

EXPLORING RABBIT SEMEN CRYOPRESERVATION AND THE CRUCIAL ROLE OF CRYOBANK: UNVEILING ADVANTAGES AND PROMISING PERSPECTIVES

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Abstract: Over the years, efforts have been made to establish a reference protocol for the first Italian rabbit semen bank. Here, we will provide an overview of our group's key discoveries, which have led us to identify an effective freezing protocol for rabbit semen. However, a drawback of our protocol involves diluting the semen with the freezing media at fixed dilution ratios, resulting in significant variability in the sperm number per straw. Addressing this limitation underscores the need to standardise the freezing protocol by investigating the effect of sperm concentrations within straws on the reproductive performances of cryopreserved rabbit semen. In this regard, in an *in vivo* trial, 192 multiparous receptive rabbit does were randomly assigned to six treatment groups, including a control group. The remaining groups were inseminated with doses containing 15, 25, 35, 55 and 75×10⁶ sperm, respectively. Sperm concentrations of 25, 35 and 55×10⁶/straw exhibited higher reproductive performances compared to other tested concentrations and were akin to fresh semen. Through the adoption of freezing protocols with standardised sperm concentrations, cryobanks can safely preserve rabbit population genetic resources, offering invaluable support for future breeding and research efforts.

Key Words: rabbit, cryopreservation, cryobank, sperm concentration.

INTRODUCTION

Rabbits have always been indispensable for human life because they are valuable for agriculture, for biomedical research and because of their docile nature they make popular pets. The rabbit's short life cycle, brief gestation period, prolificacy and high feed conversion capacity (Lebas *et al.*, 1997) make it theoretically ideal for meat production. Breeding rabbits for human consumption is largely dependent on artificial insemination (AI) programmes. In rabbit farms, AI predominantly employs fresh or cooled semen, as opposed to frozen semen, due to the compromised fertility observed following thawing and/or for highly variable results (Mocé and Vicente, 2009; Lavara *et al.*, 2017; Kubovicova *et al.*, 2021).

Frozen rabbit semen is crucial for international export, research and conservation of genetic resources (endangered breeds or high-value males). Semen cryopreservation, which is part of the ex-situ *in vitro* strategy, is a valuable tool for safeguarding animal genetics via cryobanks, particularly in rabbits, offering a cost-effective alternative to embryo preservation (Mocé and Vicente, 2009). However, the limited survival rate of rabbit sperm following cryopreservation represents a significant challenge that hinders the widespread adoption of frozen semen in AI programmes. It also poses as a barrier to the effective preservation of genetic resources through the establishment of a sperm cryobank. Several studies have investigated methods to improve the cryopreservation protocol of rabbit sperm, examining

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factors including freezing methods and the types and concentrations of cryoprotectants (Mocé and Vicente, 2009; Nishijima *et al.*, 2021; Viudes-de-Castro and Vicente, 2023). Despite substantial technical enhancements made in recent years (Kubovicova *et al.*, 2021; Viudes-de-Castro and Vicente, 2023), the lack of a firmly established standard freezing procedure for rabbits persists due to inconsistent and divergent outcomes observed across various studies.

One factor that may account for the inconsistent outcomes in rabbit sperm cryopreservation could be the variability in sperm quality (Kubovicova *et al.*, 2021). Another significant aspect contributing to the fluctuation in results is the unpredictability of the final sperm concentration within the straws, as freezing protocols typically involve diluting the semen with freezing media at fixed dilution ratios. Additionally, it is acknowledged that the success of freezing rabbit sperm differs among various breeds (Kuliková *et al.*, 2017) and among donors (Mocé *et al.*, 2005).

Over the years, we have also dedicated significant efforts to developing a reference semen freezing protocol for establishing the first Italian bank that is a milestone within the “MiPAAF” project aiming to conserve and promote Italian rabbit breeds.

