



IMPROVING THE RABBIT SEMEN CRYOPRESERVATION PROTOCOL: COMPARISON BETWEEN TWO EXTENDERS AND INSEMINATING DOSES*

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Abstract

This study has been designed to optimize the semen freezing protocol in rabbits. In this regard we compared a Tris-citrate-glucose (TCG) extender with a commercial one (Cortalap[®]), that to the best of our knowledge has never been used up to now on the *in vitro* freezability and fertilizing ability of cryopreserved rabbit semen. Two different inseminating semen doses were considered. Five pooled semen samples were divided into two subsamples and each of them were diluted to a ratio 1:1 (v:v) with a freezing extender composed of TCG or Cortalap[®] containing 16% of dimethylsulfoxide and 0.1 mol/L of sucrose. The extended semen was filled into 0.25 mL plastic straws and frozen above a liquid nitrogen surface. After thawing (50°C/10 seconds) we determined sperm motility, viability, membrane functionality, acrosome and DNA integrity. Our results showed that the Cortalap[®] extender significantly improved the *in vitro* post-thaw sperm quality, in comparison to TCG. When we compared the extenders *in vivo*, no significant differences in the reproductive performances were observed independently by inseminating doses used. In this study we demonstrated that Cortalap[®] extender can be used as an alternative to TCG. Thus, the Cortalap[®] being a ready to use extender, implies a reduction of time, mistakes and microbial contaminations during its preparation. This discovery is significant because it provides an important contribution to the creation of the first Italian semen cryobank of rabbit breeds and also for livestock rabbit farms based on artificial insemination (AI) program.

Key words: rabbit sperm, based extender, fertility, prolificacy, inseminating dose

*This research was funded by Cun-Fu project: “La cunicoltura del futuro: benessere e sostenibilità degli allevamenti cunicoli italiani?”. National Rural Development Program (PSRN) 2014/2020. Characterization of animal genetic resources of zootechnical interest and protection of biodiversity. Sub-measure: 10.2.

The role of rabbit breeds selected for meat production, has dramatically decreased (Jochová et al., 2017). This is due to the fact that the world production of rabbit meat is mainly based on commercial hybrid lines. According to the FAO guidelines (FAO, 2007) most rabbit breeds have been included in the national programs of genetic resources conservation. The value of these breeds has encouraged many conservation programs based on *in situ* and *ex situ* strategies at global and European levels. Even in Italy a conservation and valorisation project of Italian rabbit breeds is in progress thanks to the financial support of the Ministry of Agricultural, Food and Forestry Policies (MiPAAF). This project also involves the creation of the first Italian semen cryobank of rabbit breeds. In this regard, semen cryopreservation is a valuable tool for the conservation of animal biodiversity when it comes to facilitating the storage of gametes in a gene bank using thus the *ex situ in vitro* strategy (Blesbois, 2007; Leroy et al., 2011; Zaniboni et al., 2014; Iaffaldano et al., 2016).

Semen cryobank offers several benefits: allowing the backup of populations preserved *in vivo* in the event of genetic problems (inbreeding and genetic drift), therefore increasing the effective population size; providing the possibility to reconstruct breeds in the event of extinction or drastic reduction in the population. In this regard, the establishment of the semen cryobank assumes the identification of a reference procedure for rabbit semen cryopreservation.

Furthermore, the use of frozen semen could be advantageous, beyond safeguarding the rabbit breed biodiversity, but also for livestock rabbit farms based on artificial insemination (AI) program. To date, in rabbit farms AI is performed with fresh or cooled semen rather than frozen semen because of the poor fertility results after thawing (Mocé and Vicente, 2009; Lavara et al., 2017).

