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Comparison of different semen extenders and cryoprotectant agents to enhance cryopreservation of rabbit spermatozoa

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Abstract: The purpose of this research was to find a suitable protocol to enhance frozen rabbit sperm preservation analysing the role that seminal plasma (SP) plays and the effect of different cryoprotectant agents on sperm quality 0 and 2 h after thawing. Sperm samples were pooled and divided in eight fractions. Four of them were diluted with BotuCrio® (extender A), INRA 96® plus 6% glycerol (extender B), 6% N, N-dimethylformamide (extender C) and 6% N-methyl-2-pyrrolidone (extender D), respectively. The other four fractions were centrifuged and the supernatant was discarded in order to eliminate SP. Each sample was then resuspended with extender A, B, C and D. Samples were cooled progressively, loaded into 0.5 ml freezing straws and frozen with liquid nitrogen vapour. Thawing was performed by placing the straws into a bain-marie at 37°C for 21 s. Straws were dried and sperm samples placed into Eppendorf tubes to be analyzed by ISAS software, vitality test, HOS test and acrosome integrity test. The best motility and velocity parameters were obtained by extender A ($P < 0.050$) even when the motility parameter was compared with previous studies using other diluents. Additionally, sperm quality decreased over incubation time ($P < 0.050$) and no differences were found in samples processed with or without SP. This research revealed that BotuCrio® could be used for rabbit sperm cryopreservation and moreover the improvement of the cryopreservation process of rabbit sperm due to the demonstration that SP removing is not required.

Keywords: Botucrio®; cryoprotectant; dimethylformamide; glycerol; N-methyl-2-pyrrolidone; rabbit sperm preservation

Assisted reproductive technologies in mammals have been steadily progressing over the years for maintaining and preserving breeds on the verge of extinction. Moreover, a necessity of genetic resources transport all over the world instead of live animals leads to development of an alternative technology. A possible alternative may be sperm cryopreservation which is an economy method able to preserve gene resources and carry sperm between remote destinations. However, due to the issues associated with the use of freeze-thawed sperm (lower fertility and prolificacy than of cooled sperm), artificial insemination with cryopreserved rabbit sperm has not been used for commercial purposes at present (Lopez and Alvarino 1998; Moce and Vicente 2009).

During the freeze-thawing procedure intracellular ice crystals are formed in mammalian

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sperm cells causing cell destruction and membrane damage (Fuller and Paynter 2004; Hernandez et al. 2012). To avoid this severe irreversible damage it is necessary to enhance cryopreservation protocols by adding new suitable cryoprotectant agents (CPA).

Glycerol has been the main CPA used to preserve domestic or wild animal sperm (Curry et al. 1995). Nevertheless, glycerol has not been the first CPA of choice to preserve rabbit semen due to its toxicity, which may result in osmotic stress, protein denaturation, alteration of actin interactions and induction of protein-free membrane blister that leads to worse fertility (Alvarino 1993; Gilmore et al. 1995; Iaffaldano et al. 2012). Negative effects of glycerol may be related with the high molecular weight which leads to permeating the cell membrane more slowly than other CPA (Gilmore et al. 1995). Consequently, the ideal CPA should have low molecular weight and high permeability (Darin-Bennet and White 1977). Amide or methyl groups have lower molecular weight and greater water solubility than glycerol being able to reduce osmotic damage (Curry et al. 1995; Moce and Vicente 2009).

An amide solvent that could be used as an alternative to prepare freezing extenders and reduce membrane damage caused during the procedure is N, N-dimethylformamide (DMF). To date, no studies have been performed using DMF as CPA for rabbit sperm cryopreservation. Nevertheless, the efficacy of DMF to cryopreserve sperm has been studied on stallion (Olaciregui et al. 2014), boar (Malo et al. 2009), dog (Lopes et al. 2009), goat (Bezerra et al. 2011) and fowl (Chalah et al. 1999).

Another amide solvent commonly used in chemical reactions is N-methyl-2-pyrrolidone (NMP). Despite having characteristics similar to DMF, no previous studies have been performed.

