EFFECT OF GELATIN ADDITION TO FREEZING EXTENDER ON RABBIT SEMEN PARAMETERS AND REPRODUCTIVE PERFORMANCE

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ABSTRACT

An improvement in reproduction management has been associated to the wide use of artificial insemination in rabbits. The use of frozen semen in this species is greatly limited due to its low fertility rates. Moreover, in politocous species, prolificacy is a very important trait and it is particularly affected when semen is frozen. Since gelatine addition to fresh semen extenders improves and prolongs motility and viability sperm parameters, its use in semen freezing could enhance sperm efficiency after thawing.

The aim of this study was to assess the effects of gelatine addition to freezing extender using a commercially available extender (MIII) and a tris-based extender (TGC). Four experimental extenders were used: TGC extender with gelatine, the same without gelatine and MIII extender with and without gelatine. In order to evaluate seminal parameters, motility was assessed using the Computer Assisted Sperm Analysis, while viability rate was calculated using flow cytometry. To evaluate *in vivo* frozen-thawed semen, 273 females were inseminated. No significant difference was found among the four freezing extenders when motility, viability fertility or prolificacy were considered. Motility rates ranged between 22.4% and 35.1%, while viability varied between 22.5 and 30.9%. Fertility rates oscillated from 80.8% to 86.2% and the total born ranged from 7.9 to 8.5. While *in vitro* results were lower than those obtained in other studies, *in vivo* results were quite favourable. In conclusion, gelatine addition did not improve sperm motility and viability after thawing nor fertility and prolificacy after insemination with frozen-thawed semen, using MIII or TCG extender.

Key words: Rabbit**,** Gelatine, Freezing extender, Semen, Reproductive performance.

INTRODUCTION

Artificial insemination (AI) is widely used in rabbit farms in order to facilitate reproductive management. Even if fertilizing ability of fresh semen can be maintained at 15 ºC through 48 h or even 72 h (Roca *et al.,* 2000; López-Gatius *et al.,* 2005), the use of frozen semen would be interesting in order to prolong storage time and extend genetic improvement. However, although several studies have been done in order to improve reproductive efficiency when frozen semen is used, a reduction in motility, viability and fertility or prolificacy after insemination is generally evidenced (Mocé *et al.,* 2003; Si *et al.,* 2005; Kashiwazaki *et al.,* 2006; Castellini *et al.,* 2006).

Even if Tris-buffers are commonly used to dilute rabbit fresh semen (Viudes de Castro, 1996 and 1997; Castellini *et al.,* 2000; Roca *et al,.* 2000; Lavara *et al.,* 2005) commercially available porcine extenders have been used without decreasing the seminal parameters quality (Nagy *et al.,* 2002; Rosato *et al.,* 2006; Piglet® and MIII®, respectively). Since these extenders are cheaper and easier to prepare than those based on tris buffers, their use in farms would be very useful.

Moreover, in order to enhance sperm motility parameters and fertility when fresh semen is stored up to five days, gelatine has been added to extenders in different species such as rabbit (Nagy *et al*. 2002; López-Gatius *et al.*, 2005), sheep (Yániz *et al.*, 2005) or goat (Salvador *et al.*, 2006).

The aim of this study was to evaluate the effect of gelatine addition to two different freezing extenders (one based on Tris and one commercially available), assessing sperm parameters after thawing and fertility and prolificacy after insemination.

MATERIALS AND METHODS

Animals

Four bucks belonging to a line selected for growth rate and 273 crossbred females selected on the basis of litter size at weaning were used. Does were in different reproductive status (multiparous lactating, multiparous non-lactating and nulliparous). Animals were housed at the experimental farm of the CITA-IVIA (Castellón, Spain), under photoperiod of 16L:8D, in individual flat deck cages and fed with a commercial diet and provided water *ad libitum*.

Semen collection and evaluation

Each fortnight, one ejaculate per male was collected using an artificial vagina. Only white colour and normal density ejaculates were accepted and pooled. A total of twenty one pools were frozen during 40 weeks.

Volume was measured in a graduated tube. In each session, all ejaculates were mixed forming a pool. One half part was diluted (1:50) in a tris-citric-acid extender (TCG) and the other one in a commercial extender (MIII) and motility was measured subjectively. After dilution of one pool sample in a gluatraldehyde solution (1:50), concentration was estimated using a Thoma-Zeiss counting cell chamber. Sperm abnormalities and the status of the acrosome were analyzed with a differential interference contrast microscope (Nomarsky contrast).