In our previous papers many variables of a rabbit semen freezing protocols have been studied (Iaffaldano et al., 2012, 2014; Kuliková et al., 2015; Di Iorio et al., 2018). Summing up the results obtained in the above mentioned works, we obtained the following best semen freezing protocol: cooling at 5°C for 90 min, dilution 1:1 with the freezing extender (tris-citric acid-glucose – TCG) containing 16% of dimethylsulfoxide (DMSO) and 0.1 M of sucrose, equilibration time of 45 min at 5°C, exposure to liquid nitrogen vapour at 5 cm above the nitrogen. This protocol allowed us to reach similar reproductive performances with frozen semen to those recorded with fresh semen. The inseminating dose/straw (0.25 mL) contained a frozen sperm concentration ranging from 40 to 75×10^6 /mL for each doe (Iaffaldano et al., 2012, 2014; Di Iorio et al., 2018).

So far, one of the parameters that we have not yet evaluated in the freezing protocol is the type of extender. In this regard, we have used Tris-based extender (TGC) in our previous research but it is also very frequently used in the world of rabbit sperm cryopreservation (Cortell and Viudes de Castro, 2008; Mocé and Vicente, 2009; Mocé et al., 2010, 2014; Hall et al., 2017; Zhu et al., 2015, 2017).

Here we are going to test the Cortalap[®], a commercial extender that would facilitate the semen freezing protocols. The choice of the Cortalap[®] is driven by the fact that in one of our previous studies it was the best among four extenders (Cortalap[®], TCG, Lepus[®] and Merk III[®]), in terms of the *in vitro* preservability of rabbit spermatozoa stored for 72 hours at 5°C (Di Iorio et al., 2014).

Up to now, to the best of our knowledge the Cortalap® has never been used in the cryopreservation protocol of rabbit semen. Therefore, this study has been designed to compare a commercial extender (Cortalap®) with TCG on the *in vitro* freezability and *in vivo* reproductive performances of cryopreserved rabbit semen. Two different inseminating semen doses have been also considered.

Material and methods

Chemicals

All chemicals used were of the highest commercially available purity. Unless stated otherwise, all of the chemicals were purchased from Sigma Chemical Co. (Milan, Italy).

Animals

In this research 30 adult bucks (8 months old) and 150 does (8–9 months old) of the Bianca Italiana breed were used. Rabbits were kept at the Central Breeding Farm of Italian Rabbit Breeders Association (ANCI-AIA, Volturara Appula (FG), Italy). Animals were placed in individual flat-deck cages, subjected to a photoperiod of 16 h light/day, fed with commercial standard breeder diet and allowed free access to water. The experiments were carried out in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments. The approval request number was 30003 of 20-12-2017 (2017-UNMLCLE-0030003).

Semen collection and macroscopic evaluation

Ejaculates were collected using a teaser doe and pre-heated artificial vagina. Only ejaculates that exhibited a white colour were used in the experiment, samples containing urine and/or cell debris were discarded, when present, gel plugs was removed. To be included in the study, ejaculates were required to be at least 400 µL in volume. The ejaculates were pooled (4 ejaculates/pool) to avoid the effects of individual differences among males. In total, 5 pools were used for the experiments.

Experiment 1. Effects of different extenders on post-thaw semen quality

Semen pools were transferred from the farm to the laboratory in a polystyrene box at room temperature and within 30 min. An aliquot was withdrawn from each pool to evaluate the fresh semen quality, as described below, and the remaining pooled semen was cooled at 5°C for 90 min before freezing (Di Iorio et al., 2018).

Subsequently, each pool was divided into two equal aliquots, and each of them was diluted to a ratio 1:1 (v:v) with a freezing extender composed of TCG (250 mmol/L Tris-hydroxymethylaminomethane, 88 mmol/L citric acid, and 47 mmol/L glucose) or Cortalap® (IMV-Technologies), both extenders containing 16% of DMSO (as permeating cryoprotectant) and 0.1 M of sucrose (as non-permeating cryoprotectant). The extended semen was filled into 0.25 mL plastic straws and equilibrated

at 5°C for 45 min; in total, 200 straws were used (20 straws for each treatment × 2 treatments × 5 replicates). Then, the straws were allocated horizontally 5 cm above a liquid nitrogen surface for 10 min and plunged into liquid nitrogen for storage.

The straws were thawed after storage period of at least 1 month by transferring them into a water bath at 50°C for 10 seconds.

