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First pregnancies in jennies with vitrified donkey semen using a new warming method

M. Diaz-Jimenez [a,b], A. Rota [b], J. Dorado [a], C. Consuegra [a], B. Pereira [a], F. Camillo [b], D. Panzani [b], D. Fanelli [b], M. Tesi [b], D. Monaco [c], M. Hidalgo [a,*]

[a] Veterinary Reproduction Group, Department of Animal Medicine and Surgery, University of Cordoba, 14071 Cordoba, Spain
[b] Department of Veterinary Sciences, University of Pisa, 56121 Pisa, Italy
[c] Department of Veterinary Medicine, University of Bari Aldo Moro, 70121 Bari, Italy

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ABSTRACT

Sperm vitrification has been recently developed, but fertility trials have not been performed yet in equine species. In this study, a new warming technique for vitrified donkey semen was developed and the uterine inflammatory response and fertility were compared to conventional freezing. In Experiment 1, sperm was vitrified in straws and warmed in 3 ml of extender or in a water bath at: 37 °C/30 s; 43 °C/10 s; and 60 °C/5 s. Sperm motility, plasma and acrosome membranes and DNA integrity were compared between treatments. In Experiment 2, jennies were inseminated twice (500×10^6 sperm) in the uterine body either with vitrified or frozen semen (2 cycles/jenny). Pregnancy rates and the uterine inflammatory response (polymorphonuclear neutrophil concentration; **PMN**) were evaluated after artificial insemination (**AI**). No differences between warming in extender/water bath were found and 43 °C/10 s was better than lower temperatures in terms of total (53.8 ± 13.2%) and progressive sperm motility (41.4 ± 11.4%). No differences in PMN concentration ($\times 10^3$ PMN/ml) were found between vitrified (276.8 ± 171.6) or frozen (309.7 ± 250.7) semen after AI. However, PMN decreased faster ($P < 0.05$) using vitrified semen. Pregnancy rates were greater for vitrified (22%) than frozen semen (10%) but not statistically different. In conclusion, donkey sperm vitrified in straws could be directly warmed in a water bath at 43 °C/10 s, reducing the uterine inflammatory response obtained after AI and promoting positive pregnancy outcomes. These findings confirm the possibility to use vitrified semen as an alternative for AI in jennies.

Implications

Pregnancy rates in jennies after artificial insemination with frozen–thawed donkey semen are poor. The permeable agent presence was suggested to provoke high post breeding endometritis affecting fertility. However, no studies have been performed using vitrified semen without permeable agents for artificial insemination in equines. Double pregnancy rates in jennies inseminated with vitrified donkey semen in comparison to frozen–thawed semen were obtained. Vitrified semen also reduced the post breeding uterine inflammatory response. A new methodology for warming vitrified straws was also developed, making intrauterine artificial insemination in field conditions possible, avoiding post-warming dilution and centrifugation, and improving the methodology in terms of cost effectiveness and time to process.

Introduction

During the last century, the donkey population has considerably diminished due to the industrial revolution and the mechanization of agriculture (Kugler et al., 2008; Camillo et al., 2018). Actually, although these animals are still employed in some regions (mostly in less industrialized countries), the majority of the European donkey breeds are threatened by extinction; as stated by the Food and Agriculture Organization (FAO), 2018. Given the importance of biodiversity and domestic animal resource preservation, research should be focused on reproductive procedures aiming to preserve genetically valuable donkey breeds. The use of frozen semen in artificial insemination (**AI**) of domestic species has numerous advantages in comparison to fresh and chilled semen (Loomis and Graham, 2008; Oliveira et al., 2016). Among them, the availability of semen at any time, semen shipping worldwide (which also increases genetic variability) and the long-term preservation of genetic material (through the creation of genetic banks) of valuable males or endangered breeds (Sanchez et al., 2009).

Cryopreservation of donkey semen has been traditionally performed using slow freezing techniques and glycerol is largely applied as a

* Corresponding author.
E-mail address: mhidalgo@uco.es (M. Hidalgo).

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permeable cryoprotectant agent (**CPA**). Recent studies analyzed the effect of: other permeable agents and combinations (Oliveira et al., 2006; Acha et al., 2015), addition of different nonpermeable substances (Oliveira et al., 2014; Álvarez et al., 2019), and even cryopreservation with the sole use of nonpermeable agents (Diaz-Jimenez et al., 2018a). Although the excellent quality of donkey sperm is usually obtained after thawing, with sperm motility percentages up to 65% (Ortiz et al., 2015a; Oliveira et al., 2016) and pregnancy rates in mares of 53% (Canisso et al., 2011); pregnancy rates in jennies after AI with frozen–thawed donkey semen are still very disappointing (Vidament et al., 2009; Serres et al., 2014). The presence of glycerol in the insemination dose has been hypothesized to be the cause of the poor results obtained with frozen semen in jennies (Trimeche et al., 1998). Vitrification is a cryopreservation method recently developed as an alternative to conventional sperm freezing. The kind of vitrification technique employed for sperm is different than that used for embryos or oocytes, where high concentrations of permeable cryoprotectants are needed and both the intracellular milieu and the extracellular environment must become vitrified (Katkov et al., 2006). This technique has been named "kinetic vitrification" as it requires high cooling and warming rates but avoiding the use of permeable CPAs. Moreover, it does not imply that the extracellular medium is vitrified (Shaw and Jones, 2003). However, there is still some controversy surrounding the use of this terminology when compared to the conventional term for vitrification associated with oocytes and embryos (Pradiee et al., 2015; Hidalgo et al., 2018). Sperm vitrification has been employed by several authors in different species in the last few years (Merino et al., 2011; Sánchez et al., 2011; Rosato and Iaffaldano, 2013; Jiménez-Rabadán et al., 2015; Pradiee et al., 2016; Isachenko et al., 2017; Swanson et al., 2017; Arraztoa et al. 2017b and 2017c; Caturla-Sánchez et al., 2018; Pradieé et al., 2018; Consuegra et al., 2018b; Diaz-Jimenez et al., 2018b). Different procedures have been developed (cryoloops, spheres, straws), and among them, vitrification using 0.25 into 0.5 ml straws is an aseptic technique that has showed satisfactory post-warming sperm parameters in humans (Sanchez et al., 2012; Mansilla et al., 2016; Schulz et al., 2017; Uribe et al., 2017), stallions (Consuegra et al., 2018a), and donkeys (Diaz-Jimenez et al. 2017 and 2018b). Despite this growing interest, there are only a few studies in which AI has been performed with vitrified semen with no permeable CPAs added: rabbits (Rosato and Iaffaldano, 2013), humans (Sanchez et al., 2012), and mouflons (Pradiee et al., 2016), but it has not been performed in any equine species yet.

