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Review

Use of antioxidants to augment semen efficiency during liquid storage and cryopreservation in livestock animals: A review

Mohsen Ghaleb Al-Mutary *

Department of Basic Sciences, College of Education, Imam Abdulrahman Bin Faisal University, P.O. Box 2375, Dammam 14513, Saudi Arabia
Basic and Applied Scientific Research Center, Imam Abdulrahman Bin Faisal University, P.O. Box 1982, Dammam 31441, Saudi Arabia

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ABSTRACT

Despite the widespread use of frozen or refrigerated mammalian spermatozoa, their quality remains low as they are highly sensitivity to injuries during preservation and thawing. Moreover, *in vitro* conditions during spermatozoa storage may affect sperm quality and thereby oocyte fertilization, cleavage, and blastocyst development. Recently, antioxidants have been employed during semen extenders for livestock in different storage protocols to compensate for the depletion of endogenous antioxidant concentration in seminal plasma due to dilution as well as to counteract *in vitro* oxidative stress and minimize free radical generation. The present article reviews the most effective enzymatic or non-enzymatic antioxidants used during livestock spermatozoa preservation.

Contents

1. Introduction

Artificial insemination (AI) with preserved semen is the preferred mode of breeding over normal mating for livestock animals. AI has several advantages, such as the prevention of sexually transmitted diseases, establishment of a germplasm bank, and avoidance of male transfer. However, the success of AI largely depends on the efficiency of *in vitro* semen storage protocols in maintaining spermatozoa quality (Lone et al., 2017; Skidmore et al., 2018).

* Address: Department of Basic Sciences, College of Education, Imam Abdulrahman Bin Faisal University, P.O. Box 2375, Dammam 14513, Saudi Arabia.
E-mail address: mgalmutary@iau.edu.sa
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Semen is preserved in an extender containing Tris, glycerol, glucose, citric acid, water, egg yolk, and antibiotics at refrigerated temperatures for several days during liquid storage (Al-Bulushi et al., 2019) and at −198 °C for several months or even years during cryopreservation (Gangwar et al., 2018; Prell et al., 2020). Typically, seminal plasma contains both enzymatic and non-enzymatic antioxidants; however, their protective actions against oxidative stress are significantly weakened following semen dilution in the extender prior to storage (Peris-Frau et al., 2020). Furthermore, high polyunsaturated fatty acid levels render the spermatozoon plasma membrane highly susceptible to oxidative stress and particularly to lipid peroxidation by reactive oxygen species (ROS) (Özer Kaya et al., 2018), due to the imbalance between ROS levels and natural antioxidant activity of sperm (Hamilton et al., 2016). The detrimental effects of ROS on spermatozoa decrease sperm viability and motility, impair fertilization, reduce implantation and pregnancy, minimize the cleavage rate, lower embryo quality, and inhibit blastocyst formation (Selvaraju et al., 2008; Osman et al., 2015; Simon et al., 2017) (Fig. 1). Consequently, the use of exogenous antioxidants to maintain ROS balance and protect spermatozoa from oxidative damage during preservation has recently garnered increased interest (Souza et al., 2019; Al-Mutary et al., 2020). Nonetheless, the use of antioxidants for maintaining sperm quality during semen storage remains debatable. Therefore, the objective of this review is to summarize the most effective and frequently used antioxidants during spermatozoa preservation.

2. Methodology

Experimental studies in the field of semen storage, which reported the use of antioxidants to increase semen efficiency, were searched for in major databases, such as Science Direct, Scopus, PubMed, Google Scholar, and Web of Knowledge. Only publications between January 2000 and June 2020 were included to reflect the latest developments in this field. Combinations of keywords that would represent semen storage and antioxidants, such as "cryopreservation" and "liquid storage with "antioxidants," were used. The selected studies on livestock animals are summarized in Table 1.

