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Effect of chelating and antioxidant agents on morphology and DNA methylation in freeze-drying rabbit (*Oryctolagus cuniculus*) spermatozoa

Francesca Mercati[1] | Paula Domingo[2] | Rolando Pasquariello[3] | Cecilia Dall'Aglio[1] | Alessandro Di Michele[4] | Katia Forti[5] | Paolo Cocci[6] | Cristiano Boiti[1] | Lidia Gil[2] | Massimo Zerani[1] | Margherita Maranesi[1]

[1]Dipartimento di Medicina Veterinaria, Università di Perugia, Perugia, Italy

[2]Departamento de Patología Animal, Universidad de Zaragoza, Zaragoza, Spain

[3]Dipartimento di Scienze Agrarie e Ambientali - Produzione, Territorio, Agroenergia, Università di Milano, Milano, Italy

[4]Dipartimento di Fisica e Geologia, Università di Perugia, Perugia, Italy

[5]Istituto Zooprofilattico Sperimentale Umbria e Marche 'Togo Rosati' Perugia, Perugia, Italy

[6]Scuola di Bioscienze e Medicina Veterinaria, Università di Camerino, Camerino, Italy

Correspondence
Rolando Pasquariello, Dipartimento di Scienze Agrarie e Ambientali - Produzione, Territorio, Agroenergia, Università di Milano, Milano, Italy.
Email: rolando.pasquariello@unimi.it

Massimo Zerani, Dipartimento di Medicina Veterinaria, Università di Perugia, Perugia, Italy.
Email: massimo.zerani@unipg.it

Abstract

Freeze-drying (FD) has been exhaustively tried in several mammalian species as an alternative technique to sperm cryopreservation, but few studies have been done in rabbits (*Oryctolagus cuniculus*). The main objective of this study was to compare the protective effect of various antioxidants added to EDTA medium on structural and functional components of FD rabbit spermatozoa and on their status of global DNA methylation. FD media used were composed of basic FD medium (10 mM Tris-HCl buffer and 50 mM NaCl) supplemented with either 50 mM EDTA alone (EDTA) or added with 105 μM of rosmarinic acid (RA, EDTA-RA) or 10 μM of melatonin (MLT, EDTA-MLT). The effect of each medium on the preservation of FD spermatozoon structure was evaluated with light and scanning electron microscopy (*SEM*). Global DNA methylation was quantified in all FD sperm samples as well as in fresh spermatozoa. Morphologically, fracture points were evidenced in the neck, mid and principal piece of the spermatozoon tail. No differences in spermatozoon fracture points were evidenced among FD treatments: intact spermatozoa were the largest ($p < .01$) category, whereas the most frequent ($p < .01$) injury was the neck fracture, resulting in tailless heads. At *SEM*, the head of spermatozoa showed a well-conserved shape and intact membrane in all treatments. DNA methylation status was the same in all FD treatments. In conclusion, supplementation of EDTA, EDTA-RA and EDTA-MLT during FD preserved rabbit sperm morphological integrity and methylation status as well. Therefore, the difficulty of getting viable offspring using FD semen is likely unrelated to the impact of the lyophilization process on DNA methylation and morphology of lyophilized spermatozoa.

KEYWORDS

EDTA, lyophilisation, melatonin, rabbit sperm, rosmarinic acid, scanning electron microscopy

1 | INTRODUCTION

Artificial insemination was the first assisted reproductive technology used in domestic animals. Since the first successful experimented in 18th century by Lazzaro Spallanzani, many steps forward were achieved in the field of assisted reproduction: in vitro fertilization, in vivo embryo transfer, embryo sexing, cloning and intracytoplasmic sperm injection (Galli & Lazzari, 2008). These techniques have

positively affected genetic merit and production of livestock, as well as treatment of human infertility.

In recent years, the development of an alternative method to sperm cryopreservation, such as freeze-drying (FD) or lyophilization, has facilitated the storage and maintenance of genetic merit of several species, including rabbits (Keskintepe & Eroglu, 2015; Liu et al., 2004), and bio-banking of endangered species (Anzalone at al., 2018). FD is a method in which frozen material is dried by ice sublimation, thereby involving a direct transition from a solid (ice) to a vapour (gas) phase (Keskintepe & Eroglu, 2015). The purpose of FD is the removal of water that does not allow the growth of pathogens or contaminants (Bielanski, Nadin-Davis, Sapp, & Lutze-Wallace, 2000), ensuring the preservation of the samples. One of the main advantages of lyophilized semen is its conservation and management, which is much simpler and cheaper. In fact, FD semen can be stored temporarily at room temperature (Kaneko, 2015), thereby reducing storage, facility and shipping costs.

