Ameliorative effects of plant extracts on ram semen preservation and its fertilization ability

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Abstract

The liquid storage and cryopreservation of ram semen are key steps in the success of artificial insemination. However, semen handling generally results in a gradual decrease in its quality and fertilization ability. In both preservation methods, cold and osmotic shock as well as the inappropriate choice of the extender could lead to the production of reactive oxygen species-induced oxidative damage to the spermatozoa. Therefore, previous studies focused on the supplementation of extenders by exogenous antioxidants to limit oxidative stress produced during ram semen preservation. Recently, plant extracts have been investigated as cheap and natural sources of additives to preserve the ram semen. This paper aims to present in detail the factors influencing ram semen quality during liquid storage and cryopreservation. In addition, study findings investigating the use of biomolecules originating from plants with their appropriate doses for ram sperm preservation are also discussed. Precisely, an update regarding their effects on sperm quality and its *in vivo* or *in vitro* fertilization ability is provided in this review.

Keywords: ram semen, liquid storage, cryopreservation, plant extracts.

Les effets amélioratifs des extraits de plantes sur la préservation du sperme de béliers et sa capacité de fertilisation

Résumé

La conservation à l'état liquide ainsi que la cryoconservation de la semence de bélier sont des étapes clés dans la réussite de l'insémination artificielle. Cependant, la manipulation de la semence entraîne généralement une diminution progressive de sa qualité et de sa capacité de fécondation. Dans les deux méthodes de conservation, les chocs thermique et osmotique ainsi que le choix inapproprié du dilueur pourraient conduire aux dommages oxydatifs induits par les espèces réactives de l'oxygène sur les spermatozoïdes. Par conséquent, des études antérieures se sont concentrées sur la supplémentation de dilueurs par des antioxydants exogènes afin de réduire le stress oxydatif produit lors de la conservation du sperme de bélier. Récemment, des biomolécules provenant de produits naturels ont été additionnés au dilueurs. Cet article vise à présenter en détail les facteurs influençant la qualité de la semence de bélier lors de la conservation en sperme frais ou congelé. En outre, les résultats des études portant sur l'utilisation des extraits ou des biomolécules naturelles à différentes doses sont discutés. Précisément, une mise à jour concernant leurs effets sur la qualité du sperme et sa capacité de fécondation *in vivo* ou *in vitro* est fournie dans cette revue.

Mots-clés : Semence de bélier, stockage à l'état liquide, cryoconservation, extraits de plantes.

التأثيرات التحسينية المستخلصات النباتية في الحفاظ على الحيوانات المنوية للأكباش وقدرتها على التخصيب

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ملخص

يعتبر حفظ السائل المنوي عن طريق التبريد أوالتجميد من الخطوات الرئيسية في نجاح التلقيح الاصطناعي .لكن يؤدي العمل على السائل المنوي عمومًا إلى انخفاض تدريجي في جودته وقدرته على الإخصاب .في كلتا طريقتي الحفظ، يمكن أن تؤدي صدمة التبريد والصدمة التناضحية وكذلك الاختيار غير المناسب للمخفف إلى إنتاج أضرارتأكسدية للحيوانات المنوية عن طريق مركبات الأكسجين التفاعلية . لذلك ركزت الدراسات السالفة على إضافة مضادات الأكسدة للحد من الإجهاد التأكسدي الناتج أثناء حفظ السائل المنوي عند الكبش .في الآونة الأخيرة ، تمت إضافة المستقلبات الثانوية المستخلصة من المنتجات الطبيعية إلى المخففات كمصادر اقتصادية وطبيعية للحفاظ على الحيوانات المنوية المحفوظة .هدفت هذه الورقة إلى تقديم تفاصيل العوامل التي تؤثر على جودة السائل المنوي للكبش أثناء الحفظ بالتبريد و التجميد .بالإضافة إلى ذلك ، تمت مناقشة نتائج الدراسات التي قامت باستخدام المستقلبات الثانوية الطبيعية بجرعاتها المناسبة للحفاظ على الحيوانات المنوية عند الكبش .على وجه التحديد ، تم تقديم تحديث بشأن آثارها على جودة الحيوانات المنوية وقدرتها على التخصيب في هذه المراجعة.