3. The most effective antioxidants used during spermatozoa storage

3.1. Resveratrol (RSV)

RSV is a natural plant polyphenol that is commonly used as an antioxidant and a therapeutic agent (Javkhedkar et al., 2015). Recently, various concentrations of RSV were used during semen storage. Addition of 10 or 50 μM RSV to the semen extender during goat semen cryopreservation improved post-thaw sperm viability, acrosome integrity, mitochondrial activity, membrane integrity, and overall motility by inhibiting ROS generation (Lv et al., 2019). In addition, Al-Mutary (2019) and Al-Mutary et al., (2020) reported that addition of 200 and 400 μM RSV to the Triladyl extender for ram semen during chilled storage improved spermatozoa quality, relieved oxidative stress, enhanced *in vitro* fertility, and increased longevity. Furthermore, addition of 50 μM RSV to the freezing extender for buffalo semen maintained sperm membrane integrity, decreased oxidative stress, reduced capacitation-like changes, and enhanced *in vitro* fertility (Longobardi et al., 2017). Furthermore, addition of 50 μM RSV to the boar semen extender protected spermatozoa from post-thaw ROS generation and improved their quality by enhancing AMP-activated protein kinase phosphorylation (Zhu et al., 2019). Addition of 50 and 100 μM RSV to the buffalo semen extender improved post-thaw spermatozoa quality parameters, antioxidant state, and fertilizing potential as well as prevented DNA fragmentation and lipid peroxidation (Ahmed et al., 2020). Recently, Sun et al. (2020) reported the beneficial actions of 50 and 150 μM RSV on boar sperm parameters as well as mitochondrial and oxidative states during liquid storage and cryopreservation. Finally, addition of 50 μM RSV during cryopreservation prevented early apoptosis of buck spermatozoa (Falchi et al., 2020).

3.2. Glutathione (GSH)

GSH minimizes or even prevents ROS-induced intracellular damage (Pompella et al., 2003) and plays pivotal roles in mitochondrial function, cellular membrane stabilization, and oxidative stress alleviation in mammalian spermatozoa (Llavanera et al., 2020). Addition of 0.5 mM GSH to the bull semen extender

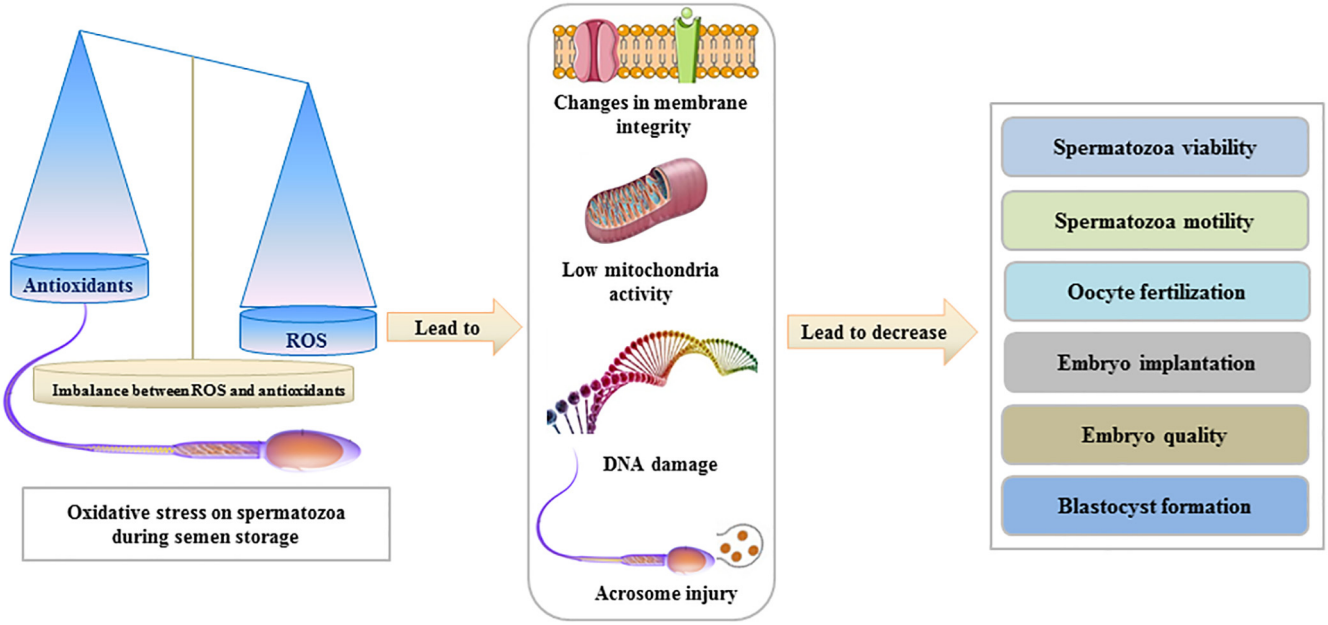

Fig. 1. Effects of oxidative stress during semen storage on spermatozoa quality.