However, current FD techniques make spermatozoa non-viable and motionless at rehydration (Keskintepe & Eroglu, 2015), but the handicap of the lost motility was easily overcome by their direct injection into the oocytes (Salamone, Canel, & Rodríguez, 2017). Those findings demonstrated that nuclear and cellular viability are not equivalent: the lyophilized spermatozoa are dead, but still capable of interacting with oocytes and giving rise to normal and live offspring. At the end of the 50s, Yushchenko (1957) reported, for the first time, the birth of a rabbit following artificial insemination with FD sperm. Using intracytoplasmic lyophilized sperm injection, normal offspring were obtained in rabbits (Liu et al., 2004) and in other mammals: sheep (Loi, Iuso, Czernik, Zacchini, & Ptak, 2013), pigs (Das, Gupta, Uhm, & Lee, 2010), horses (Choi, Varner, Love, Hartman, & Hinrichs, 2011), hamsters (Muneto & Horiuchi, 2011), mice (Ono, Mizutani, Li, & Wakayama, 2008; Wakayama & Yanagimachi, 1998) and rats (Hirabayashi, Kato, Ito, & Hochi, 2005). In other species, embryonic development did not go further from blastocyst (Saragusty & Loi, 2019).

However, FD method may affect negatively spermatozoa not only by altering their morphology membrane and acrosome, but also by causing molecular damages on DNA and centrosome, due to snap-freezing and drying stresses (Liu et al., 2004). Among all these sperm aberrations, DNA fragmentation of spermatozoa following FD is one of the main causes of embryonic development failure (Palazzese, Gosálvez, Anzalone, Loi, & Saragusty, 2018). In particular, endonucleases may induce oxidative stress due to the release of reactive oxygen species (ROS) (Kusakabe, Szczygiel, Whittingham, & Yanagimachi, 2001). The chelation of calcium seems to be important for inhibition of endonucleases that are involved in DNA integrity; in particular, chelating agents prevent DNase activation, as observed in mouse spermatozoa, whose DNase is activated by the presence of Ca^{2+}, resulting in DNA injury (Kaneko & Nakagata, 2003).

Calcium chelators, such as EDTA or EGTA, are used in different species to preserve sperm from the lyophilization process and obtain vital offspring (Kaneko & Nakagata, 2006). In fact, spermatozoa are highly sensitive to several types of chemical/physical stress, due to reactive oxygen species, osmotic changes and different temperatures applied during FD procedure for freezing, warming and thawing (Koshimoto & Mazur, 2002).

Rosemary (*Rosmarinus officinalis*) and its extracts, such as rosmarinic acid (RA), are natural antioxidants that show antiproliferative, antiradical and protective properties against oxidant-induced stress effect (Ho, Wang, Wei, Huang, & Huang, 2000). In several mammals (Luño et al., 2014; Olaciregui, Luño, Domingo, González, & Gil, 2017; Olaciregui, Luño, González, et al., 2017; Yeni et al., 2018), including rabbits (Amidi, Pazhohan, Shabani Nashtaei, Khodarahmian, & Nekoonam, 2016; Domingo, Olaciregui, González, De Blas, & Gil, 2018), addition of RA in FD or post-thawing medium (Motlagh et al., 2014) protected spermatozoa against oxidative stress and improved sperm preservation. In contrast, in a sperm in vitro study, Lv et al. (2018) found a decreased intracellular Ca^{2+} concentration compromising sperm function after the addition of RA in a dose-dependent manner.

Melatonin (MLT) is a key regulator of the circadian wake–sleep cycle and reproduction in animals characterized by photoperiod (Chemineau et al., 2008); it has also a relevant role in sperm biology (Cebrián-Pérez et al., 2014). In fact, MLT contrasts a variety of ROS species due to its well established antioxidant activity (Jang et al., 2010). According to various studies, melatonin improves semen characteristics of goats (Ramadan, Taha, Samak, & Hassan, 2009), rats (Sönmez, Yüce, & Türk, 2007), boars (Jang et al., 2010), rams (Ashrafi, Kohram, Naijian, Bahreini, & Poorhamdollah, 2011), mice (Sarabia, Maurer, & Bustos-Obregon., E., 2009) and men (Ortiz et al., 2011). By converse, no study on the role of MLT has been carried out in rabbit spermatozoa. In addition, to the best of our knowledge, no studies evaluated the combined effects of RA and MLT added to EDTA on rabbit sperm during FD.

Semen conservation methods can affect DNA methylation, thus influencing gene expression and animal fertility (Aurich, Schreiner, Ille, Alvarenga, & Scarlet, 2016; Verma et al., 2014). Alteration of DNA methylation has been associated with several pathologies, leading to abnormal embryo development and increased incidence of early embryonic loss (Al-Khtib, Blachère, Guérin, & Lefèvre, 2012). For these reasons, assessment of DNA methylation represents a new, relevant approach to evaluate the ability of spermatozoa to fertilize the oocyte and lead to normal blastocyst and embryo development (Benchaib et al., 2003).