The lack of efficient cryopreservation protocols in rabbits along with the few studies carried out on the glycerol effect in rabbit sperm cryopreservation and the absence of studies on preserving frozen rabbit sperm with DMF or NMP, led to the investigation on the effect of these three CPA. Therefore, the purpose of this research was to find a suitable protocol to enhance frozen rabbit sperm preservation analysing the role that SP plays and furthermore the effect of different CPA on sperm quality at 0 to 2 h post-thaw.

MATERIAL AND METHODS

Chemicals. Unless stated otherwise, all chemicals were from Panreac Quimica S.L.U, Spain.

Animals. The study was performed following approval by the Veterinary Ethical Committee of the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive No. 86/609 on the protection of animals used for experimental and other scientific purposes.

Eight Californian–New Zealand White hybrid bucks previously selected from a commercial AI centre (Técnicas Cunícolas S.A., Spain) were used as donors. Males were housed in individual cages with 12 h light/dark cycle at room temperature of 22–24°C and relative humidity of 60–70%. All rabbits were fed a commercial pellet diet according to their reproductive condition and fresh water was provided *ad libitum*.

Semen collection and processing. Semen was collected using artificial vagina (IMV Technologies, France) at 50°C and any gel plug was removed. Before dilution, microscopic analysis of motility and macroscopic analysis to evaluate colour and volume of the sample were performed in the farm. Only white colour ejaculates of at least 0.2 ml volume and 85% motility were used for the research. All ejaculates were pooled to eliminate individual differences.

Experimental design. Figure 1 illustrates the experimental design. Pooled semen samples were divided into eight fractions, four of them were diluted (1 : 5) at room temperature with four different semen extenders: BotuCrio® (Nidacon, Sweden: 1% glycerol and 4% methylformamid) (extender A), INRA 96® (IMV Technologies, France: purified fraction of milk micellar proteins) supplemented with 6% glycerol (extender B), INRA 96® supplemented with 6% DMF (extender C), and INRA 96® supplemented with 6% NMP (extender D). The other four fractions were centrifuged once at 700 *g* for 10 min at 37°C, and the supernatant was discarded to eliminate SP. Then each pellet was resuspended (1 : 5) with extender A, extender B, extender C and extender D.

Freezing and thawing protocol. Cryopreservation procedure was performed as previously described (Alvarino 1993; Moce and Vicente 2009). Briefly, samples were cooled progressively from 37°C to

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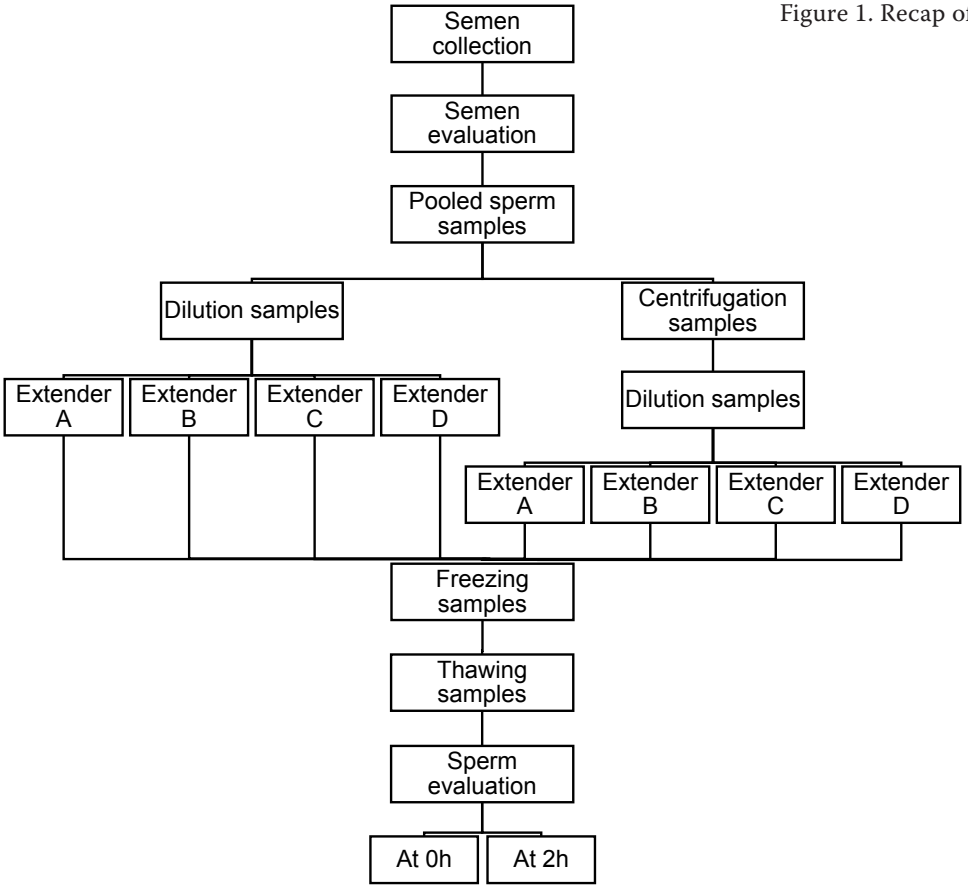