Here, we provide an overview of our group’s research, which has led to the development of an effective freezing protocol. On one hand, this protocol has enabled us to achieve reproductive performances comparable to those observed with fresh semen (Laffaldano *et al.*, 2012, 2014; Di Iorio *et al.*, 2018, 2020). On the other hand, we encountered a significant variability in the number of sperm present in each straw, due to the use of a fixed dilution ratio. Consequently, our focus has shifted towards determining the precise sperm concentration in straws, with the aim of standardising rabbit cryopreservation protocols and reducing result variability.

In our recent study, Di Iorio *et al.* (2024) specifically examined the influence of different sperm concentrations within straws on *in vitro* semen quality during the cryopreservation process. Therefore, in order to offer a more comprehensive insight into the impact of sperm concentration, our present report also includes additional investigations that evaluate the effects of different concentrations (15, 25, 35, 55 and 75×10^6) within straws of cryopreserved semen on reproductive performances in rabbit.

MATERIAL AND METHODS

Animals and experimental design

In the last decade, our research endeavours have focused on establishing an effective semen freezing protocol for rabbits. This comprehensive investigation entailed evaluating the impact of various permeable and non-permeable cryoprotectants, determining their optimal combinations, equilibrium time and cooling time prior to freezing, as will be below reported in Table 2 (Laffaldano *et al.*, 2012, 2014; Di Iorio *et al.*, 2018, 2020).

While the cryopreservation protocol resulting from these studies yielded satisfactory outcomes, standardisation was necessary due to the variability in the number of spermatozoa within each straw caused by the utilisation of a fixed dilution ratio.

Consequently, Di Iorio *et al.* (2024) investigated the impact of sperm concentration/straws on *in vitro* sperm quality of the main step of the cryopreservation procedure (see below Table 3). To conclude our study, an AI trial was conducted herein to evaluate the *in vivo* effectiveness of semen cryopreserved at various sperm concentrations per straw (15, 25, 35, 55 and 75×10^6) on reproductive performance. The semen doses utilised in the *in vivo* trial were developed in our previous research (Di Iorio *et al.*, 2024).

In this regard, in Di Iorio *et al.* (2024), 80 adult rabbit bucks (7-9 mo old) of the Bianca Italiana breed, from the Central Breeding Farm of Italian Rabbit Breeders Association (ANCI-AIA, Volturara Appula (FG), Italy) were used. The rabbits were individually housed in flat-deck cages and provided with a 16 h light/8 h dark photoperiod. A commercial standard breeder diet and free access to water were provided. Ejaculates were collected using a pre-heated artificial vagina. Ejaculates were pooled to avoid the effects of individual differences among males; in total, eight pooled semen samples were used. Semen samples were transported from the farm to the laboratory within 30 min, at a temperature of approximately 30°C in a polystyrene box. Upon arrival at the laboratory, a portion of each sample was promptly assessed for fresh semen quality, which included testing sperm motility, membrane integrity and concentration.

Table 1: Dilution process of fresh rabbit semen (example of initial concentration: 720×10^6 spermatozoa mL^{-1}) with TCG and freezing extender to achieve final sperm concentrations in the straws of 15, 25, 35, 55 and 75×10^6 .

Initial fresh sperm concentration ($\times 10^6 \text{ mL}^{-1}$)	Sperm concentration after pre-dilution with			Semen volume (mL)	TCG volume (mL)	F.E. volume (mL)	Final volume (mL)	Sperm concentration after dilution with	
	TCG ($\times 10^6 \text{ mL}^{-1}$)	Dilution rate	F.E. 1:1 ($\times 10^6 \text{ mL}^{-1}$)					Sperm concentration per straw ($\times 10^6$)	
720	120	6.00	0.42	2.08	2.50	5.00	60	15	
720	200	3.60	0.69	1.81	2.50	5.00	100	25	
720	280	2.57	0.97	1.53	2.50	5.00	140	35	
720	440	1.64	1.53	0.97	2.50	5.00	220	55	
720	600	1.20	2.08	0.42	2.50	5.00	300	75	

TCG: tris, citric acid, glucose; F.E.: freezing extender composed of TCG, 16% dimethyl sulfoxide, and 0.1 M sucrose.