The aim of this study was to determine the protective capacity of chelating agent EDTA, either alone or with the addition of RA or MLT as antioxidants, throughout the sperm FD process in the rabbit (*Oryctolagus cuniculus*). Therefore, we evaluated the morphological structure of lyophilized spermatozoa by light and scanning electron microscopy (*SEM*) as well the DNA methylation status.

2 | MATERIALS AND METHODS

2.1 | Reagents and media

Unless noted otherwise, all chemicals were from Sigma-Aldrich Co. (Alcobendas). Freeze-drying medium used was composed of basic medium (10 mM Tris-HCl buffer and 50 mM NaCl) supplemented

with 50 mM EDTA. Two more experimental FD media were prepared by adding 105 μM RA to EDTA (EDTA-RA) and 10 μM MLT to EDTA (EDTA-MLT). The final pH of the solutions was adjusted between 8.2 and 8.5 (Kaneko et al., 2003).

2.2 | Animals

This study was approved by the Veterinary Ethical Committee of the University of Zaragoza. The care and use of animals were done in accordance with the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

All semen samples were collected from five sexually mature New Zealand white male rabbits (4.5–5 kg body weight and 5–8 mo of age), raised in premises owned by the University of Zaragoza. Males were maintained in individual cages under light cycle of 12 hr light/dark at a room temperature of 22–24°C and a relative humidity of 55%–60%. All animals were fed a commercial pellet diet according to their reproductive condition, and fresh water was given ad libitum.

2.3 | Sperm collection and processing

Rabbit sperm samples were collected by artificial vagina preheated at 55°C and a collector previously tempered at 37°C. After semen collection, any gel plug was removed. Immediately, each ejaculate was diluted (1:3) in pre-warmed (37°C) phosphate-buffered saline (PBS) solution. A macroscopic analysis was performed to assess colour and volume of each ejaculate; the motility was evaluated by computer-assisted semen analysis (CASA, PROISER R + D). Only ejaculates with white colour, >0.2 ml and good wave motion (at least 85% of motility) were used for the research.

Each sperm sample was divided into four falcon tubes: one tube was used as control (fresh sperm) and the other three tubes for three freeze-drying procedures (EDTA, EDTA-RA, EDTA-MLT). Afterwards, the samples were centrifuged twice to remove seminal plasma as much as possible. The first centrifugation was performed at 500 × g for 10 min; thereafter, seminal plasma was removed, and samples were diluted with 1 ml of PBS. Straightaway, a second centrifugation was carried out at 500 g for 5 min and the supernatant was again removed leaving a drop of it to suspend the pelleted spermatozoa into 200 μl of final volume. Then, 1.800 μl of PBS was added to the samples and mixed slowly. The number of spermatozoa of each sample was counted using a Neubauer chamber, and the concentration was adjusted to 10^6 spermatozoa per sample by adding PBS. Lastly, 1 ml of sperm solution was placed into an Eppendorf tube and centrifuged at 500 g for 5 min; the supernatant was then discarded.

2.4 | Fresh sperm samples processing

Fresh sperm samples were diluted with DNA/RNA shield solution (Zymo Research) to a final volume of 200 μl and then frozen at −20°C.

2.5 | Freeze-drying and rehydration procedure

Each sample was suspended in the three different FD media: EDTA, EDTA-RA and EDTA-MLT to have 150 μl of final volume. Freeze-drying procedure was performed as reported by Wakayama and Yanagimachi (1998). Sperm suspensions from each group were placed into 1 ml glass cryovials (Labcon North) and then plunged into liquid nitrogen (LN_2) for 5 min. Immediately, the frozen samples were transferred onto the shelf (−50°C) of a programmable freeze-drier (Millrock Technology). Two dryings were performed for freeze-drying the samples: a first drying at 0.053 mbar of pressure and at −68°C and a second drying at 0.018 mbar of pressure and at −20°C of temperature. After the FD process, cryovials were sealed with rubber cups and parafilm.

The rehydration of FD spermatozoa was performed by adding 300 μl of 0.1 M PBS (pH 7.2). Rehydrated spermatozoa were centrifuged once at 1,000 g for 2 min, and the supernatant was removed.