Figure 1. Recap of the experimental design

4°C over 90 to 120 min and loaded into 0.5 ml freezing straws (Minitube Iberica, Spain). Freezing straws were frozen horizontally in racks placed 4 cm above the surface of liquid nitrogen (LN_2) for 20 min and then plunged into LN_2 (–196°C) for storage. After 1 month, samples were thawed by placing the straws into a bain-marie at 37°C for 21 s. Straws were dried and sperm samples placed into Eppendorf tubes located into a bain-marie at 37°C. Thawed samples were maintained at 37°C and analyzed immediately after thawing and 2 h post-thaw.

Evaluation of spermatozoa. Sperm motility, kinematic parameters, vitality, membrane integrity and acrosome integrity of all the samples were assessed immediately after thawing and 2 h post-thaw.

Assessment of sperm motility and kinematic parameters. Sperm motility and kinematic parameters were evaluated by ISAS software (PROISER R+D, Spain) following the default setting specifically for rabbits. Five microliters were placed on a slide and covered with 20 × 20 mm coverslip. Five fields were randomly captured at ×10 magnification by phase-contrast microscope. Up to 200 frames/s were acquired selecting particles sizing 10–70 μm^2. The linearly motile sperm were deviated < 45% from a straight line. The analyses provided information about the percentage of motile spermatozoa (MOT, %), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN = VSL/VCL, %), straightness (STR = VSL/VAP, %), wobble (WOB = VAP/VCL, %), amplitude of lateral head displacement (ALH, μm), and beat cross frequency (BCF, Hz).

Assessment of vitality. Eosin-nigrosin stain was used to evaluate vitality of the spermatozoa following the protocol described by Bjorndahl et al. (2003). An aliquot of semen was put over a glass slide, mixed with an equal volume of eosin-nigrosin suspension and a smear was made on a glass slide. Immediately after the smear was dry, 100 spermatozoa in each replicate were examined by microscope at ×400 magnification and counted with the aid of a laboratory counter. Live spermatozoa had white heads and dead spermatozoa had red or dark pink heads.

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Assessment of sperm plasma membrane integrity. Hypo-osmotic swelling test (HOS test) was performed following the protocol established by Jeyendran et al. (1984). Briefly, 90 µl of HOS test solution (100 mM of sodium citrate) was placed into an Eppendorf tube to warm at 37°C for 5 min. Once it was heated, it was added to 10 µl of semen, and the mixture was mixed gently with the pipette and kept at 37°C for least 30 min. Subsequently, 100 µl of 2% glutaraldehyde solution was added to fix the sample. Ten microlitres of the mixture were placed onto a glass slide and covered with a coverslip to examine 100 spermatozoa in each replicate by phase-contrast microscope at ×400 magnification. Spermatozoa with intact membranes allowed an influx of water inside them resulting in swollen spermatozoa with coiled tail (Amorim et al. 2009).