الكلمات المفتاحية: السائل المنوي عند الكبش ، الحفظ بالتبريد ، الحفظ بالتجميد ، المستخلصات النباتية

Introduction

Sheep farming is a sector which occupies a primordial place in many countries around the world (Reddy, 2020). This species is one of the earliest to be domesticated in Southwest Asia approximately 11,000 years ago, and is now raised around the world (Kawęcka *et al*., 2022). The sheep farming sector is an essential income source for many families in resource-poor areas (Odintsov Vaintrub *et al*., 2021). According to the FAO database (FAO, 2018), the total sheep population worldwide was 1,173 million with 43.6% in Asia, 30% in Africa, 11.2% in Europe, 8.1% in Oceania, and 7.1% in America. Sheep are considered a major source of meat and milk. In fact, sheep meat production globally is close to 9 million tons per year and ranked fourth after pork, poultry, and beef (FAO, 2018). Moreover, sheep represents 1.3% of the total milk production in the world, and it is more in demand as many people, mainly infants, have allergic reactions to cow's milk and their products (Mazinani and Rude, 2020). Hence, the implementation and effective use of reproductive technologies especially artificial insemination is increasingly important to enhance the production of the sector.

Artificial insemination serves as an essential technique for breed improvement. It allows the dissemination of genetic progress through the fecondation of a large number of females using the semen of a single male selected for its genetic qualities. It helps also in limiting health risks such as sexually transmitted diseases *(*Gibbons *et al*., 2019). However, the success of this technique is mainly based on the quality of semen after preservation in liquid or in freezing forms (Allai *et al*., 2018). The semen storage leads to a gradual decrease in its quality (Allai *et al*., 2018). In addition to the initial cold shock (Szymanowicz *et al*., 2019), dilution of sperm in the extender weakens the antioxidant system of seminal plasma (Selvaraju et al., 2008). Therefore, the reactive oxygen species produced by the metabolism are one of the primary causes that reduce the sperm quality *(Menegat et al*., 2017). Furthermore, the membrane of ram spermatozoa is particularly rich in polyunsaturated fatty acids (Kameni *et al*., 2021), which makes them very sensitive to oxidative stress (Özer Kaya *et al*., 2017), leading to a decrease in sperm motility, viability, functional integrity of ram sperm membranes and fertilization ability (Selvaraju *et al*., 2008; Menegat *et al*., 2017; Said, 2020; Kameni *et al*., 2021). Therefore, previous studies have been oriented to supplement the semen extenders by antioxidant to ensure the proper functioning of the cells, and to have a balance between the formation of ROS and the protective action of the antioxidant system (Allai *et al*., 2018; Zarei *et al*., 2020). Recently, biomolecules extracted or purified from plants have emerged as a cheap and natural source of additives to preserve and improve sperm function during semen storage (Pintus and Ros-Santaella, 2021). The purpose of the current paper is to present in details the factors influencing ram semen quality during liquid storage and cryopreservation. Moreover, the use of natural biomolecules compounds with their appropriate doses for ram sperm preservation was also discussed. Precisely, an update regarding their effects on sperm quality is provided in this review.

Factors influencing ram semen preservation

Semen preservation is the process of extending the viable lifetime of spermatozoa by preserving their biochemical, ultrastructural, and functional characteristics (Salamon and Maxwell, 1995). It allows to spread the ejaculate of an elite ram over a large number of ewes, which eventually speeds up genetic progress (Rizkallah *et al*., 2022). There are two main methods currently used in sperm preservation, liquid storage and cryopreservation. Liquid storage slows biochemical function by chilling the semen at a temperature of 5 or 15 °C, respectively (Evans and Maxwell, 1987). During ram semen preservation, sperm quality could be impacted by several factors, including temperature, storage concentration and extender. However, in this case, the fertilization ability of stored semen declines with an average of 10–35% per day using cervical insemination. This prevents the transport of the stored semen collected from an elite ram over a long distance (Kameni *et al*., 2021). To face this problem, producers rely on cryopreservation, which consists of the complete shutdown of biochemical functioning (Kameni *et al*., 2021), genraly by chilling at -196°C in liquid nitrogen.

