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Factors affecting rabbit sperm cryopreservation: a mini-review

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Review Article

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Summary

Rabbits are an important animal species for meeting the nutritional requirements of the world's growing population due to the high conversion rate of feed. In most countries, the rabbit industry currently relies on artificial insemination with fresh or chilled and frozen–thawed spermatozoa. Various factors during the freezing process, including diluents, sperm preparation and freezing techniques, antioxidants, sudden temperature changes, ice formation and osmotic stress, have been proposed as reasons for the poor sperm quality post thaw. Despite the extensive progress reached in the field of rabbit sperm cryopreservation, new methodological approaches that could overcome problems in sperm cryopreservation are necessary. The aim of this review was to describe the factors that affect the cryopreservation of rabbit sperm.

Introduction

Advances in reproductive science have brought some advantages, such as cryopreservation of sperm and embryos in liquid nitrogen, that allow their stable maintenance for long periods at low cost.

Sperm cryopreservation is considered a secure method for the *ex situ* preservation of biodiversity in different animal species (Zaniboni *et al*., 2014) by facilitating the storage of their gametes in a gene bank. The semen cryobanks provide the opportunity to preserve representative samples and further reconstruct the original strain, population, or diversity (Martínez-Páramo *et al*., 2009).

Due to their high conversion rate of feed, rabbits are an important animal species for meeting the nutritional requirements of the world's growing population. In most countries, the rabbit industry currently relies on artificial insemination (AI) of fresh or chilled spermatozoa stored for no more than 3 days (Roca *et al*., 2000) but more frequently up to 12 h (Di Iorio, 2014). This is because the cryopreservation of rabbit semen has so far shown to result in poor post-thaw sperm quality, leading to low pregnancy rates and litter sizes (Mocé and Vincente, 2009; Mocé *et al*., 2015). At this time, cryopreserved spermatozoa are routinely used in the AI of animals (Kopeika *et al*., 2015). However, despite numerous achievements in sperm cryobiology, the search for methods for successful sperm cryopreservation continues.

In market rabbit production, fresh semen is appropriate for routine AI when stored for a short time, but to prolong storage time frozen semen is required. AI can contribute to efficient rabbit production, because the use of diluted semen from only one male makes it possible to fertilize more than 10 female rabbits at one time. The combination of AI and cryopreservation of rabbit semen can be a useful technique for the maintenance of rabbit breeds.

The susceptibility of spermatozoa of farm animals to cryoinjury varies between several different species, e.g. bulls (Liu *et al*., 1998), mouse (Sztein *et al*., 2000), boar (Waterhouse *et al*., 2006), poultry (Long, 2006) and rabbits (Mocé *et al*., 2003). This different susceptibility is probably due to variations in the reaction to cold shock, although the reasons for such variation are not clear. Bull sperm can be frozen with a certain success (Liu *et al*., 1998), whereas boar (Rodriguez-Martinez and Wallgren, 2010) and ram (Salamon and Maxwell, 1995; Ollero *et al*., 1998) spermatozoa usually undergo extensive cryodamage, despite the development of appropriate protocols.

The species–species differences in membrane lipid composition cannot explain the major differences in post-thaw survival and fertility between species such as bull and boar, or the variation between breeds, and the male-to-male variation within breeds (Holt, 2000). Furthermore, species differences in the female reproductive tract and differences in the number of sperm needed for successful fertilization also determine the fertility of frozen semen.

A widely accepted explanation for the variability between species proposes that sperm membrane properties differ, therefore their permeabilities to water and cryoprotectants also differ: in

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boar – 0.84 μm/min/atm, in rabbit – 0.28 μm/min/atm, and in ram 2.79 μm/min/atm (Curry *et al.*, 2000).

There is a paucity of research into defining a cryopreservation protocol for rabbit spermatozoa that might ensure commercially acceptable pregnancy rates. Despite the extensive progress that has been made in this field, the biological and biochemical mechanisms involved in cryopreservation have not been thoroughly elucidated to date. Various factors during the freezing process, including sudden temperature changes, ice formation and osmotic stress, have been discussed as reasons for poor sperm quality post thaw. The aim of this review was to describe the factors that affect the cryopreservation of rabbit sperm.

