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CRYOPRESERVATION OF RABBIT SEMEN: IMPACTS OF PERMEABLE AND NON-PERMEABLE MIXTURE OF CRYOPROTECTANT, MALE GROUP INDIVIDUALITY, FREEZING RATE, SEMEN PACKAGE SIZE AND ANTIOXIDANT BOVINE SERUM ALBUMIN ON RABBIT SEMEN FREEZABILITY

Kamel M. Mohammed, Gamal M. Darwish, Zaher M. Rawash, Amani M. Taha

Animal Reproduction Research Institute (ARRI), 5 Hadaek Research Centre St., Giza, Egypt.

Abstract: In the present study, three experiments were designed to identify the most appropriate technique for freezing rabbit semen. Experiment 1 aimed to determine the optimal levels of dimethyl sulfoxide (DMSO) contents in freezing medium and their effects on individual bucks. Semen ejaculates for each buck (n=15 bucks) were mixed and split into three portions for extension with a freezing medium containing varying concentrations of DMSO (0.75, 1.0, and 1.4 M). Diluted semen samples were packaged in 0.25 mL straws and suspended above liquid nitrogen (LN) for 10 min, then dipped in LN. A few days after freezing, post-thaw semen evaluation was assessed, and according to the results, six bucks and an extender containing 0.75 M of DMSO were used for experiments 2 and 3. In experiment 2, the pooled semen from 6 bucks was divided into two portions for packaging in two straw sizes (0.25 and 0.50 mL). Each straw size was divided into five groups and suspended at different heights above LN (2, 4, 6, 8, and 10 cm) for 10 minutes before being preserved in LN. In experiment 3, the pooled semen was divided into four portions for dilution with freezing medium containing different concentrations of bovine serum albumin (BSA; 0, 2.5, 5.0, and 7.5 mg/mL). Semen samples were packaged in a 0.50 mL straw and suspended 10 min, 4 cm above LN for freezing. Pre-freezing and post-thawing, semen samples were evaluated for semen quality. Results showed that the extender containing 0.75 M DMSO had higher significant values for post-thaw sperm motility, longevity, acrosome integrity and sperm plasma membrane permeability. Bucks' individuality had significant effects on post-thaw motility, acrosome and sperm plasma membrane integrity. A significant interaction was recorded between DMSO concentrations and bucks' individuality on sperm longevity. Semen package sizes had no significant effects on the evaluated parameters. Semen was frozen at 2 and 4 cm above LN had significantly better post-thaw quality. BSA at concentrations 5 and 7.5 mg/mL improved recovery rates of acrosome integrity and sperm membrane permeability. DMSO 0.75 M and freezing 4 cm above LN seem to be more adequate for rabbit semen cryopreservation. The appropriate level of DMSO differs between bucks, as the post-thaw sperm longevity is affected. BSA enhanced acrosome and sperm membrane integrity. Results obtained will need further investigation to be confirmed in the field.

Key Words: rabbit, semen, cryopreservation, dimethyl sulfoxide, bovine serum albumin.

INTRODUCTION

Recently, the rabbit industry has sparked a lot of attention in most countries, as the animal is distinguished for its high quality meat and fur production, so artificial insemination (AI) application programmes in commercial rabbit farming systems have garnered a great deal of interest (Kubovicova *et al.,* 2022). Fresh diluted chilled semen is currently used for AI in this species. However, the storage of chilled rabbit semen for more than 48 h degrades the

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performance of the spermatozoa and reduces the fertility rate (Rosato and Laffaldano, 2013). The use of frozen sperm is severely limited due to its unsatisfactorily low fertility rate. Nevertheless, cryopreservation of semen from male rabbits and other species of high genetic value would gain importance in terms of prolonging storage time and extending genetic improvement (Nishijima *et al.,* 2021). Although many attempts have been made to optimise rabbit sperm cryopreservation protocols, there is still a need for reliable protocols to improve rabbit sperm cryopreservation. Cryoprotective agents (CPAs) and their concentrations, freezing/thawing rates, straw package size and freezing extender composition are all potential factors that have a significant impact on the quality of post-thaw rabbit semen and, as a result, fertilising ability (Mocé *et al.,* 2015). Rabbit buck sperm has some peculiarities unlike other animal species in that they have a low water permeability coefficient and high activation energy (Curry *et al.,* 1995). CPAs play a critical role in sperm protection against freezing stress. Consequently, for rabbit semen cryopreservation, an ideal cryoprotectant should have low molecular weight, high permeability,and high osmolarity (Curry *et al.,* 1995). Despite the fact that glycerol has become a popular CPA for cryopreservation of almost all domestic animals' sperm, it is not suitable for rabbit sperm due to poor sperm plasma membrane permeability and a high activation energy requirement. (Domingo *et al.,* 2019). On the other hand, studies have shown that dimethyl sulfoxide (DMSO) and/or amides are the cryoprotectants of choice for rabbit semen (Mocé *et al.,* 2010), as they can stabilise the sperm membrane during freezing/thawing processes (Mocé and Vicente, 2009). Whereas Rosato and laffaldano (2013) showed that high concentrations of DMSO are more suitable for rabbit sperm preservation with the same conditions of treatment, other researchers indicated that high concentrations of DMSO have adverse effects on sperm quality in terms of motility and acrosome integrity (Si *et al.,* 2006). However, slow and fast freezing rates have likely exacerbated detrimental effects on rabbits' semen cryopreservation (Morris *et al.,* 2012; Mocé *et al.,* 2015), whereas Mocé *et al.* (2015) stated that the medium freezing rate provided a better quality of post-thawing rabbit's semen. The current research is an attempt to figure out the best protocol to freeze rabbit semen. In three experiments, the effects of different concentrations of DMSO (permeable) mixed with egg yolk and sucrose (non-permeable) as cryoprotective agents, freezing rates, straw package size, bovine serum albumin (BSA) as an antioxidant and the male's individuality on the freezability of rabbit semen were investigated.