For each fresh semen sample pool, the sperm concentration was assessed through spectrophotometric analysis. This involved measuring the optical density at 530 nm of samples diluted 1:200 in a 0.9% NaCl solution. Sperm concentration was calculated through interpolation on a previously calibrated calibration curve and expressed in millions/mL. Each semen pool was split into five aliquots. The aliquots were prediluted with Tris-citrate-glucose extender (TCG; 250 mmol/L Tris-hydroxymethylaminomethane, 88 mmol/L citric acid, and 47 mmol/L glucose, pH 6.8) and then with TCG supplemented with cryoprotectants (CPAs) (freezing extender) until reaching the pre-determined concentrations to assure the following final number of spermatozoa inside the straws: 15, 25, 35, 55 and 75×10^6 sperm/straw, respectively, as reported by Di Iorio *et al.* (2024) (Table 1). Thus, each semen sample was processed and cryopreserved using the freezing technique as outlined by Iaffaldano *et al.* (2012). To prepare for freezing, the semen samples that were prediluted with TGC were cooled at 5°C for 90 min (Di Iorio *et al.*, 2018). After cooling, they were diluted to a ratio of 1:1 (v:v) with a freezing extender composed of TCG containing 16% dimethyl sulfoxide (DMSO) as a permeable CPA and 0.1 mol/L sucrose as a non-permeable CPA. The diluted semen was aspirated into 0.25 mL plastic straws, equilibrated at 5°C for 45 min (equilibration time) and frozen by exposure to liquid nitrogen vapour 5 cm above the liquid nitrogen surface (temperature was approximately $-125/-130^\circ\text{C}$) for 10 min. Finally, the straws were plunged into liquid nitrogen for storage at -196°C .

Evaluation of *in vitro* sperm quality

The semen quality was immediately evaluated after dilution of fresh semen and thawing. The frozen semen doses were thawed in a water bath heated to 50°C for a duration of 10 s.

Sperm motility was assessed using a computer-aided sperm analysis system linked to a phase-contrast microscope (Nikon Eclipse model 50i; negative contrast, Firenze, Italy) with Sperm Class Analyzer (SCA) software (version 4.0, Microptic S.L., Barcelona, Spain) (Rusco *et al.*, 2022). Samples of semen were diluted in 0.9% NaCl to achieve a sperm concentration of $50 \times 10^6/\text{mL}$. After a 5-min incubation at 37°C, 3 μL of the prepared sample were placed on a prewarmed 20-micron Leja slide (Leja Standard Count, Nieuw Vennep, The Netherlands) and examined under the microscope at 100 \times total magnification. Various parameters were recorded, including total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, $\mu\text{m/s}$), straight-line velocity (VSL, $\mu\text{m/s}$), average path velocity (VAP, $\mu\text{m/s}$), linearity (LIN, %) and straightness (STR, %). A minimum of 500 spermatozoa per sample were examined across three distinct microscopic fields.

Sperm membrane integrity (SMI) assessment was conducted using the Muse[®] Cell Analyzer (Luminex Corporation, Austin, TX, USA) according to the manufacturer’s guidelines. Semen samples were initially diluted in phosphate-buffered saline (PBS) to achieve a concentration ranging from 1×10^5 to 1×10^6 spermatozoa/mL. A 20 μL portion of this suspension was mixed with 780 μL (dilution factor 1:40) of a Muse Count and Viability Kit[®] in an Eppendorf tube (Luminex Corporation). After 5 minutes of incubation at room temperature in the absence of light, the Eppendorf tubes were subjected to flow cytometry analysis. The software generated two dot plots: one for nucleated cells, which helps in the distinction of cells with a nucleus from debris and non-nucleated cells, and another for viability, which identifies viable (non-staining living cells) from non-viable (staining dead or dying cells) based on membrane integrity.

***In vivo* experiment to test the effect of different sperm concentrations**

Regarding the AI trial, 192 multiparous (31 d postpartum) receptive rabbit does were randomly assigned to 6 treatment groups: a control group (inseminated with 0.5 mL of fresh semen diluted 1:10), while the other groups were inseminated with 15, 25, 35, 55 and 75×10^6 spz/dose from frozen semen, respectively. Each group consisted of 32 rabbits. Before insemination, all does were synchronised following a biostimulation protocol based on flushing and changing cages (3 d before insemination) and increasing the photoperiod from 16 to 24 h of light (2 d before insemination). At the moment of insemination, each female received an intramuscular injection of buserelin acetate at a dosage of 1 microgram per doe to induce ovulation.