Freezing protocol

Only ejaculates with, at least, 70% of motile sperm were used. Ejaculates with more than 15% of abnormalities or damaged acrosome were excluded also.

All the chemicals to prepare extenders were purchased from Sigma-Aldrich (Madrid, Spain). Two different extenders were used to dilute fresh sperm: TCG (0.25 M Tris[hydroxymethyl]aminomethane, 88mM citric acid anhydrous, 47 mM glucose) and MIII (commercial extender, MIII, Minitub®, Landshut, Germany). Four freezing extenders were used:
-TCG-C: TCG supplemented with 3.5 M dimethyl sulfoxide (DMSO) and 0.1 M sucrose.
-TCG-gel: TCG-C supplemented with 2% gelatine.
-MIII-C: MIII extender supplemented with 3.5 M DMSO and 0.1 M sucrose.
-MIII-gel: MIII-C extender supplemented with 2% gelatine.

Concentration was fixed in fresh semen to 140 x 10^6 spermatozoa/ml. Then semen was diluted in the corresponding freezing extender (1:1) and was packaging in 0.5 ml straws at room temperature and cooled at 5ºC during 1 hour, then straws were placed 5 cm above liquid nitrogen level for 10 minutes. Finally, straws were plunging into the liquid nitrogen. Semen was thawed at 50ºC for 10 seconds.

Post-thawing motility and viability assessment

Thawed ejaculates motility was assessed using the Computer Assisted Sperm Analysis (ISAS, Version 1.0.17, Proiser, Valencia, España) at 37ºC at 10x in negative phase contrast immediately after thawing.

The percentage of total motile sperm and progressive sperm were measured. In the software settings, frame to frame rate (s) was 25-25 and the minimum data point was 15 μm.

For sperm viability assessment immediately after thawing, 0.5 μl of SYBR-14 (Invitrogen, Barcelona, Spain) and 2 μl of propidium iodide (PI) (Sigma-Aldrich, Madrid, Spain) were added to 500 μl of semen, containing approximately 70 x 10^6 spermatozoa/ml. After 10 min. 250 μl of semen were added to 750 ml of extender 1 or 2 and were introduced in Flow Cytometer. To identify sperm populations, only those stained were selected. Then, three populations appeared: one population with red fluorescence (dead cells), another one with green fluorescence (live cells) and the last, with both of them (live-dead cells).

Artificial insemination

Two hundred and seventy three inseminations were performed with 0.5 mlof semen; each female was randomly assigned to one freezing extender. Only those females with red colour of vulvar lips were inseminated. Ovulation was induced with 1 μg of Busereline Acetate (Suprefact, Hoechst) i.m. at the insemination time.

Statistical analysis

Extender freezing effect on sperm motility and viability were analyzed by means of General Lineal Model. Freezing extender was contemplated as categorical factor and each pool was introduced as random factor. Total and alive born were analyzed also by means of General Lineal Model; in this case, the freezing extender and reproductive status were introduced as categorical factor and the session of artificial insemination as random factor. Tukey's test was used for means comparison if P was lower than 0.05.

Fertility and kindling rate were compared using logistic regression, introducing freezing extender, reproductive status and artificial insemination session as categorical factors.

Analyses were performed with Statgraphics®Plus 5.1 (Statistical Graphics Corp., Rockville, MO, USA).

RESULTS AND DISCUSSION

Post-thawing, no difference for kinetic parameters (progressive motility and total motility) and cell viability after thawing were found among freezing media (Table 1). These results are in agreement with the finding of Olivares *et al*. (2005). In the present study, progressive and total motility ranged from 6.8 to 16.2% and from 22.4 to 35.1% respectively. These rates were lower than those achieved in other experiments in which progressive motility ranged between 17 and 40% in case of using egg yolk extenders (Chen *et al.*, 1989; Si *et al.*, 2006; Kashiwazaki *et al.*, 2006) or between 35 and 60% when tris-based extenders were used (Vicente and Viudes de Castro 1996; Mocé *et al.*, 2002; Mocé *et al.*, 2003; Mocé *et al.*, 2005; Viudes de Castro *et al.*, 2005).