2.6 | Light and *SEM* microscopy

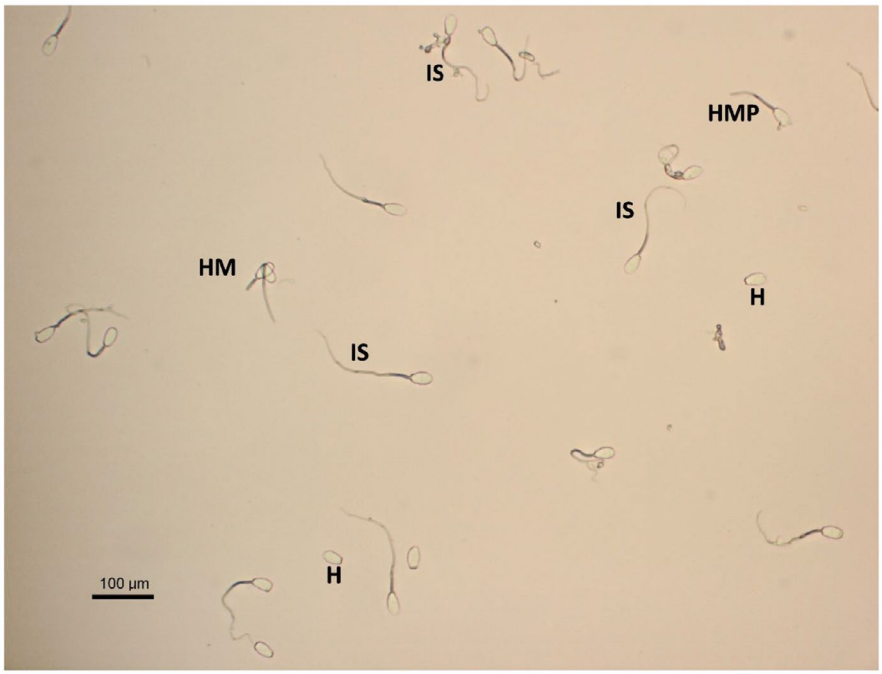
After rinsing, spermatozoa were fixed for 1 hr at room temperature in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Then, they were washed for 10 min in the same buffer and dehydrated in an increasing concentration series of ethanol solutions until absolute. Finally, a drop of solution was gently dropped onto poly-L-lysine-coated slides to spread the spermatozoa.

A total of 300 cells/animal were analysed for each treatment at light microscopy. The spermatozoa were classified according to their integrity and the region where the damage was detected: (a) intact spermatozoa (IS) (b) head, middle tail and partial principal tail (HMP), (c) head and middle tail (HM) and (d) head only (H).

To evaluate sperm ultrastructure integrity, *SEM* was performed on samples (30 cells/animal) from all treatments. Spermatozoa, fixed, dehydrated and layered on slides as above described, were sputter-coated with gold and examined with a Field Emission Scanning Electron Microscopy (FEG-LEO 1525 ZEISS; Carl Zeiss) at an accelerating voltage of 2 kV. The spermatozoa were classified as for light microscopy: (a) intact spermatozoa (IS) (b) head, middle tail and partial principal tail (HMP), (c) head and middle tail (HM) and (d) head only (H).

2.7 | DNA methylation analysis

Genomic DNA was isolated using Quick-DNA™ MicroPrep according to the manufacturer's instructions (Zymo Research). The DNA concentration from white blood cells, fresh sperm and FD sperm (EDTA, EDTA-RA, EDTA-MT) was quantified using 2000 NanoDrop spectrophotometer. Furthermore, integrity of DNA samples was assessed using agarose gel electrophoresis. Afterwards, quantification of global DNA methylation was examined using the colorimetric MethylFlash™ Methylated DNA Quantification Kit (Epigentek Group Inc.) following the manufacturer's instructions. A total of 100 ng DNA per sample was analysed in duplicate. Briefly,