Table 1
Primary effects of antioxidants during semen storage on spermatozoa quality of livestock animals.

Antioxidant	Antioxidant Concentration	Storage Type	Species	Primary Effects	References
Resveratrol (RSV)	10 and 50 μM RSV	CRY	Goat	• Improves sperm parameters • Improves mitochondrial activity and inhibits ROS production	Lv et al. (2019)
	200 and 400 μM RSV	LIQ	Ram	• Improves sperm parameters • Improves oxidative state of stored spermatozoa • Enhances *in vitro* fertility	Al-Mutary (2019), Al-Mutary et al. (2020)
	10, 20, 50, and 100 μM RSV	CRY	Buffalo	• Maintains the integrity of spermatozoa membrane and acrosome • Reduces capacitation-like changes • Enhances fertilizing capability and oxidative state • Improves mitochondrial state and ATP levels • Alleviates DNA fragmentation in spermatozoa	Longobardi et al. (2017), Ahmed et al. (2020)
	50 μM RSV	CRY	Boar	• Enhances AMP-activated protein kinase phosphorylation • Decreases ROS generation	Zhu et al. (2019)
	50 μM RSV	LIQ	Boar	• Improves sperm parameters	Sun et al. (2020)
	150 μM RSV	CRY	Boar	• Maintains sperm mitochondrial membrane potential • Decreases ROS and MDA levels • Increases total antioxidant capacity and B-cell lymphoma 2 levels	
Iodixanol (Id)	1.25, 2.5, and 5% Id	CRY	Buffalo	• Prevents lipid peroxidation of the spermatozoon membrane	Swami et al. (2017a)
	2.5% Id	CRY	Bovine	• Improves progressive motility and livability • Maintains plasma membrane and acrosome integrity • Does not improve the success of IVF and AI	Saragusty et al. (2009), Chuawongboon et al. (2017)
	10% Id	CRY	Bovine	• Protects spermatozoon plasma membrane	Marqui et al. (2018)
	5% Id	CRY	Ram	• Maintains sperm motility and membrane integrity • Prevents acrosomal and morphological damage	Cirit et al. (2013)
Cysteamine (CYM)	1 and 2 mM CYM	CRY	Buffalo	• Does not produce any beneficial effects	Swami et al. (2017b)
	5 mM CYM	CRY	Buffalo	• Adversely affects spermatozoa	
	1 and 2 mM CYM	LIQ	Ram	• Increases spermatozoa motility and viability • Improves oxidative and mitochondrial states	Peker Akalin et al. (2016)
	2.5–7.5 mM CYM	CRY	Bovine	• Decreases DNA damage and MDA levels • Activates antioxidants enzymes (superoxide dismutase and glutathione peroxidase)	Sarıözkan et al. (2015)
Glutathione (GSH)	0.5 mM GSH	CRY	Bovine	• Improves motility, sperm viability, and acrosome integrity	Gangwar et al. (2018)
	10 mM GSH	CRY	Canine	• Maintains acrosome integrity	Lucio et al. (2016)
	2 mM GSH	CRY	Boar	• Improves motility, membrane integrity, and nuclear stability	Estrada et al. (2017)
	1 and 5 mM GSH	CRY	Deer	• Enhances mitochondrial function • Improves spermatozoon kinematics	Anel-López et al. (2015)
