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Freezing and Fertilizing Capacity of Frozen Rabbit Semen Extended with Gelatin Addition

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ABSTRACT

The use of frozen semen in rabbits is greatly limited due to its low fertility rates. The current study aimed to evaluate the effect of gelatin addition to semen extender on motility, livability, abnormality and acrosome integrity of rabbit spermatozoa during freezing process and on fertility after Artificial Insemination (AI). Pooled semen collected from bucks (n = 15) belonging to a line selected by Animal Production Research Institute, Egypt (APRI). Semen was processed in tris-buffer extender with gelatin addition at levels of 0, 1, 2 and 3% (g/100 mL extender) at a rate of 1:5, and frozen in liquid nitrogen. Results showed that when compared with control semen, 2% gelatin addition had positive ($p<0.05$) effect on percentages of motility, livability, abnormality and intact acrosome o f spermatozoa in post-diluted (73 vs. 65.5%; 73.9 vs. 66.9%; 12.2 vs. 15% and 81 vs. 70%), post-equilibrated (71 vs. 60%; 71.7 vs. 60.4%; 12.6 vs. 15.1% and 80.2 vs. 67.5%) and post-thawed (54.5 vs. 31%; 54.6 vs. 31.4%; 18 vs. 21.4% and 71.4 vs. 56%) semen, respectively. Semen with 2% gelatin yielded the highest ($p<0.05$) kindling rate (75%) and litter size (6.93) as compared to control semen (65% and 6.08/doe), respectively. Adding 2% gelatin to tris-buffer extender of APRI rabbit semen enhanced freezing ability, efficiency and fertility of spermatozoa in thawed semen.

Key words: Frozen semen, fertility, gelatin, rabbit, sperm function

INTRODUCTION

The methods of improving the semen quality for Artificial Insemination (AI) were studied by several authors to estimate the male fertility. When AI is used in rabbit farms, semen quality can affect the fertility and prolificacy of rabbit does (Alvarino, 2000). In addition, the male genetic value should be considered because the maternal or growth traits have a direct effect on the productive traits of the offspring (Safaa *et al*., 2008). In Egypt, the use of AI in rabbits is not practiced on a very limited scale (Zeidan *et al*., 2002), although AI is used in most European countries.

In market rabbit production, fresh semen is appropriate for routine AI stored at a short time (Morrell, 1995; Castellini, 1996). To prolong storage time, frozen semen is required but freezing process is associated with a reduction in motility, viability and fertility or prolificacy after AI (Kashiwazaki *et al*., 2006; Castellini *et al*., 2006). Several studies have been done in order to improve quality and fertility of stored fresh semen or frozen semen. In this respect, motility and viability of spermatozoa are maintained for a longer time, when gelatin is added to fresh rabbit

semen extenders (Lopez-Gatius *et al.*, 2005; Raga-Ayat *et al.*, 2012). However, gelatin addition to frozen semen had no positive effects on sperm motility and viability after thawing or fertility and prolificacy of rabbits after AI (Cortell and de Castro, 2008). Many authors used tris-buffer extenders in dilution of rabbit fresh semen (Lavara *et al.*, 2005; Raga-Ayat *et al.*, 2012) and these extenders were effective for 2-3 days preservation of rabbit semen (Roca *et al.*, 2000). There was a negative correlation between the percentages of sperm abnormality or spermatozoa with acrosome damage with rabbit fertility (Lavara *et al.*, 2005).

The objective of this study was to assess the effect of gelatin addition (1, 2 and 3%) to tris-buffer extender on motility, livability, abnormality and acrosome integrity of spermatozoa as well as fertility and prolificacy after insemination with frozen/thawed semen.

MATERIALS AND METHODS

The present study was carried out at the International Livestock Management Training Center (ILMTC), Sakha, Kfrelsheikh governorate, belonging to Animal Production Research Institute (APRI) Agricultural Research Center, Ministry of Agriculture, Egypt; during the period from March to May 2011.

Experimental animals: Fifteen adult rabbit bucks from APRI line (a line selected by Animal Production Research Institute, Egypt, from males of local Red Baladi×females of V-line) with the same weight (3.5 kg) and 80 multiparous lactating rabbit does from the same line were used for fertility trails. Animals were kept at the farm of Sakha Experimental Station, Egypt, under the same condition of housing in individual wire cages (50×60×30 cm) fed on a commercial diet and provided water *ad libitum*. Photoperiod during the experimental period was 16 L:8 D.