Cooling storage of rabbit semen

Rabbit spermatozoa are more sensitive to hypertonic solutions, causing a reduction in storage ability and consequently a decrease in kindling rates (Riad Rowida *et al.*, 2004; Seleem and Riad, 2005). The use of cooled semen for AI depends upon the ability of the extenders to provide a suitable environment for spermatozoa metabolism. Different buffers have been tested to evaluate their ability to preserve chilled rabbit semen during storage (Castellini, 1996).

Cooling storage of semen is used to reduce metabolism and to maintain sperm viability over an extended period of time. During this extended storage period, spermatozoa face the risk of exposure to high levels of reactive oxygen species (ROS) and free radicals due to the relative high content of unsaturated fatty acids in the phospholipids of the sperm membrane.

Successful AI with cooled semen depends on the ability of the extender to provide an optimal environment for sperm storage, refrigeration temperature and spermatozoa concentration. El-Gaafary (1994) using a Tris–egg-yolk extender found that rabbit spermatozoa cooled and stored at 5°C for 24 h had a mean motility of 45% and after 48 h fell to 25%, with a significant decline in fertility. The decrease in the evaluated parameters could have been caused by the reduced temperature at which the sperm was refrigerated during the storage time. Castellini (1996), comparing various temperatures, concluded that a temperature of 15°C is more appropriate than 5°C for rabbit semen storage. Roca *et al.* (2000) evaluated the viability and fertility of rabbit diluted spermatozoa stored at 15°C, and concluded that this may be a suitable temperature.

Puente *et al.* (2018) showed that the Tris–citric acid–glucose (TCG) extender supplemented with ascorbic acid or α-tocopherol is effective in preserving rabbit semen under refrigeration at 15°C for 24 and 48 h.

Di Iorio *et al.* (2014) compared different extender effects (Cortalap® extender with TCG, Lepus® and Merck III®) on the *in vitro* preservability of rabbit spermatozoa stored at 5°C for 72 h. The results showed that Cortalap was the best extender to preserve the quality of rabbit semen *in vitro* over 72 h compared with the other extenders. Then, Cortalap was used for an AI trial for fresh semen. The fertility and prolificacy rates of the does inseminated with chilled semen were significantly lower compared with those inseminated with fresh semen. Although Cortalap proved to be the best extender to preserve semen quality *in vitro* after 72 h at 5°C, the *in vivo* results showed that its use is not recommended in AI programmes. El-Kelawy *et al.* (2012) suggested that cooled rabbit semen preservation does not significantly lower the conception rate, and the litter size at birth and at 21 or 28 days. Pregnancy length was significantly higher for cooled semen (30.8 vs 30.5 days, $P < 0.01$). These results also indicated that most offspring traits (kits weight at birth, 28 days, litter weight gain and pre-weaning mortality) were not affected by using cooled semen.

Cryopreservation of rabbit semen

Sperm cryopreservation, an alternative to cooling, is an effective method able to preserve gene resources and carry sperm between remote destinations. However, due to the issues associated with the use of frozen–thawed sperm (lower fertility and prolificacy than of cooled sperm), AI with cryopreserved rabbit sperm has not been used for commercial purposes (López and Alvariño, 2000; Mocé and Vicente, 2009).

DNA integrity is affected during cell freezing because cryopreservation easily changes mitochondrial membrane properties and increases the production of ROS, which may subsequently result in the oxidation of DNA, causing single- or double-stranded DNA breaks and induce apoptosis, which can lead to a reduction in sperm viability.

Sperm preservation protocols vary among species due to their inherent characteristics (size, morphology, membrane and acrosome composition). Spermatozoa from different species could have different tolerances to cryodamage. Several factors during the freezing process, including sudden temperature changes, ice formation and osmotic stress, have been proposed as the main causes for poor sperm quality after thawing. Regular assessment of frozen–thawed semen may assist in facilitating long-term storage of frozen rabbit semen (Kulíková *et al.*, 2017).