Fertility rate (number of pregnant does/number of inseminations), kindling rate (number of does giving births/number of inseminations), prolificacy (total born/kindling) and number of kids born alive (total liveborn/kindling) were considered when assessing the reproductive performance. The fertility rate was determined by abdominal palpation performed on each doe 17 d after AI, while the other factors were registered at parturition.

Statistical analysis

A comparison of the reproductive performance (fertility rate, kindling, prolificacy and number of kids born alive) of the does that received the six different insemination treatments was carried out by an analysis of variance, followed by Duncan's comparison test. All statistical tests were performed using the software package SPSS (IBM SPSS Statistics 23.0 for Windows, 2020; SPSS, Chicago, IL, USA). Significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Over the years, our research group has achieved satisfactory results in developing an effective freezing protocol for rabbits, substantiated by the findings and validated through both *in vitro* and *in vivo* assessments of frozen semen, as depicted in Table 2. Many variables of rabbit semen freezing protocols were studied, such as cryoprotectant (CPA) and its concentration, freezing extender and initial cooling time (laffaldano *et al.*, 2012, 2014; Kuliková *et al.*, 2017; Di Iorio *et al.*, 2018, 2020). Summing up the results obtained in the above-mentioned work, the protocol involving the dilution of semen with freezing media at fixed dilution ratios allowed us to reach similar reproductive performances with frozen semen as those recorded with fresh semen. During the cryopreservation process, the spermatozoa undergo various types of stress caused by ice formation, chemical toxicity and oxidative stress, which mainly injures cytoplasm membrane, consequently leading to a lower post-thawed quality and fertility (Khan *et al.*, 2021). To shed light on molecular damage mechanisms impacting post-thaw sperm quality, we recently studied the proteome of fresh and frozen rabbit semen to identify proteins altered during cryopreservation. The protein alterations make sperm more susceptible to stressors during and after cryopreservation, leading to disturbances in the fertilisation process (Rusco *et al.*, 2022).

Intracellular ice crystals formed during cryopreservation can lead to cell destruction. This can be prevented by dehydrating cells, using a permeable CPA in the freezing solution (laffaldano *et al.*, 2012). Permeable CPAs may also harm sperm by destabilising membranes and denaturing proteins and enzymes, with toxicity linked to CPA concentration and exposure time. Including non-permeable CPAs in the freezing medium mitigates cryodamage caused by permeating CPAs, reducing the amount required (Swain and Smith, 2010). Hence, selecting the appropriate CPA was crucial when developing an effective freezing protocol for rabbit semen.

The most widely used CPAs to preserve rabbit semen are a combination of permeable compounds such as DMSO or acetamide and non-permeable compounds such as lactose, sucrose, raffinose, trehalose or egg yolk (Mocé and Vicente, 2009; Viudes-de-Castro and Vicente, 2023).

The choice of CPA is certainly among the most important factors for an effective rabbit semen freezing protocol. Our previous findings indicate that the permeable CPA used and its concentration, the speed of cryopreservation, and the presence and particular combination of nonpermeating CPA, affected the cryosurvival of rabbit semen (laffaldano *et al.*, 2012; Rosato and laffaldano, 2013). The main points established were:

- DMSO and CPAs containing amide groups resulted in better cryosurvival rates of rabbit sperm than glycerol (Rosato and Iaffaldano, 2013) in accordance with other authors (Kashiwazaki *et al.*, 2006). On the contrary, methanol, ethylene glycol and propylene glycol showed an immediate toxic effect after 5 minutes of incubation with sperm, even at low concentrations (Rosato and Iaffaldano, 2013);
- CPA concentration and equilibrium time affected the survival rate of rabbit sperm after cryopreservation and DMSO was better than DMA at preserving the quality and fertility of rabbit sperm (Iaffaldano *et al.*, 2012; Rosato and Iaffaldano, 2013). This agrees with the findings of Holt (2000), who reported that the toxicity of penetrating CPAs increases with its concentration, exposure temperature and exposure time.

