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EFFECT OF ANTIOXIDANT SUPPLEMENTATION ON PIG AND HORSE GAMETE STORAGE

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Abstract

Abstract

Liquid storage and cryopreservation are the usual techniques actually utilized for gamete storage of mammalian domestic animals.

It is known that decreasing temperatures reduce or stop cellular metabolic activity, which is restored after warming. Liquid cold storage allows to maintain semen viable for a short period (from 24h to few days, according to different species), while freezing storage is used to preserve semen and oocytes for a long period of time. At present many studies have been performed to improve the efficiency of gamete liquid and cryopreservation techniques, in order to increase the percentage of viable cells after storage and to enhance their quality and fertilizing ability.

According to recent studies, the antioxidant supplementation on gamete processing and/or storage solutions improves gamete quality parameters, after cooling or storage at sub zero temperature. Indeed, the oxidative stress is one of the main reasons of cellular cryoinjuries: the increase of reactive oxygen species (ROS) during gamete storage provokes irreversible membrane and intracellular damage, with a consequent loss of physiological cellular functions.

The aim of the present study was to investigate the effects of antioxidant supplementation on pig and horse gamete storage. The work includes:

- the study of resveratrol (RESV) addition on in vitro pig oocytes maturation medium (IVM) and vitrification warming solution
- the study of RESV supplementation during liquid storage of stallion semen, cooled at 4°C and 10°C
- the study of reduced glutathione (GSH) and L-ascorbic acid (AA) supplementation on freezing and/or thawing solutions during boar semen cryopreservation

Vitrification with the Cryotop method is actually the most frequently used technique for oocyte cryopreservation. Compared with other domestic species, the high intracellular lipid content and the wide cell volume make porcine oocytes more susceptible to storage at low temperature, with a consequent decrease of oocytes survival rate and apoptotic progression after thawing. RESV a polyphenolic compound present in several vegetal sources, has been reported to exert, among all its other biological effects, an anti-apoptotic action.

The aim of the first work was to determine the effects of RESV on the apoptotic status of porcine oocytes vitrified by Cryotop method, evaluating phosphatidylserine (PS) exteriorization and caspases activation. RESV (2μM) was added during: IVM (A); 2 h post-warming incubation (B); vitrification/warming and 2 h post-warming incubation (C); all previous phases (D). The obtained data demonstrate that RESV supplementation in various

steps of IVM and vitrification/warming procedure can modulate the apoptotic process, improving the resistance of porcine oocytes to cryopreservation-induced damage.

In the second work different concentrations of RESV (10, 20, 40, and 80 µM) were added during liquid storage of stallion sperm for 24 hours at either 10°C or 4°C, under anaerobic conditions. Several previous studies demonstrated that cooling storage induces oxidative stress, caused by abnormal ROS production, leading to several cellular damages, collectively called "cold shock". In our study, sperm quality parameters, such as viability, motility, acrosome status and chromatin structure, were assessed immediately after RESV supplementation (0 hours) and after 24 hours of storage. Our findings demonstrate that RESV supplementation does not enhance sperm quality of stallion semen after 24 hours of storage. Moreover, the highest RESV concentrations tested (40 and 80µM) could damage sperm functional status, probably acting as pro-oxidant.

Finally, in the third work other two antioxidants, AA (100 µM) and GSH (5mM) were added on boar freezing and/or thawing solutions. Boar semen is more sensitive respect to other species to cryopreservation procedure. To improve cryopreservation technology for boar semen, many studies have been focused on trying to understand the mechanisms underlying cryodamage. From these reports, the most evident damage from freeze-thawing procedures has been demontrated to affect plasma and acrosome membranes, mitochondrial midpiece, and axonema. In our study various sperm parameters (levels of free cysteine residues in sperm nucleoproteins, sperm viability, acrosome membrane integrity, intracellular ROS, and total and progressive motility) were evaluated before freezing and at 30 and 240 minutes after thawing. Our results showed that GSH and AA significantly improved boar sperm cryotolerance when they were separately added to freezing and thawing media. However, the highest improvement was recorded when both freezing and thawing media were supplemented with 5 mM of GSH plus 100 µM of AA. This improvement was observed in sperm viability and acrosome integrity, sperm motility, and nucleoprotein structure. Although ROS levels were not much increased by freeze-thawing procedures, the addition of GSH and AA to both freezing and thawing extenders significantly decreased intracellular peroxide levels and had no impact on superoxide levels.

Therefore, according to our results, we can conclude that:

- RESV can act as pro-oxidant or antioxidant in a concentration and in species-specific manner. Indeed, while low doses of RESV (2µM) improve and optimize the quality and the resistance of IVM porcine oocytes to cryopreservation, probably exercising antioxidant action,

higher doses of RESV (40 and 80 µM) could probably induce an oxidative and/or proapoptotic damage in stallion sperm.

-Supplementation of freezing and thawing media with both GSH and AA has a combined, beneficial effect on frozen-thawed boar sperm, which is greater than that obtained with the separate addition of either GSH or AA.

Abbreviations

AA	Acorbic Acid
AI	Artificial Insemination
CPA	Cryoprotectants
DMSO	Dymethylsulfoxide
EG	Ethylene Glycol
GSH	Glutathione
GV	Germinal Vescicle
IVM	In Vitro Maturation
LPO	Lipid Peroxidation
RESV	Resveratrol
ROS	Reactive oxigen species

Introduction

Gamete cryopreservation techniques

Cryopreservation of gametes is a crucial step for the spread and conservation of animal genetic resources. Indeed, haploid cell cryopreservation is used to preserve rare breeds, well adapted to environmental changes, and threatened species, in order to conserve the livestock genetics and to maintain the biodiversity (Holt 1997; Myers et al. 2000). For this, the importance to create germinal plasm cryobanks was discussed in these years and European and international consultative forums were created within the European Union, European Association for Animal Production (EAAP) and Food and Agricultural Organization of the United Nations(FAO). Semen banks are currently developed especially for rare domestic breeds (cattle, sheep, goats and pigs) and for non domestic species, for as much as, despite the management and conservation of threatened species is being promoted extensively the approach is too costly and requires greater technical expertise(Mara et al., 2013). Germplasm cryopreservation is also important to solve problems of human infertility, life threatening diseases and preservation of DNA samples (Wildt, 2000; Barbas and Mascarenhas, 2009).

