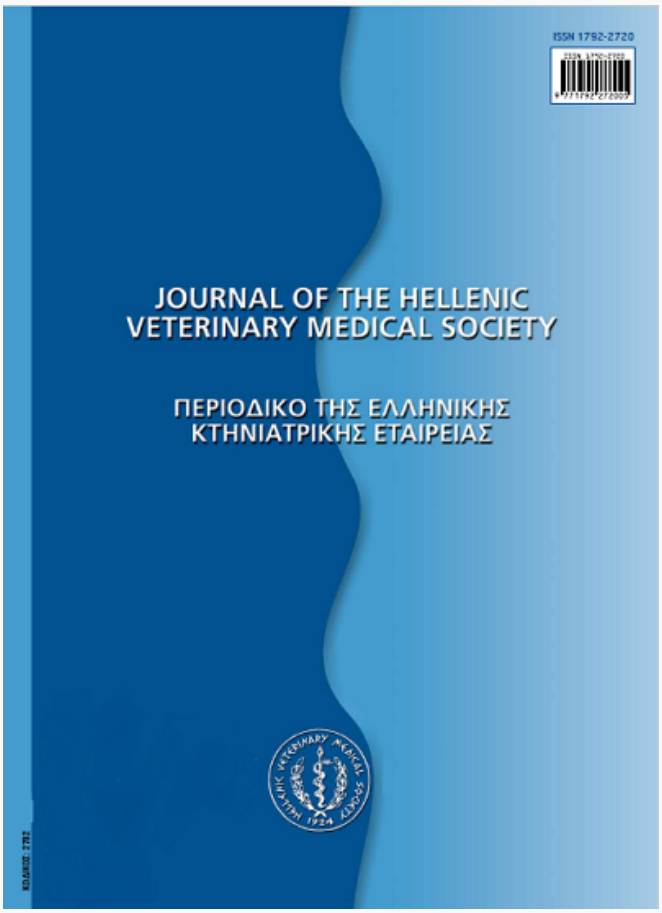
Journal of the Hellenic Veterinary Medical Society

Vol 69, No 2 (2018)



Current status and advances in ram semen cryopreservation

A. NTEMKA, I. A. TSAKMAKIDIS, E. KIOSSIS, A. MILOVANOVIĆ, C. M. BOSCOS

doi: 10.12681/jhvms.18014

J HELLENIC VET MED SOC 2018, 69(2): 911-924
ΠΕΚΕ 2018, 69(2): 911-924

Review article
Ανασκόπηση

Current status and advances in ram semen cryopreservation

A. Ntemka[1], I.A. Tsakmakidis [1], E. Kiossis[1], A. Milovanović[2], C.M. Boscos[1]

[1] *Farm Animals Clinic, School of Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece*

[2] *Scientific Veterinary Institute "Novi Sad", Novi Sad, Republic of Serbia*

Παρούσα κατάσταση και εξελίξεις στην κρυοσυντήρηση του σπέρματος του κριού

Α. Ντέμκα[1], Ι.Α. Τσακμακίδης[1], Ε. Κιόσης[1], A. Milovanović[2], Κ.Μ. Μπόσκος[1]

[1] *Κλινική Παραγωγικών Ζώων, Τμήμα Κτηνιατρικής, Σχολή Επιστημών Υγείας, Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης, Θεσσαλονίκη, Ελλάδα*

[2] *Επιστημονικό Κτηνιατρικό Ινστιτούτο «Νόβι Σαντ», Νόβι Σαντ, Σερβία*

ABSTRACT. Ram semen cryopreservation contributes to genetic improvement through artificial insemination, eliminates geographical barriers in artificial insemination application and supports the preservation of endangered breeds thus the conservation of biodiversity. Sperm freezing process induces ultrastructural, biochemical and functional changes of spermatozoa. Especially, spermatozoa's membranes and chromatin can be damaged, sperm membranes' permeability is increased, hyper oxidation and formation of reactive oxygen species takes place, affecting fertilizing ability and subsequent early embryonic development. Aiming to improve ram frozen-thawed semen's fertilizing capacity, many scientific investigations took place. Among them the composition of semen extenders, was a main point of interest. Semen preservation extenders regulate and support an environment of adequate pH and buffering capacity to protect spermatozoa from osmotic and cryogenic stress. Therefore, permeating (glycerol, dimethyl sulfoxide) and non-permeat-

Corresponding Author:
Ioannis A. Tsakmakidis,
School of Veterinary Medicine, 11 St. Voutyra str., 54627, Thessaloniki, Greece
Email address: iat@vet.auth.gr

Αλληλογραφία:
Ιωάννης Α. Τσακμακίδης
Τμήμα Κτηνιατρικής, Στ. Βουτυρά 11, 54627, Θεσσαλονίκη, Ελλάδα
Ηλεκτρονικό ταχυδρομείο: iat@vet.auth.gr

Date of initial submission: 1-6-2017
Date of revised submission: 17-7-2017
Date of acceptance: 17-7-2017

ing (egg yolk, skimmed milk) cryoprotectants, sugars (glucose, lactose, trehalose, raffinose), salts (sodium citrate, citric acid) and antioxidants (amino acids, vitamins, enzymes) have been added and tested. Moreover, semen dilution rate, storage temperature, cooling rate and thawing protocol, are also some key factors that have been studied. The research results of this scientific topic are encouraging, not only about the freezing and thawing procedures, but also about the improvement of the additives' properties. However, further research is needed to enhance the fertilizing ability of ram frozen-thawed semen, making its use practical in sheep reproductive management by the application of cervical artificial insemination.

Keywords: cryopreservation, ram semen, frozen-thawed, cryoprotectants

ΠΕΡΙΛΗΨΗ. Η κρυοσυντήρηση του σπέρματος του κριού αποσκοπεί στη γενετική βελτίωση μέσω της τεχνητής σπερματέγχυσης, στην άρση των γεωγραφικών περιορισμών, στην προστασία απειλούμενων με εξαφάνιση φυλών και στη διατήρηση της βιοποικιλότητας. Η διαδικασία κατάψυξης όμως, επιφέρει δομικές, βιοχημικές και λειτουργικές μεταβολές στα σπερματοζωάρια. Συγκεκριμένα, βλάπτονται οι κυτταρικές μεμβράνες και αυξάνεται η διαπερατότητά τους, μεταβάλλεται η δομή της χρωματίνης τους και προκαλείται υπεροξείδωση καθώς παράγονται δραστικές μορφές οξυγόνου. Οι αλλαγές αυτές, επηρεάζουν αρνητικά τη γονιμοποιητική ικανότητα των σπερματοζωαρίων του κριού και την πρώιμη εμβρυική ανάπτυξη. Στη βιβλιογραφία εντοπίζονται πολυάριθμες επιστημονικές προσπάθειες βελτίωσης της γονιμότητας του κρυοσυντηρημένου σπέρματος του κριού. Πρωταρχικό και κοινό σημείο διερεύνησης, υπήρξε η σύνθεση των αραιωτικών μέσων. Τα αραιωτικά του σπέρματος πρέπει να έχουν, μεταξύ άλλων, κατάλληλη ρυθμιστική ικανότητα και pH ώστε να προστατεύουν τα σπερματοζωάρια από την οσμωτική και τη θερμική καταπόνηση. Υπό αυτό το πρίσμα, έγινε προσθήκη και μελέτη πληθώρας ουσιών με ποικίλες ιδιότητες και αποτελέσματα. Μεταξύ αυτών, χρησιμοποιήθηκαν κρυοπροστατευτικά που διαπερνούν (γλυκερίνη, διμεθυλο-σουλφοξείδιο) ή όχι (κρόκος αυγού, αποβουτυρωμένο γάλα) την κυτταρική μεμβράνη, σάκχαρα (γλυκόζη, λακτόζη, τρεχαλόζη, ραφινόζη), άλατα (κιτρικό νάτριο, κιτρικό οξύ) και αντιοξειδωτικά (αμινοξέα, βιταμίνες, ένζυμα). Άλλοι παράγοντες που μελετώνται εκτενώς, αφορούν στη θερμοκρασία και στο χρόνο συντήρησης στα επιμέρους στάδια της κατάψυξης, στο ρυθμό πτώσης της θερμοκρασίας και στο πρωτόκολλο αναθέρμανσης. Τα αποτελέσματα των ερευνών είναι ενθαρρυντικά, όχι μόνο ως προς τις διαδικασίες κατάψυξης/αναθέρμανσης του σπέρματος, αλλά και ως προς τις ωφέλιμες ιδιότητες των προσθετικών. Παρά την πρόοδο που επιτεύχθηκε μέχρι σήμερα, εξακολουθούν να υφίστανται κενά, μέχρις ότου επιτευχθεί η διευρυμένη χρήση του κρυοσυντηρημένου σπέρματος του κριού στην αναπαραγωγική διαχείριση των ποιμνίων.

