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Evaluation of dextran for rabbit sperm cryopreservation: effect on frozen–thawed rabbit sperm quality variables and reproductive performance

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ABSTRACT

Effects were analysed of dextran supplementation to Me_2SO and acetamide rabbit semen freezing extenders on quality characteristics of rabbit spermatozoa and reproductive performance. The final concentration of cryoprotectants in pooled semen samples was 12.4% Me_2SO for the A extenders, 10.7% Me_2SO and 2.9% acetamide for the D extenders and 8.9% Me_2SO and 2.9% acetamide in F extenders, with a supplementation of 1.7% sucrose in all cases. There was not inclusion of dextran in the A0, D0, F0; while 5% dextran was included in A5, D5, F5 and 10% dextran in A10, D10 and F10 extenders. Sperm motility and viability rates were similar with use of the different extenders. Acrosome integrity after the freeze-thawing processes, however, was markedly greater when there was dextran supplementation of D and F extenders. Prolificacy was affected by extender composition. When there was artificial insemination (AI) using semen cryopreserved in the A extenders, number of kits born was similar to when there was AI with fresh semen when there was inclusion of 5%

dextran for cryopreservation, while there was no effect on prolificacy when there was cryopreservation of semen using the D and F extenders. In conclusion, dextran supplementation of extenders containing Me_2SO and acetamide resulted in greater acrosome integrity. Furthermore, when there was AI using sperm preserved in cryo-diluents containing an intermediate concentration of Me_2SO, combined with inclusion of 5% dextran, there was a marked beneficial effect on rabbit doe reproductive performance.

Keywords: Sperm; Rabbit; Cryopreservation; Dextran

1. Introduction

Currently, there is use of fresh or refrigerated sperm for artificial insemination (AI) of rabbit does using semen from paternal selected lines for meat production (Viudes de Castro et al., 1999; Roca et al., 2000; Lavara et al., 2005). Sperm cryopreservation, however, is an excellent way to store valuable gametes for a long period of time and with use of this technique there is facilitation of operations at insemination centers by reducing the need of sperm collection at specific times (Bailey et al., 2000; Curry, 2000, Bailey et al., 2003). Unfortunately, the use of frozen-thawed semen for rabbit meat production is greatly limited due to its variable results, because it has been difficult to evaluate associations between values for seminal quality variables and quantitative response in terms of fertility and prolificacy. Many of the methods used for semen assessment for assisted reproduction are only of limited value for prediction of fertility because there are a multitude of factors and interactions that affect semen quality (Rodriguez-Martinez, 2003). Sperm structure and maturation, seminal plasma, extenders and device used for insemination, female receptivity status and methodology or farming practices can interact to affect sperm transport, capacitation and, ultimately

fertilization, embryo development and prolificacy (Foote, 2003). Furthermore, semen cryopreservation contributes, both during the freezing and thawing processes, to the deleterious effects on structure and functions of spermatozoa, and as a result an undetermined number of spermatozoa will be sensitive to osmotic stress, have a short lifespan or have sublethal dysfunctions. There are, therefore, relatively small numbers of viable spermatozoa that are fully functional in the population (Watson, 2000). The integrity of sperm membranes is necessary for spermatozoa to remain functional during storage in the female reproductive tract and penetration of the oocyte (Holt, 2000). In rabbits, there is an overall reduction in the percentage of motile and viable spermatozoa after sperm cryopreservation and thawing and, in general, results on fertility and prolificacy are not comparable to those obtained when fresh semen is used for AI (Mocé and Vicente, 2009). Cryoprotectants are used to minimise the damage caused by ice crystal formation in cells during the freezing-thawing process and promote the formation of either an amorphous state or a eutectic solid (crystalline) state in cells during the cooling–cryostorage–warming cycle (Takahashi et al., 1998). Fertility when there is use of frozen-thawed semen for AI varies depending on experimental approaches. The effectiveness of rabbit semen cryopreservation and maintenance of spermatozoa viability is dependent on different factors such as cryopreservation protocol, cryoprotective agent (CPA), genetic line of males, number of spermatozoa used for AI and those aspects associated with the individual *per se*, probably related to the proteome of both the seminal plasma and the sperm membrane (Mocé et al., 2014, 2015; Viudes de Castro et al., 2014a; Casares-Crespo et al., 2018, 2019). The main CPAs currently used for preservation of rabbit semen are a combination of permeating compounds such as acetamide or dimethyl sulphoxide (Me_2SO) and non-permeating compounds, such as lactose, sucrose, raffinose or trehalose. In addition, egg yolk is