Cryopreservation is a process where cells or tissue are preserved by cooling to sub-zero temperatures. At this conditiions any chemical or enzymatic activities are stopped and restart only after thawing (Mazur 1984). All cryopreservation methods include temperature reduction, cellular dehydration, freezing and thawing procedures (Medeiros et al., 2002). During freezing step, as medium with cellsis cooled below the freezing point, microscopic ice crystals start to nucleate. What remains between the growing ice masses is the so-called unfrozen fraction, in which all cells and all solutes are confined. As the temperature decreases, the extracellular concentrations of sugars, salts and cryoprotectants (CPA) (e.g. glycerol) increases, provoking a consequent efflux of water from cells. As cooling continues, the viscosity of the unfrozen fraction ultimately becomes too high for any further crystallization. The remaining unfrozen fraction turns into an amorphous solid that contains no ice crystals(Rapatz and Luyet, 1960; Benson et al., 2012). During freezing, cells are faced with very high concentrations of solutes in the unfrozen fraction. The high salt concentration in medium may result in loss of stability in the membranes and denaturation of proteins and it may cause extracellular salts to enter the cells, a process known as “solute loading” (Lovelock, 1953; Daw et al., 1973; De Loecker and Penninckx, 1987). Moreover the fast efflux of water causes a rapid decrease in the volume of the cells to approximately 50 percent of their original volume, leading to structural deformation of the cells. Further mechanical stresses may be

caused by cells being confined in very narrow channels of unfrozen solution and squeezed between growing masses of ice (Rapatz and Luyet, 1960).

To avoid physical and chemical stresses derived from cooling, freezing and thawing of sperm several cryoprotectans are utilized (Purdy, 2006). CPA operate by increasing the solute concentration (salts plus non-electrolytes), reducing salt concentration in the unfrozen fraction and inside the cells and thus decreasing the extracellular and intracellular ice formation (Mazur and Rigopoulos, 1983). They are generally divided in two categories: permeating and non-permeating. Permeating cryoprotectans (propylene glycol, ethylene glycol, glycerol and dimethylsulphoxide) are small molecules that readily penetrate the membranes of cells, form hydrogen bonds with intracellular water molecules and lower the freezing temperature of the resulting mixture, preventing ice crystallization (Holt 2000). On the other hand, non permeating CPA (egg yolk, non-fat skimmed milk, trehalose, aminoacids, dextrans, sucrose) remain extracellular, drawing free water from within the cell and causing dehydration of the intracellular space. Moreover they act as a solute, lowering the freezing temperature of medium and decreasing extracellular ice formation (Aisen et al. 2000; Benson et al. 2012; Kundu et al. 2002).

Two methods can be used for gamete cryopreservation: slow freezing and vitrification. Slow freezing utilzes slow cooling rates and low concentrations of CPA. On the contrary vitrification is a rapid method based on an high concentration of CPA that inhibit the formation of ice crystal and lead to the development of solid glasslike system, in which water is solidified but not crystallized(Benson et al., 2012; Arav, 2014). The only difference with slow freezing is that, thanks to the rapid cooling, ice cannot form in any part of the sample. The vitrified state and the associated physico-chemical conditions obtained using vitrification methods, are to some extent similar to those obtained by slow cooling, but the way of reaching this point is quite different. The optimal cooling rate for the cryopreservation success is determined from the interactions between two effects: intracellular ice formation or hyperosmotic solution injury (Mazur, 1990). Al low cooling rates the higher concentration of extracellular media causes the exosmosis of water and the influx of permeating CPA into the cell, with a consequent increase in intracellular osmolarity and a cellular dehydration. In this situation the likelihood of intracellular ice formation is minimized, but the prolonged exposure to high concentration of salts of extracellular media causes irreversible membrane damage. On the other hand at high cooling rates the intracellular ice formation is the dominant factor in cell damage, while the toxic effect of the solution is minimized. Therefore, the combination of these two effects implies that there will be an inverted “U” shaped survival curve (Fig. 1) and

an optimal cooling rate that minimizes both the toxic solution effects and intracellular ice formation (Mazur et al., 1972;Fiser and Fairfull, 1986;Muldrew, 1994; Benson et al., 2012). Different cells or other biological materials (embryos, tissue pieces) may have different optimal cooling rates. The optimal cooling rate of cells is largely determined by their volume, their membrane surface area (volume to surface area ratio), and by the permeability of the membrane to water and to cryoprotectant (Gosden, 2011).