MATERIALS AND METHODS

Rabbit bucks and location

This study was carried out on 18 sexually mature apparently healthy crossbreed (New Zealand×Chinshella) rabbit bucks, aged 14.0±0.84 mo and weighing 3.4±0.07 kg. Bucks were housed individually in flat-deck metal wire cages at the Animal Reproduction Research Institute's experimental farm in Giza province (located at latitude of 30°00'29"N, longitude of 31°12'39"E, altitude of 30 m above sea level). The study was carried out during the months from early October to late February (2020-2021). The animals were exposed to a natural photoperiod with temperature ranging from 20 to 25°C, with no special feeding regime. Bucks were fed commercial pellets containing 18% crude protein, 3% fat, 19% crude fibre, calcium 1%, phosphorus 0.5% and vitamins, with free access to fresh water. Each rabbit buck was vaccinated and medicated according to the normal rabbit farm schedule. This research was approved by the institution's ethics committee for using experimental animals at Animal Reproduction Research Institute.

Artificial vagina preparation

Due to the lack of an artificial vagina (AV) for rabbit semen collection in the Egyptian market, our research team devised an AV using readily available products and modified it according to Naughton *et al.* (2003). As shown in Figures 1 and 2, it consists of a polyvinyl chloride (PVC) cylinder tube 35 mm in diameter and 65 mm in length. To serve as a lining sheath, a catheter condom is stretched over the pipe's two ends and secured with a rubber band. The end of the condom was connected to a graduated Eppendorf tube (1.5 mL) for semen collection. For gel plug separation, a sterile filtered disc mesh was fixed between the condom cone and the Eppendorf tube. Hot water (45°C) was introduced and filled the AV between the outer pipe and the inner sheath through the tube's middle opening, which was connected outside to the Foley catheter valve. When the appropriate inner temperatures and pressures of the AV were achieved, sperm collection was performed.

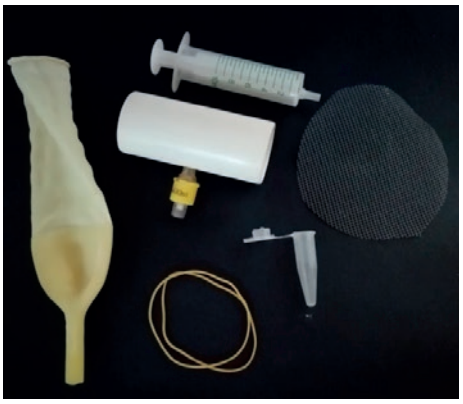

Figure 1: Components used for Rabbit AV.

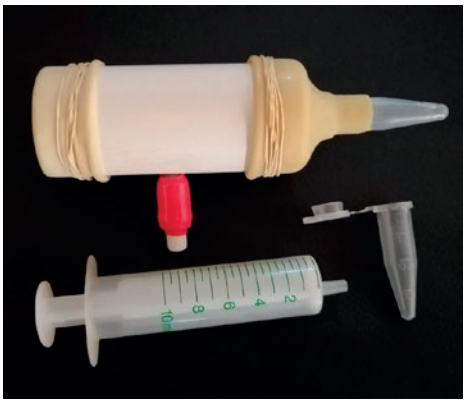

Figure 2: Handmade AV used for semen collection from rabbit.

Semen collection

Two weeks before the start of the experiment, semen samples were collected from experimental bucks using the handmade AV to ensure its efficiency and suitability for semen collection. During these weeks, semen ejaculates were collected twice a week from each male and tested for quality; only rabbit males with good ejaculates, high motility (>70%) and sperm concentrations averaging 170×10^6/mL were considered. As a result, 15 out of 18 bucks were used in the study, with three bucks being excluded due to poor semen quality.

Experimental design

Three experiments were designed in this work to study the influence of different factors on the freezability of rabbit buck semen, bearing in mind that the best outcome from the previous experiment is used to meet the goals of the following experiment.

Experiment 1: Influence of DMSO concentrations and male group individuality

This experiment aimed to determine the most appropriate concentration of DMSO as a cryoprotectant used for rabbit semen cryopreservation while considering the effects of bucks' individuality.

