fresh or after thawing (Ahmed *et al.*, 1997). On the other hand, Singh and Purbey (1996) found that the decline in acrosomal integrity of buck's spermatozoa due to freezing-thawing process was less in semen diluted in Tris diluent (29 %) than in semen diluted without Tris (52 %). Sinha *et al.* (1991) found that post-thawing motility of buck's spermatozoa diluted in a Tris-based extender was higher than that in skimmed milk or egg yolk-citrate extenders. Addition of glycerol to the Tris-based extender was proven to improve motility and live sperm counts in goat semen (Misra *et al.*, 1996). The beneficial cryoprotective role of glycerol in extender was outlined by Mazur (1980). The presence of an optimum level of glycerol minimizes occurrence of intracellular ice and exposure to high solutes during freezing and thus improve spermatozoal freezability (Rapatz, 1966).

In the present study, it was planned to investigate the optimum glycerol level, thawing rate and time of packaging straws to obtain the best buck's spermatozoal freezability and post-thawing longevity. Progressive motility and acrosomal maintenance were used to evaluate freezing-thawing spermatozoal viability

MATERIALS AND METHODS

This study was carried out, during autumn, at Maryout Research Station. One ejaculate from each of 12 healthy Barki bucks was collected at 3-days interval, using an artificial vagina. Each ejaculate was

divided into three equal parts to be diluted at 5, 7.5 and 10 % glycerol levels in a Tris-based extender (Evans and Maxwell, 1987). This was conducted to achieve that one ejaculate from each used buck was diluted once at one of the used three glycerol level. Dilution was carried out to give spermatozoa concentration of $50x10^6$ motile cells/dose (0.25ml.).

Diluted semen was divided into two portions: one of which was filled at room temperature (20°C) in 0.25 ml French straws using an automatic filling and sealing machine (MMP 133 - Minitüb Co., Germany) and then equilibrated at 4°C for 6 hrs. inside a cold handling cabinet (Minitüb Co., Germany). The other portion of diluted semen, contained in a test tube, was placed into a conical flask containing 100ml of water at 37°C and transferred into the cooling cabinet for equilibration at 4°C for 6 hrs, then filled in 0.25 ml straws.

Freezing was carried out using an electronic temperature controller semen freezer (HN - Minitüb Co., Germany) adjusted at –80°C. Straws were holded horizontally on steel racks in static liquid nitrogen vapor for 10 minutes, then plunged into liquid nitrogen for storage.

Few days later, four straws per treatment and within each ejaculate were thawed at 40°C for 20 seconds or at 65°C for 5 seconds using a temperature controller semen-thawing unit (Minitüb Co., Germany). Immediately after thawing, the contents of the four straws were pooled in 75x12mm test tubes incubated in water bath at 37°C.

Progressive motility (%) and spermatozoa with intact acrosomes (%) were estimated at 0, 2, 4 and 6 hrs of incubation. Motility evaluation was made using a phase contrast microscope equipped with a stage warmer at 37°C. Acrosomal integrity was evaluated by fixing 10 µl. of the incubated sample in 200 µl. of 0.2 % glutaraldehyde solution (Johnson *et al.*, 1976) and estimating spermatozoa with intact acrosomes by phase contrast microscopy using a 100x oil immersion objective and white light.

Data were analyzed, after arcsine square root % transformation, using Statgrph program (Version 5).

RESULTS AND DISCUSSION

The averaged values of raw seminal parameters of the used bucks (Table 1) were found to be relatively higher than that reported earlier on Egyptian baladi bucks (Hemaeda, 1972; El-Sayed *et al.*, 1983 and Abdel-Fatah, 1990). The improved seminal quality in the present study may be due to the controlled environmental and managerial conditions prevailing inside barns of males belong to the AI center.

Table 1. Raw seminal quality of Barki bucks (mean of 36 ejaculates ± S.E.)