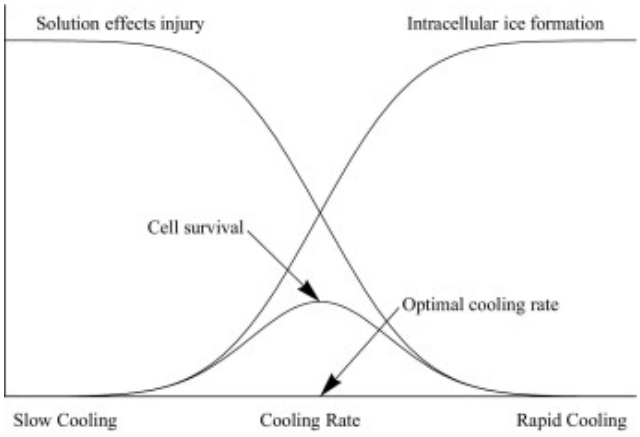


Fig. 1 Plot of the interaction between cooling rate, "solution effects" injury, ice formation injury, and cell survival. At low cooling rates "solution effects" are the dominant factor in cell damage, but as cooling rates increase and exposure time decreases, these effects are minimized. Conversely, at high cooling rates intracellular ice formation is the dominant factor in cell damage, and as cooling rates are decreased, the likelihood of intracellular ice formation decreases. The combination of these two effects imply that there will be an inverted "U" shaped survival curve and an optimal cooling rate that minimizes both the solution effects and intracellular ice formation (Benson et al., 2012; redrawn from Muldrew, 1994).

Semen cryopreservation of domestic animal species

Spermatozoa were the first mammalian cells to be successfully cryopreserved. This success was due to the serendipitous discovery by Polge and co-workers (1949) of the cryoprotective effect of glycerol. The principal technique used in domestic animal sperm preservation is slow cryopreservation method using freezing rate of 0.5-100°C/min. Indeed, vitrification is not performed for semen preservation because heat transfer in sperm cells is too slow to permit vitrification without the risk of solution effects of crystallization (Watson and Martin 1975; Arav et al., 2002). The extenders used for semen preservation on domestic animal species must have an adequate pH and buffering capacity, suitable osmolarity and should protect sperm cells from cryogenic injury (Salamon and Maxwell, 2000). Generally, sperm cryopreservation extenders includes a non permeating cryoprotectant (milk or egg yolk), a penetrating cryoprotectant (glycerol, ethyilen glycol or dimetil sulfoxide), one or more sugars (glucose, lactose, raffinose, saccharose , or trehalose), salts (sodium citrate, citric acid) and antibiotic (penicillin, streptomycin) (Barbas and Mascarenhas 2009). The role of sugars, that are not able to diffuse across the plasma membrane, is to create an osmotic pressure that induces cell dehydration and lowers the incidence of intracellular ice formation. Glycerol is the principal permeating cryoprotectant used for semen cryopreservation. When cells are brought into a glycerol medium, after a short period of equilibrium, cells regain their original volume. Indeed, the initial hyperosmotic pressure of glycerol medium, which would induce water efflux from cells, is later counteracted by glycerol cell entry. Moreover part of the extracellular and intracellular water is replaced by the glycerol, resulting in a reduction of ice formation and cellular shrinkage. Glycerol is used within the range of 4-6% because upper levels can induce osmotic stress and toxic effects that vary according to the species. Moreover glycerol may be added initially, just after semen collection (one step method) or later in a separated fraction (glycerolated medium), which is joined after semen refrigeration and before semen freezing (two step method) (Salamon and Maxwell, 1995; Holt, 2000; Barbas and Mascarenhas, 2009).

Another component of semen extenders is egg yolk, protecting the sperm against cold shock. The low density lipoproteins and the phospholipids of egg yolk avoid the cellular membrane damage during cooling, freezing and thawing (Medeiros et al., 2002; Purdy 2006).

The protocols for semen cryopreservation differ according to the species, in the extenders used and in cooling/freezing/thawing rate employed. Generally after ejaculation semen is diluted of 2-5 folds with the extender. After dilution, semen is cooled from room temperature to 4°C. It was demonstrated that rapid cooling from 30 to 15°C may not affect sperm survival

(Leboeuf et al., 2000); to the contrary fast cooling from 15 to 10,5 or 0°C decreases thawed sperm motility and fertility (Fiser et al., 1987; Salamon and Maxwell, 1995). The refrigeration step takes place in a refrigeration chamber at 4°C or with a controlled cooling rate during average 4h. Glycerol, according to the species can be added immediately after semen dilution or in a second moment, after semen refrigeration (Barbas and Mascarenhas, 2009). Semen after refrigeration is packed in 0,25 or 0,50 ml mini-straws that are cooled by cold nitrogen vapor in quite simple, non-programmable, freezers or using more sophisticated, programmable, freezers where the temperature inside the cooling chamber can be accurately controlled and the time course of the temperature can be programmed. In order to avoid cellular damage from -10°C to -60°C cooling rate must to be faster than 50°C/min, while afterwards can slow at 20-30°C/min until freezing is completed (Byrne et al., 2000; Anel et al., 2003). Optimal freezing rates for sperm, from 4°C to -140-150°C, vary according to species from 1 to 10°C/min for humans and 50 to 100°C/min for bulls (Woelders 1997). After cooling and freezing steps, semen is plunged into liquid nitrogen and stored in nitrogen liquid can. The obtained straws, can be stored in liquid nitrogen for a long period, until their employment. The warming phase is also important for the survival of sperm as well as the cooling phase (Fiser et al., 1987) and in this phase semen will also across the critical temperature between -60 and -15°C. As the cooling rate, thawing must be sufficiently high to prevent intracellular recrystallisation and low enough to permit adequate cell dehydratation. Straws of semen are generally thawed in a water bath at 37-41°C during 20-30 sec because it was demonstrated that a fast thawing permits a brief exposition to the concentrate solutes and CPA and a more rapid restoration of intracellular and extracellular osmotic equilibrium than a slower thawing (Fiser et al., 1987). Thereafter the extremities of straws are cut and semen is diluted into a thawing solution. A part from the differences among species, that involve the utilization of specific cryopreservation protocols for each one, there are individual dissimilarities within the same species that divide donors of semen in good and poor freezers (Knop et al., 2005; Hernández et al., 2006; Nichi et al., 2007; Barbas and Mascarenhas, 2009). However, predictive parameters of semen freezability are still investigated (Blesbois et al., 2008; Casas et al., 2009).