Temperature

When the ambient temperature surrounding the sperm cell is reduced, spermatozoa undergo cold shock, which disrupts the intramembrane components organization (Holt and North, 1984). This reorganization leads to the formation of lipid–lipid agglutinations, disruption of cell signaling, and creation of particle-free zones (Watson, 2000). These later decrease membrane integrity and selective permeability, allowing unrestricted ionic transport across the membrane (Rizkallah *et al*., 2022). The sensitivity of sperm to cold shock depends on the cholesterol/phospholipid molar ratio (CHO/PL) (Rizkallah *et al*., 2022). For instance, ram sperm is more sensitive to cold shock compared to a bull because of its a lower CHO/PL. The reason being that, intramembrane cholesterol protects against cold shock by promoting membrane stability via impeding particle movement within the membrane during the cooling process, thereby reducing membrane destabilization events (Flesch *et al*., 2001; Kessel *et al*., 2001).

Storage concentration

Several studies demonstrated the harmful effects of high dilutions on preserved spermatozoa. The dilution of sperm in the extender results in osmotic stress to the spermatozoa, and this weakens the endogenous antioxidants of seminal plasma (Ros-Santaella and Pintus, 2021). As it was demonstrated, when ram sperm was diluted to 100×10^6 sperm/ml, it recorded lower membrane damage and oxidative stress compared to spermatozoa diluted to 25×10^6 sperm/ml (Rizkallah *et al*., 2022). Similar results were recorded with various sperm concentrations in cattle (Patil *et* al., 2020), bucks (Sadeghi *et al*., 2020), and boar (Caballero *et al*., 2006). Therefore, to maintain osmotic balance between the spermatozoa and the changing external environment, the slowing down of the dilution rate by using a stepwise dilution process and the selection of appropriate extenders are necessary (Salamon and Maxwell, 1995; Leahy *et al*., 2010).

Extenders

Extenders are media that provide the nutrients and energy needed for sperm survival through the stages of sperm preservation. They stabilize the plasma membrane and maintain its intracellular and intramembrane ionic concentrations, thus reducing the harmful effects caused by cold and osmotic shock (Forouzanfar *et al*., 2010; Albiaty *et al*., 2016; Arando *et al*., 2019). However, unsuccessful selection of extenders could negatively influence sperm quality parameters during preservation. Depending on the species, sperm cells differ biologically, which requires the use of specific compounds for their protection. In rams, the pH range of sperm is between 7.3 and 7.5 leading to reach a maximal respiration (Evans and Maxwell, 1987; Leahy *et al*., 2010). While in bulls, the pH range of their sperm is between 6.5–7 (Arando *et al*., 2017). Additionally, the needs in energy, antioxidants, antibiotics, and antifreeze shock of the spermatozoa also differ according to the species. On the other hand, the choice extenders is also influenced by the breed species and storage temperature (Ben Moula *et al*., 2018; Acharya *et al*., 2019).

Despite taking the aforementioned factors into account, it was demonstrated that sperm quality parameters are reduced during liquid storage and cryopreservation (Sharma *et al*., 2015; Allai *et al*., 2018). Hence, for further sperm quality amelioration, studies were oriented to supplement the extenders by exogenous antioxidants to limit oxidative stress produced during ram semen preservation (Zarei *et al*., 2021). Recently, biomolecules extracted or purified from natural products (plants and microalga) have recently emerged as a cheap and natural source of additives to preserve and improve sperm function during semen storage (Leisegang, 2021; Pintus and Ros-Santaella, 2021).