Analysis of sperm quality variables

The sperm quality parameters evaluated in both fresh and thawed semen were: sperm motility, viability, membrane functionality, and acrosome and DNA integrity. Moreover, for each pool of fresh semen the sperm concentration was evaluated using a Neubauer hemocytometer. Semen was diluted 1:100 (v:v) with 3% NaCl (w:v) solution and spermatozoa were counted in the central grid of the hemacytometer. Both chambers of the hemocytometer were counted and averaged. Sperm concentration was expressed by million per milliliter (no. spz × 10⁶/mL).

Sperm motility was evaluated by subjectively visual estimation. Briefly, 5 µL of diluted semen (1:10 in NaCl 0.9%) were delivered on a glass slide prewarmed at 37°C and covered with a coverslip. The slides were examined at ×400 magnification using a phase-contrast microscope (Leica Aristoplan; Leitz Wetzlar, Heidelberg, Germany). At least five fields were observed and percentage of total motility (TM, spermatozoa showing any type of sperm head movement) and forward progressive motility (FPM, spermatozoa showing linear movement) were scored.

Sperm viability was estimated using two fluorescent stains SYBR-14 and propidium iodide (PI) (LIVE/DEAD Sperm Viability Kit-Molecular Probes, Inc.). This test was performed on 5 µL of semen, which were added to 39 µL of TCG containing 1 µL SYBR-14 (diluted 1:100 in DMSO), incubated at 37°C for 10 min, and then were added 5 µL propidium iodide (diluted 1:100 in the TCG diluent) followed by incubation of a further 5 min at the same temperature. Lastly, 5 µL of this solution were placed on microscope slides and examined at a magnification ×1000 using a ×100 oil immersion objective under epifluorescence illumination (blue excitation filter $\lambda = 488$ nm). For each sample, minimum 200 sperm cells were counted in duplicate aliquots. Spermatozoa showing green fluorescence were marked as alive and those showing red fluorescence as dead. The percentage of viability was calculated as the number of green cells × 100 divided by the total number of sperm counted.

The membrane functionality of the rabbit sperm was assessed by studying the swelling reaction of sperm tail when suspended in a medium of distilled water (hypo-osmotic swelling test; HOST) as described by Lomeo and Giambersio (1991). This test is effective in measuring changes in sperm membrane functional status and permeability when exposed to hypo-osmotic conditions. The analysis was conducted by mixing 10 µL of semen with 80 µL of distilled water, incubating for 5 min at 37°C, then 10 µL were deposited on a clean glass slide and examined under a phase-contrast microscope (magnification ×400). The typical sperm osmotic “coiled tail” reaction was easily detected, and the number of hypo-osmotic water test-positive cells was recorded by counting spermatozoa showing this feature among 200 cells examined (Rosato and Iaffaldano, 2011).

Duplicate smears were realized using a drop of semen from each sample to evaluate acrosome integrity. After fixation in methanol for 30 min, the slides were washed with water, air-dried and incubated with the *Pisum sativum* agglutinin FITC (fluorescein isothiocyanate) conjugate (FITC-PSA) for 30 min at room temperature (Mendoza et al., 1992). In each slide, assessment was made of 200 sperm at a magnification $\times 1000$ using an oil immersion objective under epifluorescence illumination. This stain intensely labels the acrosomal region of acrosome-intact sperm, which emit a uniform apple-green fluorescence, while acrosome-damaged spermatozoa show scarce or no green fluorescence in the anterior part of the head. The percentage of acrosome-intact spermatozoa was calculated as a fraction of the total.

Sperm DNA integrity was evaluated following the method developed by Gandini et al. (2006) using acridine orange (AO). Ten μL of semen were smeared onto a microscope slide, air-dried and fixed overnight in a 3:1 methanol:glacial acetic acid solution. Slides were then stained with an AO solution (0.2 mg/mL in water). After an incubation period of 5 min in the dark at room temperature each smear was observed using a fluorescence microscope with a 490 nm excitation light and 530 nm barrier filter. Nuclei in at least 200 spermatozoa per slide were examined and scored as green or yellow-orange-red fluorescing (intact DNA or damaged DNA, respectively) and the percentage of normal and abnormal chromatin condensation calculated.