Assessment of acrosome integrity. For evaluationg morphology and integrity of acrosome membrane, the protocol developed by Pursel and Johnson (1974) based on the fixation of the spermatozoa was used. Ten microlitres of semen were immediately fixed in 90 µl of glutaraldehyde 2% solution, right after 10 µl of the mixture was placed onto a glass slide and covered with a coverslip to examine 100 spermatozoa in each replicate by phase-contrast microscope at ×1000 magnification and immersion oil. Acrosomes were differentially categorized into two classes: intact acrosome (normal apical ridge) and damaged acrosome (damaged apical ridge and/or missing apical ridge).

Statistical analysis. The study was replicated three times. Data were analysed using IBM SPSS 19.0 software for Windows (SPSS, USA). Results were expressed as means ± SEM. The effect of the extender, SP and time elapsed after thawing on all the parameters were analysed by a General Linear Model (GLM) using a complete factorial design with interactions between factors. Duncan's post-hoc test was used to evaluate the effect of the extender. The level of significance was set at $P < 0.050$.

RESULTS

A comparison between the treatments showed that extender A obtained the highest results ($P < 0.050$) on kinematic parameters (MOT, VCL, VSL, VAP, ALH and BCF – Table 1) and also on other sperm quality parameters like vitality and acrosome integrity (Figure 2). Nevertheless, as shown in Figure 2, the greatest protection of sperm plasma membrane provided extender B and samples diluted with extender D showed the worst data on sperm quality.

As expected, almost all sperm parameters lessened 2 h post-thaw. Kinematic parameters like MOT (32.5 ± 1.4% at 0 h; 15.4 ± 1.4% at 2 h), VCL (53.1 ± 1.5 µm/s at 0 h; 32.8 ± 1.5 µm/s at 2 h), VSL (17.1 ± 0.4 µm/s at 0 h; 13.1 ± 0.4 µm/s at 2 h), VAP (29.8 ± 0.7 µm/s at 0 h; 21.5 ± 0.5 µm/s at 2 h), ALH (2.7 ± 0.1 µm at 0 h; 2.4 ± 0.1 µm at 2 h) and BCF (5.6 ± 0.3 Hz at 0 h; 3.2 ± 0.3 Hz at 2 h) significantly decreased ($P < 0.050$) 2 h post-thaw, nonetheless, other kinematic parameters as LIN (32.8 ± 0.9% at 0 h; 41.2 ± 0.9% at 2 h), STR (57.6 ± 0.9% at 0 h; 61.1 ± 0.8% at 2 h) and WOB (56.7 ± 0.9% at 0 h; 67.2 ± 0.8% at 2 h) raised significantly ($P < 0.050$) 2 h post-thaw. Sperm vitality and plasma membrane were affected by the time elapsed after thawing ($P < 0.050$) (Figure 3). Conversely, integrity of acrosome membranes was maintained 2 h post-thaw.

Table 1. Effect of extenders A–D on kinematic parameters of freeze-thawed rabbit spermatozoa from seminal plasma (SP) and non-SP groups