Makarevich *et al.* (2008) analyzed apoptosis occurrence in rabbit sperm using an annexin-V assay in relation to cryopreservation. Sperm samples were arranged into five groups as following: (1) fresh semen immediately after collection; (2) semen incubated in an extender (1:1) for 90 min at room temperature −RT; (3) semen incubated in a cryoprotective medium (0.5 M DMSO + 0.014 M sucrose) at a 1:1 dilution; (4) semen in 500 μl straws, placed into a floating chamber with liquid nitrogen; and (5) semen mixed with a cryoprotective medium and frozen using manual procedure: 90 min at 5°C, 20 min at −6°C, 20 min at −18°C, and plunging into liquid nitrogen. Higher rates of apoptotic cells were observed in both cryopreserved groups (4, 5) when compared with groups 1, 2 and 3 ($P < 0.05$). The highest conception rate of rabbit females was obtained with semen doses from groups 3, 2 or 1 used for the insemination. Conversely, conception rate was minimal when sperm was frozen in a floating chamber (group 4; 16.67%), when using the sperm frozen in a manual regime (group 5) none of 13 inseminated females was parturiated. The authors found a negative linear dependence between the rate of annexin-V^+ cells and female conception rate.

Techniques of cryopreservation

The classic technique for cryopreservation is the slow freezing method using Polge's protocol (Polge *et al.*, 1949). After collection, semen is mixed with freezing solution containing cryoprotectants. Commonly used sperm cryopreservation solutions for slow freezing contain 20% glycerol in hen egg-yolk–citrate buffer (20% of egg yolk). Slow freezing cryopreservation procedure in rabbit have been described in detail also by Mocé and Vicente (2009).

Vitrification is an alternative method used for the storage of spermatozoa without the use of permeable cryoprotectants. Sperm vitrification is described mainly in human sperm vitrification. Using this method, the sperm suspension is plunged directly

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into liquid nitrogen and the sperm cells are cooled in an ultrarapid manner known as kinetic vitrification (Isachenko *et al*., 2003). Rossato and Iaffaldano (2013), using vitrification of the rabbit semen diluted in the absence of permeable CPAs by dropping directly into a liquid nitrogen, obtained low or null cryosurvival of post-vitrified sperm (0.16 ± 0.4%, 1.8 ± 1.6%).

Higher numbers of motile and membrane-intact cells were observed when semen was vitrified with bovine serum albumin (BSA) alone or with BSA and sucrose (0.1 and 0.25 M) or BSA and trehalose (0.25 M), and the best recovery of DNA-intact sperm was recorded for BSA plus sucrose, compared with semen vitrified without osmoprotectants ($P < 0.05$).

In recent years, there has been an increased interest in new preservation techniques that facilitate sperm storage and distribution, freeze drying (FD) has been proposed as an alternative method for sperm preservation and maintenance of genetic resources in different animal species. Freeze drying is a method in which frozen material is dried by sublimation of ice, therefore involving a direct transition from a solid (ice) to a vapour (gas) phase and FD of spermatozoa is a preservation method in which liquid nitrogen is not required. Lyophilized freeze-dried sperm can be kept at 4°C and transported at room temperature (Keskintepe *et al*., 2002; Kusakabe *et al*., 2008; Keskintepe and Eroglu, 2015).

Freeze drying or lyophilization has been used for preserving viruses, bacteria, yeasts and fungi for easy storage and transportation (Leibo and Songsasen, 2002). Recently, FD has been applied to preserve mammalian spermatozoa (Kaneko and Serikawa, 2012a, 2012b) including cryopreservation of rabbit sperm (Liu *et al*., 2004). Liu *et al.* (2004) showed, for the first time, that freeze-dried rabbit spermatozoa retained their chromosomal integrity and were able to initiate embryo development of oocytes after microinjection and proper activation treatments.