often included as an additional protective additive in rabbit sperm-freezing extenders (Mocé and Vicente, 2009; Hall et al., 2017). There, however, have been no studies conducted where there were comparisons of the efficacy for preservation of viable rabbit spermatozoa with the combined use of acetamide and Me_2SO. The choice of extender components has marked effects on sperm viability (Holt, 2000).

Even though there have been technical improvements in cryopreservation regimens used in recent years (Mocé et al., 2014, 2015; Di Lorio et al., 2018), there has not been much progress on development of synthetic frozen extenders. Components of animal origin, such as egg yolk or BSA, included in many of the semen cryo-diluents (Chen et al., 1989; Dalimata and Graham, 1997; Rosato and Iaffaldano, 2013; Hall et al., 2017). Unfortunately, products of animal origin usually have batch-to-batch variability and there is a greater biosecurity risk with use of these compounds. The elimination of products of animal origin from the cryo-diluents reduces the risk of contamination with animal pathogens and decreases the inherent variability in composition of these products (Gil et al., 2003; Marco-Jiménez et al., 2004). There is a need to develop a freezing extender that when used addresses biosafety requirements, avoiding extender components that are of animal origin. Synthetic polymers could be an effective alternative to meet these requirements. It has been reported that aqueous solutions of polymers reduce the crystallization temperatures and the equilibrium melting points and they can easily be supercooled (Kimizuka et al., 2008). In a previous study, the addition of 10% dextran to vitrification medium resulted in an improved preservation of the essential membranes of rabbit embryos at the 3-day developmental stage (Viudes de Castro et al., 2010).

The purpose of the present study was to investigate the protective action of dextran in combination with the two most widely used membrane-permeable

cryoprotectants in rabbit semen cryopreservation (Me_2SO and acetamide) and its effects on values for semen quality variables and on kindling rate and prolificacy of does. The hypothesis was that inclusion of dextran in the semen extender that also contained Me_2SO and acetamide would have a protective effect, reducing sperm membrane susceptibility to the deleterious effects of cryopreservation and thawing.

2. Material and methods

The chemicals used in this study were purchased from Sigma-Aldrich (Merck Life Science S.L.U. Madrid, Spain), except for SYBR-14, propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA), which were purchased from Invitrogen (Thermo Fisher Scientific, Madrid, Spain).

2.1. Animals

Males and females were sexually mature New Zealand White rabbits. All experimental animals were assigned randomly to treatment groups. All does used in this experiment were receptive non-lactating females having produced at least three previous litters of kits. Animals were housed individually in flat deck cages with a 16 hours' light/8 hours' dark photoperiodic regimen being imposed at the experimental farm of the Animal Technology and Research Center (CITA-IVIA, Segorbe, Castellón, Spain). There was feeding of a standard diet *ad libitum*, and all animals had free access to water. Animal housing, care, and the procedures used for semen collection and artificial insemination were approved by the Animal Care and Use Committee of Centro de Tecnología Animal, Instituto Valenciano de Investigaciones Agrarias (Protocol #2018/VSC/PEA/0116) and were consistent the European regulations for the care and use of animals for scientific purposes (European Commission Directive 2010/63/European Union).