The warming protocol commonly employed after sperm vitrification in the two-straws method consisted on the immersion of the inner straw in 2–5 ml of pre-warmed semen extender (37–43 °C), and then centrifuged. This procedure was first described by Sanchez et al. (2012), and quite good sperm quality after vitrification has been obtained. Even so, the warming procedure is one of the major drawbacks of this method of vitrification, as it could be expensive and time consuming. For one single insemination in equines, a high quantity of warming extender and a great number of thawed straws would be required, which must be centrifuged separately after warming. Despite its unfeasibility, no one to the best of our knowledge has researched alternative warming procedures.

During the last year, the sperm vitrification process has been optimized in donkeys regarding sperm concentration, volume, and package (Diaz-Jimenez et al., 2019a), and different warming rates have been tested for vitrification in large volumes (0.5 ml) in the same species (Diaz-Jimenez et al., 2019b). Nevertheless, warming approach and rates in the two-straws method of vitrification have been poorly studied: there is only one study performed in human sperm (Mansilla et al., 2016), but there are no reports in any equine species yet. In this paper, we analyzed a new approach of warming after sperm vitrification in order to simplify the process: direct immersion of the inner straw into a water bath. We hypothesized that this method would maintain the rate of warming and guarantee warmth to be distributed equally in all the straw surface. It is easier, quicker, and cheaper, as the use of extender for warming and following centrifugation is avoided. The dose would be ready to use right after warming. To our knowledge, there are no previous reports testing this technique in sperm vitrification.

As previously stated, the presence of glycerol in the AI dose seems to be toxic to the sperm cell (Watson, 2000), but also a high post breeding endometritis is provoked in jennies, having a detrimental effect on fertility (Vidament et al., 2009). In this regard, different strategies have been developed in order to improve pregnancy rates when AI with jack frozen semen is performed in jennies: glycerol removal before insemination (Trimeche et al., 1998); glycerol susbstitution with dimethylformamide (Vidament et al., 2009); or its reduction by combination with other permeable CPAs: dimethylsulfoxide, dimethylformamide, and dimethylacetamide for sperm freezing (Oliveira et al., 2006); and performing deep horn AI or increasing the number of inseminations (Oliveira et al., 2016). However, to the best of our knowledge, there are no previous studies of AI with cryopreserved jack semen in the absence of permeable agents. To date, the highest overall jennies pregnancy rate described in the literature using frozen–thawed jack semen is still 36%, reported by Rota et al. (2012). They reported a tendency to obtain higher pregnancy results when glycerol was used as permeable agent and post-thaw extended with seminal plasma (**SP**). In this sense, the role of glycerol in the low success of AI with frozen–thawed sperm in jennies is not so clear, as was previously suggested by Vidament et al. (2009). Consequently, it is advisable to further research in jennies AI using jack semen cryopreserved with the sole use of nonpermeable agents. This would contribute to increase the knowledge about those poor pregnancy results in jennies and if the cause of it is related to the freezing extender composition.

Therefore, the aims of this study were to: 1) evaluate the effect of warming technique and temperature after donkey sperm vitrification in straws and 2) compare the post breeding uterine inflammatory response and fertility of jennies after AI with vitrified or frozen jack semen.

Material and methods

Animals

Four Andalusian and two Amiata donkeys (5 to 9 years old) were used as semen donors. Andalusian donkeys were individually housed in stalls in the Equine Sports Medicine Centre (**CEMEDE**) of the University of Cordoba (Spain) and Amiata donkeys were housed in the same conditions at the Department of Veterinary Sciences of the University of Pisa (Italy). A total of thirteen cyclic Amiata jennies, aged from 7 to 13 years and in good body condition, were used for the AI protocol. Males and females used in the study were known to be fertile. Animals were fed with meadow hay and a commercial feed for horses and water was freely available.

Semen collection and processing

In order to deplete the extragonadal sperm reserves, an initial semen collection was performed in sexually rested donkeys (daily collection for five days). Thereafter, a total of 32 ejaculates were used in this study: six ejaculates from each jack in first experiment ($n = 24$) and four ejaculates per jack in the second one ($n = 8$). Semen collection was performed on a regular basis (twice a week) using an artificial vagina in the presence of a jenny in estrus. Gel-free semen volumes were measured in the graduated collector, sperm concentration was determined using a spectrophotometer, and morphology and acrosome integrity were assessed on stained smears as previously described (Hidalgo et al., 2017; Diaz-Jimenez et al., 2019b). Thereafter, semen was extended (INRA-96, IMV Technologies, L'Aigle, France) and processed as described in each experiment.

Sperm vitrification

Semen samples were vitrified following an optimized protocol described for donkeys (Diaz-Jimenez et al., 2019a). Briefly, after collection and preliminary evaluation, semen was extended 1:1 (v:v) with INRA-96 and centrifuged (7 min/400 ×g/22 °C) in a corning-adapted centrifuge (Eppendorf, model 5702 RH, Eppendorf AG, Hamburg, Germany). The supernatant was removed and the sperm pellet resuspended in a skimmed milk-egg-yolk base extender without glycerol (Gent, Minitüb GmbH, Tiefenbach, Germany) supplemented with sucrose 0.1 M until a concentration of 300×10^6 sperm/ml. Thereafter, samples were equilibrated 10 min at room temperature (22 °C) and slowly cooled at 5 °C for 1 h into an Equitainer (Hamilton Research, Inc. Ipswich, Massachusetts, USA). Vitrification was performed applying the two-straw technique: a 160 μl aliquot of the cooled extended semen was packed in 0.25 ml French plastic straws; these were horizontally placed in 0.5 ml straws and hermetically sealed for both ends. Filled straws were then vitrified in a horizontal position by direct plunging into liquid nitrogen. After at least 24 h of storage, straws were warmed as subsequently described in the experimental design.