AI with frozen-thawed semen is daily used from the 1950s in bovine species with satisfactory results (Bratton et al 1955): good fertility rates (50-60%) are obtained with doses containing $60x10^6$/ml spermatozoa (to achieve $15x10^6$ of viable progressive spermatozoa), making AI commercially available (Watson 2000). On the contrary, AI with frozen-thawed semen is rarely used in pig and horse. Stallion and boar semen are ejaculated at a relatively low sperm

concentration as compared to bull semen. In addition, it has been demonstrated that seminal plasma is detrimental to semen survival during cryopreservation. For these reasons in these species extended semen is centrifuged and the obtained pellet is diluted in freezing media (Mazur et al., 1972; Fraser et al., 2007; Ramires Neto et al., 2013). Preservation of boar semen was developed in the 1970s, however, the method used was different to the that utilized in the other species (Pursel and Johnson 1975). Boar semen is highly sensitive in the period immediately after semen collection, while is more resistant to cold shock after 18-24h of preservation at 16°C (Pursel and Johnson 1975). Therefore the ideal time for boar semen cryopreservation is 18-24h after collection. Boar semen is usually cooled to 5°C at rate of 1°C/min in a programmable freezer. Thereafter 2% glycerol on the total medium is added and semen is packed in 0,25 or 0,50 ml mini-straws that are cooled in programmable freezer with liquid nitrogen (Fig. 2). Pig sperm cryosurvival is considerably low in comparison to the other species and fertility is high (70-80%) when artificial insemination (AI) is done during the 4h preceding ovulation (Waberski et al. 1994; Thurston et al., 2002) . Therefore the correct identification of estrous is fundamental to successful AI . For this problem and due to the relatively low numbers of insemination doses obtained from each ejaculated, freezing techniques on boar semen are less attractive in production systems (Rodríguez-Martínez et al. 2008). As regarding small ruminants, semen is deposited in cervix with low fertility results (17-20%) (Barbas and Mascarenhas 2009). Ram sperm concentrations used for cryopreservation varies between 80 and 500x10^6/ml (Ritar et al., 1990).

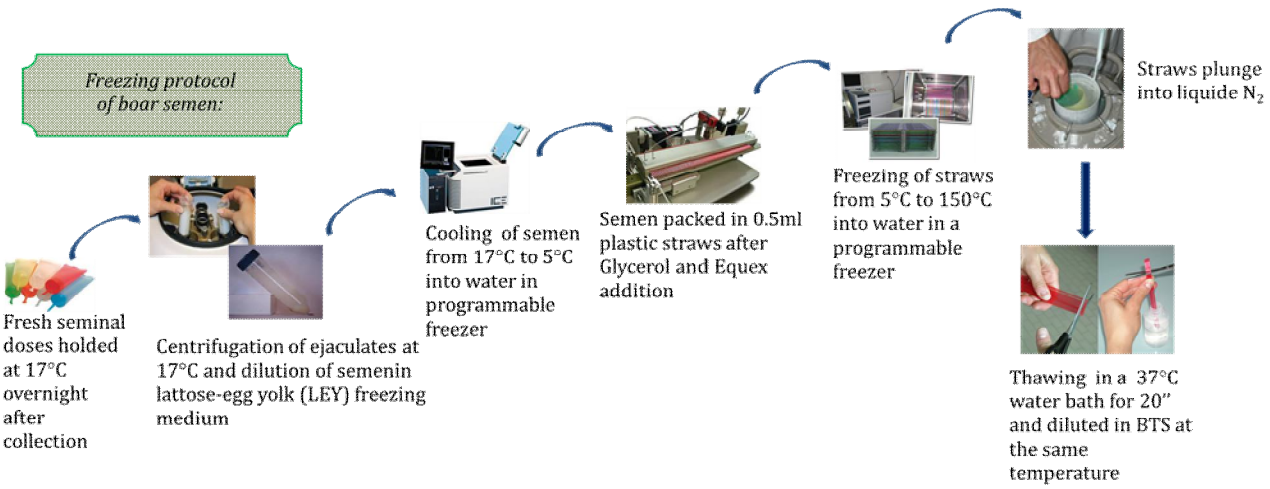


Fig. 2 Freezing procedure of boar semen (Westendorf et al., 1975).

Oocyte Vitrification

While semen and embryos are the most reliable materials to be cryopreserved, oocytes are extremely sensitive to chilling (Woods et al., 2004; Mara et al., 2013). Indeed the large size of oocytes and the low surface to volume ratio (the volume of the mammalian oocytes is in the range of ten orders of magnitude larger than that of the spermatozoa), make dehydration and penetration of CPA difficult to achive (Prentice and Anzar, 2010). Despite the cryopreservation technique is not yet established as in semen or embryos, offsprings have been born from cattle, sheep, horse and recently also pig frozen-thawed oocytes (Maclellan et al., 2002; Vieira et al., 2002; Woods et al., 2004; Dang-Nguyen et al., 2011; Somfai et al., 2014). In these last several years, almost all advancements in oocyte cryopreservation have been made using vitrification techniques (Vajta and Kuwayama, 2006)(Fig. 3).

Vitrification is defined as the solidification of a solution brought about not by crystallization but by extreme elevation in viscosity during cooling (Fahy et al., 1984). The glass state has the ionic and molecular distribution of the liquid state, thus avoiding both chemical and mechanical damage.