	MOT (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
Extender A	38.5 ± 2.0^{a}	48.6 ± 2.2^{a}	17.8 ± 0.6^{a}	29.2 ± 1.1^{a}	37.3 ± 1.3^{a}	60.9 ± 1.2^{a}	61.2 ± 1.2^{a}	2.8 ± 0.1^{a}	5.8 ± 0.4^{a}
Extender B	21.2 ± 2.0^{b}	42.1 ± 2.2^{b}	15.5 ± 0.6^{b}	26.1 ± 1.1^{ab}	37.9 ± 1.3^{a}	60.0 ± 1.2^{a}	62.9 ± 1.2^{a}	2.5 ± 0.1^{ab}	4.1 ± 0.4^{ab}
Extender C	23.4 ± 1.9^{b}	42.9 ± 2.1^{b}	14.8 ± 0.6^{b}	25.0 ± 1.0^{bc}	36.3 ± 1.2^{a}	59.6 ± 1.2^{a}	60.7 ± 1.2^{a}	2.7 ± 0.1^{a}	4.6 ± 0.4^{a}
Extender D	12.5 ± 1.9^{c}	38.2 ± 2.0^{b}	12.5 ± 0.6^{c}	22.3 ± 1.0^{c}	36.5 ± 1.2^{a}	56.9 ± 1.1^{a}	62.9 ± 1.2^{a}	2.2 ± 0.1^{b}	2.9 ± 0.4^{b}
P-value	< 0.001	0.008	< 0.001	< 0.001	0.781	0.092	0.405	0.012	< 0.001

MOT = spermatozoa motility, VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BFC = beat cross frequency
$^{a–c}$different letters within each column denote statistical differences ($P < 0.050$); data are means ± SEM

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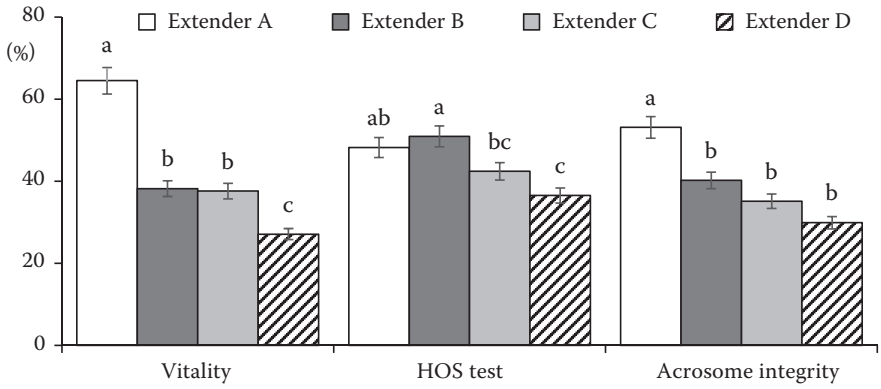

Figure 2. Percentage of vitality, HOS test and acrosome integrity of freeze-thawed rabbit sperm diluted with extenders A, B, C and D from seminal plasma (SP) and non-SP groups (data are mean ± SEM)

[a–c]different letters within each diagnostic test denote statistical differences ($P < 0.05$)

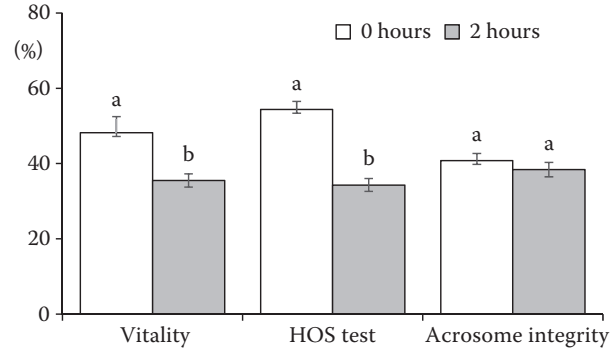

Figure 3. Percentage of vitality, HOS test and acrosome integrity of rabbit sperm analysed at 0 and 2 h after thawing from the four extenders (A–D), seminal plasma (SP) and non-SP groups (data are mean ± SEM)

[a,b]different letters within each diagnostic test denote statistical differences ($P < 0.05$)

Table 2. Rabbit spermatozoa motility percentage 0 and 2 h post-thaw diluted with extenders A–D from seminal plasma (SP) and non-SP groups

	Time after thawing (h)	MOT (%)
Extender A	0	52.9 ± 2.8[a]
	2	24.3 ± 2.8[bc]
Extender B	0	25.6 ± 2.8[b]
	2	16.8 ± 2.8[c]
Extender C	0	35.0 ± 2.8[b]
	2	11.9 ± 2.7[d]
Extender D	0	16.4 ± 2.7[c]
	2	8.7 ± 2.7[e]

[a–e]different letters within each column denote statistical differences ($P < 0.050$); data are means ± SEM

The interaction between the extender and the time after thawing was analysed. There was found a significant difference ($P < 0.001$) in the percentage of motile spermatozoa between sperm immediately post-thaw and sperm after a 2-hour post-thaw incubation in each group (extender) (Table 2). The highest percentage of motile spermatozoa was reached by extender A at 0 h after thawing (52.9 ± 2.8%). Extender D got the worst values at 0 and 2 h post-thaw.