Λέξεις ευρετηρίασης: κρυο-συντήρηση, σπέρμα κριού, ποιοτικά χαρακτηριστικά σπέρματος

INTRODUCTION

Ram semen cryopreservation is of high interest, particularly in European countries, aiming to increase productive parameters by animal genetic improvement in selected flocks. The worldwide use of ram frozen-thawed semen eliminates the geographical barriers, supports the preservation of endangered breeds and conserves the biodiversity (Andrabi and Maxwell, 2007). Additionally, the need for widespread performance of sheep artificial insemination (AI) over extended periods or at different times of the year, stimulated more research on semen cryopreservation scientific topic.

It has been comprehensively reported that sperm freezing process induces ultrastructural, biochemical and functional changes of spermatozoa (Salamon and Maxwell 1995b). Especially, spermatozoa's plasma and acrosome membranes are cryosensitive, so cells' membrane permeability is increased, sperm motility, morphology and chromatin integrity may be affected (Salamon and Maxwell, 2000; Gandini et al., 2006). Moreover, hyper oxidation and formation of reactive oxygen species takes place, damages of mitochondrial sheath and tail axoneme can be detected (Álvarez

and Storey, 1993).

These cryogenic changes have been associated with decreased sperm viability, membranes' functional integrity, and impaired transport and survival of spermatozoa into the female reproductive tract, affecting fertilizing ability and early embryonic development (Salamon and Maxwell 2000). Freezing - thawing processes can induce irreversible damage to ram spermatozoa. According to Medeiros et al. (2002), a relatively high proportion (40-60%) of ram spermatozoa preserve their motility after freeze-thawing, but only about 20-30% remain biologically functional.

Analysis of ram semen provides valuable information about the function of spermatozoa, with a reasonable degree of certainty about the outcome of AI (Tsakmakidis, 2010). However, interacting factors affect the results of AI with frozen-thawed ram semen; such are the technique of insemination, environmental elements and female-related parameters like estrous synchronization method, estrous detection and insemination time (Anel et al., 2005). Although, lower fertility rates are generally accepted owing to cryopreservation effect on ram spermatozoa (O'Meara et al., 2005), laparoscopic insemination of ram frozen-thawed semen is proposed as the selective technique to improve the outcome of AI in small ruminants (Milovanović et al., 2013). In spite of the fact that satisfying success rates are obtained by laparoscopic insemination, it is an expensive operation that has been criticized on welfare grounds (Fair et al., 2005). Additionally, it is not a practical method of fertilization, because it cannot be performed in routine.

Thus, attempts are being developed in cryopreservation methods and techniques with the goal to improve fertility after cervical insemination with frozen-thawed ram semen. This article summarizes and presents the current state of the topic.

BACKROUND AND CURRENT STATUS

The scientific efforts to improve ram frozen-thawed semen quality and fertilizing ability are classified in two major categories: a) modifications and specificities of freezing methods, protocols and semen packaging, b) additives and modification of the extenders' contents.

α) "Modifications and specificities of freezing methods, protocols and semen packaging"

In summary, the common ram semen freezing process includes semen collection, two steps of dilution by supplemented extenders with egg yolk (first step) and glycerol (second step), cooling to a temperature close to 5° C and equilibration for some time to reduce cellular metabolic activity and increase life span of sperm cells. Semen is either packaged in straws of 0.25 and 0.5 ml for freezing and storage, or frozen as pellets on shallow depressions in dry ice (Ritar and Ball, 1993). Straws are placed above liquid nitrogen vapors or into a programmable bio-freezer machine. In the first traditional method, the freezing rate is regulated by the distance between the straws and the level of liquid nitrogen. Thus, it is advised to be placed 4-6 cm above the liquid nitrogen surface, as semen is being cooling according to a parabola-shaped curve (Salomon & Maxwell, 2000).

Many years ago, it was reported that the freezing rate must be slow enough to allow water to leave the cells by osmosis and fast enough to allow extracellular water freezing because intracellular ice formation causes irreversible damages to spermatozoa (Fisher et al., 1986). Additionally, a crucial temperature range (approximately from -5 to -50° C) has been confirmed as the range when ice crystal formation and spermatozoa dehydration take place (Kumar et al., 2003). Therefore, a lot of studies tried to improve ram frozen-thawed semen quality by freezing rate modifications, but controversial results can be found in the literature.

According to Lebouef et al. (2000) rapid cooling of extended semen from 30 to 15° C may not affect sperm survival. However, Watson (2000) reported that the fast cooling from 30 to 10, 5 or 0° C, causes injuries in some sperm cells, called "cold shock". This phenomenon is more pronounced in boar, but it is also occurs in ruminants' spermatozoa. During cooling process, the temperature range of 5–15° C is the most critical for cells' damage, because it is more related to changes in plasma membranes' fatty acid composition and lipid class ratios, whilst the uptake of calcium contributes to similar to acrosome reaction, capacitation changes (Drobnis et al., 1993). Additionally, the equilibration of ram semen at 5° C is necessary to secure freezing efficiency. Purdy et

al. (2006; 2010) reported that ram semen should not be cryopreserved immediately after collection, while when it is held at 5° C for either 48 h or 24 h prior to freezing, no injurious effects on post thaw motility, membrane integrity and fertilizing potential, are induced. These results were similar to those obtained by Câmara et al. (2011) and O'Hara et al. (2010), who demonstrated that the integrity of sperm membrane remained relatively stable for up to 12 or 72 h of equilibration at 5° C, respectively.

In practice, freezing rates from 50 to 100° C/ min are usually selected for ram semen. According to Anel et al. (2003), used rates from -10 to -60° C, should be faster than 50° C/min, but then slower rates (20–30° C/ min) may be applied up to the completion of freezing. In agreement with this indication, Vichas et al. (2017) also found that a slow freezing rate (25° C/ min from -8° C to -130° C) of Chios ram breed semen, provides higher motility and plasma membrane integrity compared to a fast freezing rate (50° C/ min from -8° C to -130° C) after thawing, as well as, after 3 h of incubation at 37° C.

Not only cooling/freezing, but also thawing conditions affect spermatozoa's survival. Thawing rate depends on whether cooling rate was sufficiently high to induce intracellular freezing or sufficiently low to produce cell dehydration. Thawing involves a reversal of these effects, so in the first situation, fast thawing is required to prevent re-crystallization of intracellular ice. During the semen freezing process, intercellular water is the first part of the sample which will be frozen, whilst ice formation under uncontrolled velocity and morphology may damage spermatozoa (Arav et al., 2002). Some efforts were made to avoid that issue. Arav et al. (2002) developed a device (Multi-Thermal-Gradient'-MTG®) to control temperature seeding and morphology of created ice crystals, achieving high rates of post thaw semen motility. Recently, Murawski et al. (2015) used an innovative technology preparing the commercial semen extender Triladyl® (Minitube, Germany) with water declusterized in the low-temperature plasma reactor, called "nanowater". This process led not only to lower crystallization rate of water but also to a substantial improvement in the fertilizing ability of frozen-thawed ram semen and higher conception rates after laparoscopic AI of ewes.