Vitrification method requires three important conditions: 1) R*apid cooling and warming rate* (approximately is necessary a cooling rate of 107°C/s to vitrify pure water). This high cooling rate is performed through the direct transfer of oocytes from 37°C to -196°C of liquid nitrogen and conversely for the worming rate. 2)*Minimum volume of cryo-solutions* (small drops with minimum size around 8 to 10 μm). 3) *Viscosity*, which is reached through high concentration of CPA (Arav, 2014). Combining these three factors can result in the following general equation for the probability of vitrification:

Probability of vitrification=cooling and warming rates × viscosity/volume

Decreasing the vitrified volume and increasing the cooling rate allow a moderate decrease in CP concentration so as to minimize its toxic and osmotic hazardous effects (Arav, 2014).

Apart from the total elimination of ice crystal formation, the increased cooling rate of vitrification decreases chilling injury that normally occurs during oocyte slow freezing, (i.e. damage of the intracellular lipid droplets, lipid-containing membranes and the cytoskeleton structure), passing rapidly through the dangerous temperature range between +15° and −5°C (Martino et al., 1996; Dobrinsky, 1996; Isachenko et al., 2001; Mara et al., 2013). On the other hand, the toxicity of this high concentration of CPA (5-7M) require a cooling rate such that the cell can be exposed to the cryoprotectant solutions for a very short period of time and in a

minimum volume of the solutions, which maintains them at a liquid state at a subfreezing temperature (Arav, 2014). For this purpose, different carrier tools were applied to minimize the volume and to submerge the samples quickly into the liquid nitrogen, using surface or tubing devices such as open pulled straw (OPS)(Vajta et al., 1997), cryotops and cryotips (Kuwayama et al., 2005). The open system, such as the Cryotop method, permits to reach a high cooling rate by the direct immersion of the cryotop device in liquid nitrogen, while high warming rates are achieved by direct exposure to the warming solution. However, high concentration of permeating cryoprotectors, such as dymethylsulfoxide (DMSO) and ethylene glycol (EG), commonly used in combination, are required for successful vitrification (Arav, 2014). Therefore non-permeating cryoprotectors, such as the charboidrates trehalose and sucrose, are usually added in cryosolutions in order to decrease the concentration of permeating cryoprotectors and consequently their toxic effects on cells and also for their stabilizing effect on membranes (Arav, 2014). In the past 15 years, several different combinations of CPA have been proposed for vitrification of mammalian oocytes and embryos, with an immense amount of variations regarding the concentrations, incubation times and other conditions (Vajta, 2000).

Oocytes may be cryopreserved after maturation presenting the second meiotic spindle or in germinal vescicle phase (GV). Nevertheless, several studies reported that oocytes in GV stage are more sensitive to chilling injury (Agca 2000; Ledda et al. 2001; Rojas et al. 2004).

Encouraging results have been obtained from vitrification of bovine oocytes (Papis, Shimizu, and Izaike 2000; Vieira et al. 2002; Zhou et al. 2010). Instead, porcine oocytes are much more cryoinstable than bovine ones, due to the higher intracellular lipid content (Isachenko et al., 2001) and, only in the last year, the first offspring born from swine oocytes vitrification has been obtained (Somfai et al., 2014).

As regards the other species, equine immature oocytes undergo significant damage during freezing, while mature oocytes exhibit spindle abnormalities and poor developmental competences after devitrification (Tharasanit et al., 2006; 2009). Finally, at the present time a limited number of studies have been done on the vitrification of small ruminant oocytes (Succu et al., 2007).

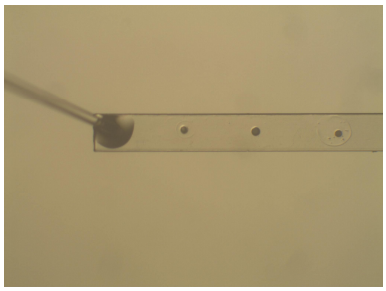
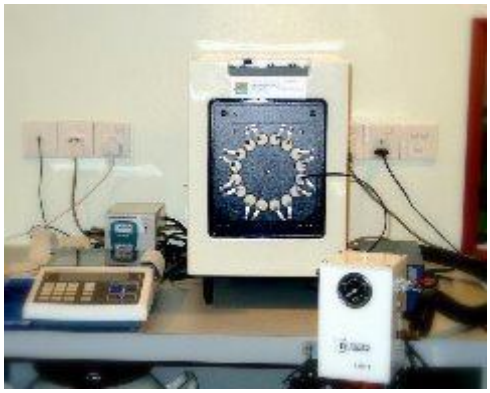
Vitrification	Conventional slow freezing
• Simple, fast, less then 10 min • Inexpensive, no machine needed • Sample volume: 1-2 uL • No ice crystal formation • Less mechanical damage • More chemical damage • Opened or closed system • Higher CPA concentration	• More than 3 h • Expensive, needs freezing machine • Sample volume: 100-250 uL • Accompanied with ice crystal formation • More mechanical damage • Less chemical damage • Closed system only • Lower CPA concentration
 Drops of medium with oocytes placed on a Cryotop.  Example of Cryotop devices.	 Programmable freezing planer for slow freezing procedure.

Fig. 3 Vitrification versus conventional slow freezing.

Sperm liquid storage

The main method of semen preservation in a liquid state is storage at reduce temperatures (0-5°C or 10-16°C), in order to extend sperm survival by reversal metabolic inactivation of spermatozoa. As regard stallion, cooled semen is generally stored either at 5°C or 10°C for 24-48h; however after that time, pregnancy rate decreases dramatically (Batellier et al. 2001). Although several new extenders are being adopted in these years in order to prolong lifespan of spermatozoa, other aspects, such as semen collection and processing, must be taken into account as they affect the quality of equine spermatozoa during cooled storage and limit the preservation of semen for a longer time (Aurich 2008). Indeed the number of mounts and the time necessary to obtain the semen collection influence semen composition and quality and the probability of semen contamination with bacteria (Loomis, 2006; Aurich and Spergser, 2007; Price et al., 2008). Stallion semen extenders actually used are principally based on milk or egg-yolk, which stabilize sperm plasma and acrosomal membrane against cold shock (Pagl et al. 2006). Moreover, an important practice to improve quality of cooled-stored stallion semen is to reduce seminal plasma concentration to 5-20%, as greater concentrations decrease semen motility and fertility (Loomis, 2006).