2.2. Semen collection and evaluation

Semen from 12 adult males was collected twice a week in six replicates, using an artificial vagina. Sperm evaluation was performed to assess the initial seminal quality. Only ejaculates with a white colour and sperm motility rate greater than 70% were used in the experiment. To eliminate individual differences, all sperm samples were pooled. A 20 µL aliquot was diluted 1:50 with 0.25% glutaraldehyde solution to calculate the concentration and percentage of abnormal sperm (at least 200 spermatozoa per pool were examined in random fields). Concentration measurements were conducted in a Thoma chamber using phase contrast microscopy at a magnification of 400X.

The sperm motility characteristics (percentage of total motile sperm), were evaluated using a computer-assisted sperm analysis (CASA) system operating at 30 video frames/s (30Hz), with particle area settings from 20 to 80 µm and a search radius of 13 mm) were determined as described by Viudes-de-Castro et al. (2015). Briefly, sperm samples were adjusted to 7.5 x 10^6 sperm/mL with TCG (Tris-Citric Acid-Glucose) extender (Viudes-de-Castro and Vicente, 1997) supplemented with 2 g/L BSA and motility was assessed at 37 ºC using the negative phase contrast objective at a magnification of 100X (NIKON Eclipse 90i microscope, Nikon Corporation Instruments Company; IZASA, Barcelona, Spain) connected to the computer through a monochrome Basler A312f video camera (Basler AG, Ahrensburg, Germany). Four microscopic fields were evaluated for each sample. Individual sperm tracks were visually assessed to eliminate possible debris and misdiagnosed tracks.

Acrosome integrity and viability were evaluated using a Coulter Epics XL cytometer (Beckman Coulter, IZASA, Barcelona, Spain). The fluorophores were excited by a 15-mW argon ion laser operating at 488 nm. The cytometer was calibrated

daily using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. A total of 10,000 gated events (based on the forward scatter and side scatter of the sperm population recorded in the linear mode) were collected per sample Flow cytometric data were analysed with the software Expo32ADC (Beckman Coulter Inc.). Samples were diluted to 30 x 10^6 sperm/mL with TCG extender. All dilutions were performed at 22 ºC. The percentage of viable sperm was determined using a dual fluorescent staining with SYBR-14/PI according to Viudes-de-Castro et al. (2014b). Briefly, the samples were stained for flow cytometric analysis by transferring 0.1 mL aliquots into tubes containing 0.4 mL of TCG extender, 2.5 μL of SYBR-14 (10 μM solution in DMSO), and 2.5 μL of PI (1.5 mM solution in purified water). The final concentration of samples was 6 × 10^6 sperm/mL. These samples were incubated (10 minutes, 22 ºC), filtered through a 40-mm nylon mesh (to remove large clumps of cells and debris) and analysed using the flow cytometer. Only the percentages of live sperm were considered in the results (SYBR-14-positive and PI-negative). The status of the acrosome was determined using a dual fluorescent staining with FITC-PNA/PI according to Viudes de Castro et al (2014b). Four sperm sub-populations were detected: live sperm with intact acrosome, live sperm with damaged acrosome, dead sperm with intact acrosome and dead sperm with damaged acrosome. Percentage of normal apical ridge (NAR) was calculated as the proportion of acrosome intact sperm.

2.3. Extender compositions

The solution used as a carrier for the cryoprotectants was TCG extender (pH 6.8, 300 mOsm/kg). Final concentrations of cryoprotectants in pooled semen samples are included in Table 1. All freezing extenders contained 0.1M sucrose as a non-permeable cryoprotectant. The permeable cryoprotectants used were Me_2SO and acetamide. For

the A extenders 3.5 M Me_2SO, and 10% or 20% of dextran (60-90 kDa) were added to the A5 and A10 extender, respectively. For the D extenders, there was supplementation of the extender with a combination of 3.0 M Me_2SO and 1 M acetamide, as well as 10% or 20% dextran were added to the D5 and D10 extenders, respectively. The F extenders were supplemented with 2.5 M Me_2SO and 1 M acetamide and 10% or 20% dextran were added to the F5 and F10 extenders, respectively.