Semen collection: Semen was collected twice weekly using artificial vagina for rabbits. Semen was collected before feeding at 8.00 a.m. Only ejaculates (13-15 ejaculates for each collection day) with ≥70% mass motility, ≥10% abnormality, ≥10% damage a crosome spermatozoa and ≥300×10^6 cells/mL sperm concentration were accepted and pooled. Gel plug was removed immediately after collection of semen and keep at 35-37°C in water bath. The collection period endured for five weeks (130-150 ejaculates).

Semen dilution and treatment: In each session on day of collection, all ejaculates were mixed forming a pool, which was divided into 4 parts and diluted with 4 types of tris-citric-acid extender containing 4 levels of gelatin (0, 1, 2 and 3 g/100 mL extender).

Semen was diluted at a rate of 1:5 in heated (37°C) tris-extender. Each 100 mL of the extender was prepared with tris (3.208 g), citric acid (1.675 g), fructose (1.25 g), glycerol (2%), egg-yolk (10 mL), streptomycin (0.5 g), lincomycin (0.01 g) and completed to 100 mL with distilled water, then mixed and kept at 37°C.

Freezing protocol: Semen was filled in 0.25 mL straws at room temperature and cooled at 5°C for 4 h, then straws were transferred into processing container and located horizontally in static nitrogen vapor 4 cm above the surface of liquid nitrogen for 10 min. Thereafter, the straws were placed vertically in a metal canister and immersed completely in liquid nitrogen container for storage at -196°C.

Semen evaluation: Semen extended with each gelatin level was evaluated in post-dilution, post-equilibrated (42 h) and post-thawed semen. Various sperm parameters including progressive

motility, livability, abnormality and acrosome integrity, were determined, using a hot microscope stage adjusted at 37°C. The percentage of motile spermatozoa (progressive motility) was assessed using research microscope with warmed stage (37°C) under the high power magnification (400x) according to Amman and Hammerstedt (1980). Sperm livability percentage was determined using eosin-nigrosin mixture stain according to Hackett and Macpherson (1965). Live spermatozoa (unstained ones) and dead spermatozoa (stained ones) were counted in field of a total of 200 spermatozoa. Then, percentage of live spermatozoa was calculated. Sperm abnormalities percentage was determined during the examination of live/dead sperm percentage at a high power magnification (400x), according to the classification adopted by Blom (1983). The percentage of acrosome integrity was conducted as indicated by Watson (1975) using a light microscope (100x). A total of 400 spermatozoa were counted and classified based on membrane integrity of the sperm head, tail and acrosome (Nagy *et al.*, 1999). For each sperm parameter except for sperm abnormality, post-thaw recovery rate was calculated as follows:

$$\text{PTRR (\%)} = \frac{\text{Sperm parameter (\%) in post} - \text{thawed}}{\text{Sperm parameter (\%) in post} - \text{diluted semen}} \times 100$$

Artificial insemination (AI) of rabbit does: About 48 h before AI, 80 primiparous and multiparous does were subcutaneously injected with 75 IU of PMSG: (Folligon, Intervet, Holland) and ovulation was induced with 100 IU of a HCG analogue (Suprefact, Hoechst Roussel, Madrid, Spain) given in at the time of insemination. Total of 20 does were inseminated by semen extended with each level of gelatin and control semen immediately post-thawing (at 37°C for 30 sec) using filled plastic AI gun close to the cervical canal, 11 days after parturition. Pregnancy diagnosis was performed by abdominal palpation on day 12 after AI and parturition was subsequently recorded. All inseminations were conducted by the same inseminator. After parturition, kindling rate, litter size and average kid weight were recorded at birth.

Statistical analysis: Data, after arcsine transformation of the percentages, were analyzed by one way ANOVA using a repeated-measures general linear model to evaluate the effect of gelatin level (0, 1, 2 and 3%) on each of sperm parameters. Data of kid performance parameters were analyzed using one way design (ANOVA), while kindling rate were analyzed using Chi-square test. Data were statically analyzed using SAS (2004). When ANOVA revealed a significant effect, values were compared using Duncan's multiple range test at $p<0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of gelatin addition on sperm characteristics during freezing process: Results presented in Table 1 showed significantly ($p<0.05$) positive effect of gelatin only at a level of 2% on percentages of motility, livability, abnormality and intact acrosome of spermatozoa in post-diluted semen as compared to other gelatin levels or control semen. However, the effect of 1 or 3% gelatin on sperm characteristics studied in post-diluted semen was not significant.