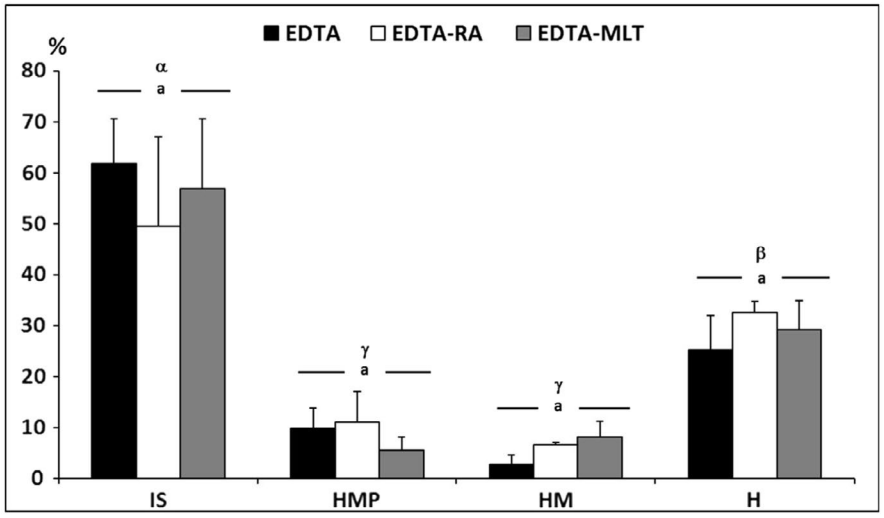


FIGURE 1 Light microscopy analysis of FD rabbit spermatozoa. Upper figure: intact spermatozoa (IS); tail principal piece fracture point comprising head, middle tail and partial principal piece tail (HMP); tail middle piece fracture point comprising head and tail middle piece (HM); and head only (H). Lower figure: lesion percentage of rabbit spermatozoa after FD. Spermatozoa were collected from five rabbits. Experimental groups: EDTA alone; EDTA-RA, EDTA supplemented with rosmarinic acid; EDTA-MLT, EDTA supplemented with melatonin. Values are the means ± *SD* of five replicates. Different letters above the bars indicate significantly different values ($p < .01$): Greek letters among experimental groups; Latin letters in the same experimental group

all samples were added to 96-well plates using an input DNA concentration of 100 ng. The global DNA methylation was detected by specifically measuring levels of 5-methylcytosine (5-mC) using capture and detection antibodies. The methylated DNA fraction was quantified using colorimetric analysis by reading the absorbance (450 nm) in a microplate spectrophotometer using a standard reference curve. The quantification of the methylation status was expressed as percentage of methylated cytosines (5-mC) using the formula provided by the manufacturer and described by Cocci et al. (2018).

2.8 | Statistical analysis

Shapiro–Wilk tests certified the distribution normality of data that were analysed by two-way ANOVA (treatments vs. spermatozoa integrity, Table S1) followed by Student–Newman–Keuls post hoc *t* test.

3 | RESULTS

3.1 | Light microscopy evaluation

Spermatozoa were clearly detectable allowing the assessment of their integrity. Sperm samples from EDTA treatment were easily diluted in PBS. In contrast, those from EDTA-RA and EDTA-MLT treatments were somewhat agglutinated and difficult to dissolve.

The three media (EDTA, EDTA-RA and EDTA-MLT) affected similarly spermatozoa integrity (Figure 1). The percentage of IS group was higher ($p < .01$) compared with the other three groups; H group was higher ($p < .01$) with respect to HMP and HM ones (Figure 1).

3.2 | Scanning electronic microscopy

Spermatozoa heads were 7–8 µm long and 4 µm wide, while tails were 0.35 µm thick in the principal piece (Figure 2a). In all