Experiment 2. Comparing the *in vivo* reproductive performance of rabbit does inseminated with semen cryopreserved using TCG and Cortalap® and two different volumes of inseminating dose

In this experiment, we tested the efficacy of semen frozen in presence of TCG and Cortalap® (using two different inseminating doses) with fresh semen in an artificial insemination trial. In total 150 multiparous (31 days postpartum) receptive rabbit does were used, that were divided into five treatment groups (30 does for each group):

- 1) fresh semen (control group), each doe received a 0.5 mL dose of fresh semen diluted 1:10 with Cortalap® extender (approximately 35×10^6 sperm),
- 2) TCG/0.25 mL: each doe was inseminated with one straw of thawed semen (approximately 75×10^6 sperm),
- 3) TCG/0.18 mL: each doe received 0.18 mL of thawed semen (approximately 55×10^6 sperm),
- 4) Cortalap®/0.25 mL: each doe was inseminated with one straw of thawed semen (approximately 75×10^6 sperm),
- 5) Cortalap®/0.18 mL: each doe received 0.18 mL of thawed semen (approximately 55×10^6 sperm).

All of the does underwent the following biostimulation protocol to synchronize their estrus: flushing, that is, increasing feed amount/doe from 180 g/day to *ad libitum* (3 days before insemination), changing cages (3 days before insemination), and increasing the photoperiod from 16 to 24 hours of light (2 days before insemination). At the time of insemination, each female received an intramuscular injection of buserelin acetate to induce ovulation (1 $\mu\text{g}/\text{doe}$).

Fertility (number of pregnant does/number of inseminations) was determined by abdominal palpation performed on each doe 17 days after artificial insemination. At parturition, the factors: kindling rate (number of does giving birth/number of inseminations), number of kits born (total born/kindling), and number of kits born alive (total live-born/kindling) were assessed.

Statistical analysis

Sperm quality (motility, viability, membrane functionality, acrosome and DNA integrity) and reproductive performances (fertility, kindling, prolificacy and kits born alive) data were compared among treatments by ANOVA, followed by Duncan's comparison test. All statistical tests were performed using the software package SPSS (SPSS 15.0 for Windows, 2006; SPSS, Chicago, IL, USA). Significance was set at $P \leq 0.05$.

Results

Effects of TCG and Cortalap® on *in vitro* post-thaw semen quality

The average sperm concentration of fresh semen was $610 \pm 15 \times 10^6$ spermatozoa/mL. The results of fresh and post-thaw semen quality in terms of TM, FPM, viability, membrane functionality, acrosome and DNA integrity are provided from Figure 1 to Figure 6, respectively. Better values of all sperm parameters (except for DNA integrity) were obtained in fresh semen ($P \leq 0.05$) in respect to those cryopreserved, even if higher TM, FPM, viability, membrane functionality and acrosome integrity were recorded in semen cryopreserved in presence of Cortalap® ($P \leq 0.05$).

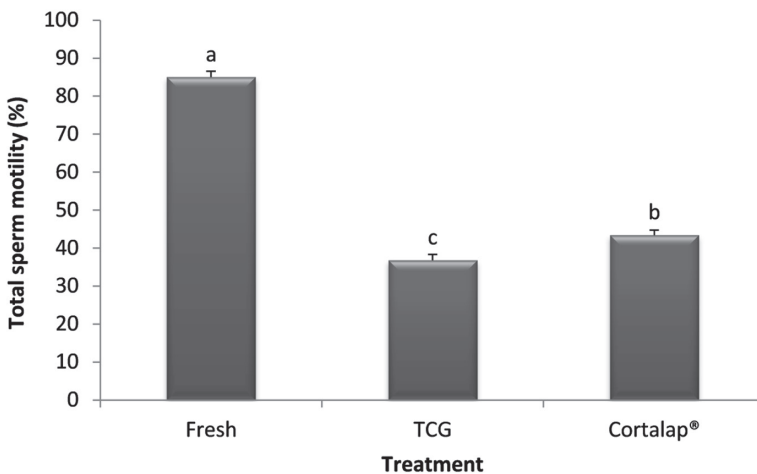


Figure 1. Total motility of fresh and frozen rabbit semen cryopreserved using TCG or Cortalap®. Bars represent means \pm SEM. Different lowercase letters on bars indicate a significant difference ($P \leq 0.05$)