Diluent Preparation and semen processing

Freezing extender used in this study consists of basic tris-citric acid-glucose (TCG); supplemented with 20% egg yolk, 10 mM sucrose and DMSO. The components of TCG; 250 mM tris (hydroxymethyl amino methane), 88 mM citric acid, 47 mM glucose, gentamycin (125 µg/dL) and ciprofloxacin (62.5 µg/dL) (Safaa *et al.,* 2012; Viudes de Castro *et al.,* 2014; Amany, 2018; Fadl, 2020). Fifteen bucks were selected according to the previous initial semen evaluation. Two ejaculates per buck were collected each week with interval times of 15-30 min. Semen ejaculates were evaluated and only ejaculates exhibiting a white colour, opalescent appearance and a motility rate higher than 70% were used in the experiment. Good ejaculates for each individual buck were mixed and split into three comparable portions, which were then initially extended (v:v; 1:1) at 37°C with a freezing extender containing various concentrations of DMSO (0.75, 1.0, and 1.4 M). The diluted semen was gradually cooled down to 5°C within one hour. Further dilution was carried out at 5°C at 2 steps (10 min interval) to obtain a final dilution of (v:v; 1:10), then held for 2 h for equilibration. The equilibrated diluted semen samples were packaged at 5°C in 0.25 mL plastic straws (IMV, L´Aigle, France) and thermally sealed. The straws were suspended horizontally in a foam box 5 cm above liquid nitrogen (LN)for 10 min (Kulíková *et al.,* 2017) before being immersed in LN for storage. Three to five days after freezing, the semen samples

for each buck were thawed in a water bath at 40°C for 30-45 s and semen quality was assessed. Three replicates were conducted for each buck.

Evaluation and calculation of pre-freeze and post-thaw semen quality

Progressive forward sperm motility, the permeability of sperm plasma membrane (hypo-osmotic Swelling Test, HOST), acrosome integrity and their recovery rates for each treatment were evaluated. Sperm longevity (viability index) for post-thaw samples was also assessed. The assessment was carried out using a phase-contrast microscope with a hot stage set at 37°C and objective lenses of ×20 and ×40 magnification.

Evaluation of semen motility

According to Yendraliza *et al.* (2019), the percentage of forwarding progressive motility was calculated using the following equation, with the motility range being 0-100% on a 5-point scale.

$$\text{Motility (\%)} = \frac{\text{number of motile sperm}}{\text{number of counted sperm}} \times 100$$

Evaluation of semen longevity (viability index)

After thawing, semen samples were incubated at 37°C and the motility of sperm was measured immediately (zero hours) and every hour for 3 consecutive hours of thermal stress. According to Milovanov (1962), the viability index was determined and calculated using the equation below.

$$\text{Viability index (VI)} = \frac{\text{Motility\% at 0h}}{2} + \text{Motility\%(1st h+ 2nd h+3rd h)}$$

Evaluation of acrosome integrity

Sperm plasma membrane permeability and acrosome integrity were evaluated using Eosin and Trypan blue stain and according to Aksoy *et al.* (2008) and Duran *et al.* (2015), with minor modification. Simply, the stock stain was made by thoroughly mixing one part of Eosin yellow 1% (w/v in Phosphate buffer saline solution; PBS) with five parts of Trypan blue 5% (w/v in PBS). Semen samples were washed twice using a basic tris extender and centrifuged at 500 rpm for 10 min. After the last wash, the semen pellet was re-suspended in 100 µL of tris buffer. On a glass slide, one drop of semen sample suspension was mixed well with two drops of stock stain (previously prepared). Double slide smears for each sample were prepared and dried at 40°C. More than 100 spermatozoa were counted in various fields using an oil immersion objective lens magnification ×1000 of the phase-contrast microscope. In the case of an intact acrosome, the outline membrane was stained with light purple without disconnection, whereas the default acrosome with an abnormal shape appeared interrupted according to the degree of deformities. The post acrosomal region of dead sperm was stained red, whereas the post acrosomal region of live sperm was unstained, appearing white against a blue background.

Evaluation of sperm plasma membrane permeability

As previously stated, sperm samples were washed twice and the pellets re-suspended in 900 µL of hypo-osmotic solution (60 mOsm fructose) and incubated at 37°C for 30 min. After the incubation period, one drop of semen suspension was mixed well with one drop of Eosin 5% (w:v) and two drops of Nigrosin 10% (w:v) onto a glass slide. Double slide smears were dried at 40°C. More than 100 spermatozoa were counted regardless of whether the sperm were alive or dead. HOST positive sperm cells had a curling tail, regardless of the degree of coiling; that is, the sperm plasma membrane was intact (Aksoy *et al.,* 2008). According to Rosato and Iaffaldano (2011), the percentages of the sperm plasma membrane and acrosome integrity are calculated as follows:

$$\text{\% of intact seminal membrane} = \frac{\sum \text{Counted sperm intact plasma membrane}}{\sum \text{Total sperm counted}} \times 100\%$$

$$\% \text{ of intact acrosome} = \frac{\sum \text{Counted sperm with intact acrosome}}{\sum \text{Total sperm count}} \times 100\%$$

According to Hussein (2018), the recovery rates of estimated parameters are calculated by the following equation.

$$\text{Recovery rate (\%)} = \frac{\% \text{ of evaluated parameter post-thawing}}{\% \text{ of evaluated parameter pre-freezing}} \times 100\%$$

Experiment 2: Influence of freezing rate and size of semen package

Referring to experiment 1, according to the result of post-thaw semen evaluation, bucks were sorted in ascending order by post-thaw sperm motility. This parameter was selected for ranking bucks because it is simple, easy, requires less time and is more field-applicable. Besides, the results showed a positive correlation between post-thaw sperm motility on one side and viability index, as well as post-thaw acrosome integrity on the other side. Data for each three consecutive bucks were pooled as one group and randomly named with a letter, thus forming five groups of bucks (A, B, C, D, and E) to study the effect of buck group individuality on frozen semen. Depending on the post-thaw sperm motility, two buck groups B and C (6 males) were used for experiments 2 and 3. In addition, DMSO at a concentration of 0.75 M was chosen as the freezing medium used for experiments 2 and 3, based on the results of post-thaw sperm motility, viability index, acrosome integrity and sperm membrane permeability.