Post-warming sperm evaluation

Sperm motility

Sperm motility was objectively evaluated using the Sperm Class Analyser system (SCA v5.4, Microptic S.L., Barcelona, Spain). The system consists of an optical phase-contrast microscope (Eclipse 50i; Nikon, Tokyo, Japan), a warm plate at 37 °C (OK 51–512, Osaka, Digifred SL, Barcelona, Spain), and acquisition was performed using high-speed digital camera (A312fc, BaslerTM AG, Ahrensburg, Germany). The settings parameters of the software analysis were as follows: frame rate of 25 frames/s and 25 images captured, cell size from 15 to 75 μm^2, connectivity 12, spermatozoa were considered motile with a mean average path velocity (VAP) > 10 μm/s, and progressive with a straightness coefficient (VSL/VAP × 100) greater than 75%. For each evaluation, two 5 μl drops and three random microscopic fields per drop including a minimum of 200 spermatozoa were evaluated. The trajectory of each spermatozoa was determined by the software and the following parameters of sperm motion were measured: total motility (TM; %); progressive motility (PM, %); curvilinear velocity, or total distance traveled by the spermhead per unit time (VCL; μm/s); straight line velocity, or net distance gain of the sperm head per unit time (VSL; μm/s); average path velocity, or length of a derived "average" pathof sperm head movement per unit time (VAP; μm/s); wobble (WOB, as VAP/VCL × 100), beat cross frequency, number of times the curvilinear path crosses the average path per unit time (BCF; Hz), and amplitude of lateral head displacement, or width of the head movement envelope (ALH; μm).

Plasma membrane integrity

Sperm plasma membrane integrity was examined with fluorescence microscopy using the VitalTest stain (Halotech DNA S.L.), as previously described (Dorado et al., 2014). Briefly, an aliquot of 10 μl of diluted semen was mixed with 1 μl propidium iodide stock solution and 1 μl of acridine orange stock solution and evaluated under epifluorescence microscopy (Olympus BX40, Tokyo, Japan) using a U-ND25-2 filter (a 460–490 nm excitation filter). At least 200 sperms were counted and those with green emission (spermatozoa with an intact plasma membrane) were recorded. The results are expressed as plasma membrane integrity percentage (PMI; %).

Acrosome membrane integrity

For sperm acrosome evaluation, the double stain propidium iodide (PI)/peanutagglutinin–fluorescein isothiocyanate (FITC-PNA) (Sigma-Aldrich, Sant Louis, USA) was used as described by Diaz-Jimenez et al. (2019b). In short, a mixture of 10 μl PI and 20 μl FITC-PNA was spread over pre-permeabilized smears of the samples and slides were incubated in a dark, moist chamber at 4 °C for 30 min. Thereafter, a minimum of 200 spermatozoa were evaluated under epifluorescence microscopy, and sperm acrosomes were classified as follows: intact (spermatozoa displaying intensively bright green fluorescence of the acrosomal cap) and damaged (spermatozoa displaying disrupted fluorescence, fluorescent band at the equatorial segment or no fluorescence, indicating damages to the outer acrosomal membrane). The percentage of acrosome-intact sperm was recorded (AIS, %).

Sperm DNA analysis

Sperm DNA integrity was assessed with the sperm chromatin structure assay (**SCSA**). Data were acquired and analyzed using a CytoFLEX S high-resolution flow cytometer (Beckman Coulter, Life Sciences Division Headquarters, Indianapolis, USA) with subsequent data analysis using CytExpert software (Beckman Coulter Life Sciences, Indianapolis, IN, USA). Sperm samples were managed before analysis as described by Diaz-Jimenez et al. (2018b): an aliquot from each treatment was stored at −80 °C (Jackson et al., 2010). The individual semen samples were thawed in a water bath at 37 °C prior to DNA assessment. A 5 μl aliquot of semen was diluted in 195 μl of a buffered solution and then mixed with 400 μl of a low pH solution for 30 s. Then, 1.2 ml of a stock solution (4.0 g/ml) of acridine orange was added to the sample and immediately processed using flow cytometer (Salazar et al., 2011). The analysis was restricted to the sperm cells based on size and granularity using forward (**FSC**) and side scatter (**SSC**), respectively, and uniformity of the cell suspension. Each sample was allowed to pass through the flow cytometer system in a flow rate of 10 μl/min, and at least 10 000 cells/sample were studied, after exclusion of the non-sperm cells. The percentage of sperm cells with fragmented DNA was recorded as DNA fragmentation Index (**DFI**, %).

Experimental design

Experiment 1. Effect of warming technique and temperature after donkey sperm vitrification in straws

This experiment was performed in order to improve and simplify warming protocol after vitrification in straws by testing different variables: warming procedure and warming rate.

Comparison of different methods of warming and centrifugation effect after sperm vitrification

A comparison between a previously described warming protocol (Diaz-Jimenez et al., 2017; Diaz-Jimenez et al. 2018b and 2019a) and a new direct method of warming was performed. First, the covering straw was opened with forceps and each 0.25 straw with the vitrified sperm was pulled out. The inner straw was either (i) immersed into a tube containing 3 ml of extender (INRA-96) at 43 °C and then centrifuged (Control; as described by Diaz-Jimenez et al. (2019a)) or directly warmed in a water bath at 43 °C during 10 s. Since the inner straw was not sealed, immersion was done in an upside-down way in order to avoid contact with water (Fig. 1). After warming in the water bath samples were (ii) directly diluted for evaluation (WB) or (iii) subjected to centrifugation as performed in the control (WB + centrifugation). Finally, sperm parameters were evaluated as described above and compared between protocols.

Effect of different warming rates after donkey sperm vitrification

The effect of different temperatures and warming rates on donkey sperm quality after vitrification in the two-straws method was evaluated. The warming approach with the most desirable results obtained was employed. For that purpose, the covering straw was opened with forceps and each 0.25 ml straw with the vitrified sperm was pulled out and directly immersed in a water bath at (i) 37 °C during 30 s; (ii) 43 °C during 10 s; and (iii) 60 °C during 5 s. Warmed samples were not centrifuged and directly re-extended with INRA-96 for sperm evaluation as described in the followings sections.