The final concentration of cryoprotectants in pooled semen samples was 12.4% Me_2SO for the A extenders, 10.7% Me_2SO and 2.9% acetamide for the D extenders and 8.9% Me_2SO and 2.9% acetamide for the F extenders. Furthermore, there was supplementation with 1.7% sucrose to all extenders and 0% dextran to the A0, D0, and F0 extenders. There was 5% dextran supplementation to A5, D5, and F5 extenders and 10% dextran in A10, D10 and F10 extenders.

2.4. Freezing and thawing procedures

After semen evaluation, each pool was split in nine equal fractions and diluted 1:1 with all freezing extenders at room temperature (20 to 23 ºC), packaged in 0.50 mL plastic straws (IMV Technologies, L'Aigle, France) and sealed with polyvinylalcohol (IMV Technologies, L'Aigle, France). These straws were then placed at 5 °C for 45 min. Cooled straws were suspended horizontally in liquid nitrogen vapour 5 cm above the liquid nitrogen surface for 10 min before being plunged in liquid nitrogen for storage. Thawing was conducted by immersing the straws in a water bath at 50 °C for 12 s. After the freezing-thawing process, sperm motility and status of the acrosome were determined in the same way as for fresh semen.

2.5. Artificial insemination

A total of 559 inseminations were performed using six different batches. Multiparous non-lactating does (n = 208) were randomly assigned to one of the nine

experimental groups. Only receptive females (identified by red colour of vulvar lips) were inseminated using standard plastic curved pipettes (22 cm). Each insemination was performed with 40 x 10^6 of total spermatozoa. Females were induced to have ovulations by administering 1 μg of busereline acetate intramuscularly at the same time as insemination. At parturition, percentage of kindling rate (number of does giving birth/number of inseminated does) and prolificacy (number of total kits born) were recorded.

2.6. Statistical analysis

A GLM including was conducting considering freezing extenders as fixed effects using the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA, 2002). Shapiro-Wilk tests were conducted using the SPSS Explore procedure to assess normality of distribution of the residuals (Gaussian distribution) and homogeneity of variances was evaluated using the Levene test. For the kindling rate, a probit link with binomial error distribution was used, whereas for semen-quality variables (motility, viability, and acrosome integrity) an ANOVA was used. Differences between groups were assessed using the Bonferroni's test. There were considered to be mean differences when there was a P <0.05. Data are expressed as the least square mean ± standard error of means (LSM±SEM).

3. Results

In the fresh semen samples, there was an average sperm concentration of 380 ± 17.7 million sperm/mL with there being 76.0 ± 2.2% total motile sperm, 83.3 ± 0.33% live sperm and 90.0 ± 0.87 % sperm with a normal apical ridge (data not presented in tables).

3.1. Effect of freezing extender on seminal characteristics

The data for post-thawing seminal characteristics with the different extenders are reported in Table 2. Data regarding sensitivity of frozen-thawed sperm to osmotic shock are shown in that table. There were 7 x 10^6 sperm/mL used for the motility assessment with a CASA system. At this concentration, the dilution rate used after thawing the samples was greater than 1:20 for all samples, and there was very little total sperm motility.

Results indicated sperm motility and viability rate were similar when semen was stored in the different extenders. The acrosome integrity after the cryopreservation and thawing processes was markedly greater when dextran was included in the extender composition. Results from evaluation of the type D and F extenders indicated there was a lesser percentage acrosome integrity when these extenders were not supplemented with dextran. The percentages of acrosome integrity were markedly greater when there was supplementation with the largest quantity of dextran (60.1% compared with 80.1% for the D0 and D10 extenders, respectively; 52.1% compared with 79.8% with the F0 and F10 extenders, respectively; $P<0.05$; Table 2). , There were no differences in acrosome integrity when there was semen cryopreservation using the A extenders, however, the trend in values was similar with there being a lesser percentage acrosome integrity when the extender did not contain dextran.