The purpose of this experiment was to determine the most suitable level of semen freezing above LN, as well as to compare the effect of different semen package sizes on semen freezability. Good semen ejaculates were pooled and equally diluted (v:v; 1:1) at room temperature using a tris extender containing 0.75 M of DMSO. Semen was processed as in experiment 1, and further dilution (v:v; 1:10) was assessed at 5°C. After equilibration time (2 h), semen samples were divided into two portions. The first portion was packaged in 0.25 mL plastic straws with five different colours, while the second portion was packaged in 0.50 mL straws in five different colours. Straws were suspended horizontally in LN vapour at heights of 2, 4, 6, 8, and 10 cm for 10 min. Each straw colour indicates the level of freezing. Pre-freezing and post-thaw semen samples were evaluated for motility, longevity and motility recovery as mentioned above. This experiment was replicated five times.

Experiment 3: Influence of Bovine serum albumin as an antioxidant on semen freezability

The objective of this experiment was to figure out what level of BSA to use as an antioxidant to improve the freezing ability of rabbit semen. Based on the results of post-thaw sperm motility, viability index and acrosome integrity in experiment 2, freezing at level 4 cm above LN was selected to be used in this experiment. Although there were no significant differences between straw sizes of 0.25 and 0.50 mL, the latter size was selected for semen package in this experiment because the viability index is slightly higher (87.51 *vs.* 90.69%). In addition, the straw is easier to handle, easier to read and less likely to break during storage.

In this experiment, the pooled semen samples were divided into four equal portions. Each portion was partially diluted (v:v; 1:1) at 37°C with an extender containing 0, 2.5, 5.0, and 7.5 mg/mL BSA, respectively. As in previous experiments, diluted semen samples were cooled and extended. For freezing, the equilibrated semen samples were packaged in 0.50 mL straws and suspended 4 cm above LN for 10 min. The same parameters were used to evaluate pre-freeze and post-thaw semen samples as in experiment 1. Seven replicates were conducted for this experiment.

Statistical analysis

Statistical tests were carried out using the SPSS Version 16.0 for Windows software package (SPSS Inc., Chicago, IL, USA). Each experiment was statistically analysed independently of the other experiments. ANOVA (two-way for experiments 1 and 2) was used to compare all estimated parameters for semen quality. The fixed effects of the various treatments on sperm quality, as well as their interactions between the main effects, were conducted for each experiment. One way ANOVA was used for experiment 3. Generalised linear model (GLM) procedure, followed by post hoc and Duncan tests were used for means comparison. The data are presented as a mean with standard error. The significance level was set at $P<0.05$.

RESULTS

Experiment 1: Effect of DMSO concentrations and buck groups on the cryopreservation of rabbit semen

Regardless of the buck groups, post-thaw sperm motility, longevity, acrosome integrity and plasma membrane permeability, the recovery rates of motility and acrosome integrity showed higher significant mean values ($P<0.05$) for extenders containing 0.75 M DMSO than the other two concentrations (1.0 and 1.4 M). Buck groups B and C had significantly higher recovery rates for motility, acrosome, and sperm plasma membrane integrity ($P<0.05$) than the other groups (Tables 1 and 2). There were no significant differences in pre-freeze sperm motility, acrosome integrity, or sperm plasma membrane permeability for the different levels of DMSO. On the other hand, there were significant differences ($P<0.05$) in pre-freeze acrosome integrity and sperm plasma membrane permeability between buck groups (Table 1). The interaction between DMSO concentrations and male groups had a highly significant ($P<0.01$) effect on sperm longevity, but did not affect the other traits. The data obtained showed that post-thaw sperm motility was highly significantly positive correlated with viability index (r=0.58; $P<0.001$) and post-thaw acrosome integrity (r=0.36; $P<0.01$).

Table 1: Influence of dimethyl sulfoxide (DMSO) concentrations in tris extender and rabbit buck individuality on pre-freezing and post-thawing semen parameters (mean±standard error).