The reason why rabbit sperm cells were able to better withstand DMSO than DMA during the cryopreservation process is unknown, but several explanations can be proposed. DMA and DMSO share many common physical-chemical properties, but they differ in molecular weight and molecular structure. These differences may influence permeability through the cell membrane, which could explain why DMSO provides better cryoprotective protection (Iaffaldano *et al.*, 2012). Subsequently, our efforts focused on selecting the best non-permeable CPA. Through testing different concentrations of lipoproteins (LDL) in comparison with sucrose and egg yolk, we found that sucrose was the most suitable non-permeable CPA for freezing rabbit semen (Iaffaldano *et al.*, 2014). These findings underscore that sucrose's effectiveness as a non-permeable CPA for rabbit sperm cryopreservation is attributed to its dehydration ability at high subfreezing temperatures, inhibiting ice crystal growth and allowing rapid cooling. Sucrose also has a higher glass transition temperature, enabling long-term storage at high subzero and suprazero temperatures (Woelders *et al.*, 1997; Gómez-Fernández *et al.*, 2012).

By investigating the effect of initial cooling time during the semen cryopreservation process, we showed that 90 min of initial cooling (at 5°C) before freezing resulted in better post-thaw semen quality and reproductive performance compared to the semen cooled for 45 min. The longer cooling time prepares the sperm more effectively for the addition of the CPAs (Di Iorio *et al.*, 2018). In addition, we also compared a TCG extender with a commercial one (Cortalap[®]) regarding the *in vitro* freezability and fertilising ability of cryopreserved rabbit semen. No significant differences in reproductive performances were found, showing that Cortalap[®] could be a valid alternative to TCG. Being a ready-to-use extender, it entails a reduction in time, mistakes and microbial contaminations during its preparation (Di Iorio *et al.*, 2020).

Integrating the outcomes of these studies, we devised an optimal protocol for freezing semen, which encompassed cooling sperm at 5°C for 90 min, diluting it with a freezing extender (TCG containing 16% of DMSO and 0.1 M of sucrose), 45-min equilibration time at 5°C, and exposing it to liquid nitrogen vapour at 5 cm above the nitrogen. This protocol, based on a fixed semen extender dilution rate of 1:1, enabled us to achieve reproductive outcomes with frozen semen that were comparable to those observed with fresh semen (Iaffaldano *et al.*, 2012, 2014; Di Iorio *et al.*, 2018, 2020). Our protocol's major drawback was the wide variability in sperm numbers per straw. Additionally, it should also be noted that most of the protocols developed by other authors are based on fixed sperm-to-extender ratios, ranging from 1:1 to 1:10 (Mocé and Vicente, 2009; Mocé *et al.*, 2014; Viudes-de-Castro *et al.*, 2014, 2021; Nishijima *et al.*, 2015; Lavara *et al.*, 2017; Domingo *et al.*, 2019; Fadl *et al.*, 2019; Kùçük *et al.*, 2021; Mohammed *et al.*, 2022).

Therefore, identifying the optimal sperm concentration within the straw is a crucial aspect that has received little attention to date. This goal is essential to standardise rabbit cryopreservation protocols, reducing result variability and enhancing freezing techniques. For example, the dairy bull industry is considered a model in the context of sperm cryopreservation due to its highly standardised protocols, which involve consistent and specified sperm concentrations in each straw.

Our *in vitro* study demonstrated that sperm concentration plays a significant role in specific phases of the cryopreservation process (dilution of fresh semen, cooling and equilibration, immediately after and 30 min post-thawing) (Di Iorio *et al.*, 2024).

Our findings highlighted the significant impact of sperm concentration per straw on key stages of the cryopreservation process, particularly during equilibration and post-freezing phases. Additionally, the final sperm concentration in the straws influences the post-thaw motility of cryopreserved rabbit sperm. We observed that mid-range sperm

Table 2: An overview of the *in vitro* quality and reproductive performance outcomes with cryopreserved rabbit semen, obtained by our research group over the last decade.