Many researchers studied the effect of thawing in relation to the types of semen packaging, taking different results. Paulenz et al. (2004) indicated that cervical AI using mini tubes resulted in the highest lambing rate, superior to mini straws. Regarding the thawing temperatures, there was a significantly higher lambing rate for 70° C/ 5 sec compared to 35° C/ 12 sec. However, another field study of the same research team didn't reveal significant differences on fertility after vaginal insemination of 719 Norwegian crossbred ewes with different packaging types (mini straws or mini tubes) and thawing protocols (mini tubes were thawed at 70° C for 8 sec and mini straws either at 50° C for 9 sec or at 35° C for 12 sec) (Paulenz et al., 2007). Nordstoga et al. (2009) also reported no significant differences between straw types (medium or mini) and thawing protocols (medium thawed at 35° C for 20 sec or at 70° C for 8 sec, mini straws thawed at 35° C for 15 sec) and neither a significant difference in non-return-to estrus or lambing rate, after vaginal insemination. A dissimilar approach was selected by Awada and Graham (2004), who demonstrated that cryopreservation of ram spermatozoa is more efficient in pellets which were frozen on the cold surface of cattle fat than in straws or pellets frozen on paraffin wax. *(Table 1)*

b) "Additives and modification of the extenders' contents"

Semen cryopreservation requires extension in specific diluents. The extenders must have adequate pH, buffering capacity and suitable osmolality to protect spermatozoa from cryogenic injury, by reducing the physical and chemical stress that induce both freezing and thawing processes (Purdy, 2006). Sperm cryopreservation extenders contain a penetrating cryoprotectant (glycerol, ethylene or propylene glycol, dimethyl sulfoxide), a buffer (Tris or TES titrated with Tris), one or more saccharides (glucose, lactose, raffinose, saccharose, or trehalose), salts (sodium citrate, citric acid) and antibiotics (penicillin, streptomycin). Penetrating cryoprotectants cause membrane lipid and protein rearrangement, resulting in increased membrane fluidity, greater dehydration at lower temperatures, reduced intracellular ice formation, and increased cryosurvival (Holt, 2000). On the other hand, a non-permeating cryoprotectant acts

Table 1: Modifications and specificities of freezing methods, protocols and semen packaging

Methods	Results	Publication
Rapid cooling from 30 to 15° C	Sperm survival may not be affected	Lebouef et al., 2000
Semen held at 5° C for either 48 h or 24 h prior to freezing	No effects on motility, membrane integrity, fertilizing potential	Purdy et al., 2006; 2010
12 or 72 h of equilibration at 5° C	Stable membrane integrity	-Câmara et al., 2011 -O'Hara et al., 2010
-From -10 to -60° C: faster rates than 50° C/ min -From -60 to -196° C: slower rates (20–30° C/ min) -Two-step glycerol addition	Kinetic parameters Conception and lambing rate	Anel et al., 2003
Control of temperature seeding and morphology of created ice crystals	Motility	Arav et al., 2002
Preparation of the extender Triladyl® with "nanowater"	Water crystallization rate Fertilizing ability Conception rates	Murawski et al., 2015
-Types of semen packaging -Thawing temperatures	-Mini tubes: the highest lambing rate -70° C/ 5 s: the higher lambing rate	Paulenz et al., 2004
-Semen frozen in pellets on the cold surface of cattle fat or on paraffin wax -Frozen in straws on paraffin wax	More efficient cryopreservation: pellets on the cold surface of cattle fat	Awada and Graham, 2004

extracellularly without crossing of plasma membrane (Aisen et al., 2000). Therefore, this may act as a solute, reducing the freezing temperature of the medium and decreasing the extracellular ice formation.

1. *Research concerning "egg yolk", properties and alternatives*

Egg yolk is the most common component of Tris-based used diluents for semen cryopreservation. It is of animal origin and protects spermatozoa from cold shock (Holt et al., 1992). The constituents that protect the sperm membranes' phospholipids integrity during cryopreservation are the low density lipoproteins (LDL) of egg yolk (Moussa et al., 2002). The LDL adhere to the surface of sperm plasma membrane, reinstate the phospholipids and prevent the damage of the membrane (Bispo et al., 2011). Many researchers studied the role of different species and concentrations of egg yolk, aiming to improve its benefits to ram semen cryopreservation.

A recent alternative investigation (Alcay et al., 2015) indicated that a modified extender containing lyophilized egg yolk provided similar cryoprotective effects with the fresh egg yolk extender. Additionally, Moustacas et al. (2011), who evaluated the suitability of using natural or lyophilized low density lipoproteins, concluded that in terms of post-thaw kinetics, total and progressive sperm motility, natural but not lyophilized LDL were appropriate for cryopreservation of ram semen. A different approach was followed by Del Valle et al. (2013), who used biologically safer components (casein, palm or coconut oil) as alternatives to egg yolk in ram freez-

ing process, but none of them was superior to egg yolk. Interesting information were provided from the study of Kulaksiz et al. (2010), who aimed to determine the effect of different egg yolk species, namely the domestic chicken (Gallus gallus domesticus), goose (Anatidae anser), turkey (Meleagris gallopavo), duck (Anatidae anas platyrhynchos), Japanese quail (Coturnix japonica) and chucker (Alectoris chukar), on ram semen cryopreserved by a Tris-citric acid-glucose extender containing 15% avian egg yolk and 5% glycerol. The most important finding was that ram frozen semen with 15% chucker egg yolk recorded higher sperm quality compared to the other tested egg yolks. Moreover, Gil et al. (2003) recommended that egg yolk concentration not higher than 5-10% supports frozen-thawed ram semen characteristics when milk-based extenders are used, whereas Bioexcell®, a free of animal origin additives extender (IMV, L' Aigle, France) which contains 6.4% glycerol, may be alternative to the conventional milk extender that contains 5% egg yolk. Taking into account that soybean lecithin is safer than egg yolk as the risk of microbial contamination is significantly lower (Bousseau et al., 1998) and not any cytotoxic effect of it has been reported (Fiume, 2001), it is widely used as an alternative of egg yolk in ram semen diluents. An improvement of sperm motility, viability and membrane integrity has been referred in soybean concentrations between 1-1.5% (Emamverdi et al., 2013; Forouzanfar et al., 2010). Supporting this theory, Masoudi's et al. (2016) showed that 1% soybean lecithin extenders have similar effects on post-thawed sperm quality with 20% egg yolk extenders. Notwithstanding the abovementioned positive properties of soybean lecithin, other surveys have noticed some detrimental effects of higher soy lecithin concentrations on post thaw ram semen quality (Mata-Campuzano et al., 2015; Valle et al., 2012). Although it is effective in protection of basic sperm quality characteristics against freezing-induced damage, high concentration of it (3.5%) induces loss of mitochondrial membrane potential without any significant reflection in motility (Valle et al., 2012). Mitochondrial membrane potential is a parameter that has been considered as a good indicator of sperm functionality which affects fertilizing capacity. In agreement with the aforementioned study, Mata-Campuzano et al. (2015) found a decrease of mitochondrial activity by soy lecithin extender at concentration of 3.5%. However, this study stated a protective efficiency of soy lecithin on sperm chromatin integrity, but it was attributed to its greater antioxidant, not cryo-protective, effect compared to egg yolk. Independently of the controversial results, the beneficial effects of soybean lecithin have been utilized in semen extenders' industry. Two commercial soybean lecithin-based extenders, AndroMed (Minitub, Tiefenbach, Germany) and BioXcell (IMV Technologies, L' Aigle, France), were evaluated in a field trial by Khalifa et al. (2013). Two hundred six ewes were laparoscopically inseminated with frozen-thawed ram semen and the authors reported that BioXcell is superior to AndroMed in preserving the fertilizing potential. Nonetheless, other studies did not demonstrate any significant variation among pregnancy rates of ewes inseminated with semen stored in AndroMed, milk, and egg yolk extenders (Fukui et al., 2008). Similarly, there was no difference in pregnancy rates after intracervical AI of ewes with frozen-thawed semen in milk-egg yolk and BioXcell extenders (Gil et al., 2003).

1) Research concerning "glycerol" concentration and addition methodology

Glycerol is one of the most widely used cryoprotectants. It is able to permeate the sperm cell, induce osmosis and prevent ice crystals formation. It can be added to semen during a two-step dilution in the second phase, or in one step as an essential component of the appropriate extender (Salamon and Maxwell, 1995a).