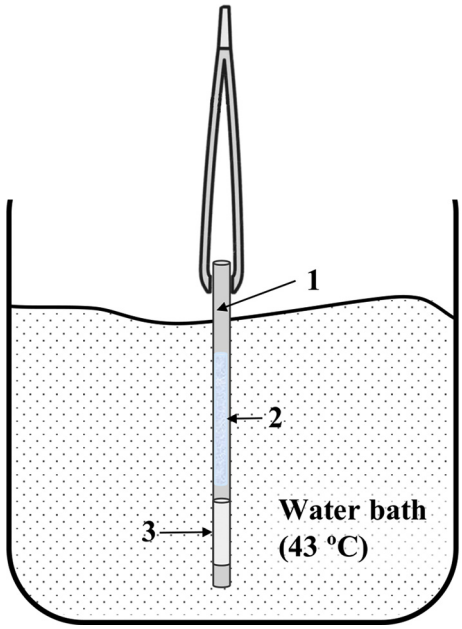

Fig. 1. Scheme of the mode of warming in an upside-down way: (1) opened 0.25 ml straw, (2) vitrified donkey sperm suspension, (3) cotton plug.

Experiment 2. Comparison between the post breeding uterine inflammatory response and fertility of jennies after artificial insemination with vitrified and frozen jack semen

The second experiment consisted of the comparison between pregnancy rates and post breeding uterine inflammatory response of jennies inseminated with vitrified sperm in comparison to a control using conventionally frozen sperm.

Sperm processing

After semen collection and evaluation, each ejaculate was divided into two aliquots and subjected to vitrification and conventional slow freezing.

Vitrification was performed using the two-straws method (as described before) followed by the optimized warming protocol from Experiment 1: in a water bath at 43 °C/10 s with no centrifugation (WB). The slow freezing and thawing procedures were performed exactly as described by Rota et al. (2012), without modifications. In brief, extended semen was centrifuged (10 min/600 $\times g$/22 °C) and pellet was resuspended in two steps until a final concentration of 168 $\times$ 10^6 sperm/ml. The freezing extender was previously prepared using INRA-96 containing 2% egg yolk and 2.2% of glycerol. Sperm suspension was maintained at 4 °C during 1 h and loaded in 0.5 ml straws. Freezing was performed in a programmable freezer (Mini-Digitcool, IMV Technologies) at 60 °C/min from 4 to 140 °C and then straws were plunged into liquid nitrogen. Straws were thawed in a water bath at 37 °C for 30 s.

Artificial insemination and breeding management

A total of 20 estrus cycles from 10 jennies were used for inseminations. Two estrus cycles per animal were randomly submitted to AI: one cycle with the vitrified and the other with the frozen semen, from the same ejaculate.

Ovarian activity was monitored weekly by transrectal palpation and ultrasonography using a linear transducer (Mindray DP30, Shenzhen, China) until a growing follicle bigger than 28 mm was detected. Then, ultrasound evaluations were performed daily: when the dominant follicle reached 35 mm, in the presence of uterine edema and estrous behaviour, ovulation was induced with 0.4 ml sc of GnRH agonist (buserelin acetate, Suprefact, Sanofi Spa, Milano, Italy).

wA fixed-time insemination protocol previously described by Rota et al. (2012) was followed. Jennies were inseminated twice, 18 and 38 h post-induction. Each breeding dose consisted of approximately 500 $\times$ 10^6 sperm of pooled sperm from both jacks, in order to avoid male effect. Therefore, ten 0.25 ml straws from vitrified (five straws from each jack) or six 0.5 ml straws from frozen semen (three straws from each jack) were used in each AI. Post-thaw re-extension was performed, for frozen or vitrified semen, with respectively either 2 or 3 ml of INRA-96, to obtain a final insemination volume of 5 ml in both treatments. All inseminations were performed in the uterine body using an equine insemination pipette (IMV Technologies) after subjective motility evaluation of thawed doses under a phase contrast microscope. The uterus was flushed after each AI for cytological examination, as described in section "*Uterine inflammatory response evaluation*".

The schedule regarding breeding management was previously described (Rota et al., 2012) and it is showed in Table 1. Cycles in which jennies ovulated before 14 h or later than 62 h post-induction were not included in the study. If a jenny had ovulated by 24 h post-induction of ovulation, she was not reinseminated at 38 h.

Uterine inflammatory response evaluation

Six hours after the last AI, jennies were examined ultrasonographycally for evaluation of the uterine edema, intrauterine fluid accumulation and follicle diameter or ovulation. Uterine lavages for endometrial cytology were performed 6 and 10 h after the first and second AI, respectively (Table 1). The jenny's uterus was infused with 1 l of Ringer Lactate (Galenica Senese, Siena, Italy) using a sterile one-way 30 cm French equine embryo flushing catheter (Bivona, Kruuse, Denmark). Fluid was recovered back to the bag and concentration and proportion of polymorphonuclear cells (**PMN**) was recorded.

For that purpose, the bag was first mixed to homogenise cell suspension and obtain a representative sample of cells. A 10 ml sample of the fluid was drawn with a syringe and cell concentration was measured using a Thoma counting chamber and number of cells per ml was recorded. For a proportion of PMN evaluation, the bag was suspended for 30 mins to help cell precipitation. Thereafter, 20 ml were collected from the bottom of the bag and centrifuged (10 min/600 $\times g$). The supernatant was discarded except for a small quantity in which the pellet was re-suspended. Smears were prepared onto glass microscope slides, air-dried and stained with Diff-Quick (Dade Berhing SPA, Milano, Italy). At least 200 cells were counted and the proportion of PMN was calculated (PMN, %). The PMN concentration ($\times 10^3$ cells/ml, [PMN]) was calculated as described by Rota et al. (2012), as follows:

$$[\mathrm{PMN}] = cellconcentration \times \frac{\mathrm{PMN}\%}{100}$$

Table 1
Breeding management schedule of jennies inseminated with cryopreserved donkey semen.

Time (h)	Procedure
0	Induction of ovulation (buserelin)
14	Ultrasound
18	Ultrasound and artificial insemination
24	Ultrasound and uterine flushing
38	Ultrasound and artificial insemination
42	Ultrasound
48	Ultrasound and uterine flushing
62	Ultrasound

Pregnancy diagnoses

Pregnancy diagnosis was made by ultrasonography 14 days after ovulation using a linear transducer (Mindray DP30, Shenzhen, China). After being rechecked at 16 days to confirm the pregnancy, luteolysis was induced with 3 mg (intramuscular) of alfaprostol (Gabbrostim, CEVA, VETEM, Milan, Italy) to bring all jennies back into estrus. Pregnancy rates (%) were calculated as the total number of pregnancies divided by the total number of oestrus cycles bred (Vidament et al., 2009).