Reference	Breed	Cooling	Freezing protocol				Post-thaw <i>in vitro</i> quality				Reproductive performance			
			Freezing extender composition	Dilution rate	Sperm viability (%)	Motility (%)	Fertility (%)		Prolificacy (mean ± SEM)					
							Fresh	Frozen	Fresh	Frozen				
laffaldano <i>et al.</i> 2012	Hybrid of Centro genetica Martini	90 min at 5°C	TCG	1:1 (v:v)	36.1±1.6	TM: 30.9±2.5 PM: 23.5±1.3	81.6	47.4	8.6±0.3	6.7±0.4				
			12% DMA 2% sucrose TCG		47.1±1.8	TM: 42.6±2.1 PM: 35.4±1.9		79.8		7.7±0.3				
Rosato and laffaldano 2013	Hybrid of Centro genetica Martini	90 min at 5°C	TCG	1:1 (v:v)	40.0±1.1	TM: 44.4±1.0	84.0	52.0	8.8±2.1	7.4±2.8				
			10% DMSO 0.5% BSA 0.1 M trehalose TCG		36.5±1.2	TM: 41.1±1.8		77.0		8.1±2.5				
laffaldano <i>et al.</i> 2014	Bianca Italiana breed	90 min at 5°C	TCG	1:1 (v:v)	43.5±1.0	TM: 38.9±1.4 PM: 29.7±1.7	93.3	86.7	10.1±0.5	9.2±0.5				
			16% DMSO 0.1M sucrose TCG		39.9±1.1	TM: 35.4±0.9 PM: 27.9±1.1		66.7		8.2±0.6				
Di Iorio <i>et al.</i> 2018	Bianca Italiana breed	45 min at 5°C	TCG	1:1 (v:v)	36.8±1.8	TM: 30.5±1.0 PM: 22.4±0.7	74.0	64.0	8.7±0.6	8.2±0.6				
			16% DMSO 0.1 M sucrose 90 min at 5°C		42.5±1.0	TM: 37.3±1.1 PM: 27.8±1.1		76.0		8.3±0.6				
Di Iorio <i>et al.</i> 2020	Bianca Italiana breed	90 min at 5°C	TCG	1:1 (v:v)	44.7±2.0	TM: 36.8±1.5 PM: 30.2±1.9	80.0	86.7	7.7±0.7	7.4±0.8				
			16% DMSO 0.1M sucrose Cortalap® 16% DMSO 0.1M sucrose		52.5±1.8	TM: 43.4±1.4 PM: 36.5±1.1		76.7		9.4±0.6				

TCG: Tris-citrate-glucose; DMSO: dimethyl sulfoxide; DMA: dimethylacetamide; LDL: low-density lipoproteins; BSA: bovine serum albumin; TM: total motility; PM: progressive motility; data are presented as mean±SEM; standard error of the means.

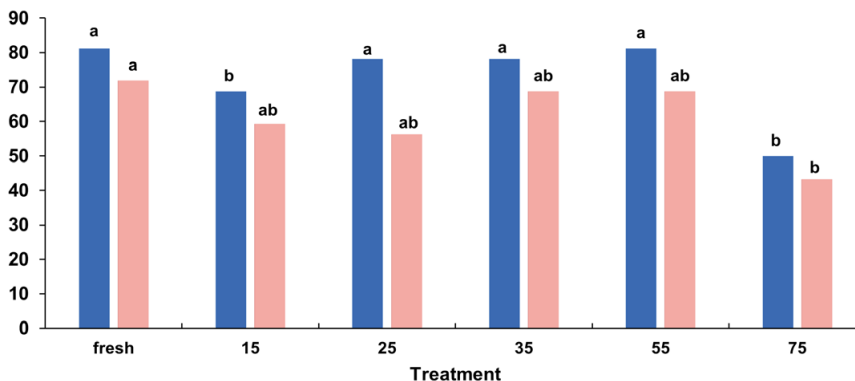


Figure 1: Fertility and kindling rate (%) obtained with fresh and frozen semen (with 15, 25, 35, 55 and 75×10⁶ sperm/straw, respectively). ■ Fertility; ■ kindling. Different lowercase letters indicate a significant difference (P<0.05).

concentrations (25 and 35×10⁶/straw) showed greater sperm quality immediately after thawing compared to other concentrations (Table 3). Importantly, after a 30-min post-thawing period, semen quality no longer exhibited a discernible influence on sperm concentration (Di Iorio *et al.*, 2024).