Thus, although centrifugation has been shown to be critical for the sperm plasma membrane as it may induce peroxidation, it is used as an alternative to dilution of the ejaculate with a suitable extender to a ratio of more than 1:3, in order to reduce the negative effect of seminal plasma. In addition, another important factor affecting the longevity of equine spermatozoa during storage is the individual differences between stallions, which may be classified as “good” and “bad” coolers (Brinsko et al., 2000). These differences depend on the quality of native semen, on the composition of seminal plasma and sperm plasma membranes (Aurich, 2005a). For these reasons it could be important to find the optimal semen extender combination for stallions with poor semen quality (Aurich 2008). Especially in “poor cooler” stallions, centrifugation and partial removal of seminal plasma result in reduced decrease in the percentage of progressively motile spermatozoa, even after storage times of more than 24 h (Brinsko et al., 2000; Aurich, 2005).

It was demonstrated that 5°C is the optimal storage temperature for maintenance of motility and fertility of stallion semen, irrespective of oxygen exposure, while storage at higher temperatures as 15°C requires aerobic conditions and antibiotics additions (Batellier et al., 1998; Aurich and Spergser, 2007; Price et al., 2008). The principal critical step of cooling rate is from 18 to 8°C , resulting in “cold shock” for sperm plasma membrane (Varner et al., 1989).

The injury is reduced if cooling rate is slow (<0.3°C/min) (Francl et al., 1987; Amann and Waberski, 2014). The most desirable cooling rate around the above-mentioned parameter and the optimal maintenance of temperature are provided by the "Equitanier"(Hamilton-Thorn Rsearch, Danvers, MA,USA), used from the large artificial insemination centers. Although the improvements obtained in these years, in the majority of stallion, fertility cannot be maintained for periods longer than 48h (Aurich, 2008).

As regards liquid storage of ram and bull semen the common diluents used contain Tris (hydroxymethyl)aminomethane (Tris)–based medium added with milk or egg yolk, glucose, saline buffers and antibiotics. Bull diluted semen can be refrigerated at 5°C for 2-4 days, while ram semen stored for more than 24 h has a considerable decline of fertility, at rate of 10-35% per day of storage (Salamon and Maxwell, 2000; Vishwanath and Shannon, 2000).

Boar spermatozoa are more susceptible to cold shock than other domestic animal species, especially when rapid cooled at temperatures below 15°C. For this reason, boar semen is usually stored at temperature of about 15°C (Johnson et al., 2000). Cold shock is related to the lipid composition of the membrane bilayer affecting the fluidity of membrane at low temperatures. Indeed, the lower percentage of sature fatty acid and higher percentage of insature ones on plasma membrane, respect of the other species, induce a restriction of lateral movement, that results in a transition from a fluid to a gel phase (De Leeuw et al., 1990). Additionally, the lower percentage of cholesterol, especially in the inner monolayer of the membrane, determines a reorganization of membrane particles, resulting in permeability increase, reduction of enzymatic activity and changes in lateral motion channels (De Leeuw et al. 1990). Moreover the increase of permeability could stimulate the ingress of free calcium ions into the cell, inducing the capacitation process (Ortman and Rodriguez-Martinez, 1994).

Boar semen is preserved at pH between 7,2 and 7,5. The common diluents utilized for boar semen liquid storage, such as BTS and Androhep, contain glucose, saline buffers such as sodium bicarbonate or sodium citrate and potassium chloride, zwitteronic organic buffer (TES or HEPES), ethilenediamine-tetra-acetic acid (EDTA) and antibiotics (Waberski et al., 2011). The Androhep diluent, containing HEPES and BSA, is used for up 5 days of semen storage in routine AI and has therefore been called a long-term extender (Dubé et al.,2004). HEPES, heavy metal chelant agent and EDTA, divalent metal ions (Ca++) chelant, are added in order to control pH and to prevent capacitation and the acrosome reaction, respectively (Johnson et al., 2000).

Oxidative stress during liquid storage and cryopreservation of gametes

The oxidative stress is one of the most important factors that contribute to poor quality mammalian gametes after liquid storage or cryopreservation (Curry, 2007; Kadirvel et al., 2009). According to several studies, the sub-lethal damage to cellular structures during gamete preservation is due to the increase of reactive oxygen species (ROS). ROS, produced by oxidation–reduction (REDOX) reactions, include the superoxide anion (•O2–), hydrogen peroxide (H_20_2), the hydroxyl radical (OH•)(Veal et al., 2007). The effects of ROS are dose-dependent: while physiological levels of ROS are fundamental for physiological reproductive processes, such as sperm capacitation, acrosome reaction, and maintenance of fertilizing ability and gamete interaction, increased amounts of reactive oxygen species (ROS) may cause cytotoxic damage to gametes (de Lamirande and Gagnon, 1995; Attaran et al., 2000; Desai et al., 2009; Gonçalves et al., 2010). It has been demonstrated that cooling procedure can damage plasma membrane structure, resulting in loss of sperm motility and/or fertilizing ability (Aitken and Baker 2006). The cholesterol/phospholipid ratio and the concentration of polyunsaturated fatty acids, which differ between species, but also between individual males within a species and between individual ejaculate of a single male, determine a different ROS sensibility and thus affect the suitability of the ejaculates to long-term (freezing) and short-term (liquid) storage (Gadella et al., 2001; Aurich, 2005). The excessive generation of ROS can be generated from leukocyte contamination during semen processing and from immature spermatozoa present in the ejaculate, which excess residual cytoplasm (Walczak-Jedrzejowska et al., 2013). In addition spermatozoa themselves produce intracellular ROS through mithocondrial activity (Amaral et al. 2013). Brouwers and Gadella (2003) showed that lipid peroxidation (LPO) in bull spermatozoa occurs in mid piece and tail regions, mainly in the inner leaflet, suggesting an endogenous ROS source identified in the mitochondria. The lipid oxidation process leads to a loss of membrane integrity and an increase in its permeability which results in inactivation of cellular enzymes, structural DNA damage, decreased sperm motility, due to ATP depletion because of mitochondrial malfunctions, decreased sperm viability and cell apoptosis (Kodama et al., 1996; Nichi et al., 2007; Kadirvel et al., 2009).