The use of plant extracts in ram semen preservation

Biomolecules with antioxidant properties extracted or purified from natural products have been recently studied in order to improve the quality of stored or cryopreserved ram semen (Carrera-Chávez *et al*., 2020). Plants and microalgae produce a wide range of biomolecules to defend against biotic stress and pathogens such as bacteria, fungi and viruses (Ros-Santaella and Pintus, 2021). Therefore, these biomolecules have a broad spectrum of activities such as antioxidant, antibacterial, antifungal and antiviral activities (Seca and Pinto, 2019). In recent decades, they have been extensively used as supplements of the semen extenders in both liquid storage and cryopreservation (Ros-Santaella and Pintus, 2021).

Liquid storage

To reduce the harmful effects associated to sperm liquid storage, an appropriate extender could not only provide the necessaire energy, nutrients and buffers, but also prevent the generation of free radicals generated by stressors (Kameni *et al*., 2021). In this regard, several investigations have focused on the supplementation of extenders by plant extracts. Therefore, a detailed description of the effects of biomolecules originated from four plant species including *Alnus incana, Opuntia ficus-indica*, *Argania spinosa* and *Salvia verbenaca* belonging to *Betulaceae*, *Cactaceae*, *Sapotaceae* and *Lamiaceae* families, respectively, was presented. Moreover, the extracts of propolis and *Spirulina platensis* (microalgae) were also highlighted.

A study realized by El-Harairy *et al*. (2018) demonstrated that soybean lecithin extender supplemented with 1 mg/ml of propolis ethanolic extract improved motility and chromatin integrity of Rahmani ram semen stored at 5°C for 48 h. The same study reported that this extract contains considerable levels of chlorogenic acids, catechin, protocatechuic and pyrogallol which play a crucial role in free radical scavenging (McCann *et al*., 2015; Ozturk Sarikaya, 2015). On the other hand, Oregonin extracted from *Alnus incana* bark and added to Tris-glucose-glycerol-egg yolk extender improved sperm motility, mitochondria activity and *in vivo* fertilization ability in ram semen stored at 5˚C for to 48 h (Abadjieva *et al*., 2020). Furthermore, Allai *et al*. (2016) reported that skim milk and Tris-egg yolk extenders enriched by 1% of *Opuntia ficus-indica* acetone extract improved total and progressive motilities, viability, membrane integrity, and decreased the abnormalities, lipid peroxidation, and DNA fragmentation in Boujaâd ram semen stored at 5°C for 72 h as compared to the control group. The phenolic compounds profile revealed that this extract is rich in quercetin, hyperoside, rutin, hesperidin and high level of tr-Aconitic, malic and quinic acids which possess a high antioxidant activity. Similar results were obtained with the inclusion of the essential oil of *Argania spinosa* and *Opuntia ficus-indica* seed oil to Tris-egg yolk and skim milk extenders (Allai *et al*., 2015; Allai *et al*., 2017). Recently, a study conducted by Ben moula *et al*. (2023) reported that the inclusion of *Spirulina platensis* and *Salvia verbenaca* extracts improved semen quality and its fertilization ability in Sardi rams. After 24 h of liquid storage, the study revealed that skim milk supplemented with 1.25 µg/ml *Spirulina platensis* acetonic extract and 3.75 µg/ml *Salvia verbenaca* acetonic extract increased total and progressive motilities, viability and membrane integrity, and decreased also lipid peroxidation levels and sperm abnormalities, however, sperm fertilization ability was not significantly influenced (Ben Moula *et al*., 2023). Similarly, in Djallonké rams, Leugoué *et al*. (2022) showed that the ethanolic extract of *Spirulina platensis* reduced sperm abnormalities and increased total and progressive motility, viability, membrane integrity and antioxidant activity after liquid storage. The ameliorative effect of *Spirulina platensis* in sperm quality parameters could be explained by its richness in phenolic compounds particularly phenolic acids, phenolic diterpenes, and flavonoids (Rahim *et al*., 2021a). Furthermore, this microalga includes distinctive natural pigments, such as c-phycocyanin which possesses a strong antioxidant activity (Soni *et al*., 2008). Recently, a study realized in our laboratory reported that grade analytical c-phycocyanin purified from *Spirulina platensis* possess a remarkable ameliorative effect on the stored (5°C) Boujaâd ram semen quality and *in vivo* fertilization ability (Rahim *et al*., 2021b). As results of this study, skim milk supplemented with 2.4 µg/ml enhanced total motility, progressive motility, viability, abnormalities and antioxidant activity of semen after 0, 4, 8 and 24 h of storage. Furthermore, the same dose of c-phycocyanin improved the *in vivo* fertilization after artificial insemination (Rahim *et al*., 2021b).