Preliminary in vivo results obtained by testing different sperm concentrations/straw

Regarding the reproductive performance of post-thawed semen presented for the first time here (Figures 1 and 2), no significant differences for fertility and kindling rate among fresh and frozen semen with 25, 35, 55×10⁶ concentrations were registered. The concentration of 75×10⁶ showed the worst value of fertility (P<0.05) compared to fresh semen and the concentrations of 25, 35, 55×10⁶ respectively, while the kindling rate was significant only in respect to fresh semen. No significant difference was found among fresh and frozen semen for all concentrations tested on total born and liveborn. The lowest reproductive outcomes were observed with 75×10⁶ compared to other concentrations, aligning with *in vitro* findings (Di Iorio *et al.*, 2024), possibly due to a dose-dependent reduction in cryoprotective effectiveness as noted by Contri *et al.* (2012). Additionally, it has been suggested by other authors, such as Lahnsteiner (2000), that increased sperm numbers per straw could lead to cellular compression due to limited intercellular space, consequently reducing post-thaw sperm quality. Based on these findings, our freezing protocol using concentrations

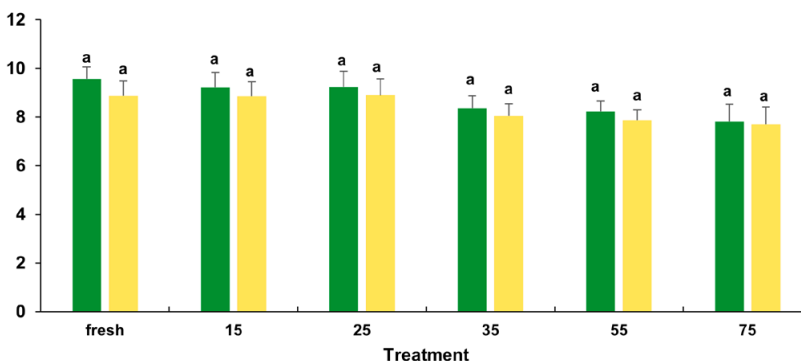


Figure 2: Total and live born kids (mean±standard error of mean) obtained with fresh and frozen semen (with 15, 25, 35, 55 and 75×10⁶ sperm/straw, respectively). ■ Total born; ■ live born. Different lowercase letters indicate a significant difference (P<0.05).

Table 3: Effect of different straw sperm concentrations on quality of freshly diluted and thawed rabbit semen (data extracted from Di Iorio *et al.*, 2024).