For the future, FD technology provides a substantial advantage for biobanking and the maintenance of genetic diversity in laboratory, domestic and wild animal species (Kaneko *et al*., 2014). This method has been proposed to overcome the disadvantages of the current cryopreservation techniques and to achieve the ability to store sperm doses indefinitely at ambient temperature or in ordinary refrigerators.

Cryoprotectants and extenders used for rabbit spermatozoa

Rabbit sperm cells represent a low water permeability coefficient and a high activation energy (Curry *et al*., 1995). This low water permeability value is consistent with the need to use CPAs of lower molecular weight and higher permeability (such as DMSO or amides) compared with glycerol or the egg yolk for rabbit sperm cryopreservation (Curry *et al*., 1995). It was also demonstrated that diluent for the cryopreservation of rabbit sperms needs to be optimized according to concentrations of penetrating and nonpenetrating cryoprotectants included in the diluent (Hall *et al*., 2017).

The egg yolk, as demonstrated already by Pace and Graham (1974), can contain substances able to interfere with cellular respiration and subsequently can lead to the loss of motility. The soybean lecithin is a valuable plant-based source of phospholipids that is included in commercial extenders used for freezing of mammalian sperm. In a recent study, Nishijima *et al.* (2015) concluded that soybean lecithin (1.5%) can be used as a substituent for egg yolk in the semen extenders for freezing of rabbit semen.

Substitution of egg yolk with soybean lecithin may reduce hygienic risks in extenders. Despite the favourable efficiency of the egg yolk in an extender, the use of egg yolk has many disadvantages that are attributed mainly to hygiene and the risk of bacterial contamination (Aires *et al*., 2003; Masoudi *et al*., 2016), which may consequently interfere with semen quality (Ansari *et al*., 2010). The egg-yolk biochemical composition varies depending on the source and then could give variable results. The use of a non-animal origin cryoprotectant instead of egg yolk in semen cryopreservation has been of growing interest in recent years (Fukui *et al*., 2008; Masoudi *et al*., 2016).

Glycerol has not been the first CPA of choice to preserve rabbit semen due to its toxicity, which may result in osmotic stress, protein denaturation, alteration of actin interactions and induction of protein-free membrane blister that leads to worse fertility (Iaffaldano *et al*., 2011, 2012). The negative effects of glycerol may be related to its high molecular weight, which leads to permeation of the cell membrane more slowly than other CPA. Consequently, the ideal CPA should have a low molecular weight and high permeability.

Dimethyl sulfoxide (DMSO) prevents intracellular and extracellular crystals forming in cells during the freezing process. Hall *et al.* (2017) showed that DMSO (7%) in Tris–glucose–glycerol–saccharide (TGGS; combination of 25 mM glucose and 25 mM sucrose) diluent containing 17% egg yolk was the most efficient method for cryopreserving White New Zealand rabbit sperm in terms of post-thaw motility, viability and *in vitro* fertilization of oocytes with frozen–thawed sperm. Viudes-de Castro *et al.* (2014) reported that DMSO, used as a permeable cryoprotectant in combination with sucrose, gave better results for rabbit sperm cryopreservation compared with egg yolk.