Measured Criteria	Mean ± S.E.
Ejaculate volume (ml.)	0.9 ± 0.06
Progressive motility (%)	89.6 ± 1.44
Concentration (x 10^9 spermatozoa / ml.)	2.954 ± 0.23
Spermatozoa with intact acrosome (%)	88.5 ± 1.49
Primary abnormalities (%)	7.4 ± 0.96
Secondary abnormalities (%)	12.3 ± 0.69

The averaged values of post-thawing progressive motility (PPM %) and of spermatozoa with intact acrosome (PIA %) are presented in Tables (2) and (3). The results revealed that glycerol level significantly ($P<0.01$) affected post-thawing PPM and PIA. The overall means of PPM were 24.6, 25.4 and 22.8 % in semen diluted at 5, 7.5 and 10 % glycerol levels, respectively. The corresponding values of PIA were 43.9, 41.7 and 37.3%, respectively. These values reflected that dilution of buck's semen in the Tris-based extender at 5 or 7.5 % glycerol level significantly ($P<0.05$) improved PPM (8-11 %) and PIA (12-18 %) as compared to dilution at 10 % glycerol level. The present results are so close to that obtained by Dutta *et al.* (1996) who found that 6.4 % glycerol level was superior in maintaining higher percentage of post-thawing motility, live spermatozoa, morphologically normal spermatozoa and intact acrosomes in buck's semen diluted in a Tris-based extender as compared with 9 % glycerol level. In respect of testing freezability, the average of 0-hr. incubation values of PPM in semen diluted at glycerol levels 5, 7.5 and 10 % were 48.2, 48.6 and 44.9 %, respectively. The corresponding values of PIA were 65.5, 62.7 and 58.0 %. These results revealed that freezability of buck's spermatozoa diluted in the Tris-based extender at 5 or 7.5 % glycerol level was relatively higher than that of semen diluted at 10 % glycerol level. Prasanth and Mathai (1996) reported that maximum motility after glycerol treatment (67.85 ± 1.39 %) and freezing (42.0 ± 1.84 %) was obtained with 6 % glycerol, which is so close to the present finding.

Table 2. Effect of glycerol level, packaging method and thawing rate on post-thawing percent progressive motility of buck's spermatozoa incubated at 37° C. (Mean percent of 12 ejaculates ± S.E.)

Glycerol level (%)	Packaging Method	Thawing Rate	Incubation Time (hours)				Overall Means		
			0	2	4	6			
5	Bef. Equ.	At 40° C for 20 sec.	45.2 ± 1.42 ^{cd}	21.6 ± 1.33 ^{d}	10.0 ± 0.81 ^{d}	2.8 ± 0.39 ^{cd}	19.9 ± 2.40 ^{d}	25.3 ± 1.79 ^{a}	
	Bef. Equ.	At 65° C for 5 sec.	53.9 ± 1.13 ^{a}	35.3 ± 1.26 ^{a}	24.5 ± 1.12 ^{a}	9.2 ± 0.95 ^{a}	30.7 ± 2.44 ^{ab}		
	Aft. Equ.	At 40° C for 20 sec.	44.5 ± 1.41 ^{d}	26.8 ± 1.13 ^{c}	14.6 ± 1.12 ^{c}	3.4 ± 0.51 ^{c}	22.3 ± 2.29 ^{cd}	24.0 ± 1.66 ^{b}	24.6 ± 1.22 ^{a}
	Aft. Equ.	At 65° C for 5 sec.	49.0 ± 1.34 ^{b}	30.4 ± 1.13 ^{b}	17.6 ± 0.93 ^{b}	5.4 ± 0.63 ^{b}	25.6 ± 2.41 ^{bc}		
7.5	Bef. Equ.	At 40° C for 20 sec.	41.7 ± 1.60 ^{d}	24.8 ± 1.43 ^{d}	12.7 ± 1.41 ^{d}	2.8 ± 0.37 ^{cd}	20.5 ± 2.21 ^{d}	23.1 ± 1.67 ^{b}	
	Bef. Equ.	At 65° C for 5 sec.	50.7 ± 1.27 ^{b}	29.3 ± 1.02 ^{bc}	17.1 ± 1.01 ^{b}	5.9 ± 0.42 ^{b}	25.8 ± 2.47 ^{bc}		
	Aft. Equ.	At 40° C for 20 sec.	42.9 ± 1.88 ^{cd}	24.9 ± 2.01 ^{cd}	12.9 ± 1.52 ^{cd}	2.6 ± 0.43 ^{d}	20.8 ± 2.32 ^{cd}	27.7 ± 1.92 ^{a}	25.4 ± 1.28 ^{a}
	Aft. Equ.	At 65° C for 5 sec.	59.1 ± 1.99 ^{a}	43.0 ± 1.71 ^{a}	24.7 ± 1.41 ^{a}	11.6 ± 0.88 ^{a}	34.6 ± 2.73 ^{a}		
10	Bef. Equ.	At 40° C for 20 sec.	39.8 ± 1.75 ^{d}	22.7 ± 1.78 ^{d}	10.4 ± 1.10 ^{d}	2.2 ± 0.42 ^{d}	18.8 ± 2.17 ^{d}	21.4 ± 1.62 ^{b}	
	Bef. Equ.	At 65° C for 5 sec.	46.2 ± 1.69 ^{bc}	29.8 ± 1.56 ^{b}	14.7 ± 1.33 ^{bc}	5.3 ± 0.64 ^{b}	24.0 ± 2.36 ^{bc}		
	Aft. Equ.	At 40° C for 20 sec.	41.7 ± 1.78 ^{cd}	25.1 ± 1.68 ^{cd}	13.3 ± 1.52 ^{cd}	2.3 ± 0.48 ^{cd}	20.6 ± 2.24 ^{cd}	24.2 ± 1.71 ^{a}	22.8 ± 1.18 ^{b}
	Aft. Equ.	At 65° C for 5 sec.	52.0 ± 1.38 ^{a}	33.0 ± 1.38 ^{ab}	18.9 ± 1.31 ^{ab}	7.7 ± 0.74 ^{a}	27.9 ± 2.49 ^{ab}		