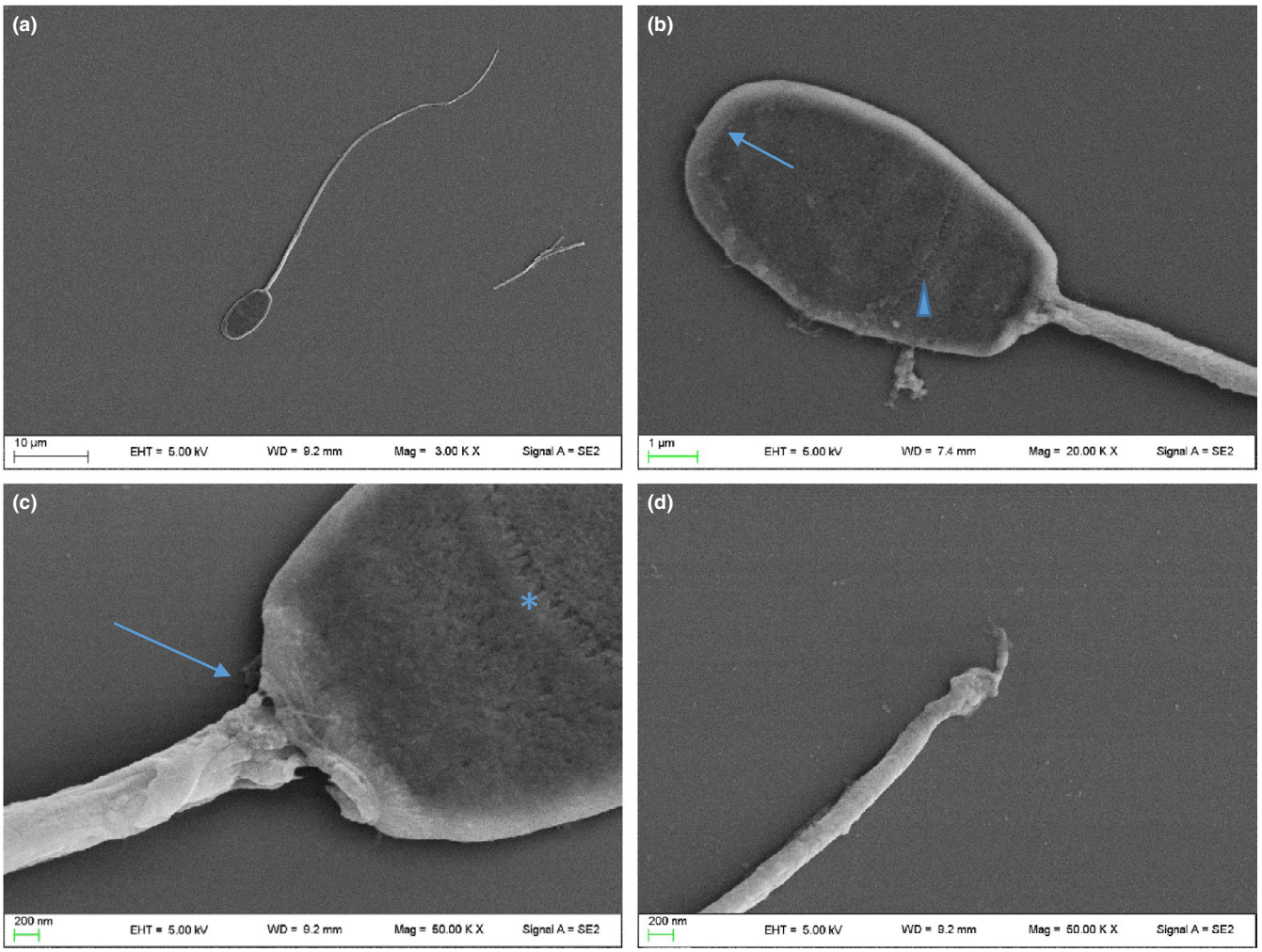


FIGURE 2 Spermatozoa at *SEM* after lyophilization. (a) An intact spermatozoon. (b) A head, characterized by a typical paddle shape form showing a swollen rim on the top of the head (arrow) and a curved ridge (arrowhead) delimitating the post-acrosomal region. (c) The neck of a spermatozoon with a visible fracture point (arrow). The asterisk points out the serrations in the post-acrosomal region. (d) A swollen end of the tail

treatments, the head of spermatozoa showed a well-conserved paddle-shaped form with an intact membrane (Figure 2b), while membrane lesions were occasionally observed in the tail. Membrane acrosome and acrosomal and post-acrosomal regions separated by a slightly curved ridge were identified; serrations in the anterior border of the post-acrosomal region were usually observed in the head (Figure 2b) such as a characteristic swollen at the end of the tail (Figure 2b). The results of *SEM*, confirmed light microscopy one, in fact, the three used media (EDTA, EDTA-RA, EDTA-MLT) affected similarly spermatozoon integrity (Figure 3). Whereas, as regard the percentage of integrity, the IS group was higher ($p < .01$) with respect to the other three groups, H group was higher ($p < .01$) with respect to HMP and HM ones (Figure 3).

3.3 | DNA methylation of lyophilized sperm

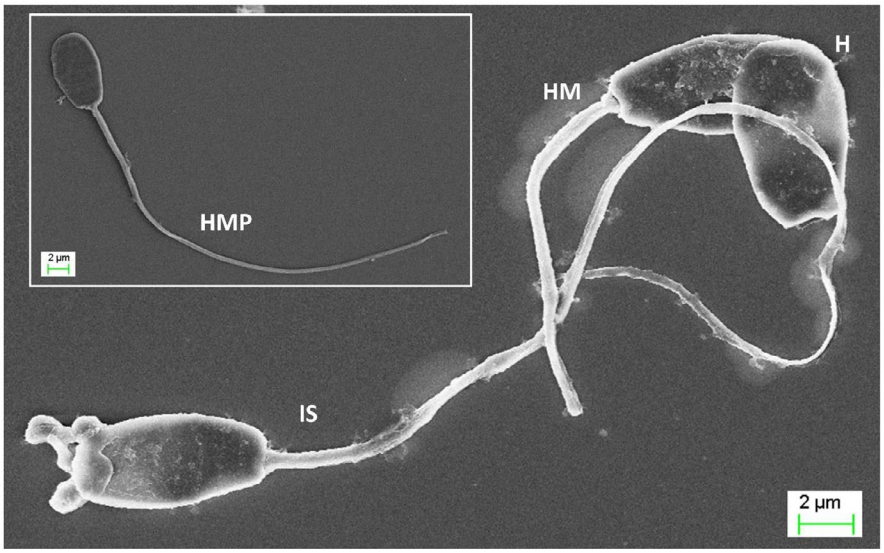
Genome integrity was not affected after FD and genomic DNA of EDTA, EDTA-RA and EDTA-MLT samples were similar to that of fresh semen after agarose gel electrophoresis (Figure 4).