An acceptable concentration of glycerol in ram frozen semen aliquots has been established. Alvarez et al. (2012) stated that glycerol concentrations higher than 8% have great toxic effect on spermatozoa cryosurvival, while higher values of motility and viability were obtained at glycerol concentrations 4 or 6%. These results are in agreement with the study of Sönmez and Demirci (2004) who noted that the highest percentage of motile spermatozoa was determined in ram semen diluted with Tris-glucose-egg yolk based extender, containing 5% glycerol (testing levels between 3% - 7%). On the other hand, Forouzanfar et al. (2010) evaluated quality and *in*

vitro fertility of frozen-thawed ram sperm diluted in a Tris-based extender consisted of egg yolk or soybean lecithin and 5% or 7% glycerol. Sperm viability, motility and cleavage rate were higher for 7% glycerol group.

Moreover, many studies dealt with the time and temperature of glycerol addition, as well as, with the one or two steps of semen dilution process. Pelufo et al. (2015) demonstrated that the best procedure for ram semen cryopreservation in hypertonic disaccharide-containing diluents is the addition of glycerol after the cooling process, at 5° C, than before it at 30° C. Differences between 30° C and 5° C for spermatozoa quality were noticed just for HOST (hypo-osmotic swelling test), as plasma membrane showed higher sensitivity to glycerol addition at 30° C. Moreover, Anel et al. (2003) suggested that freezing of ram semen in a test-fructose-egg yolk medium containing 4% glycerol in a two-step dilution (to 2% at 35° C and to 4% at 5° C) along with a programmable biofreezer (-20° C/min), results in good field fertility rate. Furthermore, according to Gil et al. (2003) the addition of glycerol in a traditional milk extender supplemented with 5% egg yolk at 15° C does not present any improvement in thawed semen quality compared to 5° C. In contrast with the two steps dilution protocol studies, Morrier et al. (2002) reported that the addition of glycerol either immediately after collection (one-step) or at 5° C (two-steps) does not affect motility and viability of frozen- thawed ram sperm.

Finally, taking into account the osmolality of a Tris-based extender containing 6% glycerol in a two-step dilution procedure, Soylu et al. (2007) concluded that glycerol based extenders with a high osmotic pressure (400 mOsm) was a better choice for ram semen cryopreservation, after testing post-thawed motility, acrosomal, morphological, and membrane integrity.

2) Research concerning "sugars" effects and properties

Sugars are non-permeating cryoprotectants that raise sperm cells dehydration by increasing the tonicity of the extender, interacting with phospholipid bilayers and contributing to plasma membrane stabilization against intracellular ice crystallization (Molinia et al., 1994). Among the most commonly supplemented sugars, trehalose (disaccharide) is supposed to be the most effective, whereas raffinose (trisaccharide) and sucrose (disaccharide) are also widely used.

It has been strongly supported that the addition of trehalose to semen extenders before freezing, improves ram semen cryopreservation. Najafi et al. (2013) demonstrated that in a soybean lecithin-based extender, the combination of 5% glycerol and 100 mM trehalose resulted in higher post-thawing sperm quality (motility, viability, morphology, acrosome and plasma membrane integrity) than other combinations, or using glycerol alone, indicating a synergistic effect. These findings agree with Jafaroghli et al. (2011), who obtained the highest post-thawing semen quality by the combination of 5% glycerol with 100 mM trehalose in a Tris-citric acid-fructose-yolk extender. Similar findings were provided by Tonieto et al. (2010) by the supplementation of Tris-egg yolk-glycerol extender with100 mM trehalose and 8% LDL, while Matsuoka et al. (2006) noticed that the addition of trehalose to an egg-yolk based ram semen diluent containing fructose, improves post-thaw sperm motility and viability. Although trehalose is the most investigated sugar additive in semen extenders, other sugars such as sucrose, raffinose and hypotaurine also resulted in higher ram semen cryoprotection (Bucak et al., 2013; Jafaroghli et al., 2011). Furthermore, a beneficial role of cyclodextrins, a family of cyclic oligosaccharides with a hydrophilic outer surface and a lipophilic central cavity, on semen has been found by Mocé et al. (2010). Specifically, they demonstrated that treating ram semen with cyclodextrins pre-loaded with cholesterol prior to cryopreservation, promotes sperm cryosurvival, since post thawed spermatozoa remain longer motile, showing increased binding capacity to zona pellucida.

3) Research concerning "seminal plasma" concentration and addition methodology

The role of seminal plasma in spermatozoa fertilization capacity is important and well known. It is produced by prostatic, epididymal and vesicular glands and provides sperm maturation, survival and fertilizing ability. It contains high concentration of

certain proteins that maintain the stability of the spermatozoa plasma membrane until capacitation begins in the female reproductive tract and also prevent cold-shock damage (Pérez-Pé et al., 2001). Considering the properties of seminal plasma, some researchers involved it in semen freezing or thawing process, with questionable results. Despite that some studies have shown an improvement of semen quality and fertility rate of ewes after cervical insemination with frozen-thawed sperm supplemented with seminal plasma (El-Rajj Ghaoui et al., 2007; Maxwell et al., 1999; Rebolledo et al., 2007; Leahy et al., 2010), some other indicated that post-thaw ram semen incubated in seminal plasma had no identifiable beneficial effect on sperm quality (de Graaf et al., 2007; Dominguez et al., 2008; Rovegno et al., 2013). Controversial results may be explained by seasonality effects on seminal plasma patterns, in which the complex of protein content and composition changes among seasons (Dominguez et al., 2008). *(Table 2)*

3) Research concerning "antioxidants" addition

Spermatozoa contain a high ratio of polyunsaturated fatty acid (PUFA) and low cholesterol to phospholipids ratio which makes them sensitive to excessive production of reactive oxygen species (ROS) with subsequent lipid peroxidation (Holt and North, 1985). Spermatozoa regulate and resist to this phenomenon by an antioxidant protective system with the cooperation of seminal plasma, membranes, and cytoplasm, including superoxide dismutase, glutathione peroxidase and catalase. However, this system is partly removed and severely altered during cryopreservation procedures (Marti et al., 2008; Forouzanfar et al., 2013). A variety of biological and chemical antioxidants are presently under investigation, aiming to avoid the detrimental effects of oxidative stress on semen quality.

i) Enzymatic Antioxidants.

Enzymatic antioxidants are also known as natural antioxidants. They prevent cellular damage by the neutralization of ROS excessive production. Enzymatic antioxidants are composed of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR) (Bansal and Bilaspuri, 2010). Two antioxidant agents that mimic superoxide dismutase (Tempo and Tempol) displayed protective effect on sperm DNA fragmentation, after their addition on ram semen extender during the cooling process (Mata-Campuzano et al., 2012; Santiani et al., 2014). Additionally, it has been shown that 150 μM of a cell-permeable superoxide dismutase mimetic and peroxynitrite scavenger (MnTBAP), reduced the oxidative stress provoked by cryopreservation, leading to higher sperm motility and improved acrosomal membrane integrity (Forouzanfar et al., 2013). Moreover, Maia et al. (2006; 2009) reported a valuable impact of catalase on frozen-thawed ram spermatozoa's motility and stability of both acrosome and plasma membranes. On the other hand, some researchers demonstrated that the supplementation of a Tris-egg yolk extender with catalase does not influence the total antioxidant capacity of semen, nor it enhances post thaw ram semen quality (Camara et al., 2011; Sicherle et al., 2011).

ii) Non-enzymatic Antioxidants

Non-enzymatic antioxidants are also known as synthetic antioxidants or dietary supplements. Vitamins, minerals, fatty and amino acids are included in non-enzymatic dietary antioxidants.