In post-equilibrate semen, extender supplemented with 1 or 2% gelatin had significantly higher ($p<0.05$) percentages of motility, livability, abnormality and intact acrosome of spermatozoa as compared to 3% gelatin and the control semen. However, the best percentages of sperm characteristics were recorded in semen supplemented with 2% gelatin (Table 2).

Table 1: Effect of gelatin level in semen extender on sperm characteristics in post-diluted rabbit semen

		Extender with gelatin addition		
Characteristics (%)	Control semen	1%	2%	3%
Sperm motility	65.5±1.27^{b}	70.0±2.15^{ab}	73.0±1.17^{a}	67.5±1.12^{b}
Sperm livability	66.9±1.49^{b}	70.3±1.83^{ab}	73.9±1.07^{a}	67.2±1.50^{b}
Sperm abnormality	15.0±0.70^{a}	13.3±0.56^{ab}	12.2±0.87^{b}	14.2±0.39^{a}
Acrosome-intact spermatozoa	70.0±2.31^{b}	75.3±1.92^{b}	81.0±1.50^{a}	70.5±1.96^{b}

Means within the same row with different superscripts are significantly different at $p<0.05$

Table 2: Effect of gelatin level in semen extender on sperm characteristics in post-equilibrated rabbit semen

		Extender with gelatin addition		
Characteristics (%)	Control semen	1%	2%	3%
Sperm motility	60.0±1.13^{b}	67.5±1.97^{a}	71.0±1.25^{a}	62.5±1.17^{b}
Sperm livability	60.4±1.09^{b}	68.0±1.79^{a}	71.7±1.07^{a}	62.7±1.28^{b}
Sperm abnormality	15.1±0.50^{a}	13.6±0.49^{b}	12.6±0.83^{b}	14.5±0.40^{a}
Acrosome-intact spermatozoa	67.5±2.27^{c}	73.9±1.57^{b}	80.2±1.12^{a}	66.9±1.97^{c}

Means within the same row with different superscripts are significantly different at $p<0.05$

In accordance with the beneficial effects of gelatin addition (2%) on sperm characteristics in post-diluted and equilibrated semen was found in this study. Nagy *et al.* (2002) and Lopez-Gatius *et al.* (2005) found that 2% gelatin addition to extender of fresh semen increased storage period of rabbit semen up to five days. Also, rabbit semen could be preserved for 48 and 72 h at room temperature (25°C) and at 5°C, respectively, with satisfied and acceptable sperm quality in terms of motility, livability, abnormality and acrosome integrity by adding 2% gelatin to tris-buffer extender (Raga-Ayat *et al.*, 2012).

Such results indicated that supplementing tris-buffer extender of rabbit semen with 2% gelatin was effective in reducing spermatozoa metabolism and movement in post-diluted and equilibrated semen, because gelatin increases the viscosity of the extender and viscosity affects the motility parameters of spermatozoa (Hirai *et al.*, 1997). Decreasing sperm metabolism probably reduces lactic acid generation in extended semen and it is known that low pH value of seminal plasma kill the spermatozoa (Echegaray-Torres *et al.*, 2004).

Data in Table 3 showed that addition of semen extender with 1 or 2% gelatin significantly ($p<0.05$) increased the percentages of motility, livability and intact acrosome of spermatozoa in post-thawed semen as compared to 3% gelatin and the control semen, being significantly ($p<0.05$) higher with 2 than 1% gelatin. However, both levels of gelatin (1 or 2%) decreased sperm abnormality percentage in post-thawed semen as compared to the control semen.

Such effect reflects significant ($p<0.05$) recovery rates of motility, livability and intact acrosome of spermatozoa in post-thawed semen diluted with 1 and 2% than 0 and 3% gelatin (Table 4).