DMSO concentrations	Buck groups	Pre-freeze motility	Post-thaw motility	Viability index (VI)	Pre-freeze acrosome integrity	Post-thaw acrosome integrity	Pre-freeze sperm membrane integrity	Post-thaw sperm membrane integrity
0.75 M	A	83.33±1.67	31.67±8.33	54.17±10.83	96.67±0.33	74.33±2.67	74.35±1.71	64.67±2.67
	B	83.33±4.41	45.00±2.89	107.50±11.27	92.00±2.52	76.00±1.53	77.55±2.99	59.87±3.74
	C	80.00±2.89	48.33±3.33	120.83±7.26	95.33±0.88	87.33±1.20	71.88±2.06	56.87±2.21
	D	85.00±2.41	41.67±1.67	110.83±6.51	86.00±6.66	70.33±4.91	77.02±2.89	62.67±1.17
	E	73.33±11.67	31.67±1.67	65.83±3.63	96.00±0.58	78.00±1.00	73.43±0.79	60.67±0.67
	Total	81.00±2.50	39.67^{B}±2.46	91.83^{C}±7.79	93.20±1.61	77.20^{C}±1.82	74.84±1.02	60.95^{B}±1.14
1.0 M	A	83.33±1.67	41.67±3.33	50.83±1.67	92.67±1.61	57.33±1.33	72.08±1.59	61.17±5.59
	B	86.67±1.67	45.00±2.89	55.83±4.64	91.67±1.33	68.00±2.52	74.61±2.06	60.92±3.55
	C	81.67±1.67	45.00±2.89	82.50±6.29	95.00±1.53	70.33±3.18	71.83±1.59	65.67±2.33
	D	83.33±3.33	43.33±4.41	66.67±7.95	85.67±9.84	66.33±3.18	76.22±2.90	63.00±1.50
	E	83.33±1.67	35.00±5.77	55.83±8.33	95.00±0.58	66.33±2.03	71.99±1.50	67.84±1.08
	Total	83.67±0.91	42.00^{B}±1.81	62.33^{B}±3.85	92.00±1.98	65.67^{B}±1.52	73.35±0.89	63.72^{B}±1.42
1.4M	A	81.67±3.33	21.67±3.33	34.17±3.33	90.67±0.67	53.00±1.00	76.51±5.30	53.00±3.00
	B	81.67±1.67	40.00±5.77	48.33±4.41	89.67±2.91	61.67±3.33	73.84±2.57	56.00±6.00
	C	75.00±12.58	38.33±7.26	72.50±2.50	97.33±0.67	65.00±2.89	72.67±1.86	53.66±0.67
	D	83.33±1.67	28.33±4.41	54.17±9.82	92.00±1.15	60.33±0.3	78.33±1.20	67.33±1.33
	E	80.00±2.89	35.00±5.00	52.50±9.46	94.33±0.67	60.00±2.89	71.74±1.75	54.67±2.60
	Total	80.33±2.41	32.67^{A}±2.71	52.33^{A}±4.14	92.80±0.93	60.00^{A}±1.39	74.62±1.28	56.93^{A}±1.88
Overall	A	82.78±1.21	31.67^{a}±4.00	46.39^{a}±4.53	93.33^{ab}±0.99	61.56^{a}±3.38	74.31^{ab}±1.79	59.61^{ab}±2.63
	B	83.89±1.62	43.33^{b}±2.20	70.56^{b}±10.02	91.11^{ab}±1.40	68.56^{b}±2.44	75.33^{ab}±1.40	58.93^{ab}±2.40
	C	78.89±3.89	43.89^{b}±2.86	91.94^{c}±7.90	95.89^{b}±0.65	74.22^{c}±3.60	72.13^{a}±0.93	58.73^{a}±2.03
	D	83.89±1.39	37.78^{ab}±3.02	77.22^{b}±9.52	87.89^{a}±3.60	65.67^{ab}±2.23	77.19^{b}±1.27	64.33^{b}±1.01
	E	78.89±3.80	33.89^{a}±2.32	58.06^{a}±4.29	95.11^{b}±0.39	68.11^{b}±2.84	72.39^{a}±0.75	61.06^{ab}±2.08

Means with capital alphabetical superscripts A, B, C for columns (DMSO concentrations) were significantly different ($P<0.05$).
Means with small alphabetical superscripts a, b and c for columns (Buck groups) were significantly different ($P<0.05$).

Table 2: Influence of dimethyl sulfoxide (DMSO) concentrations in tris extender and male individuality on recovery rates of semen motility, acrosome integrity and sperm membrane integrity (mean±standard error).

DMSO concentrations	Buck groups	Recovery rates		
		Motility	Acrosome integrity	Sperm membrane integrity
0.75M	A	37.62±9.44	76.88±2.50	86.91±1.56
	B	53.94±0.82	82.69±2.13	77.17±3.66
	C	60.56±4.57	91.65±2.09	79.09±1.75
	D	49.13±2.49	81.96±2.15	81.70±4.51
	E	46.86±11.57	81.25±1.14	82.65±1.54
	Total	49.62^{B}±3.36	82.89^{C}±1.51	81.50^{B}±1.41
1.0M	A	49.88±3.06	61.94±2.37	85.28±9.43
	B	51.96±3.53	74.36±4.09	82.00±6.88
	C	55.15±3.77	74.09±3.65	91.38±1.72
	D	52.08±5.51	78.82±6.05	82.77±1.96
	E	41.79±6.27	69.84±2.33	94.25±0.46
	Total	50.17^{B}±2.12	71.81^{B}±2.13	87.14^{C}±2.40
1.4M	A	26.27±314	58.45±0.67	69.39±0.82
	B	49.02±7.28	68.72±2.41	75.47±5.32
	C	51.09±4.19	66.79±2.98	73.91±1.53
	D	34.07±5.38	65.61±1.16	85.97±1.22
	E	43.46±5.14	63.57±2.69	76.21±3.33
	Total	40.78^{A}±3.18	64.63^{A}±1.25	76.19^{A}±1.83
Overall	A	37.92^{a}±4.54	65.75^{a}±3.00	80.53±3.93
	B	51.64^{bc}±2.46	75.26^{bc}±2.52	78.21±2.89
	C	55.60^{c}±2.50	77.51^{c}±3.98	81.46±2.70
	D	45.09^{ab}±3.64	75.46^{bc}±3.13	83.48±1.60
	E	44.04^{ab}±4.15	71.56^{b}±2.80	84.37±2.85

Means with capital alphabetical superscripts A, B, C for columns (DMSO concentrations) were significantly different ($P<0.05$).
Means with small alphabetical superscripts a, b, c for columns (Buck individuality) were significantly different ($P<0.05$).