Cryopreservation

Twelve plant species including *Artemisia incana*, *Camellia sinensis*, *Echinacea purpurea*, *Entada abyssinica, Foeniculum vulgare*, *Moringa oleifera*, *Nigella sativa*, *Punica granatum*, *Rosmarinus officinalis*, Syzygium aromaticum, *Thymus vulgaris* and *Zingiber officinale* belonging to ten families (*Apiaceae*, *Asteraceae*, *Fabaceae*, *Lamiaceae*, *Moringaceae*, *Myrtaceae*, *Punicaceae*, *Ranunculaceae*, *Theaceae* and *Zingiberaceae*) were studied until now (Table 1). Merati and Farshad. (2020) evaluated the effect of supplementing the freezing extender by 10 mg/ml of *Echinacea purpurea*

or *Zingiber officinale* aqueous extracts on the quality and *in vitro* fertility potential of ram epididymal spermatozoa after cryopreservation. After thawing, motility and velocity parameters, acrosome integrity, mitochondrial activity, antioxidant activity and *in vitro* fertility were significantly improved compared to the control (epididymal spermatozoa stored in freezing extender alone) (Merati and Farshad, 2020). In another study realized on Ghezel rams, semen was cryopreserved with soybean lecithin-based extender supplemented with 0 (control), 5, 10, or 15 mg/ml of aqueous extract of *Foeniculum vulgare*, the results revealed that the extract increased viability, motility, velocity, plasma membrane integrity, and mitochondrial activity, as well as reduced lipid peroxidation levels in sperm (Najafi *et al*., 2019). Moreover, the best results were obtained after adding a concentration of 10 mg/ml (Najafi *et al*., 2019). Moreover, the methanolic extract of *Entada abyssinica* bark (375µg/ml) has been proven to increases motility, viability, and membrane integrity, as well as decreases peroxide hydrogen concentration in Ossimi ram semen after cryopreservation (Sobeh *et al*., 2020). Motlagh *et al*. (2014) showed that soybean lecithin-based extender supplemented with 4 and 6% of aqueous extract of *Rosmarinus officinalis* leaves resulted in higher percentages of total and progressive motilities, plasma membrane integrity and viability or Chal rams' semen as compared to the control group. Whereas, the acrosomal and capacitation status were not affected by *Rosmarinus officinalis* aqueous extract (Motlagh *et al*., 2014). The same extender supplemented by 7.5 mg/l of *Punica granatum* fruit juice or 10 mg/ml of aqueous *Camellia sinensis* powder enhanced total and progressive motilities, membrane integrity, viability, mitochondria activity and total antioxidant activity in cryopreserved semen of ghazel rams (Mehdipour *et al*., 2016; Mehdipour *et al*., 2017). This dose also reduced lipid peroxidation of apoptotic sperm compared to the control group (Mehdipour *et al*., 2017). Furthermore, in hair breed ram semen, it was reported that the inclusion of 0.5 and 5 mg/ml of *Moringa oleifera* seed methanol extract in extender (Tris 6%, glycerol and 10%, egg yolk) increased sperm motility, membrane integrity and antioxidant activity in comparing with the control group (Carrera-Chávez *et al*., 2020). In addition, the best results were obtained in the cryopreserved semen with extender supplemented with 5 mg/ml *(Carrera-Chávez et al*., 2020). Moreover, the beneficial effect of *Moringa oleifera* have been also reported Awassi and Barki ram semen extended in Tris-citrate-glucose and Commercial tris base extender (BullXcell) extenders, respectively (Shokry *et al*., 2020; El-Seadawy *et al*., 2022). In another study, 75 µg/ml of ethanol extract of *Syzygium aromaticum* buds added to the extender (Tris, egg yolk, and glycerol) boosted motility, velocity, viability, and plasma membrane integrity parameters of Lori-Bakhtiyari ram semen after cryopreservation. In Merino rams, Tris-based extender supplemented with 100 g/ml of *Nigella sativa* essential oil, showed higher values of the total motility, progressive motility, velocity parameters, acrosome integrity and DNA integrity compared with the spermatozoa cryopreserved without supplementation (Miah *et al*., 2018).