3.2. Effect of freezing extenders on values for reproductive variables

The data when the different freezing extenders were used for cryopreservation are presented in Table 3. There were no differences in percentage of does kindling when there was AI with semen cryopreserved in the A extenders and percentages were similar to those when fresh semen was used for AI. There were no differences in percentage of does kindling, when there was cryopreservation of semen in the different D extenders. The percentage of does kindling was greater when there was semen cryopreservation in

the D5 extender with the percentages being similar than when fresh semen was used for AI. When there was use of F extenders for semen cryopreservation, there was no effect of dextran inclusion in the extender on percentage of does kindling, however, only with the F0 extender, without dextran supplementation, was there a similar percentage to that when there was AI with fresh semen. There was some indication that inclusion of dextran in these cryo-diluents had a detrimental effect on kindling rate, however, there was not substantiation of this effect as a result of conducting the statistical analyses.

Semen extender composition affects number of kits born (i.e., prolificacy). There was no difference in number of kits born when there was AI with semen cryopreserved in A extenders, and there were no differences, as a result of extender used for cryopreservation of semen, as compared with use of fresh semen for AI when there was supplementation with 5% dextran. The number of kits born was not different when there was use of the different D and F extenders for cryopreservation of semen used for AI. There, however, were lesser values for kits born alive when these extenders were utilised for cryopreservation of semen used for AI compared with the use of fresh semen for AI.

4. Discussion

Sperm motility is commonly believed to be one of the most important semen quality characteristics for fresh semen and sperm motility variables as determined using CASA systems, in combination with sperm morphology analyses, can be utilized to estimate the capacity for contributing to fertilization of fresh rabbit sperm (Lavara et al., 2005). In the present study, sperm motility was sub-optimal when all extenders were used for cryopreservation, with an average total sperm motility of 8% and with there being less than 4% motility when there was cryopreservation with some of the extenders

that were evaluated in this study. The results from the present study confirm there is a negative effect of diluting medium of rabbit semen that needs to be considered to increase the accuracy of *in vitro* evaluation of semen samples before CASA (Farrell et al.; 1996). It should be considered that a characteristic aspect of rabbit semen is the large abundance of semen constituents of different sizes (Castellini, 2008) that affect sperm motility resulting in evaluations being difficult when conducted using CASA systems. It, therefore, is common that there be large dilution rates (1:20 to 1:80) to correctly assess and determine values for this variable. Castellini et al. (2000) reported there was a marked decrease in values for sperm motility variables as extent of semen dilution increased to greater than 1:10. For frozen-thawed semen, in addition to the large dilution rate effects on sperm motility assessment, the mechanical stress on semen during the cryopreservation process could have contributed to the sub-optimal sperm motility in the present study. The pre-selection of the samples, based on values for semen quality variables, tended to result in a reduction in the variability of in sperm quality in the fresh semen samples.

The freezing rate of rabbit semen samples affects the quality and fertilising capacity of frozen-thawed rabbit spermatozoa. The cryopreservation process of rabbit sperm is usually performed at different distances above the static liquid nitrogen source, without the use of sophisticated freezing equipment. In a previous study, where there was use of a programmable freezer, slow (-15 ºC/min) and rapid (-60 ºC/min) freezing rates were detrimental to the quality of rabbit spermatozoa cryopreserved with 12.4% DMSO and 1.7% sucrose. With moderate freezing rates (-40 ºC/min), however, there was a similar quality and fertilising capacity of spermatozoa as compared to freezing above the surface of the liquid nitrogen source in expanded polystyrene boxes (Mocé et

al., 2015). Hence with rabbit semen cryopreservation, freezing of semen in boxes positioned above the liquid nitrogen surface is an efficient and economical technique.