Means in the same column, in each block, with different superscripts are significantly different (P< 0.05).
Bef. Equ. Before equilibration; Aft. Equ. After equilibration.

Table 3. Effect of glycerol level, packaging method and thawing rate on post-thawing percent intact acrosomes of buck's spermatozoa incubated at 37° C. (Mean percent of 12 ejaculates ± S.E.)

Glycerol level (%)	Packaging Method	Thawing Rate	Incubation Time (hours)				Overall Means		
			0	2	4	6			
5	Bef. Equ.	At 40° C for 20 sec.	64.9 ± 1.79 ^{c}	49.7 ± 2.37 ^{e}	36.4 ± 1.94 ^{e}	21.3 ± 1.57 ^{e}	43.1 ± 2.53 ^{c}	45.4 ± 1.82 ^{a}	43.9 ± 1.29 ^{a}
	Bef. Equ.	At 65° C for 5 sec.	70.3 ± 1.65 ^{b}	54.7 ± 1.79 ^{b}	39.7 ± 1.99 ^{b}	26.0 ± 2.28 ^{b}	47.7 ± 2.57 ^{bc}		
	Aft. Equ.	At 40° C for 20 sec.	55.9 ± 1.40 ^{d}	41.3 ± 1.65 ^{d}	28.9 ± 1.32 ^{d}	17.4 ± 1.59 ^{d}	35.9 ± 2.21 ^{d}	42.4 ± 1.82 ^{b}	
	Aft. Equ.	At 65° C for 5 sec.	70.8 ± 2.09 ^{ab}	55.8 ± 2.38 ^{ab}	41.5 ± 2.40 ^{ab}	27.4 ± 2.31 ^{ab}	48.9 ± 2.60 ^{ac}		
7.5	Bef. Equ.	At 40° C for 20 sec.	53.2 ± 1.52 ^{d}	38.3 ± 1.80 ^{d}	25.2 ± 1.75 ^{d}	14.3 ± 1.10 ^{d}	32.8 ± 2.25 ^{d}	36.2 ± 1.65 ^{b}	41.7 ± 1.36 ^{b}
	Bef. Equ.	At 65° C for 5 sec.	60.9 ± 1.75 ^{bc}	45.5 ± 1.55 ^{bc}	30.6 ± 1.26 ^{bc}	20.8 ± 1.41 ^{b}	39.5 ± 2.33 ^{bc}		
	Aft. Equ.	At 40° C for 20 sec.	57.2 ± 1.11 ^{cd}	41.8 ± 1.40 ^{cd}	29.0 ± 0.87 ^{c}	16.2 ± 1.24 ^{cd}	36.1 ± 2.28 ^{cd}	47.4 ± 2.01 ^{a}	
	Aft. Equ.	At 65° C for 5 sec.	79.4 ± 1.75 ^{a}	65.5 ± 1.45 ^{a}	51.6 ± 1.57 ^{a}	38.2 ± 1.72 ^{a}	58.7 ± 2.38 ^{a}		
10	Bef. Equ.	At 40° C for 20 sec.	54.3 ± 1.39 ^{cd}	39.7 ± 1.49 ^{cd}	27.6 ± 1.23 ^{cd}	16.2 ± 1.03 ^{cd}	34.4 ± 2.16 ^{cd}	37.6 ± 1.63 ^{a}	37.3 ± 1.18 ^{c}
	Bef. Equ.	At 65° C for 5 sec.	61.8 ± 1.03 ^{b}	48.4 ± 0.97 ^{b}	33.9 ± 1.22 ^{b}	19.3 ± 0.75 ^{bc}	40.8 ± 2.37 ^{b}		
	Aft. Equ.	At 40° C for 20 sec.	51.3 ± 1.44 ^{d}	36.6 ± 2.01 ^{d}	25.0 ± 1.94 ^{d}	15.3 ± 1.47 ^{d}	32.0 ± 2.14 ^{d}	37.0 ± 1.71 ^{a}	
	Aft. Equ.	At 65° C for 5 sec.	64.4 ± 1.40 ^{ab}	48.5 ± 1.84 ^{ab}	33.4 ± 1.84 ^{ab}	21.6 ± 1.73 ^{ab}	42.0 ± 2.49 ^{d}		