As specifically regarding gamete cryopreservation, cellular cryoinjuries are the result of several factor, such as ice nucleation and dehydration, which are responsible for osmotic stress and damage to membrane structure (Meryman, 1971). The increase of ROS during oocytes and sperm cryopreservation alter the cellular redox equilibrium, leading structural

and functional cellular damage (Agarwal et al.; de Lamirande and Gagnon, 1995). The protective antioxidant system in the semen is composed of enzymes, as well as non enzymatic substances, which closely interact with each other to ensure optimal protection against ROS. Antioxidant enzymes include superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR). SOD converts •O2– to H_2O_2. This compound is then transformed to water by catalase and GPx, as shown in following image (Bansal and Bilaspuri 2010).

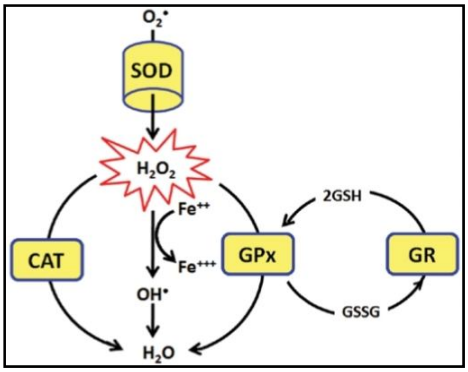


Fig. 4 Cellular antioxidant system (redrawn from Pandey et al., 2010).

Non enzymatic antioxidants include ascorbate, GSH, tocopherols, flavonids and carotenoids (Bansal and Bilaspuri 2010; Tatone et al. 2010). During cryopreservation the physiological balance between ROS production and antioxidant cellular defenses is altered because of an increase of ROS production and a parallel decrease of antioxidant cellular content. In sperm freezing-thawing, ROS generation occures predominantly in the mitochondrial midpiece of sperm cells and results from the alterations to electron transport chain in the mitochondria and to NADPH oxidase in the plasma membrane (Brouwers and Gadella 2003). Mammalian sperm, during terminal stages of differentiations, lack a significant cytoplasmic component, which contains sufficient antioxidants to counteract the damaging effects of ROS and LPO (Bansal and Bilaspuri 2010). Moreover, the removal and dilution of seminal plasma which is well endowed with enzymatic and non-enzymatic antioxidants (i.e. urate, AA, vitamine E, taurine, hypotaurine, carotenoids, pyruvate and GSH) causes a considerable reduction of spermatozoa antioxidant defence (Tatone et al., 2010). In addition, direct analysis of redox level showed that intracellular GSH decrease after oocytes and sperm cryopreservation, with a concomitant increase of hydrogen peroxide in a species-specific manner (Gadea et al. 2004;

Somfai et al. 2007; Stradaioli et al. 2007). Compared to other mammalian species, boar spermatozoa are more susceptible to peroxidative damage, due to the higher concentration of unsaturated fatty acids (UFA) in the phospholipids of the plasma membrane (Cerolini et al., 2001) and the relatively low antioxidant capacity of boar seminal plasma (Brezezinska et al. 1995).

Taken together these findings suggest a crucial and decisive involvement of oxidative stress in gamete cryoinjury process.

As concerning oocyte cryopreservation, oxidative stress induces membrane injuries, which may cause abnormal cell division during early embryonic stages, citoskeletal alterations and mitochondrial damage which may be responsible for decreased oxidative metabolism. Moreover, the CPA commonly used (propanediol, ethile glycol and DMSO) have been described to induce intracellular calcium increase with subsequent cortical granule release and zona hardening. Furthermore, in vitro maturation, fertilization and embryo culture technologies, which are indispensable for generating embryos from cryopreserved oocytes, generate high rates of abnormal fertilization (polyspermy) and additional stress in resultant embryos further compromising their developmental competence (Tatone et al. 2010; Somfai et al., 2012). The impact of oxidative stress on cryopreservation of mammalian semen has been extensively studied in these years (Bilodeau et al. 2000; Ball et al., 2001, Kim et al., 2010) and several recent investigations demonstrated considerable variations between the different species. For example the production of ROS following cryopreservation remains unclear in boar sperm. Contrary to the hypothesis that the cryopreservation process increases ROS, Awda et a (2009) reported that cryopreservation decreased intracellular radical •O2– and had no effect on H_2O_2 content in viable boar sperm. In addition, Guthrie and Welch (2012) observed that ROS formation was low (less than 4%) and did not differ between viable fresh and frozen-thawed boar sperm. On the other hand, according to Kim et al. (2011) cooling process at 15°C decreased intracellular •O2– and H_2O_2, while the freezing-thawing process increased intracellular H_2O_2, in viable cells. Therefore further studies are necessary to better understand the involvement of ROS and oxidative stress on gamete damage that occurs during different storage conditions,