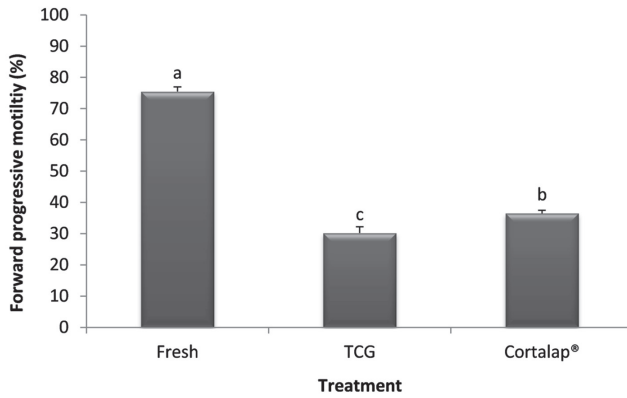


Figure 2. Forward progressive motility recorded in fresh and frozen rabbit semen cryopreserved using TCG or Cortalap®. Bars represent means \pm SEM. Different lowercase letters on bars indicate a significant difference ($P \leq 0.05$)

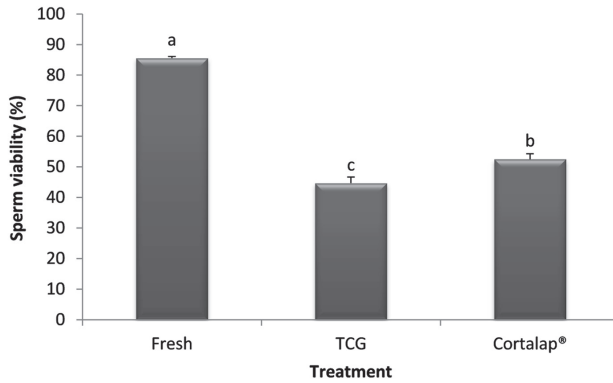


Figure 3. Sperm viability obtained in fresh and frozen rabbit semen cryopreserved in presence of TCG or Cortalap®. Bars represent means \pm SEM. Different lowercase letters on bars indicate a significant difference ($P \leq 0.05$)

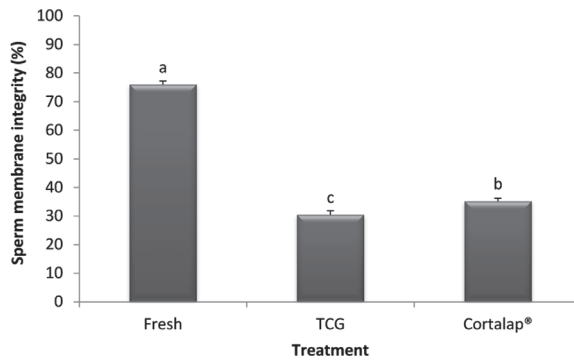


Figure 4. Sperm membrane functionality of fresh and frozen rabbit semen cryopreserved using TCG or Cortalap®. Bars represent means \pm SEM. Different lowercase letters on bars indicate a significant difference ($P \leq 0.05$)