Statistical analyses

Analyses were performed using IBM SPSS Statistics 20 (IBM Corp., Armonk, NY, USA). All data was first tested for normality of the data distribution and homogeneity of variances using the Kolmogorov–Smirnov and Levene test, respectively. When necessary, data were transformed using logarithmic scale before analysis.

The effect of warming technique and temperature after vitrification on sperm quality parameters was analysed by a general linear model (GLM) followed by the Duncan and Tukey test for *post hoc* analysis. Animals and ejaculates were considered as random factors. The percentage of pregnancy rates between vitrified and frozen semen and the relationship between uterine fluid accumulation and cryopreservation method, pregnancy diagnosis and uterine inflammatory response was analysed by Chi-square and Fisher's exact test. Differences in number of PMN between the first and the second flushing were evaluated using the paired two samples *t*-test. Differences in number of PMN between cryopreservation methods were evaluated by two-sample *t*-test. Data are shown as mean ± standard deviation (SD). In all the statistical analyses, the level of significance was set at $P < 0.05$.

Results

Average sperm parameters obtained after fresh sperm evaluation from ejaculates used in both experiments were: gel-free volume 49.7 ± 17.4 ml (range: 20–85 ml), sperm concentration 345.7 ± 216.3 × 10^6 sperm/ml (range: 153.0 – 1273.0 × 10^6 sperm/ml), total motility 88.1 ± 8.8% (range 70.0–98.1%), progressive motility 80.4 ± 9.1% (range 59.5–93.1%), normal forms 87.5 ± 11.7% (range 58.0–98.1%) and acrosome-intact sperm 70.2 ± 18.2% (range 35.7–97.0%). All males were known to be fertile and all ejaculates were within the values considered as physiologic when evaluating donkey sperm (Miró et al., 2005; Ortiz et al., 2014).

Experiment 1. Effect of warming technique and temperature after donkey sperm vitrification in straws

Comparison of different methods of warming and centrifugation effect after sperm vitrification

No significant ($P > 0.05$) differences between methods of warming nor centrifugation process were found for TM, PMI, AIS and other sperm kinematic parameters (ALH, WOB and BCF). However, warming in the extender (control) and direct warming in a water bath without centrifugation (WB) showed statistically ($P < 0.05$) higher percentages for PM and numerically greater ($P > 0.05$) sperm velocities (VSL, VCL and VAP) than direct warming and centrifugation. Direct warming in a water bath, with or without centrifugation also resulted in significantly ($P < 0.05$) lower DNA fragmentation percentage.

The direct warming protocol in a water bath without centrifugation (WB) was selected for the subsequent experiments instead of the control because the DNA results and simplicity of the technique. Results are provided in Table 2.

Effect of different warming rates after donkey sperm vitrification

Semen quality data from comparison between warming rates after sperm vitrification is provided in Fig. 2. Warming at 43 °C/10 s showed significantly higher ($P < 0.05$) results for TM (53.8 ± 13.2%) and PM (41.4 ± 11.4%) in comparison to 37 °C/30 s (TM: 40.8 ± 11.4% and PM: 30.2 ± 11.9%); but no significant differences were found when compared to 60 °C/5 s (TM: 47.3 ± 15.4% and PM: 37.5 ± 11.7%; $P > 0.05$). Parameters regarding membrane integrity (PMI and AIS) and DNA integrity showed no differences between warming rates ($P > 0.05$). Variables regarding sperm motility features are summarized in Table 3. No differences ($P > 0.05$) between 43 °C/10 s and 60 °C/5 s warming rates were found for sperm velocities (VSL, VCL and VAP), but 37 °C/30 s showed a tendency to obtain the lowest percentages. No differences ($P > 0.05$) were found for the remaining parameters assessed.

The warming protocol 43 °C/10 s was selected for the following experiment instead of 60 °C/5 s for the tendency to obtain higher total and progressive motility and practical reasons.

Experiment 2. Comparison between the post breeding uterine inflammatory response and fertility of jennies after artificial insemination with vitrified and frozen jack semen

A total of 19 cycles were considered in this study, as one cycle from one jenny was not included because failure to ovulate within 72 h after induction.

Only those straws showing at least 30% of total sperm motility after thawing were used for artificial insemination in this experiment. After

Table 2
Sperm parameters from vitrified-warmed samples ($N = 12$) using different methods of warming with and without centrifugation in donkey.

Sperm parameters	Warming procedure (43 °C)			*P*-values
	Extender + centrifugation (control)	Water Bath	Water Bath + centrifugation	
TM (%)	51.1 ± 15.8	50.7 ± 13.0	42.1 ± 15.5	>0.05
PM (%)	41.8 ± 14.8[a]	39.5 ± 10.5[a]	31.51 ± 12.3[b]	<0.05
PMI (%)	49.1 ± 8.9	48.0 ± 11.1	46.1 ± 9.9	>0.05
AIS (%)	44.0 ± 12.2	43.8 ± 11.1	49.7 ± 10.6	>0.05
VCL (μm/s)	90.9 ± 13.1[ab]	95.7 ± 19.4[a]	84.2 ± 14.1[ab]	<0.05
VSL (μm/s)	82.0 ± 11.4[a]	83.9 ± 17.8[a]	73.4 ± 12.8[b]	<0.05
VAP (μm/s)	85.0 ± 11.6[ab]	89.2 ± 18.7[a]	78.0 ± 13.4[b]	<0.05
ALH (μm)	1.9 ± 0.3	1.9 ± 0.3	1.8 ± 0.3	>0.05
WOB (%)	93.5 ± 2.1	93.1 ± 1.8	92.6 ± 1.5	>0.05
BCF (Hz)	9.0 ± 0.7	9.0 ± 0.6	8.8 ± 5.4	>0.05
DFI (%)	21.0 ± 14.6[b]	12.7 ± 4.3[a]	11.3 ± 4.7[a]	<0.05

Extender (control): vitrified sperm warmed in 3 ml of extender (INRA) at 43 °C and then centrifuged; Water Bath: vitrified sperm directly warmed in a water bath at 43 °C; Water Bath + centrifugation: vitrified sperm directly warmed in a water bath at 43 °C and then centrifuged. TM = total motility; PM = progressive motility; PMI = plasma membrane integrity; AIS = acrosome-intact sperm; VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; ALH = amplitude of lateral head displacement; WOB = wobble; BCF = beat cross frequency; DFI = DNA fragmentation index. Values are expressed as mean ± SD. [a,b]Different superscripts in the same row indicate significant differences between warming methods.