The differences in the values for frozen-thawed seminal variables could be directly associated with the freezing extender used in the cryopreservation process. Additives such as egg yolk or low-density lipoproteins from egg yolk, albumin or milk proteins are routinely included in semen extenders as a part of the cryopreservation regimen to minimise cold-shock effects. There are no reports on the use of dextran for the cryopreservation of rabbit semen. In the present study, sperm motility and viability post-thawing were similar among groups but acrosome membrane integrity was markedly greater when dextran was included in the freezing extender. The present findings are consistent with those of Kuliková et al. (2014) where the addition of a large molecular weight polymer (Ficoll 70) to the cryopreservation medium containing sucrose and Me_2SO that led to a marked increase in the number of acrosome intact sperm. The possibility that dextran inclusion in rabbit semen extenders may contribute to protection of membranes is also supported by embryo cryopreservation research findings, in which a reduction in deleterious effects on embryonic membranes occurred when large molecular weight polymers were used as an extracellular cryoprotectant (Carroll et al., 1993; Shaw et al., 1997; Nowshari and Brem, 2000; Checura and Seidel, 2007; Viudes de Castro et al., 2010). Furthermore, considering that only sperm maintaining an intact acrosome have the capacity for fertilization of an oocyte, this seminal variable could be very important in fertility outcomes when there is use of frozen thawed semen for AI. Results from the present study provide convincing evidence that the inclusion of 5% dextran to semen extenders during the cryopreservation process has beneficial effects on acrosome integrity and reproductive performance.

There is thought to be lack of effective ways to compare results from various studies where there was a focus on fertility outcomes as a result of the use of rabbit semen that had been cryopreserved for AI. This problem has resulted because of a lack of detailed descriptions of the methodology used to conduct the studies, nor was there a complete description of extender composition, either because there were only laboratory evaluations or because there were only small-scale experiments conducted, with very few does in experimental groups. The reproductive performance data from the present study when there was use of 12.4% of Me_2SO (1.75 M) and 5% of dextran are similar to those with use of fresh semen for rabbit doe AI. Use of this freezing extender allows for preservation of a sufficient quantity of intact functional spermatozoa for there to be optimal fertility and prolificacy when this semen is used for AI of rabbit does. Viudes de Castro and Vicente (1997) reported, when there was use of a conventional insemination procedure at a commercial rabbit farm, there were no negative effects on fertility and prolificacy when there was insemination with 4 million fresh spermatozoa. In the present study, when spermatozoa viability was considered with use of the different cryo-diluents, all samples used for insemination had greater than the minimal 4 x 10^6 live sperm. The fertility differences in the present study, therefore, cannot be attributed to insufficient number of live sperm in the semen samples used for AI.

There are distinct modes of Me_2SO action when there are different concentrations of this compound in aqueous solution with Me_2SO (Gurtovenko and Anwar, 2007). When there are inclusions in the range from 10% to 20% of Me_2SO in aqueous solution, there is an induced formation of water pores, in addition to progressive thinning of the phospholipid membrane. The Me_2SO concentrations included in the A and D extenders of the present study led to an enhanced permeation of solutes across the membrane and probably allowed for more rapid osmotic equilibrium

than when lesser Me_2SO concentrations were included in the extenders. At a relatively smaller concentration (less than 10% of Me_2SO), there are structural modifications in membranes, however, there were no pore formations that persisted in the cell membrane throughout the evaluation period (Gurtovenko and Anwar, 2007; de Ménorval et al., 2012). This could be a contributing factor to the encouraging results from the present study which are similar to when there was inclusion of 8% Me_2SO to semen extenders and where there was need for an additional incubation period at 5 ºC for 90 min before semen was diluted in freezing extender to minimize cold-shock damage (Iaffaldano et al., 2012, 2014; Di Lorio et al., 2018). Similarly, results from the present study indicate semen extenders in which there is inclusion of a combination of both, acetamide and Me_2SO, the further supplementation of dextran leads to improvements in acrosome integrity and reproductive performance when there was a 10.7% of Me_2SO inclusion in the semen extender. The specific mode of solvent action may contribute to the marked enhancement of cryoprotectant activity because an intermediate Me_2SO concentration (10% to 20 %) facilitates the membrane permeability while dextran would have functions as a membrane-stabilising compound, coating and protecting the cell membrane and preventing deleterious effects due to osmosis.