Previous studies investigated the effect of supplementation of rams' diet with fatty acids. Taking into account their results, fish oil significantly improves frozen-thawed ram sperm motility, viability, membrane integrity and fertilizing ability, while palm and sunflower oil don't enhance or negatively affect semen characteristics (Esmaeili et al., 2014; Masoudi et al., 2016). Low-molecular weight amino acids were widely used as additives in ram semen extenders. Among them methionine, cysteamine and cysteine were extensively studied and were indicated as supplements that can reduce malondialdehyde's levels and improve post-thaw ram semen parameters (Bucak et al., 2007; 2008; Najafi et al., 2014; Sharafi et al., 2015; Toker et al., 2016). However, there are relative to the aforementioned amino acids studies, which report negative effects of cysteamine on semen motility, mitochondrial activity and DNA integrity (Cirit et al., 2013; Mata-Campuzano et al.,

Table 2: Additives and modification of the extenders' contents

Methods	Results	Publications
Lyophilized or fresh egg yolk	Similar cryoprotective effects	Alcay et al., 2015
Natural or lyophilized low density lipoproteins	Total and progressive motility	Moustacas et al., 2011
Casein, palm oil, coconut oil or egg yolk	Egg yolk is superior	Del Valle et al., 2013
Different species' egg yolk	Sperm quality by 15% chucker egg yolk	Kulaksiz et al., 2010
-Egg yolk concentration -Bioexcell ® or egg yolk	-Optimal concentration: 5-10% -Bioexcell ® may be an alternative	Gil et al., 2003
Soybean concentrations between 1-1.5%	Motility, viability, membrane integrity	-Emamverdi et al., 2013 -Forouzanfar et al., 2010
Soybean lecithin or egg yolk	1% soybean lecithin similar with 20% egg yolk extenders	Masoudi et al., 2016
Soybean lecithin concentration	-Soy lecithin > 1% : semen quality -Soy lecithin > 3.5% : no significant effect on motility -Soy lecithin 3.5% : mitochondrial activity	-Valle et al., 2012 -Mata-Campuzano et al., 2015
BioXcell or AndroMed	BioXcell > AndroMed	Khalifa et al., 2013
-Fertility of semen stored in AndroMed, milk, or egg yolk extenders -Fertility of semen stored in BioXcell or milk-egg yolk extenders	No significant difference	-Fukui et al., 2008 -Gil et al., 2003
Glycerol concentration	-8%: toxic effect on spermatozoa cryosurvival -4 or 6%: motility and viability -5%: motility -7%: viability, motility, cleavage rate	-Sönmez and Demirci, 2004 -Forouzanfar et al., 2010 -Alvarez et al., 2012
Temperature of glycerol addition	-Best results: at 5° C -At 30° C: plasma membrane sensitivity	Pelufo et al., 2015
Temperature of glycerol addition	No improvement in semen quality at 15° C, compared to 5° C	Gil et al., 2003
One-step or at 5° C two-steps glycerol addition	No effect on motility and viability	Morrier et al., 2002
4% glycerol in a two-step dilution (to 2% at 35° C and to 4% at 5° C)	Good field fertility rate	Anel et al., 2003
Osmolality of glycerol based extenders in a two-step dilution	High osmotic pressure (400 mOsm)	Soylu et al., 2007
Glycerol alone or in combination with trehalose	5% glycerol and 100 mM trehalose: post-thawing sperm quality	Najafi et al., 2013
-100 mM trehalose with 5% glycerol -100 mM trehalose and 8% LDL	High post-thawing semen quality	-Jafaroghli et al., 2011 -Tonieto et al., 2010
Trehalose in combination with fructose	Motility and viability	Matsuoka et al., 2006
Trehalose, sucrose, raffinose, hypotaurine	High ram semen cryo-protection	-Bucak et al., 2013 -Jafaroghli et al., 2011
Cyclodextrins pre-loaded with cholesterol	Binding capacity to zona pellucida	Mocé et al., 2010
Seminal plasma	-Semen quality and fertility rate -No beneficial effect on sperm quality	-El-Rajj Ghaoui et al., 2007 -Maxwell et al., 1999 -Rebolledo et al., 2007 -Leahy et al., 2010 -de Graaf et al., 2007 -Dominguez et al., 2008 -Rovegno et al., 2013

2015), or no beneficial effect of cysteine to semen cryopreservation (Coyan et al., 2011). Furthermore, Sangeeta et al. (2015) demonstrated that the addition of 20 mM l-glutamine and 25 mM l-proline in a Tris-egg yolk-glycerol diluent, reduced lipid peroxidation and significantly improved sperm motility, viability and acrosome integrity. In contrast, supplementation of l-alanine in freezing extender resulted in lower cryoprotection, observed by a decrease of sperm motility and an increase of the immotile and non-progressive spermatozoa percentage. Moreover, Najafi et al. (2014) showed that when the freezing soybean lecithin extender was supplemented by 6 mM ergothioneine, motility, viability and membrane functionality were increased, while lipid peroxidation was decreased. However, Çoyan et al. (2011) found that ergothioneine, despite the satisfying motility results, reduces membrane integrity and mitochondrial activity. Concerning hypotaurine and taurine, their addition in Tris-based extenders enhances post thawed ram semen quality parameters (Bucak et al., 2007; 2013).

Regarding vitamins' antioxidant effects on ram frozen-thawed semen, it has been proved that when Tris extender supplemented with vitamin $B_{12,}$ viability and motility are advanced, while the morphological defects are decreased (Asadpour et al., 2012; Hamedani et al., 2013). Likewise, Mata-Campuzano et al. (2015) indicated that the supplementation of Trolox, a water-soluble vitamin E analog, in a TES-Tris-fructose extender reduces lipid peroxidation after thawing of frozen ram semen. Silva et al. (2013) reinforced the previous finding, reporting that Trolox addition to Tris-egg yolk extender at 60 and 120μM provides higher plasma membrane and mitochondria integrity, as well as, better kinematics for ram cryopreserved spermatozoa. These results confirm also Maia's et al. (2007; 2009) observations that Trolox addition at 50-100 μM to freezing extender resulted in increased semen motility. In contrast, Sicherle et al. (2011) noted no effect on any ram sperm kinematics and no sperm protection from spontaneous production of ROS after post thaw addition of 100 μM Trolox.

Furthermore, a big group of substances with antioxidant capacity are based on plant origin. Mata-Campuzano et al. (2015) indicated that crocin, a carotenoid extracted from saffron (Crocus sativus), could be beneficial for ram semen freezing, because it protects sperm DNA. Moreover, Uysal and Bucak (2007) observed that the addition of lycopene, a natural carotenoid which occurs in certain fruits and vegetables, in a Tris-based extender exhibited high protection of all tested ram semen parameters after thawing. Similar results were obtained by the supplementation of soybean lecithin extender with 4 or 6% rosemary aqueous extract (Motlagh et al., 2014).

5) *Substances with different main purposes of use, as antioxidant agents*

Cirit et al. (2013) examined the cryosurvival of electro-ejaculated ram spermatozoa in the presence of iodixanol, a drug which contains iodine a substance that absorbs x-rays. Supplementation of 5% iodixanol was optimal to increase total and progressive motility and decrease acrosomal damage and total morphological abnormalities of frozen-thawed ram semen. Moreover, many studies investigated the role of insulin-like growth factor-I (IGF-I) in a variety of reproductive purposes. It is a polypeptide hormone, which is involved in follicular development, fetal growth and spermatogenesis in ruminants (Kumar and Laxmi, 2015). Padilha et al. (2012) demonstrated that IGF-I improves sperm motility and membranes' structural integrity after its addition in Tris-extender, but this positive effect didn't reflect on the pregnancy rate after laparoscopic insemination of ewes with frozen-thawed semen. Finally, it has been reported that bovine serum albumin (BSA), usually used as a protein source in media, eliminates free radicals and protects sperm acrosome and plasma membranes' integrity from heat shock during freezing-thawing procedures (Uysal and Bucak 2007).