Treatments	Sperm concentration	Sperm variables							
		SMI (%)	TM (%)	PM (%)	VCL (µm/sec)	VAP (µm/sec)	VSL (µm/sec)	STR (%)	LIN (%)
Fresh	15	89.6±0.7 ^a	92.2±0.8 ^a	66.1±1.3 ^b	73.0±2.5 ^a	40.8±2.4 ^a	27.1±2.2 ^a	61.8±1.4 ^a	36.9±2.4 ^{ab}
	25	89.9±0.7 ^a	91.4±0.9 ^a	67.2±1.0 ^{ab}	75.4±1.9 ^a	38.2±1.8 ^a	24.4±1.3 ^a	60.9±1.2 ^a	32.7±1.5 ^b
	35	90.2±0.7 ^a	91.9±0.9 ^a	69.4±0.9 ^{ab}	76.3±1.5 ^a	40.1±1.9 ^a	26.2±1.5 ^a	61.0±0.9 ^a	33.2±1.4 ^{ab}
	55	90.8±0.8 ^a	91.4±1.0 ^a	68.1±1.6 ^{ab}	75.1±2.8 ^a	45.5±4.1 ^a	31.2±3.9 ^a	62.9±1.7 ^a	39.4±3.1 ^a
	75	91.3±0.5 ^a	91.8±0.8 ^a	70.5±1.7 ^a	75.9±2.1 ^a	41.6±1.8 ^a	26.7±1.8 ^a	61.7±1.5 ^a	35.6±1.8 ^{ab}
Frozen	15	75.5±1.1 ^a	42.2±1.6 ^{bc}	14.9±1.1 ^{bc}	41.9±1.3 ^c	20.0±0.5 ^b	10.1±0.4 ^b	45.5±0.9 ^c	22.6±0.7 ^c
	25	77.1±1.0 ^a	49.9±2.5 ^a	19.7±1.4 ^a	44.6±1.0 ^{bc}	21.5±0.4 ^{ab}	11.3±0.5 ^{ab}	49.0±1.0 ^b	24.7±0.8 ^{ab}
	35	75.8±1.7 ^a	46.2±3.0 ^{ab}	19.7±1.8 ^a	47.7±1.4 ^{ab}	22.7±0.7 ^a	12.2±0.4 ^a	50.0±1.0 ^{ab}	24.8±0.8 ^{ab}
	55	75.4±1.7 ^a	38.8±1.7 ^c	17.0±0.9 ^{ab}	49.2±1.6 ^a	22.8±0.8 ^a	12.6±0.5 ^a	50.5±0.6 ^{ab}	23.9±0.6 ^{bc}
	75	72.5±1.8 ^a	31.5±2.0 ^d	12.5±1.1 ^c	46.1±1.8 ^{abc}	22.7±0.8 ^a	12.6±0.6 ^a	51.8±0.9 ^a	26.5±0.7 ^a

Different superscripts (a,b,c,d) within the same column indicate a significant effect of sperm concentration in fresh and frozen semen. SMI: sperm membrane integrity; TM: total motility; PM: progressive motility; VCL: curvilinear velocity; VAP: average path velocity; VSL: straight-line velocity; STR: straightness; LIN: linearity.

of 25, 35 and 55×10⁶ per straw resulted in minimised damage during freezing, leading to optimal reproductive performances. Furthermore, determining the optimal freezing concentration for rabbit semen marks a significant breakthrough, addressing an aspect previously overlooked in this field.

Developing an efficient freezing protocol was crucial when establishing the first Italian rabbit cryobank, which promotes ex-situ conservation strategies for safeguarding rabbit breeds. Currently, the cryobank contains 3846 semen doses from 43 rabbit breeds.

This diversified repository not only serves as a valuable genetic resource but also provides a platform for research and breeding programmes focused on preserving rabbit diversity and genetic heritage.

The significance of semen cryobanks extends beyond storage, as they offer numerous advantages. One notable benefit is their role as a backup for populations preserved *in vivo*, providing a safeguard against potential genetic issues like inbreeding and genetic drift. This capability ensures the preservation of genetic diversity and offers the potential to reconstruct breeds in the event of extinction or a significant reduction in the population, thereby contributing to the conservation of rabbit biodiversity on a broader scale. In the context of cryobank purposes, sperm concentration controls are crucial for safeguarding the genetic integrity and viability of stored genetic material. Testing lower sperm concentrations also aligns with the potential of freezing individual donor sperm for cryobanking. Given that a single donor of these breeds may yield lower sperm concentrations, exploring this range is important for a comprehensive assessment.

CONCLUSIONS

These findings provide valuable insights for improving rabbit semen freezing techniques. Standardising sperm concentration in each straw is essential to minimise result variability and accurately determine the number of sperm received by each doe during the AI procedures, ensuring the reproducibility and accuracy of the AI technique, which is particularly crucial for successful rabbit population breeding management. Sperm concentrations of 25, 35, and 55×10⁶/straw demonstrated higher reproductive performance compared to other concentrations tested, and the results were similar to those in fresh semen.

Through the adoption of freezing protocols with standardised sperm concentrations, cryobanks can confidently preserve rabbit population genetic resources. Additionally, rabbit breeding facilities could start to take advantage of the opportunities by extensive use of doses of frozen semen.

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