Regarding all sperm parameters studied, significant differences between freeze-thawed rabbit sperm samples processed with or without SP were not evaluated (Table 3).

DISCUSSION

Sperm cryopreservation technique is used for conservation of banking resources (endangered breeds or high-value males), animal genetic diffusion between countries (semen from selected lines) and research. Due to the lack of an efficient cryopreservation method, many authors have tried to improve it by testing extenders (Lopez and Alvarino 2000; Carluccio et al. 2004) and CPA (Dalimata and Graham 1997; Rosato and Iaffaldano 2013; Johinke et al. 2014; Nishijima et al. 2015). Nevertheless, Carluccio et al. (2004) demonstrated that INRA 96® diluent maintained chilled rabbit sperm quality better than others even though it was made specifically for chilling stallion sperm preservation. To avoid cryodamage caused during freeze-thawing sperm process some CPA should be added to chilling diluent (Fuller and Paynter 2004; Hernandez et al. 2012). Additionally, there are extenders ready to use in sperm cryopreservation such as BotuCrio® which is composed of lower concentration of glycerol (1%) and amides (4%). In this study we observed that the addition of CPA as 6% of glycerol, DMF or NMP to INRA 96® (specific diluent to preserve cooled sperm) was not suitable to maintain sperm quality during freeze-thawing procedure as well as BotuCrio®

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Table 3. Effect of seminal plasma (SP) on motility, vitality, HOS test and acrosome integrity parameters of freeze-thawed rabbit spermatozoa

	MOT (%)	VCL (μm/s)	VSL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)	Vitality (%)	HOS test (%)	Acrosome integrity (%)
Without SP	24.0 ± 1.4	42.5 ± 1.5	14.9 ± 0.4	25.1 ± 0.7	37.0 ± 0.9	59.9 ± 0.9	61.6 ± 0.9	2.6 ± 0.1	4.5 ± 0.3	42.7 ± 1.5	43.7 ± 1.6	41.6 ± 2.8
With SP	23.9 ± 1.4	43.4 ± 1.5	15.4 ± 0.4	26.2 ± 0.7	36.9 ± 0.9	58.8 ± 0.8	62.4 ± 0.9	2.6 ± 0.1	4.3 ± 0.3	40.9 ± 1.5	45.2 ± 1.7	36.1 ± 2.9
P-value	0.968	0.684	0.480	0.327	0.928	0.351	0.519	0.984	0.687	0.387	0.479	0.230

MOT = spermatozoa motility, VCL = curvilinear velocity, VSL = straight-line velocity, VAP = average path velocity, LIN = linearity, STR = straightness, WOB = wobble, ALH = amplitude of lateral head displacement, BFC = beat cross frequency; data are means ± SEM

(specific diluent to preserve frozen sperm). The highest values on motility and velocity parameters (VCL, VSL and VAP) obtained by BotuCrio® confirmed the hypothesis of Salmani et al. (2014) suggesting that high concentrations of CPA may increase the viscosity of the media and therefore decrease the velocity and movement characteristics of spermatozoa. And furthermore, the different concentration of glycerol contained in BotuCrio® (1% glycerol) and extender A (6% glycerol) could be one of the reasons why BotuCrio® maintains better sperm quality than extender A, supporting other authors who demonstrated that the toxic effect of glycerol on rabbit sperm is directly related to the concentration and the time of cell exposure (Alvarino 1993; Swain and Smith 2010).