The DNA methylation of lyophilized rabbit sperm was not affected by supplementation with EDTA (1.11 ± 0.60 ng μl^{-1}), EDTA-RA (0.86 ± 0.55 ng μl^{-1}) or EDTA-MLT (1.28 ± 0.62 ng μl^{-1}) with respect to the fresh semen (1.16 ± 0.43 ng µl-$^{-1}$).

4 | DISCUSSION

This study indicates that chelating agent EDTA, either alone or supplemented with antioxidant substances (RA or MLT) added to the lyophilization medium, has beneficial effects on the morphology and methylation grade of lyophilized rabbit spermatozoa.

In previous studies, supplementation of TRIS buffer chelating agents, such as EDTA or EGTA, to the freeze-drying medium increased sperm stability (Bhowmick et al., 2003). Liu et al. (2004) produced blastocysts after intracytoplasmic sperm injection (ICSI) using FD spermatozoa using medium supplemented with EGTA at a similar rate to that of ICSI carried out using fresh sperm. Despite this relevant achievement, only a viable puppy was born and hence the



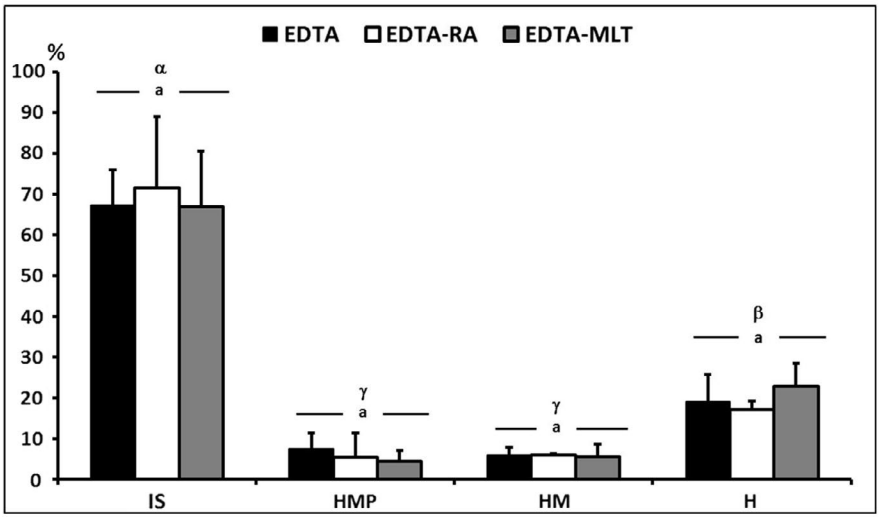


FIGURE 3 Scanning electron microscopy analysis of FD rabbit sperm. Upper figure: intact spermatozoa (IS); tail principal piece fracture point comprising head, middle tail and partial principal piece tail (HMP); tail middle piece fracture point comprising head and tail middle piece (HM); and head only (H). Lower figure: lesion percentage of rabbit spermatozoa after FD. Spermatozoa were collected from five rabbits. Experimental groups: EDTA alone; EDTA-RA, EDTA supplemented with rosmarinic acid; EDTA-MLT, EDTA supplemented with melatonin. Values are the means ± *SD* of five replicates. Different letters above the bars indicate significantly different values ($p < .01$): Greek letters among experimental groups; Latin letters in the same experimental group