Amide or methyl groups have lower molecular weights and greater water solubility than glycerol, being able to reduce osmotic damage (Mocé and Vicente, 2009). An amide solvent that could be used as an alternative to prepare freezing extenders and reduce membrane damage caused during the procedure is *N*,*N*-dimethylformamide (DMF). The efficacy of DMF to cryopreserve rabbit sperm has been studied by Kashiwazaki *et al.* (2006). The authors compared glycerol, lactamide, acetamide, and DMSO as cryoprotectants in egg-yolk diluent of ejaculated Japanese white rabbit spermatozoa for improvement of sperm cryopreservation methods. The rates of sperm plasma membrane integrity in lactamide and acetamide (35.9 ± 3.3% and 30.2 ± 3.0%, respectively) were significantly ($P < 0.05$) higher than in glycerol (17.0 ± 2.6%). These results indicated that 1.0 M lactamide and acetamide had higher cryoprotective effects than 1.0 M glycerol for cryopreservation of Japanese white rabbit spermatozoa. Similarly, Okuda *et al.* (2007) showed that 2% acetamide has a higher cryoprotective effect than 2% glycerol for sperm cryopreservation in the Japanese white rabbit. The authors observed that the rates of forward progressive motility and plasma membrane integrity of the spermatozoa frozen with acetamide (27.1 ± 8.3% and 24.5± 6.5%) were significantly ($P < 0.05$) higher than those of the spermatozoa frozen with glycerol (16.3 ±10.9% and 14.3 ±7.6%). Fadl *et al.* (2019) demonstrated that dilution of rabbit semen in INRA-82 extender supplemented with a combination of DMSO and DMF (4% each) improved sperm quality after freezing–thawing (Table 1). The authors suggested that supplementation of INRA-82 medium with DMSO or DMF alone at higher concentrations (8%) may negatively affect the quality and function of rabbit spermatozoa. Their results indicate that both DMSO and DMF could have a synergistic effect on the characteristics of rabbit semen after freezing and thawing. In the doctorate thesis, Di Iorio (2014) compared the efficiency of different concentrations of the cryoprotectants DMSO (4%, 8%) and

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Table 1 Sperm quality evaluation (%) after freezing–thawing

Breed of rabbit	Freezing medium diluent/cryoprotectant	Survivability after thawing	Reference
New Zealand White	DMRS + 16% DMSO, 4% Ficoll 70 (Minitube)	M 34–44.8%, (CASA, Minitube)	Kulíková *et al.*, 2015
Bianca Italiana breed	TCG + 16% DMSO + 0.1 M sucrose	M ≥ 30% (S)	Di Iorio *et al.* 2020
Bianca Italiana breed	Cortalap + 16% DMSO + 0.1 M sucrose	M ≥ 40% (S)	Di Iorio *et al.*, 2020
Hybrid rabbit of the Centro genetica Martiny	Tris + DMSO 4%	M 32.06%, V 38.89%, I 96.15% (CASA, Sybr 14/PI)	Di Iorio, 2014
Hybrid rabbit of the Centro genetica Martiny	Tris + DMSO 8%	M 42.63%, V 47.09%, A 97.02% (CASA, Sybr 14/PI)	Di Iorio, 2014
Hybrid rabbit of the Centro genetica Martiny	Tris + DMA 8%	M 27.13%, V 28.77%, A 95.323% (CASA, Sybr 14/PI)	Di Iorio, 2014
Hybrid rabbit of the Centro genetica Martiny	Tris + DMA 4%	M 15.19%, V 17.90%, A 95.32.15% (CASA, Sybr 14/PI)	Di Iorio, 2014
Californian–New Zealand White hybrid	BotuCryo + 1% glycerol + 4% methylformamide (Nidacon)	M 52.9% (ISAS, PROISER R+D, Spain)	Domingo *et al.*, 2019
Californian–New Zealand White hybrid	INRA 96® + 6% glycerol (IMV Technologies, France)	M 25.6% (ISAS, PROISER R+D, Spain)	Domingo *et al.*, 2019
Californian–New Zealand White hybrid	INRA 96® + 6% DMF (IMV Technologies, France)	M 35.0% (ISAS, PROISER R+D, Spain)	Domingo *et al.*, 2019
Californian–New Zealand White hybrid	INRA 96® + 6% NMP (IMV Technologies, France)	16.4% (ISAS PROISER R+D, Spain)	Domingo *et al.*, 2019
New Zealand White	INRA-82 + 4% DMSO + 4% DMF	M 42% (S)	Fadl *et al.*, 2019
New Zealand White	INRA-82 + 8% DMSO	M 33% (S)	Fadl *et al.*, 2019
New Zealand White	INRA-82 + 8% DMF	M 24% (S)	Fadl *et al.*, 2019
New Zealand White	TRIS-citric acid + 3 M DMSO + 0.1 M sucrose	M 18.1% (S)	Safaa *et al.*, 2012
New Zealand White	TRIS-citric acid +3 M DMSO + 20% egg yolk	M 11.51% (S)	Safaa *et al.*, 2012
New Zealand White	TRIS-citric acid + 2 M acetamide + 20% egg yolk	M 4.57% (S)	Safaa *et al.*, 2012

A, acrosome integrity; M, motility; S, subjective evaluation; V, viability.