(Table 3)

CONCLUSIONS

The majority of researches regarding frozen-thawed ram semen aim to improve spermatozoa's quality characteristics. On the other hand, these ones that have practically evaluated their results through artificial insemination are fewer. Nonetheless, there are seasonal, management, hygienic and individual factors, such as rams' and ewes' breed, age and inseminator's expertise, that

affect fertility results. As a consequence, although a progress on ram frozen-thawed semen has been achieved, there is a need for further research to enhance fertility results through cervical insemination of cryopreserved ram semen.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

Table 3: Addition of enzymatic and non-enzymatic antioxidants

Antioxidants	Results	Publications
Tempo and Tempol	Protective effect on sperm DNA fragmentation	-Mata-Campuzano et al., 2012 -Santiani et al., 2014
MnTBAP	Motility Acrosomal membrane integrity	Forouzanfar et al., 2013
Catalase	Motility Acrosomal membrane integrity No effect on semen quality	-Maia et al., 2006, 2009 -Camara et al., 2011 -Sicherle et al., 2011
Fish oil, palm oil, sunflower oil	Motility, viability, membrane integrity, fertilizing ability No effect on semen characteristics	-Esmaeili et al., 2014 -Masoudi et al., 2016
Methionine	Improvement of semen parameters	Toker et al., 2016
Cysteamine	Improvement of semen parameters Motility, mitochondrial activity, DNA integrity	-Bucak et al., 2007 -Najafi et al., 2014 -Toker et al., 2016 -Cirit et al., 2013 -Mata-Campuzano et al., 2015
Cysteine	Improvement of semen parameters or no beneficial effect	-Bucak et al., 2008 -Sharafi et al., 2015 -Toker et al., 2016 -Coyan et al., 2011
-l-Glutamine -l-Proline -l-Alanine	Motility, viability, acrosome integrity Immotile and non-progressive spermatozoa	Sangeeta et al., 2015
Ergothioneine	Motility, viability, membrane functionality Lipid peroxidation, membrane integrity and mitochondrial activity	-Najafi et al., 2014 -Çoyan et al., 2011
Hypotaurine and taurine	Semen quality parameters	Bucak et al., 2007; 2013
Vitamin B_{12}	Viability, motility, morphological integrity	-Asadpour et al., 2012 -Hamedani et al., 2013
Trolox	Lipid peroxidation Kinematics, plasma membrane and mitochondria integrity motility	-Mata-Campuzano et al., 2015 -Silva et al., 2013 -Maia's et al., 2007, 2009
Trolox (post-thaw addition)	-No effect on sperm kinematics -No protection from ROS production	Sicherle et al., 2011
Crocin	Sperm DNA protection	Mata-Campuzano et al., 2015
-Rosemary aqueous extract -Lycopene	High protection of all tested semen parameters	-Uysal and Bucak, 2007 -Motlagh et al., 2014

REFERENCES

Aisen EG, Alvarez HL, Venturino A, Garde JJ (2000) Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. Theriogenology 53: 1053-1061.

Alcay S, Toker MB, Gokce E, Ustuner B, Onder NT, Sagirkaya H, Nur Z, Soylu MK (2015) Successful ram semen cryopreservation with lyophilized egg yolk-based extender. Cryobiology 71: 329-333.

Álvarez JG, Storey BT (1993) Evidence that membrane stress contributes more than lipid peroxydation to sublethal cryodamage in cryopreserved human sperm: glycerol and other polyols as sole cryoprotectant. J Androl 14: 199-209.

Álvarez M, Tamayo-Canula J, Martínez-Rodrígueza C, López-Uruena E, Gomes-Alves S, Anel L, Martínez-Pastora F, de Paz P (2012) Specificity of the extender used for freezing ram sperm depends of the spermatozoa source (ejaculate, electroejaculate or epididymis). Anim Reprod Sci 132: 145-154.

Andrabi SMH & Maxwell WMC (2007) A review on reproductive biotechnologies for conservation of endangered mammalian species. Anim Reprod Sci, 99: 223-243.

Anel L, de Paz P, Alvarez M, Chamorro CA, Boixo JC, Manso A, Gonzalez M, Kaabi M, Anel E (2003) Field and in vitro assay of three methods for freezing ram semen. Theriogenology 60: 1293-1308.

Anel L, Kaabi M, Abroug B, Alvarez M, Anel E, Boixo JC, de la Fuente LF, de Paz P (2005) Factors influencing the success of vaginal and laparoscopic artificial insemination in churra ewes: a field assay. Theriogenology 63: 1235-1247.

Arav A, Yavin S, Zeron Y, Natan D, Dekel I, Gacitua H (2002) New trends in gamete's cryopreservation. Mol Cell Endocrin 187: 77-81.

Asadpour RM, Pourseif M, Moghadam G, Jafari R, Tayefi H, Mahmodi H (2012) Effect of vitamin B12 addition to extenders on some physico-chemical parameters of semen in crossbred rams. Afr J Biotechnol 11: 11741-11745.

Awada MM, Graham JK (2004) A new pellet technique for cryopreserving ram and bull spermatozoa using the cold surface of cattle fat. Anim Reprod Sci 84: 83-92.

Bansal AK & Bilaspuri GS (2010) Impacts of oxidative stress and antioxidants on semen functions. Vet Med Int 2011.

Baspinar N, Çoyan K, Bucak MN, Tuncer PB (2011) Effects of dithioerythritol on ram semen after the freeze-thawing process. Cryobiology 63: 152-156.

Bispo CAS, Pugliesi G, Galvão P, Rodrigues MT, Ker PG, Filgueiras B, Carvalho GR (2011) Effect of low and high egg yolk concentrations in the semen extender for goat semen cryopreservation. Small Rumin Res, 100: 54-58.

Bousseau S, Brillard JP, Marguant-Le Guienne B, Guerin B, Camus A, Lechat M (1998) Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. Theriogenology 50: 699-706.

Bucak MN, Atessahin A, Varıslı O, Yuce A, Tekin N, Akcay A (2007) The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen microscopic and oxidative stress parameters after freeze-thawing process. Theriogenology 67: 1060-1067.

Bucak MN, Keskin N, Taspınar M, Çoyan K, Baspınar N, Cenariu MC, Bilgili A, Öztürk C, Kursunlu AN (2013) Raffinose and hypotaurine improve the post-thawed Merino ram sperm parameters. Cryobiology 67: 34-39.

Câmara DR, Mello-Pinto MMC, Pinto LC, Brasil OO, Nunes JF, Guerra MMP (2011) Effects of reduced glutathione and catalase on the kinematics and membrane functionality of sperm during liquid storage of ram semen. Small Rumin Res 100: 44- 49.

Câmara DR, Silva SV, Almeida FC, Nunes JF, Guerra MMP (2011) Effects of antioxidants and duration of pre-freezing equilibration on frozen-thawed ram semen. Theriogenology 76: 342-350.

Cirit U, Bagıs H, Demir K, Agca C, Pabuccuoglu S, Varıslı O, Clifford-Rathert C, Agca Y (2013) Comparison of cryoprotective effects of iodixanol, trehalose and cysteamine on ram semen. Anim Reprod Sci 139: 38-44.

Çoyan K, Baspınar N, Bucak MN, Akalin PP (2011) Effects of cysteine and ergothioneine on post-thawed Merino ram sperm and biochemical parameters. Cryobiology 63: 1-6.

Del Valle I, Souter A, Maxwell WMC, Muiño-Blanco T, Cebrián-Pérez JA (2013). Function of ram spermatozoa frozen in diluents supplemented with casein and vegetable oils. Anim Reprod Sci, 138: 213-219.

Dominguez MP, Falcinelli A, Hozbor F, Sanchez E, Cesari A, Alberio RH (2008) Seasonal variations in the composition of ram seminal plasma and its effect on frozen-thawed ram sperm. Theriogenology 69: 564-573.

Drobnis EZ, Crowe LM, Berger T, Anchordoguy T, Overstreet JW, Crowe JH (1993) Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. J Exp Zool 265: 432–437.

El-Rajj Ghaoui R, Thomson PC, Leahy T, Evans G, Maxwell WMC (2007) Autologous whole ram seminal plasma and its vesicle-free fraction improve motility characteristics and membrane status but not in vivo fertility of frozen- thawed spermatozoa. Reprod Dom Anim 42: 541-549.

Emamverdi M, Zhandi M, Shahneh AZ, Sharafi M, Akbari-Sharif A (2013) Optimization of Ram Semen Cryopreservation Using a Chemically Defined Soybean Lecithin-Based Extender. Reprod Dom Anim 48: 899-904.

Esmaeili V, Shahverdi AH, Alizadeh AR, Alipour H, Chehrazi M (2014) Saturated, omega-6 and omega-3 dietary fatty acid effects on the characteristics of fresh, frozen-thawed semen and blood parameters in rams. Andrologia 46: 42-49.

Fair S, Hanrahan J, O'Meara C, Duffi P, Rizos D, Wade M, Donovan A, Boland M, Lonergan P, Evans A (2005) Differences between Belclare and Suffolk ewes in fertilization rate, embryo quality and accessory sperm number after cervical or laparoscopic artificial insemination. Theriogenology 63: 1995–2005.