5. Conclusion

In the present study, there was the first evaluation of the effects of dextran on spermatozoa when there was freezing–thawing of rabbit semen. There was a marked protective effect of dextran on spermatozoa. Results from the present study indicate dextran supplementation of extenders with Me_2SO and acetamide led to a greater rabbit acrosome integrity. Furthermore, the inclusion of 5% dextran had a marked beneficial effect on the reproductive performance when there was inclusion of intermediate

Me_2SO concentrations in the rabbit semen extender with there being similar results as those with fresh semen.

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CRediT authorship contribution statement

María P. Viudes-de-Castro: Conceptualization, Methodology, Investigation, Writing - original draft, Project administration. **Amparo G. Talaván**: Investigation, Writing - review & editing. **José S. Vicente**: Conceptualization, Methodology, Investigation, Writing - review & editing, Project administration.

Competing interest's statement

None of the authors have any conflicts of interest to declare.

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531 Table 1

532 Final concentration of cryoprotectants in pooled semen samples.

Extender	%Me_2SO	%Acetamide	%Sucrose	% Dextran
A0	12.4	-	1.7	-
A5	12.4	-	1.7	5
A10	12.4	-	1.7	10
D0	10.7	2.9	1.7	-
D5	10.7	2.9	1.7	5
D10	10.7	2.9	1.7	10
F0	8.9	2.9	1.7	-
F5	8.9	2.9	1.7	5
F10	8.9	2.9	1.7	10

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534

Table 2

Effect of freezing extenders on Frozen-thawed semen quality; Values are reported as least square mean ± standard error mean; *n*: number of pools used for artificial insemination; Values in the same column with different superscripts are different ($P<0.05$).

	n	Total Motility (%)	Viability rate (%)	Acrosome integrity (%)
A0	5	8.0 ± 1.99	28.4 ± 4.79	52.8 ± 4.30^{c}
A5	5	8.4 ± 1.99	19.4 ± 4.79	60.8 ± 4.30^{abc}
A10	5	3.2 ± 1.99	23.7 ± 4.79	63.3 ± 4.30^{abc}
D0	6	7.8 ± 1.82	22.6 ± 4.37	60.1 ± 3.93^{bc}
D5	6	12.5 ± 1.82	29.6 ± 4.37	75.1 ± 3.93^{ab}
D10	6	10.0 ± 1.82	28.4 ± 4.37	80.1 ± 3.93^{a}
F0	5	7.4 ± 1.99	14.2 ± 4.79	52.1 ± 4.30^{c}
F5	5	9.4 ± 1.99	22.7 ± 4.79	66.7 ± 4.30^{abc}
F10	5	12.2 ± 1.99	32.9 ± 4.79	79.8 ± 4.30^{ab}

Table 3

Effect of freezing extenders on values for reproductive variables; Values are reported as least square mean ± standard error mean; *n*: number of inseminated does; [a,b,c]Values in the same column with different superscripts are different ($P<0.05$).

	n	Kindling rate	Number of kits born
A0	53	72.0 ± 6.2^{ab}	7.2 ± 0.5^{bc}
A5	47	84.7 ± 5.2^{a}	8.3 ± 0.5^{ab}
A10	48	73.0 ± 6.4^{ab}	7.1 ± 0.5^{bc}
D0	59	51.0 ± 6.5^{b}	5.7 ± 0.6^{c}
D5	58	72.5 ± 5.9^{ab}	7.2 ± 0.5^{bc}
D10	59	62.7 ± 6.3^{b}	7.2 ± 0.5^{bc}
F0	63	76.4 ± 5.4^{ab}	7.1 ± 0.5^{bc}
F5	54	59.5 ± 6.7^{b}	6.8 ± 0.5^{bc}
F10	55	62.1 ± 6.6^{b}	6.6 ± 0.5^{bc}
Fresh Semen	63	88.5 ± 4.0^{a}	9.3 ± 0.4^{a}