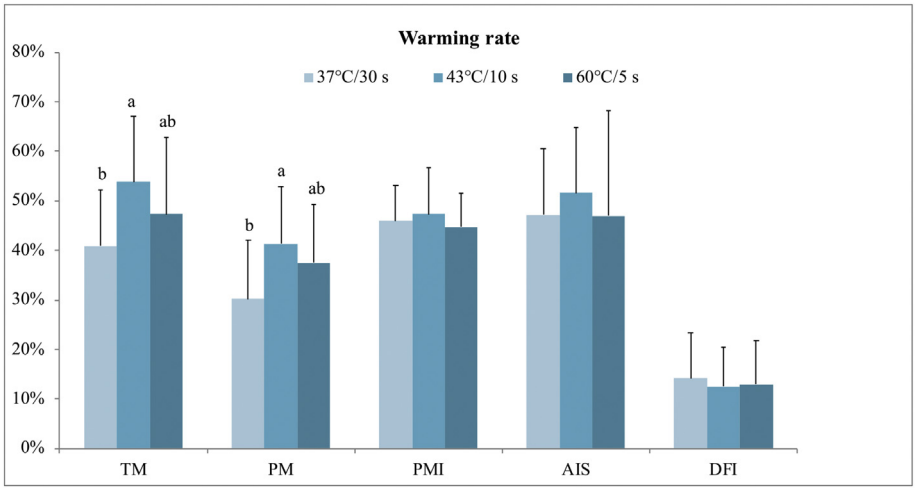

Fig. 2. Percentages of total (TM) and progressive (PM) motility, plasma membrane integrity (PMI), acrosome intact sperm (AIS) and DNA fragmentation index (DFI) from donkey sperm after sperm vitrification and warming at different rates. Different superscripts (a, b) indicate significant differences between warming rates ($P < 0.05$). Values are expressed as mean ± SD.

AI, intrauterine fluid (always <2 cm) was observed in 12/37 (32.4%) ultrasound examinations at flushing times post-AI and 6/10 (60%) jennies. In one of the positive pregnancy diagnosis uterine fluid was detected. No relation was found between uterine fluid accumulation and cryopreservation method, pregnancy diagnosis nor uterine inflammatory response ($P > 0.05$).

Pregnancy rates diagnosed at day 16 post ovulation are summarized in Table 4. AI using vitrified sperm obtained double values in positive pregnancy diagnosis percentages than frozen semen, but there were no statistical differences ($P > 0.05$).

The mean concentration of PMN is shown in Table 5. AI using vitrified semen showed a significant ($P < 0.05$) reduction in the concentration of PMN at the second flushing in comparison to the first one. No significant differences ($P > 0.05$) were found between first and second flushing using frozen semen. The comparison of PMN concentration after AI between treatments (in first and second flushing) showed no significant ($P > 0.05$) differences.

Discussion

In the present study, donkey sperm vitrification in straws has been optimized regarding the method of warming, and it has been employed for the first time in AI of jennies. To the best of the authors knowledge, there are no previous reports of AI in equine species using vitrified semen.

Sperm vitrification using the straw method has showed some advantages in comparison to other vitrification procedures. It is an aseptic technique which avoids direct contact with the liquid nitrogen and therefore preventing the risk of cross-contamination (Isachenko et al., 2017), as might occur using open carriers (open-pulled straws, cryoloops or spheres). Moreover, it allows cryopreservation of greater sperm volumes than all mentioned devices. In order to maximize the vitrified semen sample volume, vitrification in straws in larger volumes (up to 0.5 ml) has been successfully developed in humans (Isachenko et al., 2011; Slabbert et al., 2015), obtaining high sperm quality after warming. However, this technique has been recently tested in stallion (Consuegra et al., 2019; Restrepo et al., 2019) and donkey (Diaz-Jimenez et al. 2019a and 2019b) semen cryopreservation, and results were very disappointing: total motility ranged from 0 to 19%. In this sense, sperm from equine species seemed to have higher outcomes of quality when the straw system is employed. Although excellent sperm quality after vitrification in the straw method in the absence of permeable agents is obtained, the warming procedure is not quite practical; particularly when applied to an AI program: each

Table 3
Mean values of sperm motility features from vitrified samples ($n = 12$) warmed at different temperatures and times (warming rate) in donkeys.

Sperm parameters	Warming rate			*P*-values
	37 °C/30 s	43 °C/10 s	60 °C/5 s	
VCL (μm/s)	90.1 ± 15.4[b]	93.2 ± 17.2[ab]	103.2 ± 12.9[a]	<0.05
VSL (μm/s)	77.1 ± 15.1[b]	81.0 ± 14.6[ab]	90.2 ± 11.1[a]	<0.05
VAP (μm/s)	82.7 ± 15.5[b]	86.2 ± 15.2[ab]	96.5 ± 12.5[a]	<0.05
ALH (μm)	2.0 ± 0.4	1.9 ± 0.4	2.0 ± 0.3	>0.05
WOB (%)	91.6 ± 3.0	92.6 ± 3.0	93.5 ± 1.7	>0.05
BCF (Hz)	9.2 ± 0.3	9.4 ± 1.9	9.4 ± 0.6	>0.05

VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; ALH = amplitude of lateral head displacement; WOB = wobble; BCF = beat cross frequency. Values are expressed as mean ± SD. [a,b]Different superscripts in the same row indicate significant differences between warming rates.

Table 4
Pregnancy rates in jennies ($n = 10$) inseminated with vitrified or conventionally frozen donkey semen.

	Cryopreservation method		Total
	Vitrification	Conventional freezing	
Pregnancy rate	2/9 (22.2%)	1/10 (10%)	3/19 (15.8%)

Differences were not statistically significant $P > 0.05$.

Table 5
Number (showed as $\times 10^3$ cells/ml) of polymorphonuclear neutrophils on recovered post breeding fluid ($n = 32$) after artificial insemination of jennies.