	5 mM GSH	CRY	Boar	• Improves sperm viability and acrosome integrity • Maintains motility and nucleoprotein structure • Decreases intracellular peroxide levels	Yeste et al. (2014), Giaretta et al. (2015)
	100 and 200 mM GSH	LIQ	Ram	• Improves sperm motility, membrane integrity, mitochondrial activity, and antioxidant status	Shi et al. (2020)
Quercetin (QUE)	200 μM QUE	CRY	Buffalo	• Increases progressive motility • Maintains membrane, acrosome, and DNA integrity • Enhances *in vivo* fertility	Ahmed et al. (2019)
	10 μM QUE + Dimethylacetamide	CRY	Goat	• Suppresses lipid peroxidation • Improves spermatozoa motility parameters	Seifi-Jamadi et al. (2017)
	0.1 mM QUE	CRY	Equine	• Maintains spermatozoa motility	Seifi-Jamadi et al. (2016)
	0.15 mM QUE	CRY	Equine	• Enhances sperm motility and oocyte penetration • Maintains DNA integrity	Gibb et al. (2013)
	25 μg·mL-1 QUE	CRY	Bovine	• Protects spermatozoa DNA integrity	Avdatek et al. (2018)
	5 and 20 μg·mL-1 QUE	CRY	Ram	• Improves mitochondrial membrane potential	Silva et al. (2012)
L-arginine (LA)	0.5 mM LA	CRY	Ram	• Maintains membrane integrity	Özer Kaya et al. (2018)
	1 mM LA	CRY	Buffalo	• Enhances sperm motility and viability	Siddique and Atreja (2013)
	4 and 6 mM LA	LIQ	Goat	• Maintains sperm viability, membrane integrity, and motility • Inhibits MDA production • Decreases apoptotic rate	Susilowati et al. (2019)
	1 mM LA	CRY	Bovine	• Alters spermatozoa proteome abundance	Maciel et al. (2018)
Catalase (CAT)	500 IU·mL-1 CAT	CRY	Camel	• Prolongs spermatozoa survival • Does not improve fertilization rate following AI	Malo et al. (2019)
	15 IU·mL-1 CAT + antioxidants	LIQ	Equine	• Inactivates caspase-3 • Improves sperm motility and viability	Del Prete et al. (2019)
	5, 10, 15, and 20 IU·mL-1 CAT	CRY	Bovine	• Increases sperm motility and maintains plasma membrane integrity • Increases mitochondrial membrane potential • Maintains spermatozoa acrosome and DNA integrity • Decreases ROS levels and prevents lipid peroxidation • Does not improve IVF rate and embryo development	Arslan et al. (2019)
Melatonin (MEL)	0.05, 0.1, 0.2, and 1 mM	LIQ	Ram	• Maintains sperm motility, plasma membrane integrity, mitochondrial activity, and total antioxidant capacity and decreases MDA content	Dai et al. (2019)
	1 μM MEL	CRY	Equine	• Improves spermatozoa mitochondrial function	Lançoni et al. (2018)
	1 μM MEL	LIQ	Equine	• Protects spermatozoa acrosome, mitochondria, and membrane	Affonso et al. (2017)
	1.5 mM MEL	LIQ	Equine	• Improves spermatozoa motility by preventing lipid peroxidation	Izadpanah et al. (2015)
	1 mM MEL	LIQ	Ram	• Improves spermatozoa motility parameters	Ashrafi et al. (2011)