Means in the same column, in each block, with different superscripts are significantly different ($P<0.05$).
Bef. Equ.: Before equilibration; Aft. Equ.: After equilibration.

Post-thawing PPM and PIA are significantly (P<0.01) affected by packaging method. The overall means of PPM were 23.3 and 25.3 % for semen filled in straws before equilibration and that filled after equilibration, respectively. The corresponding values of PIA were 39.7 and 42.3 %. Filling straws after equilibration at 4°C for 6 hrs significantly (P<0.05) improved post-thawing PPM (8 %) and PIA (7 %) as compared with filling at 20°C (before equilibration). A highly significant interaction (P<0.01) was found between glycerol level and packaging method for the two measured criteria. Filling straws after equilibration was superior in maintaining post-thawing PPM and PIA of semen diluted at 7.5 % glycerol level as compared with filling at 20°C, however, the reverse was true in semen diluted at 5 % glycerol level. The interaction of glycerol level and packaging method was of great importance. This result reflects that as glycerol level increases diluted buck's semen must be filled in straws after equilibration to achieve maximum post-thawing PPM and PIA.

Thawing rate significantly (P<0.01) affected PPM and PIA. The overall means of PPM were 20.5 and 28.1 % for the slaw and rapid thawing rates, respectively. The corresponding values of PIA were 35.7 and 47.3 %. These results revealed significant (P<0.05) improvement in PPM (37 %) and PIA (30%) of semen thawed at 65°C for 5 seconds as compared with semen thawed at 40°C for 20 seconds. These results were in agreement with previous reports on semen of bovine (Almquist and Wiggin, 1973), buffalo (Ahmed, 1984) and ram (Fiser *et al.*, 1986 and El-Bahrawy, 2000). A thorough examination of Tables 2 and 3 at 0-hr. incubation revealed the beneficial effect of using rapid thawing to improve buck's spermatozoal freezability. Thawing at 65°C for 5 second improved 0-hr. incubation values of PPM (15, 30 and 20 %) in semen diluted at 5, 7.5 and 10 % glycerol levels, respectively, as compared with thawing at 40°C for 20 seconds. The corresponding values of PIA were 17, 27 and 20 %. The previously mentioned values of improvement in PPM and PIA revealed that dilution of buck's semen at 7.5 % glycerol level and thawing at 65°C for 5 seconds was superior in improving buck's spermatozoal freezability.

In respect of livability of buck's spermatozoa during the incubation period, a thorough examination of results in Tables (2) and (3) revealed that the least decline in the post-thawing values of the two measured criteria was reported when semen was diluted at 7.5 % glycerol level, filled in straws after equilibration at 4°C for 6 hrs. and thawed at 65°C for 5 seconds. The percent decline in PPM at 2, 4 and 6 hrs. of incubation was found to be 27,58 and 80, respectively, as compared to its value at 0-hour of incubation. The corresponding values for PIA were found to be 18, 35 and 52. These results may reflect the beneficial combination of dilution at 7.5% glycerol level (as cryoprotective agent) and using rapid thawing rate in improving buck's spermatozoa survival during the 37°C incubation.

In conclusion, the present study reflected that, for cryopreservation of Barki buck's spermatozoa for using in AI programs, it can be advised to dilute semen in a Tris-based extender at 7.5 % glycerol level, filling straws after equilibration at 4°C for 6 hrs., freezing at –80°C for 10 minutes and thawing at 65°C for 5 seconds. This sequence of processing Barki buck's semen proved to improve spermatozoa freezability and post-thawing longevity.

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