Flushing	Polymorphonuclear neutrophils concentration		*P*-values
	Vitrified	Slow frozen	
1st Flushing	459.1 ± 382.2[a]	347.6 ± 389.5	0.77
2nd Flushing	94.5 ± 76.9[b]	298.2 ± 329.5	0.08
Both flushings	276.8 ± 171.6	309.7 ± 250.7	0.74
P-values	0.03	0.79	

[a,b]Different superscripts in the same column indicate significant differences between flushings (paired *t*-test). Different superscripts within a row indicate significant differences between cryopreservation methods (two-samples *t*-test). Data are presented as mean ± SD.

inner straw must be singly warmed in a tube with semen extender, and subsequently centrifuged in order to remove vitrification medium and concentrate the sample (Sanchez et al., 2012; Uribe et al., 2017). Different extender volumes per straw have been employed in warming: from 2 ml (Sanchez et al., 2012; Schulz et al., 2017; Uribe et al., 2017) to 5 ml (Mohamed, 2015) in human; and 3 ml in stallion (Consuegra et al., 2018a) and donkey sperm (Diaz-Jimenez et al. 2017 and 2018b). This warming protocol is suitable in human reproduction, in which 3 straws with a total of 1.3×10^6 sperm are employed for intrauterine insemination (Sanchez et al., 2012). However, in equine species, approximately 10 straws would be necessary per an AI dose (500×10^6 sperm), which would imply the use of at least 30 ml of equine extender for each insemination. In the present study, an optimized and more practical warming method for sperm vitrification in the two-straws has been developed: the inner straw is directly immersed in a water bath in an upside-down way, and no semen extender is needed for warming (WB). According to our results, no differences were found between the conventionally warming protocol in 3 ml extender and the new WB method without centrifugation in terms of sperm motility or membrane integrities, but significant ($P < 0.05$) less DNA fragmentation was found after warming using WB warming method. This direct warming in a water bath had been previously tested after sperm vitrification in large volumes in sealed 0.5 ml straws, but as mentioned before, poor sperm motility results were obtained when it has been employed in equines (Consuegra et al., 2019; Restrepo et al., 2019; Diaz-Jimenez et al. 2019a and 2019b).

Interestingly, we found that the post-warming sperm centrifugation could be also avoided: progressive motility and sperm velocities (VSL and VAP) were significantly higher in uncentrifugued samples (WB) in comparison to WB + centrifugation protocol. The effect of centrifugation in comparison to direct re-extension in frozen–thawed jack semen was previously evaluated by Ortiz et al. (2015b). They reported a higher sensitivity of the sperm cell and its membranes to centrifugation after freezing, which resulted in higher sperm viability percentages in uncentrifugued samples. In our results, no differences between plasma membrane integrity were found between centrifuged or uncentrifugued samples. This could be due to the different cryopreservation method used in the present study, which has demonstrated to conserve plasma membrane integrity better than conventional slow freezing (Hidalgo et al., 2018). This warming protocol represents a clear improvement on current warming methods in the straws vitrification method in terms of cost effecteness and time-saving.

Despite the fact that the thawing process has demonstrated to influence post-thaw sperm function (Sanchez et al., 2013), the effect of warming rate after vitrification using the two-straws method has been poorly researched. In the study performed by Mansilla et al. (2016) in human sperm after vitrification in the two-straws method, authors showed that plasma membrane integrity percentage was higher at a warming rate of 42 °C/5 s, in comparison to lower temperatures (38 and 40 °C). In our experiment, no differences were found regarding plasma or acrosome membrane between warming rates employed. The importance of DNA integrity and its relation to fertility has been widely described in different animal species including equids (Evenson, 2016). In fact, it has shown to be an independent predictor of fertility (Bungum et al., 2006; Oleszczuk et al., 2013), in spite of apparently normal values of sperm motility, morphology or plasma membrane integrity. In this study, no differences between DNA fragmentation index were found among warming treatments, but in terms of sperm motility, higher temperatures (43–60 °C) were found to obtain significantly greater sperm motility and velocity percentages in comparison to the lower one (37 °C). This is consistent with motility results reported by Mansilla et al. (2016), in which percentages were improved as temperature was increased (42 °C better than 40 and 38 °C). Likewise, it supports previous findings after sperm vitrification in spheres in mouflon (Pradiee et al., 2016) and in large volumes in donkey (Diaz-Jimenez et al., 2019b) and stallion (Consuegra et al., 2019). All mentioned studies reported better sperm quality results after vitrification using high warming rates during short periods of time. This phenomenon was hypothesized by Seki and Mazur (2009) and recently reviewed by Mazur and Paredes (2016): it seems to exist an opposite relation between warming rate and necessary permeable CPA concentration, so in the absence of any permeable agent the warming rate should be high. They also showed cell survival to be highly dependent on the warming rate: a cell that has survived cooling to low sub-zero temperatures is still challenged during warming and thawing, which can exert effects on survival comparable with those of cooling (Mazur, 1984; Gao and Critser, 2000; Johnson et al., 2000). Besides, it has been reported that if a very rapid curve of temperature descent is used, the thawing should be equally fast (Cochran et al., 1984; Mazur, 1984; Gao and Critser, 2000; Johnson et al., 2000).

In the second experiment, a comparison between pregnancy rates obtained after AI of jennies in the uterine body using vitrified or conventionally frozen sperm was performed. In previous studies in other species, vitrified sperm (with and without permeable agents) has been employed for *in vitro* fertilization (**IVF**) in cat (Swanson et al., 2017), mouse (Horta et al., 2017), fish (Zilli et al., 2018) and wild goat sperm (Pradieé et al., 2018) and intracytoplasmic sperm injection in humans (Isachenko et al., 2012) and porcines (Arraztoa et al., 2017a). However, a thorough search of the literature yielded few studies in which intrauterine AI with vitrified sperm has been carried out: in human (Sanchez et al., 2012), rabbit (Rosato and Iaffaldano, 2013), wild goat (Pradiee et al., 2015) and mouflon sperm (Pradiee et al., 2016). Sanchez et al. (2012) reported the first case of children born after intrauterine insemination with vitrified semen, and Pradiee et al. (2015) obtained similar fertility outcomes using vitrified sperm as those obtained with conventionally frozen semen in earlier studies performed in wild goat (Santiago-Moreno et al., 2006). In contrast, Rosato and Iaffaldano (2013) and Pradiee et al. (2016) in their studies performed in rabbit and mouflon, respectively; reported considerably lower pregnancy rates using vitrified in comparison with fresh and/or conventionally frozen semen, but the quality of sperm after vitrification in both studies was very disappointing. Our work represents the first report in which a direct comparison using both techniques for intrauterine insemination has been performed in equine species. Results showed a trend to obtain higher pregnancy percentages using vitrified (22.2%), *versus* frozen semen (10%); but differences were not statistically different. This result is consistent with embryo production and development rates between methods after IVF in mentioned species (Horta et al., 2017; Swanson et al., 2017; Pradieé et al., 2018). In this sense, vitrified semen seems to be, at least, equally able to result in pregnancy as frozen semen is. Larger number of cycles might provide more accurate results, and possibly evince a statistically significant higher pregnancy rates using vitrified semen.