Experiment 2: Effect of freezing rate and size of semen package on cryopreservation of rabbit semen

Post-thaw semen motility, longevity, and motility recovery were all affected by the freezing rates. Semen that was frozen at 2 and 4 cm above LN had significantly ($P<0.05$) higher motility, longevity and motility recovery values than at other freezing levels, regardless of straw size. The size of the semen package did not affect the parameters studied (Table 3). Furthermore, on the estimated variables, there was no interaction between straw package size and freezing rates.

Experiment 3: Influence of Bovine serum albumin as an antioxidant on semen freezability

BSA did not improve post-thaw semen motility or longevity when added to the cryoprotectant extender (Table 4). However, BSA at concentrations of 5 and 7.5 mg/mL resulted in a significant ($P<0.05$) improvement in sperm membrane permeability and acrosome integrity recovery rates (Table 5).

DISCUSSION

Unlike the sperm of other species, the plasma membrane of rabbit sperm cells has a high cholesterol and lecithin content and a low ratio of polyunsaturated fatty acids (Curry *et al.,* 1995; Castellini *et al.,* 2006). DMSO and acetamide are more compatible for rabbit sperm cryopreservation (Mocé *et al.,* 2010), as they have lower molecular weight and higher permeability (Curry *et al.,* 1995). Sucrose and egg yolk are also required as non-permeable CPAs for rabbit

Table 3: Influence of straw package sizes and freezing level above liquid nitrogen (LN) on buck semen parameters (mean±standard error).

Straws size (mL)	Heights above LN (cm)	Pre-freeze motility	Post-thaw motility	Viability index (VI)	Motility recovery rate
0.25	2	85.00±0.97	45.54±2.15	90.45±7.66	53.74±2.55
	4	85.08±0.63	49.59±1.58	102.09±5.36	58.28±1.80
	6	85.00±0.73	43.77±1.59	82.41±5.21	51.54±1.86
	8	85.65±1.06	41.96±3.22	77.28±9.14	48.83±3.70
	10	86.67±0.94	35.42±1.99	46.46±4.03	40.96±2.46
	Total	85.22±1.29	45.27±3.22	87.51±10.72	53.17±3.76
0.50	2	85.88±0.71	46.03±2.00	90.96±7.41	53.81±2.42
	4	85.52±0.70	46.64±1.85	92.89±5.55	54.58±2.21
	6	83.60±0.61	42.89±1.82	81.45±5.75	51.17±2.11
	8	85.21±0.93	38.33±3.05	64.79±8.14	44.81±3.48
	10	85.00±0.87	36.67±2.25	54.17±5.55	43.18±2.66
	Total	84.92±0.35	43.65±1.00	90.69±5.31	51.38±1.18
Overall	2	85.42±0.61	45.77^{c}±1.46	90.69^{c}±5.31	53.78^{c}±1.75
	4	85.29±0.47	48.15^{c}±1.22	97.61^{c}±3.86	56.47^{c}±1.42
	6	84.27±0.47	43.32^{bc}±1.21	81.91^{bc}±3.88	51.35^{bc}±1.40
	8	85.43±0.69	40.11^{ab}±2.21	70.90^{b}±6.11	46.78^{ab}±2.53
	10	85.83±0.65	36.04^{a}±1.47	50.31^{a}±3.45	42.07^{a}±1.79

Straw size had not effect on the buck semen parameters controles $P<0.05$.
Means with small alphabetical superscripts a, b, c for columns (level above LN) were significantly different ($P<0.05$).

sperm membrane stabilisation during the freezing process (Mocé and Vicente, 2009). In this study, the influence of different concentrations of DMSO (0.75, 1.0 and 1.4 M) as a permeable CPA mixed with a fixed level of sucrose (10 mM) and egg yolk (20%) as non-permeable CPA on rabbit semen freezability was investigated. Unlike the other two concentrations (1.0 and 1.4 M), our findings showed that the low level of DMSO (0.75 M) in the freezing medium had a significant improvement in post-thaw sperm quality. Similarly, Rosato and laffaldano (2013) and Plagne *et al.* (2020) demonstrated, under the same conditions, that a based medium containing 4-5% DMSO was a better biological medium than one containing 10% DMSO for the cryopreservation of rabbit sperm and stem cells. In contrast, other reports have found that semen frozen in high DMSO concentrations had higher quality and viability than those frozen in low concentrations. (Mocé *et al.,* 2005; Di Iorio, 2014). It should be taken into account that this disparity might be attributable to variances in related treatments, as multiple factors and their combinations influence the quality of semen cryopreservation and fertility rate (Si *et al.,* 2006; Sally *et al.,* 2017). Understanding these

Table 4: Influence of freezing medium containing 0.75 M of dimethyl sulfoxide (DMSO) and different concentrations of BSA on pre-freezing and post-thawing semen parameters (mean±standard error).