The present results contrasted with those reported by Cortell and de Castro (2008), they found non-significant effect of 2% gelatin addition on kinetic parameters (progressive motility and total motility) and cell viability after thawing of rabbit semen. In the present study, the obtained percentage of progressive motility ranged between 31% in control semen and 54.5% by adding 2% gelatin to the extender, yielding motility recovery rate of 51.8 and 74.3%, respectively. These

Table 3: Effect of gelatin level in semen extender on sperm characteristics in post-thawed semen

		Extender with gelatin addition		
Characteristics (%)	Control semen	1%	2%	3%
Sperm motility	31.0±2.13^{c}	45.5±3.25^{b}	54.5±1.62^{a}	33.0±2.72^{c}
Sperm livability	31.4±1.83^{c}	45.7±2.88^{b}	54.6±1.74^{a}	33.3±2.65^{c}
Sperm abnormality	21.4±0.89^{a}	17.3±0.65^{b}	18.0±0.60^{b}	20.2±0.63^{a}
Acrosome-intact spermatozoa	56.0±2.01^{c}	64.4±1.75^{b}	71.4±1.92^{a}	57.7±2.01^{c}

Means within the same row with different superscripts are significantly different at $p<0.05$

Table 4: Effect of gelatin level in semen extender on recovery rate (%) of sperm characteristics in post-thawed rabbit semen

		Extender with gelatin addition		
Characteristics (%)	Control semen	1%	2%	3%
Sperm motility	51.8±3.61^{b}	68.9±4.92^{a}	74.3±4.07^{a}	52.3±3.74^{b}
Sperm livability	47.0±2.64^{b}	66.1±3.37^{a}	71.3±3.21^{a}	49.4±4.12^{b}
Acrosome-intact spermatozoa	80.2±2.01^{b}	86.0±1.20^{a}	87.5±1.87^{a}	81.7±2.14^{b}

Means within the same row with different superscripts are significantly different at $p<0.05$

percentages were higher than those achieved in the experiment of Cortell and de Castro (2008) and were within a range between 35 and 60% when tris-based extenders were used (Moce *et al*., 2005; Viudes-de-Castro *et al*., 2005).

Rate of change in sperm characteristics during freezing process: During freezing assessment, a slight reduction in motility, livability and intact acrosome spermatozoa and unchanged sperm abnormality were observed as a result of dilution and equilibration of the semen. On the other hand, freezing process resulted in deleterious effects occurred in all sperm characteristics in semen with or without gelatin addition, but 2% gelatin maintained all sperm characteristics at the best percentages. It is of interest to note that most dramatically changes during freezing were found in motility and livability of spermatozoa.

Several authors indicated a reduction in motility and viability in frozen/thawed semen (Moce *et al*., 2003; Si *et al*., 2005; Castellini *et al*., 2006; Kashiwazaki *et al*., 2006). The present study revealed that adding 2% of gelatin to tris-buffer extender of rabbit semen reflected significantly ($p<0.05$) positive effects on sperm characteristics including motility, livability, acrosome integrity and normality in post-thawed semen. Such effects were associated with improving all sperm characteristics studied in post-diluted and post-equilibrated semen. Similarly, sperm motility enhanced in post-diluted rabbit semen supplemented with gelatin (Lopez-Gatius *et al*., 2005).

Fertility and prolificacy rates: Insemination of synchronized does with frozen/thawed semen diluted with tris-buffer extender supplemented with 2% gelatin resulted in the highest significant ($p<0.05$) kindling rate (75%, Fig. 1) and prolificacy (litter size was 6.93/doe, Fig. 2) as compared to other gelatin levels or control semen.

The present fertility results in term of kindling rate were in agreement with those achieved in other experiments, ranging between 25 and 71% (Moce *et al*., 2002, 2005; Castellini *et al*., 2006) or between 77.5 and 80.8% (Cortell and de Castro, 2008) Prolificacy is also a very important trait due to its high economic impact. The obtained number of born ranged between 6.1 in control

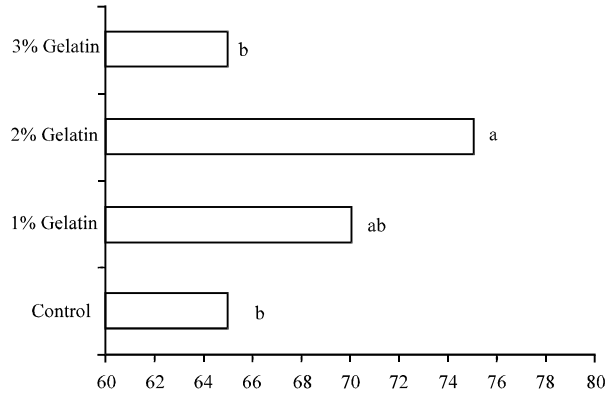

Fig. 1: Kindling rate (%), Bars with different letters are significantly different at p<0.05