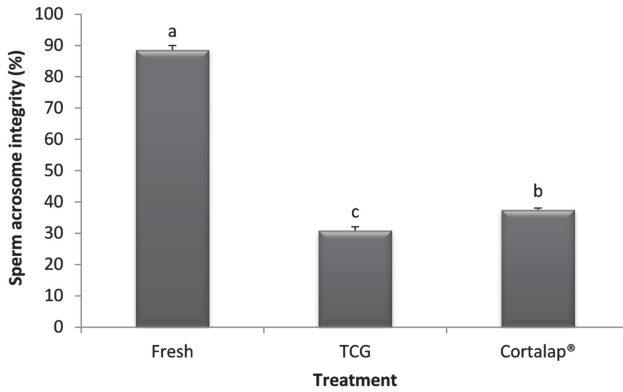


Figure 5. Acrosome integrity recorded in fresh and frozen rabbit semen, cryopreserved using TCG or Cortalap®. Bars represent means \pm SEM. Different lowercase letters on bars indicate a significant difference ($P \leq 0.05$)

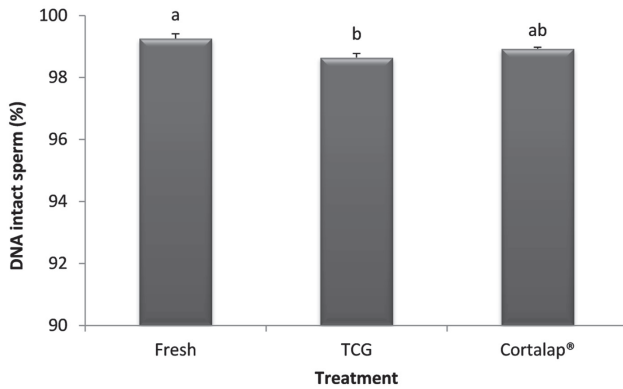


Figure 6. DNA integrity of fresh and frozen rabbit semen cryopreserved in presence of TCG or Cortalap®. Bars represent means \pm SEM. Different lowercase letters on bars indicate a significant difference ($P \leq 0.05$)

Artificial insemination

Table 1 provides the reproductive performances recorded after the artificial insemination of does that were inseminated with fresh semen or semen frozen in presence of TCG or Cortalap®.

No significant differences were observed for all reproductive performances considered among the treatments. Nevertheless, higher prolificacy and number of kits born alive were found in does fertilized with semen cryopreserved in presence of Cortalap® (0.25 mL); this based extender guarantees a higher number of total born and live born (197 and 183) compared with fresh semen (170 and 154) and semen frozen in TCG (178 and 170).

Table 1. Reproductive performance outcomes in rabbit does after insemination with fresh semen or semen frozen in presence of TCG or Cortalap® considering two inseminating doses

| Semen treatment | | | Reproductive performances | | | |
|-----------------|-----------|-------------|---------------------------|----------------|-------------------------|------------------------|
| | Extender | Volume (mL) | % Fertility (n) | % Kindling (n) | Total born (mean ± SEM) | Live born (mean ± SEM) |
| Fresh | | 0.5 | 80.0 (24) | 73.3 (22) | 7.7±0.7 | 7.0±0.7 |
| Frozen | TCG | 0.25 | 86.7 (26) | 80.0 (24) | 7.4±0.8 | 7.1±0.8 |
| | TCG | 0.18 | 73.3 (22) | 70.0 (21) | 7.8±0.5 | 7.7±0.5 |
| | Cortalap® | 0.25 | 76.7 (23) | 70.0 (21) | 9.4±0.6 | 8.4±0.9 |
| | Cortalap® | 0.18 | 70.0 (21) | 63.3 (19) | 8.4±0.9 | 8.3±0.9 |

Discussion

The purpose of the present study was to evaluate the effect of Cortalap® that is a ready to use commercial extender that could substitute the TCG in freezing protocol of rabbit semen. Premising that the TCG, as a based extender turned out to be successful for rabbit sperm cryopreservation both by us and by other authors (Mocé and Vicente, 2009; Iaffaldano et al., 2012, 2014; Zhu et al., 2015, 2017; Hall et al., 2017; Di Iorio et al., 2018), the replacement of TCG with the Cortalap® could offer practical advantages such as: reduction of time, mistakes and microbial contaminations during its preparation. Thus, the Cortalap® that is a ready to use extender would facilitate the processing of the semen cryopreservation. Here, the Cortalap® has been tested for the first time for cryopreservation of rabbit semen and the rationale of its usage is based on the satisfactory results obtained previously *in vitro* in chilled semen (Di Iorio et al., 2014). Our results showed that the based extender clearly affected the post-thaw semen quality *in vitro*. In fact, the Cortalap® extender significantly improved sperm motility, viability, membrane functionality and acrosome integrity after the freezing process in comparison to TCG. Contrary to our expectations no significant differences were observed between the two extenders for all reproductive performances considered. This is consistent with Cortell and Viudes de Castro (2008) who observed similar reproductive performances between TCG and another commercial extender (MIII-Minitube).