DMA (4%, 8%) diluted in Tris diluent during cryopreservation of hybrid rabbit sperms for the post-thaw motility and viability and showed that 8% DMSO was the most effective (motility after thawing, 42.63%; viability, 47.09%; and acrosome integrity, 92.02%).

The study by Domingo *et al.* (2019) assessed whether different cryoprotective agents (CPA) such as glycerol, *N*,*N*-dimethylformamide (DMF) and *N*-methyl-2-pyrrolidone (NMP) could improve the quality of cooled rabbit sperm stored at 4°C or 16°C. Sperm samples were diluted with INRA 96® (Extender A), INRA 96 with 6% glycerol (Extender B), 6% DMF (Extender C) or 6% NMP (Extender D) and stored at 4°C or 16°C (Table 1). Extender C showed the higher percentage of motility, vitality and HOS test compared with extenders B and D ($P < 0.05$). Sperm quality decreased over time ($P < 0.05$), however, and these data showed that the addition of DMF preserved motility and sperm plasma membrane integrity after 24 h of storage more efficiently compared with other diluents. These results suggested that the addition of DMF to INRA 96 diluent had a protective effect on the membrane of spermatozoa and improving seminal quality. The authors concluded that the differences found between DMF and NMP could be due to their molecular weights and densities. DMF and NMP are both amide solvents, and NMP has a higher molecular weight (99.13 g/mol) and density (1.028 g/cm^3) than DMF (73.09 g/mol of molecular weight; 0.944 g/cm^3 of density). These small differences in their composition might be responsible for that NMP is not a good CPA for storage of cooled rabbit semen.

Polymer Ficoll 70 as a cryoprotectant

The addition of Ficoll 70 as a non-permeable CPA into freezing extender can improve the post-thaw quality of rabbit semen. Iaffaldano *et al.* (2014) demonstrated the efficacy of Ficoll 70 as a second non-permeable CPA, in combination with sucrose, in the semen cryopreservation protocol, obtaining reproductive performances of frozen semen similar to those of fresh semen. The positive effect of the combined use of Ficoll 70 and sucrose, compared with the use of sucrose as an only non-permeable CPA, on the post-thaw semen quality *in vitro*, was described. A significant difference in the proportion of sperm with damaged plasma membrane and exposed acrosome was found between the control and Ficoll groups. Therefore, freezing medium enriched with Ficoll 70 apparently reduced damage

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to plasma membrane caused by freezing and thawing. The author postulated that the effectiveness of Ficoll 70 to protect sperm cells during cryopreservation process probably might be attributed to the affected viscosity of the freezing solution, ensuring a greater stability of the sperm membrane, reducing mechanical strain and ice crystals formation and, therefore, increasing the ability to survive cryopreservation.

Kulíková *et al.* (2015) confirmed the effect of non-permeable cryoprotectant Ficoll 70 and found that its addition to vitrification solution had a positive effect on rabbit sperm parameters (Table 1). In particular, the higher values ($P < 0.05$) of motile and progressively moving spermatozoa immediately after thawing and at 30 min following incubation at 37°C were obtained in the Ficoll group. Moreover, a higher number ($P < 0.05$) of acrosome intact sperm was found in Ficoll compared with the control group. Furthermore, no significant differences in kindling rates and numbers of pups born between frozen/thawed and fresh semen group were found.