(*continued on next page*)

Table 1 (*continued*)

Antioxidant	Antioxidant Concentration	Storage Type	Species	Primary Effects	References
Cysteine (CYS)	15 mM CYS	CRY	Ram	• Enhances spermatozoa motility	Atessahin et al. (2008)
	2.0 mM CYS	CRY	Buffalo	• Activates the antioxidant system, improves sperm motility, and enhances *in vivo* fertility	Iqbal et al. (2016)
	10 mM CYS	CRY	Boar	• Positively affects sperm viability and acrosome integrity	Malo et al. (2010)

AI, artificial insemination; CRY, cryopreservation; IVF, *in vitro* fertilization; LIQ, liquid storage; MDA, malondialdehyde; ROS, reactive oxygen species

improved post-thaw spermatozoa motility, live sperm count, and acrosome integrity (Gangwar et al., 2018). Moreover, addition of 10 mM GSH to the canine semen extender protected acrosome integrity following cryopreservation, whereas the addition of 20 mM GSH promoted mitochondrial injury (Lucio et al., 2016). Additionally, supplementation of the freezing medium with 2 mM GSH for boar spermatozoa positively affected sperm motility, membrane integrity, and nuclear stability following freeze–thaw procedures (Estrada et al., 2017). Improved mitochondrial functionality and kinematics of cryopreserved red deer spermatozoa following the addition of 1 and 5 mM GSH to the semen extender were also reported (Anel-López et al., 2015). Similarly, addition of 5 mM GSH improved the cryotolerance of poor freezability ejaculates of boar spermatozoa (Yeste et al., 2014). Moreover, addition of high GSH concentrations (100 and 200 mM) during liquid storage maintained motility, membrane integrity, mitochondrial activity, and antioxidant status of ram spermatozoa (Shi et al., 2020). Giaretta et al., (2015) reported that addition of 5 mM GSH alone or in combination with 100 μM ascorbic acid to the freeze–thaw medium improved the quality of boar spermatozoa.

3.3. Quercetin (QUE)

QUE is a flavonoid that scavenges reactive nitrogen species and ROS (Boots et al., 2008). The addition of QUE at 200 μM level improved post-thaw progressive motility; membrane, acrosome, and DNA integrity; and *in vivo* fertility of buffalo bull spermatozoa (Ahmed et al., 2019). Addition of QUE (10 μM) in combination with dimethylacetamide during freezing protected goat semen by preventing lipid peroxidation and improving spermatozoa motion kinetics (Seifi-Jamadi et al., 2017). In stallions, addition of 0.1 mM QUE during cryopreservation maintained sperm motility (Seifi-Jamadi et al., 2016). Similarly, Gibb et al. (2013) reported that the use of 0.15 mM QUE during the storage of sex-sorted, frozen stallion spermatozoa enhanced sperm motility and oocyte penetration as well as maintained DNA integrity. However, contrary to the reported benefits, addition of 25 $\mu g \cdot mL^{-1}$ QUE to the freezing extender did not improve progressive and overall bull spermatozoa motility, although it positively affected spermatozoa DNA integrity (Avdatek et al., 2018). Conversely, the use of QUE at concentrations of 5–20 $\mu g \cdot mL^{-1}$ enhanced the mitochondrial membrane potential of ram spermatozoa (Silva et al., 2012).

3.4. Iodixanol (Id)

Id exhibits antioxidant properties depending on the amount of free radical generation in an extender or a medium (Swami et al., 2017a). Addition of 1.25%, 2.5%, and 5% (v/v) Id during cryopreservation protected buffalo spermatozoa by minimizing antioxidant consumption, which in turn prevented membrane lipid peroxidation (Swami et al., 2017a). Supplementation of the semen extender with 2.5% Id improved progressive motility, viability, and plasma membrane and acrosome integrity of frozen bull spermatozoa, although it did not affect the efficiency of IVF and AI (Chuawongboon et al., 2017). However, addition of Id at a high concentration (10%) to the bull semen freezing medium protected the plasma membrane (Marqui et al., 2018). Moreover, addition of 5% Id improved the post-thaw parameters of ram spermatozoa (Cirit et al., 2013). Id likely altered the ice crystal structure and increased glass transition temperature during cryopreservation of bovine spermatozoa (Saragusty et al., 2009).

3.5. Cysteamine (CYM)

CYM functions by promoting GSH production in cells to protect them from oxidative stress (Maher et al., 2008). Peker Akalin et al. (2016) reported that 1 and 2 mM CYM enhanced ram sperm motility and viability as well as oxidative and mitochondrial states following liquid storage. Moreover, Sarıözkan et al., (2015) demonstrated that addition of 2.5 or 7.5 mM CYM to the freezing and thawing solutions for bull semen decreased DNA damage and malondialdehyde (MDA) content of spermatozoa as well as activated antioxidant enzymes (e.g., superoxide dismutase and glutathione peroxidase). However, Swami et al. (2017b) reported that low CYM concentrations (1 and 2 mM) did not protect buffalo semen from cryopreservation effects, while high CYM concentrations produced detrimental effects (5 mM).