Table 1: Frozen-thawed semen quality according to freezing extender

Freezing Extender	*In vitro* semen parameters, LSM±SE (n)		
	Progressive motility %	Total motility %	Viability %
TCG-C	11.9±2.23 (10)	25.8±4.1 (10)	30.9±3.2 (12)
TCG-gel	6.8±3.2 (7)	26.3±5.8 (7)	22.5±4.1 (10)
MIII-C	11.7±2.9 (10)	22.4±5.4 (10)	24.0±4.8 (10)
MIII-gel	16.2±3.3 (5)	35.1±6 (5)	26.3±5.3 (6)

LSM ± SE: least square means ± standard error; n: number

Results of viability after thawing among pools was significantly different (data not shown) and oscillated between 6.4% and 43.5%, similar results to those reported by other authors as Kashiwazaki *et al.* (2006) or Chen *et al.*(1989), with 17-36% and 36-52% of viable sperm cells respectively.

Neither freezing extender, female reproductive status or artificial insemination session had effect on fertility rate, kindling rate or total or live born (Table 2). These fertility results were higher than those achieved in other experiments, where results ranged between 32 and 77% in fertility rate and between 25 and 71% for kindling rate (Mocé *et al.,* 2002; Mocé *et al.,* 2005; Castellini *et al.,* 2006).

Table 2: Results after insemination with frozen sperm according to freezing extender

Freezing extender	AI	Fertility rate % (n)	Kindling rate % (n)	Total born LSM±SE (n)	Live born LSM±SE (n)
TCG-C	94	86.2 (81)	79.8 (75)	8.5±0.4 (75)	8.1±0.3 (72)
TCG-gel	107	85.9 (92)	77.6 (83)	7.9±0.3 (84)	7.8±0.3 (79)
MIII-C	26	80.8 (21)	80.8 (21)	8.2±0.6 (21)	7.6±0.6 (21)
MIII-gel	40	85 (34)	77.5 (31)	7.9±0.5 (31)	7.6±0.5 (31)

AI: artificial inseminations performed; n: number; LSM ± SE: least square means ± standard error

Prolificacy is also a very important trait due to its high economic impact. In the present study total born ranged between 7.9 and 8.5, while live born ranged from 7.6 to 8.1. These results are in agreement with the finding of Mocé *et al. (*2002, 2005) and Castellini *et al.* (2006) who reported results ranged between 4.4 and 7.1 for born alive and between 7.4 and 8 for total born.

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, our data showed that, in spite of the low motility rate achieved after thawing, fertility and prolificacy results were satisfactory. In this experiment, the number of motile sperm per insemination dose ranged between 2.24 and 15.22 × 10^6 depending on the pool, however, no differences were found in the reproductive performance. These results are in agreement with those obtained by Castellini *et al.* (2006), who appreciated that increasing motile sperm cells number from 3.75 to 20.6 × 10^6 did not enhance reproductive performance.

Moreover, these results are probably also due to the high selection of males for seminal characteristics at the beginning of the experience, in fact only 4 males from 13 were selected. A male effect has been seen on semen freezing resistance in rabbits (Chen *et al.,* 1989; Mocé *et al.,* 2005). Since semen viability average after thawing was 25.57%, insemination doses, formed by 35 million of cells, had approximately 8 million of live cells.

The use of gelatine may exert a beneficial effect avoiding sperm cell sedimentation and, therefore, avoiding changes in the composition medium and pH. Since sperm lifespan is reduced when semen is frozen, gelatine addition may improve frozen semen motility or viability. When fresh semen extender was supplemented with gelatine, while in goat and sheep semen motility parameters there were no differences immediately after semen collection(Yániz *et al.,* 2005; Salvador *et al.,* 2006); in rabbits, semen motility was enhanced (López-Gatius *et al.,* 2005). Moreover, 72 hours after semen collection, viability and acrosome integrity were improved if gelatine were added (Nagy *et al.,* 2002). However, our results with frozen-thawed semen did not indicate improvement in seminal parameters, fertility or prolificacy after insemination.

CONCLUSIONS

Gelatine addition did not improve sperm characteristics (motility and viability) after thawing nor fertility and prolificacy after insemination with frozen-thawed semen, using MIII or TCG extender.

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