Fiser PS, Fairfull RW, Marcus GJ (1986) The effect of thawing velocity on survival and acrosomal integrity of ram spermatozoa frozen at optimal and suboptimal rates in straws. Cryobiology 23: 141-149.

Fiume Z (2001) Final report on the safety assessment of lecithin and hydrogenated lecithin. Int J Toxicol 20: 21–45.

Forouzanfar M, Sharafi M, Hosseini SM, Ostadhosseini S, Hajian M, Hosseini L, Abedi P, Nili N, Rahmani HR, Nasr-Esfahani MH (2010) In vitro comparison of egg yolk–based and soybean lecithin-based extenders for cryopreservation of ram semen. Theriogenology 73: 480-487.

Forouzanfar M, Ershad SF, Hosseini SM, Hajian M, Ostad-Hosseini S, Abid A, Tavalaee M, Shahverdi A, Dizaji AV, Esfahani MH (2013) Can permeable super oxide dismutase mimetic agents improve the quality of frozen–thawed ram semen. Cryobiology 66: 126-130.

Fukui Y, Kohno H, Togari T, Hiwasa M, Okabe K (2008) Fertility after Artificial Insemination Using a Soybean-Based Semen Extender in Sheep. J Reprod Dev 54: 286-289.

Gandini L, Lombardo F, Lenzi A, Spano M, Dondero F (2006) Cryopreservation and sperm DNA integrity. Cell Tissue Bank 7: 91–98.

Gil J, Lundeheim N, Soderquist L, Rodríguez- Martínez H (2003) Influence of extender, temperature and addition of glycerol on post-thaw sperm parameters in ram semen. Theriogenology 59: 1241-1255.

Graaf SP, Evans G, Gillan L, Guerra MMP, Maxwell WMC, O'Brien JK (2007) The influence of antioxidant, cholesterol and seminal plasma on the in vitro quality of sorted and non-sorted ram spermatozoa. Theriogenology 67: 217-227.

Hamedani MA, Tahmasbi AM, Ahangari YJ (2013) Effects of vitamin B12 supplementation on the quality of ovine spermatozoa. Open Vet Sci J 3: 140-144.

Holt WV (2000) Basic aspects of frozen storage semen. Anim Reprod Sci 62: 3-22.

Holt WV, Head MF, North RD (1992) Freeze-induced membrane damage in ram spermatozoa is manifested after thawing: observations with experimental cryomicroscopy. Biol Reprod 46: 1086-1094.

Jafaroghli M, Khalili B, Farshad A, Zamiri MJ (2011) The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen. Small Rumin Res 96: 58-63.

Khalifa T, Lymberopoulos A, Theodosiadou E (2013) Association of soybean-based extenders with field fertility of stored ram (Ovisaries) semen: A randomized double-blind parallel group design. Theriogenology 79: 517–527.

Kulaksız R, Cigdem C¸ Akcay E, Daskin A (2010) The protective effect of egg yolk from different avian species during the cryopreservation of Karayaka ram semen. Small Rumin Res 88:12-15.

Kumar A & Laxmi NA (2015) Role of IGF 1 in male and female reproduction in bovines: A Review. Asia Pacific Journal of Research Vol: I. Issue XXIV.

Kumar S, Millar JD, Watson PF (2003) The effect of cooling rate on the survival of cryopreserved bull, ram, and boar spermatozoa: a comparison of two controlled-rate cooling machines. Cryobiology 46: 246-253.

Leahy T, Marti JI, Evans G, Maxwell WMC (2010) Seasonal variation in the protective effect of seminal plasma on frozen-thawed ram spermatozoa. Anim Reprod Sci 119: 147-153.

Leboeuf B, Restall B, Salamon S (2000) Production and storage of goat semen for artificial insemination. Anim Reprod Sci 62:113-141.

Maia MS (2006) Sperm viability and reactive oxygen species (ROS) generation in RAM semen cryopreserved in extenders with sodium lauryl sulfate (OEP), Trolox-c and catalase. Thesis. Faculty of Veterinary Medicine and Animal Science, UNESP, Botucatu.

Maia MS, Sicherle CC, Bicudo SD, Sousa SD, Azevedo HC (2007) Effect of Trolox addition on the motility and membrane integrity of ram spermatozoa with high and low freezability. Reprod Domest Rumin VI. SRF 64, 467-467

Maia MS, Bicudo SD, Azevedo HC, Sicherle CC, Sousa DB, Rodello L (2009) Motility and viability of ram sperm cryopreserved in a Tris-egg yolk extender supplemented with anti-oxidants. Small Rumin Res 85: 85-90.

Marti E, Marti JI, Muiño-Blanco T, Cebrián-Pérez JA (2008) Effect of the cryopreservation process on the activity and immunolocalization of antioxidant enzymes in ram spermatozoa. J Androl 29: 459-67.

Masoudi R, Sharafi M, Shahneh AZ, Towhidi A, Kohram H, Zhandi M, Esmaeili V, Shahverdi A (2016) Effect of dietary fish oil supplementation on Ram semen freezability and fertility using soybean lecithin and egg yolk-based extenders, Theriogenology, http://dx.doi.org/10.1016/j.theriogenology.2016.05.018 in press

Mata-Campuzano M, Alvarez-Rodriguez M, Alvarez M, Anel L, de Paz P, Garde JJ, Martínez Pastor F (2012) Effect of several antioxidants on thawed ram spermatozoa submitted to 37oC up to four hours. Reprod Dom Anim 47: 907-914.

Mata-Campuzano M, Álvarez-Rodríguez M, Álvarez M, Tamayo-Canul J, Anel L, de Paz P, Martínez-Pastor F (2015) Post-thawing quality and incubation resilience of cryopreserved ram spermatozoa are affected by antioxidant supplementation and choice of extender. Theriogenology 83: 520-528.

Matsuoka T, Imai H, Kohno H, Fukui (2006) Effects of Bovine Serum Albumin and Trehalose in Semen Diluents for Improvement of Frozen-Thawed Ram Spermatozoa. J Reprod Dev 52: 675-683.

Maxwell WMC, Evans G, Mortimer ST, Gillan L, Gellatly ES, Mcphie CA (1999) Normal fertility in ewes after cervical insemination with frozen-thawed spermatozoa supplemented with seminal plasma. Reprod Fertil Dev 11: 123-126.

Medeiros CM, Forell F, Oliveira AT, Rodrigues JL (2002) Current status of sperm cryopreservation: why isn't better. Theriogenology 57: 327-344.

Milovanović A, Maksimović N, Barna T, Lazarević M, Delić N (2013) Laparoscopic insemination of sheep in Republic of Serbia. Biotechn Anim Husbandry 29: 449-456.

Mocé E, Purdy PH, Graham JK (2010) Treating ram sperm with cholesterol-loaded cyclodextrins improves cryosurvival. Anim Reprod Sci 118: 236-247.

Molinia FC, Evans G, Casares PI, Maxwell WMC (1994) Effect of monosaccharide and disaccharides in Tris-based diluents on motility, acrosome integrity and fertility of pellet frozen ram spermatozoa. Anim Reprod Sci 36: 113–122.

Morrier A, Castonguay F, Bailey JL (2002) Glycerol addition and conservation of fresh and cryopreserved ram spermatozoa. Can J Anim Sci 418: 347-356.

Motlagh MK, Sharafi M, Zhandi M, Mohammadi-Sangcheshmeh A, Shakeri M, Soleimani M, Zeinoaldini S (2014) Antioxidant effect of rosemary (Rosmarinus officinalis L) extract in soybean lecithin-based semen extender following freeze–thawing process of ram sperm. Cryobiology 69: 217-222.

Moussa M, Matinet V, Trimeche A, Tainturier D, Anton M (2002) Low density lipoproteins extracted from hen egg yolk by an easy method: cryoprotective effect on frozen-thawed bull semen. Theriogenology 57: 1695-1706.