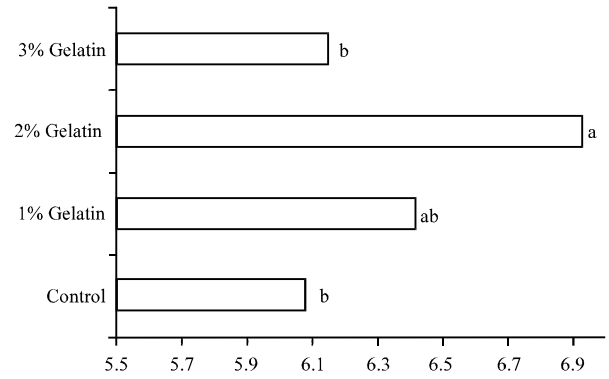

Fig. 2: Litter size/doe, Bars with different letters are significantly different at p<0.05

and 6.93 in 2% gelatin. Such results were within a range of 4.4 and 7.1 for born alive (Moce *et al*., 2005; Castellini *et al*., 2006), while were lower than a range of 7.6-8.1 (Cortell and de Castro, 2008).

The relationships between semen characteristics and fertility have been investigated in rabbits, with the general objective of predicting fertility from semen traits (Brun *et al*., 2002). Amann (1989) stressed the importance of the in-vitro fertility test through AI. In rabbit does inseminated with fresh semen, (Raga-Ayat *et al*., 2012) found significant (p<0.05) with kindling rate (90%) and litter size (7.5/doe) for APRI does inseminated with fresh semen supplemented with 2% gelatin to tris-buffer extender compared with kindling rate (75%) and litter size (6.67/doe) for the control does. Such results were higher than those recorded for the frozen semen in the present study for 2% gelatin and even for the control semen, because sperm lifespan is reduced when semen is frozen, but gelatin addition may improve frozen semen motility or viability (Cortell and de Castro, 2008). Also, when frozen semen was used, a reduction in fertility after AI generally occurred in various attempts (Moce *et al*., 2003; Si *et al*., 2005; Kashiwazaki *et al*., 2006; Castellini *et al*., 2006).

The present results indicated a positive relationship between each of kindling rate or litter size with sperm parameters in fresh or frozen semen supplemented with 2% gelatin. In this respect, Brun *et al*. (2002) and Lavara *et al*. (2005) found a positive correlation between motility of fresh semen and fertility. On the contrary, for frozen-thawed semen, the results of Cortell and de Castro,

(2008) showed that, in spite of the low motility rate achieved after thawing, fertility and prolificacy results were satisfactory. In rabbit, few authors (Foote *et al.*, 1991) have investigated the relationships between qualitative and quantitative traits of semen and reproductive performances after AI. Brun *et al.* (2002) found that of all the semen traits, mass motility had the most influence on kindling rate, while litter size seemed to be more dependent on quantitative aspects such as number of spermatozoa in the dose, via concentration. Such finding may indicate the important role of sperm cell concentration (Castellini *et al.*, 2006).

Generally, the observed variation in fertility and prolificacy results probably may be due to the high selection intensity on males for semen quality. A male effect has been seen on semen freezing resistance in rabbits (Chen *et al.*, 1989; Moce *et al.*, 2005). Also, fertility varied according to the physiological stage of the does at the time of insemination (Brun *et al.*, 2002).

One of the main constraints is the low storage ability of rabbit semen for prolonged periods with acceptable fertility. Therefore, semen preservation is a main limitation in AI of rabbits. For the fresh semen of the same line (APRI) used in this study, Raga-Ayat *et al.* (2012) found that adding 2% gelatin to tris-buffer extender of rabbit semen maintained sperm function for 72 h at 5°C, yielding the best fertility rate in terms of higher kindling rate and litter size than those supplemented with 1 or 3% gelatin and the control semen without gelatin. Also, in the present study, adding 2% gelatin to tris-buffer extender of APRI rabbit semen enhanced freezing ability, efficiency and fertility of spermatozoa in thawed semen. Since frozen semen is currently suitable for biotechnological programs to improve reproductive performance, *in-vitro* fertilization and gen banks. Using frozen semen of rabbits may facilitate the semen transport and subsequent widespread use of AI in rabbits all over the world. In conclusion, adding 2% gelatin to tris-buffer extender of APRI rabbit semen enhanced freezing ability, efficiency and fertility of spermatozoa in thawed semen.

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