To improve current freezing protocols for rabbit sperm, Rossato and Iaffaldano (2013) examined the toxicity of different permeable cryoprotectants (CPAs) used for standard conventional freezing, the feasibility of ultrarapid nonequilibrium freezing (vitrification) of sperm in the absence of permeating CPAs and the addition of BSA, alone or with sucrose or trehalose as osmoprotectants. They concluded that DMSO (10%) was the best cryoprotectant and that enriching the freezing medium with BSA and adequate amounts of sucrose or trehalose could improve the cryosurvival of rabbit sperm after conventional freezing or vitrification. BSA/sucrose was more effective than BSA/trehalose for preserving the *in vivo* fertilization capacity of rabbit sperm cryopreserved using the standard procedure.

El-Sherbieny *et al.* (2012) determined an enhanced freezing ability, efficiency and fertility of rabbit spermatozoa post thaw (positive correlation between each kindling rate or litter size and sperm parameters) after addition of 2% gelatine to the Tris buffer extender.

López-Gatius *et al.* (2005) showed that rabbit spermatozoa were effectively stored in the gelatine-supplemented extender-solid state at 15°C with fertility preserved for up to 5 days.

Recently, Di Iorio *et al.* (2020) reported the use of the commercial extender Cortalap for substitution of TCG in freezing medium for rabbit semen. They showed that Cortalap containing DMSO (16%) and sucrose (0.1 mol/l) significantly improved rabbit sperm motility, viability, membrane functionality and acrosome integrity post-freezing (Table 1).

Tris buffer extenders have been found to be useful in short-term storage (6 h) of chilled rabbit semen (Maertens and Luzi, 1995). Rabbit semen has been successfully stored in a liquid state for short periods of time at 5°C (6 h for fresh and 48 h for refrigerated semen) without serious loss of its fertilizing ability (Daader and Seleem, 1997; Seleem and Riad, 2005; Zeidan *et al.*, 2008). However, experiments to evaluate their use in prolonging sperm viability and fertility over time have been very limited. El-Gaafary (1994), using a Tris–egg-yolk extender, found that spermatozoa cooled and stored at 5°C for 24 h had a motility of 45% and after 48 h it dropped to 25%. This sharp decline in sperm viability throughout the preservation time might be due to the chilling temperature. Roca *et al.* (2000) demonstrated that 15°C can be an adequate temperature to store chilled rabbit semen when Tris buffer extenders are used. The prolonged storage of extended rabbit semen significantly decreased sperm motility, viability and induced acrosomal damages. Safaa *et al.* (2012) showed a moderate and interesting relationship between acrosomal integrity of frozen semen and fertility rate.

El-Kelawy *et al.* (2012) showed that the fructose–yolk–Tris buffer is more effective for rabbit semen storage at 5°C and for maintaining fertilizing ability compared with the glucose–egg-yolk–citrate buffer. Cooled semen preservation does not significantly depress the conception rate and the litter size at birth, at 21 and at 28 days.

Dessouki *et al.* (2016) demonstrated that adding melatonin (10^{-6} M) to rabbit semen extenders enhanced sperm motility characteristics after cooling. This effect strongly suggested an antioxidant protection effect of melatonin against injury after the cooling process. Higher concentrations of melatonin (10^{-3} M) decreased the in-line movement of spermatozoa and increased undesirable parameters such as lateral head displacement.

Effect of antioxidants

The addition of antioxidant substances reduced the effects of cold osmotic shock and stress due to an imbalance between the ROS and antioxidants (Agarwal *et al.*, 2003). Detailing this, Thuwanut *et al.* (2013) evaluated the effect of adding vitamin E, Trolox (a vitamin E analogue), or glutathione peroxidase (GPx) to thawed spermatozoa of the flat-headed cat (*Prionailurus planiceps*) and concluded that the addition of GPx reduced oxidative stress and increased the efficiency of cryopreservation. Puente *et al.* (2018) showed no significant differences in the studied parameters after the addition of ascorbic acid or α–tocopherol to refrigerated rabbit semen. For fertility and prolificacy, there were no significant differences among rabbit females inseminated using semen either with or without antioxidants. However, the total number of rabbit offspring was slightly higher when female insemination was performed in the presence of antioxidants.