Moustacas VS, Zaffalon FG, Lagares MA, Loaiza-Eccheverri AM, Varago FC, Neves MM, Heneine LGD, Arruda RP, Henry M (2011) Natural, but not lyophilized, low density lipoproteins were an acceptable alternative to egg yolk for cryopreservation of ram semen. Theriogenology 75: 300-307.

Murawski M, Schwarz T, Grygier J, Patkowski K, Oszczeda Z, Jelkin I, Kosiek A, Gruszecki TM, Szymanowska A, Skrzypek T, Zieba DA, Bartlewski PM (2015) The utility of nanowater for ram semen cryopreservation. Exp Biol Med 240: 611-617.

Najafi A, Zhandi M, Towhidi A, Sharafi M, Sharif AA, Motlagh MK, Martinez-Pastor F (2013) Trehalose and glycerol have a dose-dependent synergistic effect on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. Cryobiology 66: 275-282.

Najafi A, Kia HD, Mohammadi H, Najafi MH, Zanganeh Z, Sharafi M, Martinez-Pastor F, Adeldust H. (2014) Different concentrations of cysteamine and ergothioneine improve microscopic and oxidative parameters in ram semen frozen with a soybean lecithin extender. Cryobiology 69: 68-73.

Nordstoga AB, Soderquist L, Adnøy T, Paulenz H (2009) Effect of Different Packages and Freezing/Thawing Protocols on Fertility of Ram Semen. Reprod Dom Anim 44: 527-531.

O'Hara L, Hanrahan JP, Richardson L, Donovan A, Fair S, Evans ACO, Lonergan P (2010) Effect of storage duration, storage temperature, and diluent on the viability and fertility of fresh ram sperm. Theriogenology 73: 541-9.

O'Meara C, Hanrahan J, Donovan A, Fair S, Rizos D, Wade M, Boland M, Evans A, Lonergan P (2005) Relationship between in vitro fertilization of ewe oocytes and the fertility of ewes following cervical artificial insemination with frozen thawed semen. Theriogenology 64: 1797-1808.

Padilha RT, Magalhães-Padilha DM, Cavalcante MM, Almeida AP, Haag KT, Gastal MO, Nunes JF, Rodrigues APR, Figueiredo JR, Oliveira MAL (2012) Effect of insulin-like growth factor-I on some quality traits and fertility of cryopreserved ovine semen. Theriogenology 78: 907-913.

Paulenz H, Söderquist L, Ådnøy T, Nordstoga AB, Gulbrandsen B,

Andersen BK (2004) Fertility results after different thawing procedures for ram semen frozen in mini tubes and mini straws. Theriogenology 61:1719-1727.

Paulenz H, Adnøy T, Söderquist L (2007) Comparison of fertility results after vaginal insemination using different thawing procedures and packages for frozen ram semen. Acta Vet Scand 49: 26.

Pelufo V, LópezArmengol MF, Malcotti V, Venturino A, Aisen EG (2015) Effects of glycerol and sugar mixing temperature on the morphologic and functional integrity of cryopreserved ram sperm. Theriogenology 83: 144-151.

Pérez-Pé R, Cebrián-Pérez J, Muio-Blanco T (2001) Semen plasma proteins prevent cold-shock membrane damage to ram spermatozoa. Theriogenology 56: 425-434.

Purdy PH (2006) The post-thaw quality of ram sperm held for 0 to 48 h at 5oC prior to cryopreservation. Anim Reprod Sci 93: 114-123.

Purdy PH, Mocé E, Stobart R, Murdoch WJ, Moss GE, Larson B, Ramsey S, Graham JK, Blackburn HD (2010) The fertility of ram sperm held for 24h at 5oC prior to cryopreservation. Anim Reprod Sci 118: 231-235.

Rebolledo AD, Sierra LN, Tamayo AC, Loria AA, Denis SE, Oses RB, Parra EG, Monsreal LP, Ugalde JR (2007) Fertility in hair sheep inseminated with freeze spermatozoa rediluted with seminal plasma. Rev Cient 17:73-76.

Ritar AJ and Ball PD (1993) The effect of freeze-thawing of goat and sheep semen at a high density of spermatozoa on cell viability and fertility after insemination. Anim Reprod Sci 31: 249-262.

Rovegno M, Feitosa WB, Rocha AM, Mendes CM, Visintin JA, MEO D'AvilaAssumpco (2013) Assessment of post-thawed ram sperm viability after incubation with seminal plasma. Cell Tissue Bank 14: 333-339.

Salamon S & Maxwell WMC (1995a) Frozen storage of ram semen: I. Processing, freezing, thawing and fertility after cervical insemination. Anim Reprod Sci 37: 185-249.

Salamon S & Maxwell WMC (1995b) Frozen storage of ram semen: II. Causes of low fertility after cervical insemination and methods of improvement. Anim Reprod Sci 38: 1–36.

Salamon S & Maxwell WMC (2000) Storage of ram semen. Anim Reprod Sci 62: 77-111.

Sangeeta S, Arangasamy A, Kulkarni S, Selvaraju S (2015) Role of amino acids as additives on sperm motility, plasma membrane integrity and lipid peroxidation levels at pre-freeze and post-thawed ram semen. Anim Reprod Sci 161: 82-88.

Santiani A, Evangelista S, Sepúlveda N, Risopatrón J, Villegas J, Sánchez R (2014) Addition of superoxide dismutase mimics during cooling process prevents oxidative stress and improves semen quality parameters in frozen/thawed ram spermatozoa. Theriogenology 82: 884-889.

Sharafi M, Zhandi M, Sharif AA (2015) Supplementation of soybean lecithin-based semen extender by antioxidants: complementary flow-cytometric study on post-thawed ram spermatozoa. Cell Tissue Bank 16: 261-269

Sicherle CC, Maia MS, Bicudo SD, Rodello L, Azevedo HC (2011) Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen supplemented with catalase or Trolox. Small Rumin Res 95: 144-149.

Silva SV, Soares AT, Batista AM, Almeida FC, Nunes JF, Peixoto CA, Guerra MMP (2013) Vitamin E (Trolox) addition to Tris-egg yolk extender preserves ram spermatozoon structure and kinematics after cryopreservation. Anim Reprod Sci 137: 37-44.

Sönmez M and Demirci E (2004) The Effect of Ascorbic Acid on the Freezability of Ram Semen Diluted with Extenders Containing Different Proportions of Glycerol. Turk J Vet Anim Sci 28: 893-899.

Soylu MK, Nur Z, Ustuner B, Dogan I, Sagirkaya H, Gunay U, Ak K (2007) Effects of various cryoprotective agents and extender osmolality on post-thawed ram semen. B Vet I Pulawy 51: 241-246.

Toker MB, Alcay S, Gokce E, Ustuner B (2016) Cryopreservation of ram semen with antioxidant supplemented soybean lecithin-based extenders and impacts on incubation resilience. Cryobiology 72: 205-209.

Tonieto RA, Goularte KL, Gastal GDA, Schiavon RS, Deschamps LC, Lucia T (2010) Cryoprotectant effect of trehalose and low-density lipoprotein in extenders for frozen ram semen. Small Ruminant Res 93: 206-209.

Tsakmakidis IA (2010) Ram semen evaluation: development and efficiency of modern techniques. Small Rumin Res 92: 126-130.

Uysal O, Bucak MN (2007) Effects of Oxidized Glutathione, Bovine Serum Albumin, Cysteine and Lycopene on the Quality of Frozen-Thawed Ram Semen. Acta Vet Brno 76: 383-390.

Valle I, Gomez-Duran A, Holt WV, Muino-Blanco T, Cebrian-Perez JA (2012) Soy lecithin interferes with mitochondrial function in frozen-thawed ram spermatozoa. J Androl 33: 717-725.

Valle I, Souterb A, Maxwell WMC, Muino-Blanco T, Cebrián-Péreza JA (2013) Function of ram spermatozoa frozen in diluents supplemented with casein and vegetable oils. Anim Reprod Sci 138: 213-219.

Vichas L, Tsakmakidis IA, Vafiadis D, Tsousis G, Malama E, Boscos CM (2017) The effect of antioxidant agents' addition and freezing method on quality parameters of frozen thawed ram semen. Cell Tissue Bank (DOI 10.1007/s10561-017-9633-6).

Watson PF (2000) The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 60-61: 481-492.