BSA concentration (mg/mL)	Pre-freeze motility	Post-thaw motility	Viability index (VI)	Pre-freeze Acrosome integrity	Post-thaw Acrosome integrity	Pre-freeze sperm membrane integrity	Post-thaw sperm membrane integrity
0	81.90±1.00	50.95±2.57	98.10±9.33	91.06±0.98	72.06^{a}±1.70	75.24±1.94	53.59^{a}±4.01
2.5	81.09±1.40	47.61±2.91	86.63±10.00	90.63±1.06	77.32^{b}±1.58	73.95±2.12	51.63^{a}±2.76
5.0	81.18±0.91	51.76±3.09	98.82±9.41	91.31±1.08	81.08^{b}±1.18	77.54±1.52	63.92^{b}±1.94
7.5	80.83±1.16	48.33±3.08	86.94±10.16	90.71±1.05	78.86^{b}±1.00	78.57±1.23	59.00^{ab}±2.17
Total	81.27±0.58	49.56±1.44	92.37±4.86	90.90±0.51	77.02±0.83	76.06±0.93	56.33±1.59

Means with different alphabetical superscripts for columns were significantly different ($P<0.05$).

Table 5: Influence of freezing medium containing 0.75 M of dimethyl sulfoxide (DMSO) and different concentrations of bovine serum albumin (BSA) on post-thaw recovery rates of semen motility, acrosome integrity and sperm membrane integrity (mean±standard error).

BSA concentration (mg/mL)	Recovery rates		
	Motility	Acrosome integrity	Sperm membrane integrity
0	62.43±3.16	79.18^{a}±1.81	70.78^{a}±4.94
2.5	58.78±3.26	85.32^{b}±1.52	70.54^{a}±3.76
5.0	64.06±3.78	88.90^{b}±1.43	82.77^{b}±2.79
7.5	60.83±3.88	87.05^{b}±1.36	75.24^{ab}±2.88
Total	61.35±1.73	84.79±0.90	74.17±2.00

Means with different alphabetical superscripts for columns were significantly different (P<0.05).

factors and how they interact is crucial for optimising cryopreservation processes (Khan *et al.,* 2021). It was reported that cryodiluent containing high concentrations of DMSO in the absence of egg yolk improved the quality of rabbit sperm after thawing (Castellini *et al.,* 1992; Rosato and Laffaldano, 2013). Whereas DMSO at low concentrations required a high concentration of egg yolk to have favourable effects on post-thaw semen quality, high amounts did not (Si *et al.,* 2006). This is in line with the current findings, as DMSO at 0.75 M had a better effect than 1.4 M in the presence of the same concentration of egg yolk (20%). It is possible that egg yolk, as a non-permeable cryoprotectant, influenced the intracellular DMSO effect at a certain level, so more research is needed with DMSO alone at different concentrations versus a mixture of DMSO and egg yolk. Furthermore, Di Iorio (2014) found that DMSO concentration and equilibration duration had positive significant interactions on post-thaw sperm motility, viability and sperm plasma membrane permeability. Consequently, the success of rabbit semen cryopreservation is determined by several interdependent components, including extender composition, CPA type and concentration, egg yolk concentrations, cooling, freezing and thawing rates, equilibration time and straw packaging size (Di Iorio, 2014).

The individuality of the bucks' group was the focus of our attention in the study because AI in rabbits is done with pooled semen. Regardless of DMSO concentrations, buck groups B and C had significantly improved mean values for post-thaw semen parameters compared to the other groups. Furthermore, the most interesting aspect of our findings was the highly significant interaction between DMSO concentration and males' group on post-thaw sperm longevity *in vitro*. This indicates that buck individuality works dependently on the appropriate level of DMSO, which differs from one buck to the other. These findings indicate that a genetic component that differs between males is involved, as rabbit ejaculates have shown variability in viability and plasma membrane integrity after freezing and thawing (Kulíková *et al.,* 2017). Different lipids, proteins and other cryoprotectants are found in some males' ejaculate, making the sperm more resistant to freezing/thawing stresses and providing better fertilisation conditions than sperm from males that are not resistant to these stresses (Chen *et al.,* 1989). In the same context, Chen *et al.* (1989) and Mocé *et al.* (2005) found significant differences in fertility, prolificacy and kindling rates between males. Authors attributed these differences to sperm membranes undergoing many stresses during cryopreservation and, according to its potential for resistance to the freezing process, which differs from one male to another, semen was classified as "good" or "bad freezers" (Viudes de Castro *et al.,* 2014). With the exception of sperm longevity, there was no significant interaction between DMSO levels and male groups for the other parameters studied. This is in line with the findings of Viudes de Castro *et al.* (2014), who found that the interactions between rabbit genetic line and freezing extender were non-significant for all traits studied.

It was found in this study that there was no obvious relationship between straw size and post-thaw semen quality. These findings were unexpected and surprising, as the 0.25 mL straw has a higher surface-to-volume ratio than 0.50 mL straw, and as a result, semen containing is faster to respond to ambient temperature changes (cooling, freezing, and thawing). Similarly, straw size did not affect post-thaw semen quality in sheep (Nordstoga *et al.,* 2008), goats (Ritar *et al.,* 1990) or bovine (Johnson *et al.,* 1995). Furthermore, previous rabbit studies have shown that the size of the straw seemed to have no effect on rabbit fertility or prolificacy (Mocé *et al.,* 2010). It is worth mentioning that Johnson *et al.* (1995) and Bezerra *et al.* (2012) have documented the interaction between straw size and thawing rate, as well as their impact on post-thaw semen parameters and fertility. This means that each type of straw would

require a different thawing temperature and, as a result, a different time duration (Mocé *et al.,* 2010). However, we achieved our results using a 40°C thawing temperature for 30-45 s for both straw sizes to avoid overheating, as this thawing protocol is more appropriate under AI field conditions.