We assume that the Cortalap® works similarly to TCG during the freezing of rabbit semen. Due to the commercial interests of the Cortalap® we were only able to know the type of component of extender which includes tris, citric acid, salts, sugars and water but not their concentrations. Some of these components are also present in the glucose-based Tris extender. However, an effective comparison between two extenders is difficult because we do not know what sugars are included and/or the concentrations of different components.

Notice that the role of extenders in semen preservation is primarily to extend the fertile life of sperm (indefinitely in the case of frozen semen) and to greatly extend the genetic potential of individual males through artificial insemination (A. I.) (Foote and Bratton, 1950). An extender must first meet the basic physiological requirements

of sperm-iso-osmotic, near-neutral pH with an appropriate buffer and metabolizable substrate (Salamon and Maxwell, 2000; Layek et al., 2016). Therefore, an ideal extender should have nutrients as an energy source (glucose and fructose are most commonly used), substances that buffer against harmful changes of pH, provide a physiological osmotic pressure and concentration of electrolytes (citric acid, sodium citrate, TES and Tris).

Thus, we can speculate that the Cortalap[®] extender provided a suitable condition for rabbit spermatozoa during cryopreservation process and preserved the *in vitro* spermatic characteristics of frozen semen better.

Another interesting point studied in this research was reducing the number of spermatozoa for each doe (75×10^6 vs 55×10^6 sperm/female) in order to fertilize a greater number of females using 0.18 mL (55×10^6 sperm)/doe (in total 5.4 mL/30 female has been used). Although no significant differences were registered in the reproductive performances using two different inseminating volumes for both extenders, a decrease of about 10% of fertility and kindling rate was observed when the inseminating dose was 0.18 mL. However, this information deserves attention in further research. Thus, at the moment we can sustain that the insemination dose corresponding to 55×10^6 sperm/female turned out to be impractical during artificial insemination operations due to smaller volume dose used (0.18 mL).

Many studies have been performed to find out what the appropriate sperm dose is when using frozen semen for artificial insemination (Mocé and Vicente, 2009). Some authors worked at a fixed concentration of motile sperm ranging from 3 to 20×10^6 motile sperm/female (Maeda et al., 2012; Nishijima et al., 2015) while others implemented a fixed total sperm concentration (Arriola and Foote, 2001; Mocé et al., 2003 a, b, 2010; Viudes-de-Castro et al., 2005), or at a fixed semen dilution depending on the initial concentration of the ejaculates (Iaffaldano et al., 2012, 2014; Viudes-de-Castro et al., 2014; Di Iorio et al., 2018).

However, the optimal number of frozen spermatozoa, in order to reach effective reproductive performances, is highly variable because it largely relates to the experimental conditions and the physiological status of the does at the moment of the insemination.

Conclusions

In conclusion the results obtained here provide a contribution to the development of an effective semen cryopreservation protocol that is necessary for the creation of the first rabbit cryobank that is an important milestone within our financed “Mi-PAAF” project which promotes the *ex situ* conservation strategies for the safeguard of rabbit breeds.

Moreover, the discoveries obtained here lay out the groundwork for further challenges. In this regard we aim to find an optimal sperm concentration for a straw utilizing commercial ready to use extender that ensures safety and facilitates to field activities such as the Cortalap[®].

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors would like to thank the President (Dr. Sergio Pompa) of Italian Rabbit Breeders Association (ANCI-AIA) for allowing the use of the Central Breeding Farm.

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Received: 28 X 2019

Accepted: 14 II 2020