For many years, limiting factors affecting the low fertility results in jennies when cryopreserved jack semen is employed were explored. First investigations on this regard considered glycerol to impair the fertilizing ability of jack sperm, as pregnancy rates were higher after its removal (Trimeche et al., 1998). Later, Vidament et al. (2009) also reported lower pregnancy rates after insemination when glycerol was present in the AI dose, even with cooled semen, which were improved when it was substituted with other molecules. Surprisingly though, when a combination of those permeable CPAs was used (with and without glycerol presence) by Oliveira et al. (2006), no pregnancies were obtained. This was recently reviewed by Rota et al. (2012), who reported no differences between pregnancy rates using ethylene glycol and glycerol, but a tendency to obtain better results when glycerol was resuspended in SP. In that study, pregnancy rates obtained when glycerol was not resuspended with SP (23%) were similar to those obtained in this study. This lends support to the fact that the lack of SP addition in the present study may be the cause of the lower average mean pregnancy rates obtained (15.8%), in comparison to those previously reported by Rota et al. (2012) using treatments adding SP (36%). Authors decided to avoid post-thaw addition of SP before AI because

the main purpose of this study was to evaluate the influence of permeable agent presence in the insemination dose. Therefore, and according to our results, the role of glycerol in the low fertility rates obtained in jennies could not be confirmed yet.

More recent investigations focused research on semen dose and deposition site effect, in order to improve fertility outcomes in jennies. Oliveira et al. (2016) obtained highest pregnancy results when a dose of $1\,000 \times 10^6$ sperm (twice the dose employed in the present study) was deposited in the tip of the uterine horn. Still, only slightly higher pregnancy rates (28.3%) were achieved by Oliveira et al. (2016) using frozen semen in comparison to our results using vitrified semen (22.2%), even if female tract was monitored every 8 h until detection of ovulation. Nevertheless, the effect of a higher semen dose and deep horn insemination protocol in relation to fertility using vitrified semen needs to be further investigated.

A higher uterine inflammatory response after insemination with cryopreserved semen has been also considered to negatively affect fertility in jennies (Vidament et al., 2009; Miro et al., 2011). This physiologic response is necessary for uterine clearance, but the maintenance of inflammatory cells in the uterine lumen results in an undesirable environment, reducing fertility (Katila, 2012). In this sense, SP addition prior to AI has been described as a strategy to partially solve this problem by reducing the duration of inflammatory response in equine species, which has been reviewed for horses (Troedsson et al., 2001) and studied in donkeys *in vivo* (Rota et al., 2012). Although no differences in the uterine inflammatory response were found in previous studies after AI with cryopreserved donkey semen with or without SP addition (Rota et al., 2012), pregnancy rates were higher when AI dose was resuspended with SP. Similarly, in this study, we found no differences between uterine inflammatory response after insemination using vitrified (without permeable CPAs) or frozen semen; however, a significant reduction in PMN concentration between first and second insemination and subsequent flushing was found using vitrified sperm. In this sense, the inflammatory response of the uterus after insemination with frozen–thawed jack semen could be independent of the presence of permeable agents, but the inflammatory condition is shorter if there is no glycerol in the AI dose. The present findings might help to solve the difficulty of treating the endometritis in jennies after frozen–thawed jack sperm insemination, as previously described by Viles et al. (2013). Following our results, the uterus of the jenny could be able to clean itself easier after insemination if no permeable agents are present, in particular glycerol. Nevertheless, whether uterine inflammatory response and fertility were related is still not so obvious. This study is the first step toward enhancing our understanding of the relation between uterine inflammatory response in jennies and permeable CPAs presence.

In summary, a new methodology for direct warming of vitrified straws in a water bath (43 °C/10 s) has been devised, avoiding post-warming sperm centrifugation. Artificial insemination with vitrified donkey sperm promoted positive pregnancy outcomes and reduced the post breeding inflammatory response in jennies. These findings represent an excellent initial step toward to use vitrified semen as an effective alternative for artificial insemination with cryopreserved donkey semen.

Ethics approval

All the experiments were performed in accordance with the Ethical Animal Experimentation Committee of Cordoba University (Project No. 31/08/2017/105) according to the Spanish law for animal welfare and experimentation (Decision 2012/707/UE and RD 53/2013) and approved by the Ethical Committee of the University of Pisa (Prot. n. 45/2017).

Data and model availability statement

None of the data were deposited in an official repository.

Author ORCIDs

Maria Diaz-Jimenez: https://orcid.org/0000-0001-7100-5878.
Alessandra Rota: https://orcid.org/0000-0002-3379-2003.
Jesús Dorado: https://orcid.org/0000-0002-4310-7663.
Cesar Consuegra: https://orcid.org/0000-0002-9270-8406.
Blasa Pereira: https://orcid.org/0000-0002-7747-8358.
Francesco Camillo: https://orcid.org/0000-0001-9389-6464.
Duccio Panzani: https://orcid.org/0000-0002-3433-7918.
Diana Fanelli: https://orcid.org/0000-0003-0863-5898.
Matteo Tesi: https://orcid.org/0000-0003-2810-5835.
Davide Monaco: https://orcid.org/0000-0001-8938-9099.
Manuel Hidalgo: https://orcid.org/0000-0001-7830-7422.

Author contributions

M Diaz-Jimenez: Formal analysis, Investigation, Methodology, Writing– original draft, Writing– review & editing. A Rota: Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Writing– review & editing. J Dorado: Funding acquisition, Investigation, Resources, Supervision, Writing– review & editing. C Consuegra: Investigation. B Pereira: Investigation. F Camillo: Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Writing– review & editing. D Panzani: Formal analysis, Investigation, Methodology. D Fanelli: Investigation, Methodology. M Tesi: Investigation. D Monaco: Investigation. M Hidalgo: Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Writing–original draft, Writing– review & editing.

Declaration of interest

None.

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