In the current study, the effect of different freezing rates on semen quality was designed to optimise rabbit semen cryopreservation. According to our findings, regardless of straw size, differences in freezing rates have a significant impact on post-thaw semen parameters. Semen frozen at level 4 cm above LN has significantly higher values than other freezing levels. This level of freezing is likely to be considered moderate, making it more suitable for rabbit semen cryopreservation. Similarly, Mocé *et al.* (2015) monitored semen quality after thawing and found that samples frozen at medium rates had better fertilising ability and litter size than those frozen at fast and slow rates. Both rapid and slow freezing rates both degrade the quality of rabbit spermatozoa cell membrane (Morris *et al.,* 2012; Whaley *et al.,* 2021), as the rate of ice formation inside the sperm cell increased (Morris *et al.,* 2012; Khan *et al.,* 2021). Moreover, mechanical fragmentation of DNA, enzyme inhibition and lipid peroxidation of sperm membranes were all affected (Morris *et al.,* 2012; Viudes de Castro *et al.,* 2016). These sperm cell destructions have a detrimental effect on post-thaw semen quality, and hence fertilisation ability, kindling rate and litter size (Mocé *et al.,* 2015). The freezing rate and time are balanced in the moderate freezing protocols to achieve a negligible amount of intracellular crystallisation, which diminishes the mechanically adverse effects on spermatozoa.

Spermatozoa are particularly vulnerable to lipid peroxidation during sperm preservation due to a lower content of antioxidant enzymes and a higher content of polyunsaturated fatty acids (Gautier and Aurich, 2021). As a result, maintaining an appropriate reactive oxygen species (ROS) level is essential for the normal physiological process of sperm function (Sharma and Agarwal, 1996). On the other hand, an imbalance between ROS production and antioxidative activity has adverse implications for the sperm plasma membrane, DNA and sperm quality, all of which are linked to fertility (Sharma and Agarwal, 1996; Finelli *et al.,* 2022). Antioxidant agents act as ROS quenchers, reducing and/or eliminating the deleterious effects of excessive ROS production (Nishijima *et al.,* 2021; Mannucci *et al.,* 2022). Due to the limited antioxidant capacity of spermatozoa and seminal plasma (Aurich *et al.,* 1997), several studies have been conducted to improve frozen semen quality in rabbits by adding antioxidants to the diet (Yousef *et al.,* 2003) or to semen extender before cryopreservation (Ortiz *et al.,* 2021).

Because of its negative charge, BSA binds water, salts, fatty acids, vitamins and hormones, transporting them between tissues and cells. Furthermore, the ability of calcium ions to influx through the plasma membrane of sperm cells regulates hyperactivation and acrosome reaction is aided by the beneficial effect of BSA as a supplement in semen extenders (Kwon *et al.,* 2013). Belinskaia *et al.* (2020) recorded that BSA has the ability to bind and eliminate free radicals caused by oxidative stress and protect the membrane integrity of sperm cells, increasing their resistance to the freezing process (Amidi *et al.,* 2016). In our study, BSA was added to the freezing extender at various concentrations as an antioxidant, but it did not affect sperm motility or longevity after thawing. Similarly, Soriano *et al.* (2015) recorded that BSA at concentrations 5, 30, or 60 mg/mL did not improve rabbit semen quality parameters after thawing. However, post-thaw acrosome and sperm plasma membrane integrities were significantly improved in our study at concentrations of 5 and 7.5 mg/mL; on the other hand, the control samples showed a high rate of deformations for these parameters. This means that BSA at these concentrations can adequately protect the acrosome and sperm plasma membrane from ROS. BSA alone, even at appropriate concentrations, does not appear to be sufficient to improve post-thaw semen parameters, but there are other factors at play. Rosato and laffaldano (2013) found that when BSA was combined with sucrose or trehalose in the semen extender, it improved frozen-thawed rabbit sperm motility and DNA integrity. Sucrose is one of the semen extender contents in this study, so the contradictory results reported about BSA usage can be explained and justified.

CONCLUSION

Cryoprotectant extenders containing 0.75 M DMSO and a freezing rate of 4 cm above LN are more appreciated for rabbit semen cryopreservation, with consideration of their interaction between male groups and DMSO concentrations. This means that each male has an appropriate level of DMSO, which may differ from one animal to other. Based on the efficiency of post-thaw semen quality, there is no difference between straw sizes. Consequently, a 0.5 mL straw can be used for semen packaging as it is easier to handle, easier to read and less likely to break during storage.

Supplementation of cryoprotectant extender with 5 or 7.5 mg/mL of BSA enhanced the quality of post-thaw acrosome and sperm plasma membrane integrity.

Future studies: The satisfactory results obtained in this study will need to be confirmed in the field through the application of AI, to find out its effect on the fertility rate and foetal survival, and consequently whether this protocol can be used for commercial purposes.

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