To highlight, other studies in stallion sperm cryopreservation (Olaciregui et al. 2014) suggested that DMF should be used for cryopreservation; contrarily, in other animal species like boar (Malo et al. 2009), canine (Lopes et al. 2009), goat (Bezerra et al. 2011) and fowl (Chalah et al. 1999) it was concluded that glycerol was more suitable CPA than DMF for sperm cryopreservation. In our study no significant differences were found between glycerol or amide solvents (DMF and NMP). These results differ from those of other authors (Curry et al. 1995; Moce and Vicente 2009) suggesting that the most appropriate solvents to use in rabbit sperm cryopreservation should have low molecular weight and high permeability as amide solvents. Moreover, these results suggested that due to the differences in fatty acid composition and sterol levels of mammals' sperm membrane (Darin-Bennet and White 1977) every animal species requires a specific CPA to fit their needs.

Motility is one of the most studied parameters which is related directly to sperm quality. Dalimata and Graham (1997) studied the cryopreservation of rabbit spermatozoa using different CPA concluding that the combination of cell permeating CPA (acetamide) with cell non-permeating CPA (trehalose and methyl cellulose) was the most effective media for rabbit sperm cryopreservation reaching 46 ± 3% of motile spermatozoa after thawing. Similar results were found by Rosato and Iaffaldano (2013) using 0.1M trehalose to freeze-thawed rabbit sperm (45% of MOT) and Nishijima et al. (2015) demonstrated that 1.5% of lecithin could be used as a CPA to freeze-thawed rabbit sperm (34.3 ± 7.3% of MOT). Two interest-

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ing experiments were performed by Kulikova et al. (2015, 2017). In 2015, these researchers studied the cryoprotective effect of Ficoll (non-permeable CPA) on rabbit spermatozoa quality; 50.93% of motile sperm was reached at 0 min after thawing and 26.93% 2 h post-thaw. In 2017, they demonstrated no breed effect (Nitra and Zobor rabbit) on sperm motility after thawing, no reaching 40% of motile spermatozoa immediately post thaw. In compare with available publications, this research achieved the highest percentage of MOT (52.9 ± 2.8%) by processing rabbit sperm samples with BotuCrio®.

As other authors have published, the sperm quality decrease 2 h after thawing at 37°C (Kulikova et al. 2015; Hall et al. 2017) may be due to the peroxidation of spermatozoa during conservation (Darin-Bennet and White 1977; Castellini et al. 2006). In spite of almost all parameters decreased, there was not observed a significant alteration on the membrane of the acrosome 2 h post-thaw. This result along with the good percentage of alive and motile spermatozoa suggest that frozen sperm samples thawed during 2 h may be usable for artificial insemination. Naturally, the faster artificial insemination with thawed sperm performed, the higher fertility rate obtained.

In the present experiment the elimination of SP prior to cryopreservation did not exert a beneficial effect on sperm survival post the freeze-thawing procedure. This observation is in agreement with previous findings of Gogol (1999), reporting that freeze-thawed samples processed with SP displayed similar fertility (81%) than samples without SP (77%). Similarly, Dalimata and Graham (1997) and Aksoy et al. (2008) came to the conclusion that a detrimental effect of SP removing during cryopreservation process on sperm fertilizing ability seems unlikely. For that reason, despite SP containing beneficial components for sperm, this results suggest that it is not necessary to remove SP during cryopreservation process what would diminish and facilitate freeze-thawing sperm protocol.

CONCLUSION

To our knowledge, BotuCrio® has never been studied for freezing rabbit sperm and whilst it is a commercial diluent for stallion sperm cryopreservation, the finding of this research is very important revealing that BotuCrio® is a good extender for using in freeze-thawing rabbit sperm procedure. Anyhow, further studies should be carried out to confirm this result by *in vivo* experiments and determine the optimal concentration of glycerol, DMF and NMP cryoprotectants. Additionally, artificial insemination with frozen sperm should be performed immediately after thawing and the elimination of SP is not necessary making the cryopreservation process of rabbit sperm easier.

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