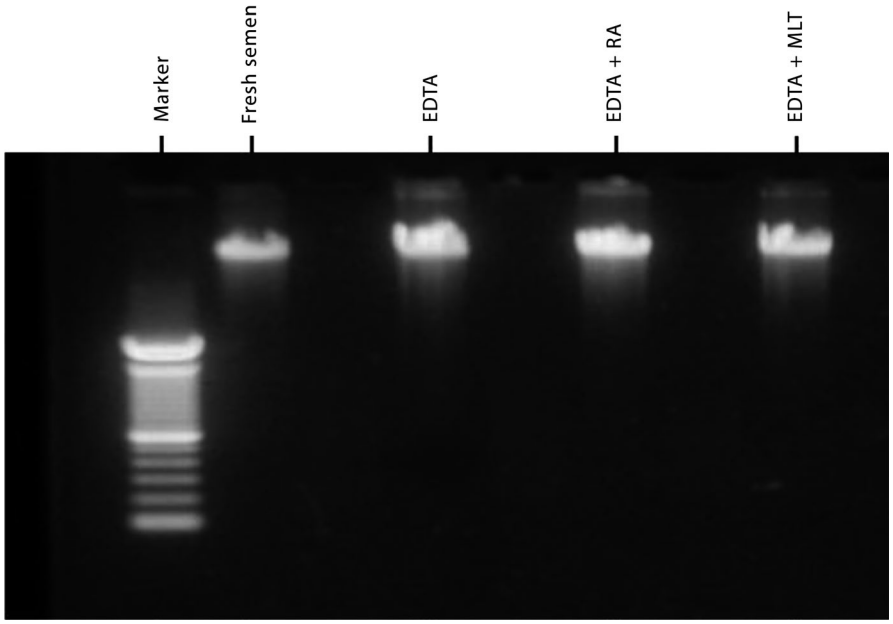


FIGURE 4 Assessment of DNA integrity. Random samples of each treatment were tested for DNA integrity using 0.8% agarose gel. Spermatozoa were collected from five rabbits. Experimental groups: EDTA alone; EDTA-RA, EDTA supplemented with rosmarinic acid; EDTA-MLT, EDTA supplemented with melatonin

need to study the possible factors that compromised the success of the technique. A later study on lyophilized rabbit sperm demonstrated that EDTA preserved DNA integrity of spermatozoa better than EGTA as well as the positive effect of RA supplementation (Domingo et al., 2018). However, the effect of chelating molecules on the morphology of freeze-dried sperm is still not well defined.

In this study, we performed an accurate morphologic assessment that allowed to evidence a normal morphology of spermatozoa according to Flechon & Bustos Obregon (1974) and Contreras and Fierro (2004). However, some fracture points were observed in specific regions of the cells, such as the neck, middle and principal pieces of the tail. Previous studies in pigs (Kwon, Park, & Niwa, 2004), rabbits (Liu et al., 2004) and mice (Wakayama & Yanagimachi, 1998) reported a detachment of the tail from the head after the FD process in agreement with our results.

In all treatments, the neck fracture was the most frequent injury; the consequent presence of tailless heads attests that the neck is the weakest point of the gamete. In contrast, Martins, Báo, Dode, Correa, and Rumpf (2007) reported a low degree of head–tail separation in lyophilized bovine spermatozoa, suggesting a greater stability of this conjunction point in this species. Taken together, these conflicting results suggest that the fragility of lyophilized spermatozoa is species-specific, independently from the medium used for the FD process. In agreement with Liu et al. (2004), a certain degree of broken tails was evidenced, confirming that plasma membrane is highly susceptible to damage due to the loss of water during dehydration. Finally, intact spermatozoa were the largest category in all the three media evaluated, attesting that they adequately protected the cell structure. Moreover, very few spermatozoa with plasma membrane damage were detected, suggesting that chelating agents plus addition of antioxidant treatments preserve DNA integrity as previously reported (Domingo et al., 2018).

Sperm from EDTA treatment was easily diluted in phosphate buffer, whereas those from EDTA-RA and EDTA-MLT treatments were quite agglutinated and difficult to dissolve, even after repeated pipetting, as observed by Martins et al. (2007). In agreement with these authors, our results showed that EDTA-treated spermatozoa appeared regularly scattered on slide surface, while the others appeared aggregated in small clusters and only few gametes were free.

DNA methylation is an epigenetic modification that has relevant importance during early embryogenesis. Indeed, DNA methylation is widely remodelled in embryonic stem cells of several species (Al-Khtib et al., 2012). In the germline development, paternal DNA methylation tracts are cancelled and again set up after waves of demethylation and methylation (O'Doherty & McGettigan, 2015). In humans, sperm DNA methylation level has been positively associated with conventional sperm parameters, like concentration and motility, whereas chromatin fragmentation and denaturation negatively influence DNA methylation (Montjean et al., 2015).

Our results demonstrated that the FD process used did not affect the overall DNA methylation status of rabbit sperm. Moreover, supplementation of EDTA as well as addition of RA or MLT to storage media did not influence the spermatozoa methylation rate with respect to the fresh medium. As reduced DNA integrity of spermatozoa is one of the main causes of failure of assisted reproductive technologies, the presence of structurally intact DNA in lyophilized samples is particularly relevant.

In conclusion, our study demonstrated that FD of spermatozoa with two different antioxidant supplementations does not affect rabbit male gamete preservation and could be beneficial for long-term sperm storage during clinical application research. Further studies are needed to understand the difficulties related to the use of FD technique in rabbits.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

FM, MM, PD and LG developed the idea, designed the research and analysed the results. PD, RP, CD, AD, KF and PC contributed to the experiments. MZ contributed to the statistical analysis. FM and MM wrote the manuscript. CB and MZ revised the final version of the manuscript, which was edited and approved by all authors.

ORCID

Rolando Pasquariello https://orcid.org/0000-0003-3191-7009
Massimo Zerani https://orcid.org/0000-0002-2476-5097
Margherita Maranesi https://orcid.org/0000-0002-1727-4118

DATA AVAILABILITY

The data sets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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