3.6. L-arginine (LA)

The use of LA is based on its potential to regulate superoxide and hydrogen peroxide levels (Scott and Bolton, 2000). *In vitro* addition of 0.5 mM LA during cryopreservation protected the membrane of ram spermatozoa and increased arginase activity in seminal plasma following freezing (Özer Kaya et al., 2018). Moreover, addition of 1 mM LA protected buffalo spermatozoa from lipid peroxidation as well as enhanced their motility and viability (Siddique and Atreja, 2013). In addition, storage of goat semen in a skim milk extender supplemented with 4 and 6 mM LA for 5 days improved spermatozoa quality by maintaining viability, membrane integrity, and motility; inhibiting MDA production; and decreasing apoptotic rate (Susilowati et al., 2019). Furthermore, supplementation of 1 mM LA to an *in vitro* capacitation medium for frozen–thawed bovine spermatozoa altered proteome abundance (Maciel et al., 2018).

3.7. Catalase (CAT)

CAT is a sensitive enzyme that can reduce hydrogen peroxide intoxication within cells by splitting it into water and oxygen molecules (Rubio-Riquelme et al., 2020). CAT supplementation (500 $IU \cdot mL^{-1}$) during thawing of dromedary camel semen extended spermatozoa survival, although it did not affect fertilization rate following AI (Malo et al., 2019). Addition of CAT (15 $IU \cdot mL^{-1}$) in combination with specific antioxidants to the chilled extender of stallion spermatozoa inactivated caspase-3 and improved motility and viability after 72 h of storage (Del Prete et al., 2019). Similarly, addition of various concentrations of CAT (5, 10, 15, and 20 $IU \cdot mL^{-1}$) to the semen extender during freezing improved bull spermatozoa quality following cryopreservation, although it did not affect IVF rate and embryo development (Arslan et al., 2019).

3.8. Melatonin (MEL)

MEL protects human spermatozoa by reducing nitric oxide levels (Du Plessis et al., 2010). In equines, addition of 1 μM MEL to the semen extender improved post-thaw mitochondrial functioning of spermatozoa (Lançoni et al., 2018). Moreover, MEL (at 1 μM) protected equine spermatozoa acrosome, mitochondria, and cellular membrane integrity after 8 h of refrigerated storage (Affonso et al., 2017). During refrigerated storage of stallion semen, 1.5 mM MEL improved sperm motility by decreasing lipid peroxidation after 48 h (Izadpanah et al., 2015). Addition of MEL (1 mM) to the ram semen extender improved spermatozoa motility parameters after storage at 5 °C for 48 h (Ashrafi et al., 2011). The best sperm motility, plasma membrane integrity, mitochondrial activity, and total antioxidant capacity and the lowest MDA content were observed following liquid preservation of ram semen in the presence of MEL (0.05, 0.1, and 0.2 mM) (Dai et al., 2019).

3.9. Cysteine (CYS)

CYS is an intracellular GSH precursor and contains a thiol group that can penetrate the plasma membrane of spermatozoa. It also act as an antioxidant (Coyan et al., 2011). The use of CYS during spermatozoa storage is limited compared to that of the other antioxidants. At 5 mM, CYS protected ram spermatozoa and enhanced their motility after thawing (Atessahin et al., 2008). Iqbal et al. (2016) reported that addition of 2.0 mM CYS during cryopreservation of buffalo spermatozoa activated the antioxidant system as well as enhanced sperm motility and *in vivo* fertility. Furthermore, higher CYS concentrations (10 mM) positively affected the viability and acrosome integrity of boar spermatozoa following cryopreservation (Malo et al., 2010).

4. Conclusion and future perspective

The present review emphasizes the positive effects of using RSV, GSH, QUE, Id, CYM, LA, CAT, MEL, and CYS on sperm quality of livestock animals during liquid storage or cryopreservation. However, the concentration of each antioxidant exclusively depends on species, semen extender or preservation medium composition, storage type, and *in vitro* stress conditions. Therefore, further studies on optimizing the conditions of *in vitro* semen storage, composition of the preservation medium, and procedures for maintaining the optimum quality of stored spermatozoa are warranted. Moreover, studies examining the fertility of stored spermatozoa (either *in vivo* or *in vitro*) would advance the field of AI. Specific studies investigating the precise mechanisms of action of the abovementioned antioxidants during semen storage are also imperative. Finally, the field of sperm cryobiology would benefit from studies on altered expression patterns of specific genes in persevered spermatozoa following antioxidant supplementation and their potential use as markers for sperm quality.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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