Table 1: The beneficial effects of biomolecules extracted from natural products on cryopreserved ram semen quality.

Plant Species	Breed	Part	Extract	Best dose	Effects	References
Echinacea purpurea	ND	Leaves	Water	10 mg/l	Improved MT, MP, VP, AI, MI, AA, and *in vitro* fertility.	Merati and Farshad (2020)
Foeniculum vulgare	Ghezel	Powder	Water	10 mg/l	Increased MT, MP, VP, PMI and MA. Reduced LP.	Najafi *et al*. (2019)
Entada abyssinica	Ossimi	Bark	Methanol	375 µg/ml	Increased MP, PMI and AA. Decrease HP.	Sobeh *et al*. (2020)
Rosmarinus officinalis	Chal	leaves	Water	6%	Increased MT, MP, PMI, LP, HP and SV.	Motlagh *et al*. (2014)
Punica granatum	Ghezel	fruit	Juice	7.5 µg/ml	Increased MP, PMI, SV, MA and AA. Reduced LP.	Mehdipour *et al*. (2017)
Moringa oleifera	hair	seed	Methanol	0.5 mg/ml	Increased MT, MP, PMI and AA.	Carrera-Chávez *et al*. (2020)
Syzygium aromaticum	Lori-Bakhtiyari	Buds	Ethanol	75 µg/ml	Increased MT, MP, VP, SV and PMI.	Baghshahi *et al*. (2014)
Nigella sativa	Merino	seed	EO	100 µg/ml	increased MT, MP, VP and DNA integrity.	Miah *et al*. (2018)
Camellia sinensis	Ghezel	powder	Water	10 mg/l	Increased MP, PMI, SV, MA and AA.	Mehdipour *et al*. (2016)
Moringa oleifera	hair	Seed	methanol	10 mg/ml	Increased MT, MP, PMI, AI, AA and *in vitro* fertility.	Guedea-Betancourt *et al*. (2022)
Thymus vulgaris	Moghani	Powder	Ethanol	4%	Increased MP, SV and PMI.	Vahedi et al. (2018)
Moringa oleifera	Awassi	Leaf	methanol	0.64 mg/ml	Increased MT, MP, SV, PMI and AI. Decreased ABN.	El-Seadawy *et al*. (2022)
Artemisia incana	Moghani	Powder	Water	4%	Increased MT and MP.	Ahadi *et al*. (2021)

ND: not determined; Mt: total motility; MP: progressive motility; VP: velocity parameters; AI: acrosome integrity; MA: mitochondrial activity, AA: antioxidant activity; PMI: plasma membrane integrity; LP: lipid peroxidation, HP: hydrogen peroxide: SV: sperm viability, ABN: sperm abnormalities; EO: essential oil.

Conclusion

In conclusion, studies addressing the effects of biomolecules extracted or purified from natural products on ram semen quality during semen preservation showed to be encouraging and promising for improving sheep farming sector in the future. The ameliorative effect of these natural products on ram semen quality have been well highlighted. However, very limited studies have investigated the effect of biomolecules on *in vivo* fertilization ability of ram semen. In addition, further studies involving fertility trials are recommended to pave the way toward the incorporation of these natural compounds into sperm extenders. Also, the strong antibacterial activity of these biomolecules wasn't properly exploited. Therefore, more scientific investigations are needed to understand the mechanisms by which these natural biomolecules improve the stored semen quality.

Declaration of Competing Interest

The authors report no declarations of interest.

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