Maya-Soriano *et al.* (2015) showed that tested antioxidants at different concentrations (retinol: 50, 100 or 200 μM, or retinyl: 0.282 or 2.82 μg/ml) did not improve rabbit sperm quality after thawing, and that retinyl supplementation appeared to be toxic. A similar observation was reported by Paál *et al.* (2017), who found no protective effect of taurine (1.5, 7, 12.5 or 50 mM) on the viability, morphology and acrosome integrity of short-term stored rabbit spermatozoa.

Zhu *et al.* (2015) suggested that the addition of vitamin E to the freezing extender led to a higher integrity of acrosome, plasma membrane and mitochondrial membrane potential, as well as to higher motility in rabbit sperm. Vitamin E protected spermatozoa by reducing ROS accumulation and lipid peroxidation during preservation. Addition of vitamin E is recommended to facilitate improvement in semen preservation for the rabbit breeding industry.

Interbreed differences in freezability of rabbit semen

The quality of fresh and frozen–thawed semen of four Slovak native rabbit breeds (Nitra, Zobor, Holic Blue and Pastel Rex) was analyzed by Kulíková *et al.* (2017), who showed interbred differences in fresh and frozen–thawed sperm quality traits. Using Ficoll as a non-permeable cryoprotectant, the authors revealed several differences in motility parameters among the rabbit breeds. Fresh sperm from Slovak Pastel Rex rabbits showed the

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lowest ($P \leq 0.05$) motility values compared with the other breeds. In frozen–thawed semen, sperm total motility was similar among Nitra, Zobor and Pastel rabbits, while the Holic rabbit showed higher ($P \leq 0.05$) total motility than the Pastel rabbit. In addition, the Pastel rabbit showed the lowest ($P \leq 0.05$) progressive movement among the breeds. Similarly, for motility analysis, the highest ratio ($P \leq 0.05$) of dead sperm and plasma membrane damage among the fresh sperm samples was found in Pastel semen. Conversely, both Nitra and Pastel rabbits showed lower ($P \leq 0.05$) viabilities for frozen–thawed semen than Holic and Zobor rabbits. This study confirmed the variability in quality parameters measured *in vitro* among four Slovak breeds of rabbit. Therefore, regular semen assessment is required to collect good-quality insemination doses from rabbit breeds.

The combination of an individual and a breed effect builds a strong argument for the view that variation in sperm freezability is, to some extent, genetically determined (Thurston *et al.*, 2002). The identification of genetic differences between individuals that may be linked to cryosurvival provides an opportunity to understand the factors that influence semen cryopreservation, allowing the selective breeding of desired traits.

Conclusion

Sperm cryopreservation is an integral part of the application of reproductive biotechnologies in livestock farming. The results obtained in our experiments contribute to the development of an effective semen cryopreservation protocol that is necessary for the creation of rabbit cryobanks. For short-term storage (up to 24 h) and transport of rabbit semen it is preferable to preserve semen by cooling at 0–5°C, however for long-term storage this technique is not satisfactory, and it is necessary to cryopreserve sperm doses by deep freezing at ultralow temperatures. The most efficient and reliable technique for sperm cryopreservation is vitrification because sperm quality after vitrification is comparable with that of standard slow-frozen and thawed spermatozoa. Moreover, this method is quick and simple, does not require special cryobiological equipment and can, therefore, be easily performed in any less-equipped laboratory. Based on our previous experience, the addition of Ficoll 70 to the freezing medium has a positive effect on the quality of sperm after thawing. Application of the combination of commercially available diluents and cryoprotectants could improve the efficiency of rabbit semen cryopreservation protocols that are necessary to create a rabbit gene bank. Cryopreserved rabbit semen represents the genetic reserve for restoration of breeds and maintenance of rabbit breeding.

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