Antioxidants and gamete storage

Numerous antioxidants have been proven to be beneficial in protecting damaging effects of ROS during gamete preservation (Zhang et al. 2012). It has been recently demonstrated that dietary supplementation of stallions with antioxidants attenuated a decline in motility and membrane integrity of cooled-stored stallion semen during winter (Schmid-Lausigk and Aurich, 2014). L-Glutamine (L-Glu) and L-cysteine (L-Cys), the precursor of intracellular GSH, has been employed in sperm cryopreservation for mammalian species. L-Glutamine (L-Glu) has been used successfully for freezing buck (Al Ahmad et al. 2008), human (Renard et al. 1996) bull (Amirat-Briand et al. 2009), stallion sperm (Khlifaoui et al. 2005). Cysteine has been shown to prevent the loss of sperm motility of frozen thawed bull, ram and goat semen, and to improve viability, membrane integrity and chromatin structure of boar sperm during cryopreservation (Kaeoket et al., 2010; Coyan et al., 2011; Memon et al., 2012; Büyükleblebici et al., 2014). GSH is a tripeptide ubiquitously distributed in living cells that has an important role as intracellular defense mechanisms against oxidative stress (Irvine 1996). Addition of GSH to the freezing and thawing extender significantly improves the quality and fertilizing ability of frozen-thawed spermatozoa, increasing farrowing rates and the number of total born piglets and alive born piglets, and counteracting the cryopreservation-induced damages inflicted on boar, bull, human and goat frozen-thawed spermatozoa (Gadea et al. 2004, 2008, 2011, 2013; Estrada et al., 2014). AA (vitamin C), a water-soluble ROS scavenger with low toxicity and high potency, has a protective effect on metabolic activity and viability of cryopreserved porcine spermatozoa (Peña et al., 2003, 2004). The addition of AA to cryoprotective medium suppressed deleterious effect of ROS and increased sperm motility and integrity of the sperm plasma and acrosome membrane of bovine semen (Hu et al. 2010). Moreover it was demonstrated that the addition of AA before cryopreservation can reduce DNA damages in infertile men (Branco et al. 2010).

Poliphenolic compounds, such as RESV, quercitin, epigallo-catechin-gallate, act as free radical scavengers and, at times, as metal chelating agents, acting both in the initiation step, as well as in the propagation of the oxidative process (Doshi et al. 2015; Shrikanta et al.y 2015). Opposite effects and species-specific actions were obtained using various concentrations of the same molecule, suggesting the existence of a “paradoxical effect”, that a substance can act as pro or antioxidant in base of the concentrations used (Calabrese et al. 2010; Murakami 2014).

For example, the addition of 5 to 20 μg/mL of either RESV or quercetin to the Tris-egg yolk-glycerol extender did not significantly affect progressive motility, vigor, acrosome or plasma membrane integrity of ram semen, while 0.15 mM quercitin significantly improved the motility and zona binding ability of cryopreserved stallion sperm, and reduced DNA fragmentation in sex-sorted, cryopreserved stallion sperm (Silva et al., 2012; Gibb et al., 2013). In the matter of female gametes, several antioxidants, such as L-carnitine (LC), lactoferrin (LF), sulforaphane (SF), insulin-transferrin-selenium (ITS) and AA were added during in vitro maturation (IVM) of porcine and goat oocytes (Hammami et al. 2013; Ishikawa et al. 2014), but no antioxidant has yet been supplemented on vitrification solutions and thawing solutions of oocyte cryopreservation. According to Spricigo et al. (2014) study, 1μM reveratrol, added on IVM medium, alone or in association with l-carnitine, was able to reduce DNA fragmentations of IVM oocytes after a vitrification/warming process. In addition, it was demonstrated that 0.5 μM RESV supplementation during embryo culture improved the quality of vitrified-warmed blastocysts (Salzano et al. 2014).

Objectives

The present work aimed to study the effects of some antioxidants supplemented during gamete storage.

The first study evaluated the effect of RESV addition during swine oocyte vitrification.

Two micromolar RESV was added on different phases of vitrification and warming procedures of swine oocytes. In order to evaluate the possible protective proprieties of RESV, caspases activation and phosphatydilserine exteriorization, two principal events of apoptotic process that usually occur during oocyte cryopreservation, were assessed.

In the second study different doses of RESV (10, 20, 40, and 80 µM) were added during stallion semen storage for 24h at two different temperatures (4° and 10°C), under anaerobic conditions. The aim was to evaluate if RESV, at these concentrations, could exert a protective antioxidant effect during cooling of stallion semen. For this purpose, the principal semen quality parameters were assessed: viability, motility, chromatin integrity, mitochondrial activity and acrosomal membrane integrity.

Finally, the third study aimed to evaluate the effect of two antioxidants, GSH (5mM) and AA (200µM), supplemented during boar semen cryopreservation. The two molecules were added, alone or together, on different phases of boar semen freezing-thawing procedure. In order to compare the possible beneficial effects of the antioxidants on the various experimental groups, the principal semen quality parameters were evaluated after semen thawing. Moreover, superoxide and peroxide levels were assessed in order to evaluate the antioxidant protective effect of GSH and AA.

Results

To accomplish the objectives listed above, a series of three set of experiments were developed. The results obtained in each set of experiments were reported in a paper. Thus, the thesis consists of a compendium of three papers, which have already been published.

1. Giaretta E., Spinaci M., Bucci D., Tamanini C., Galeati G. "Effects of resveratrol on vitrified porcine oocytes" Oxidative Medicine and Cellular Longevity. 2013; 920257.

2. Giaretta E., Bucci D., Mari G., Galeati G., Love C.C., Tamanini C., Spinaci M. "Is resveratrol effective in protecting stallion cooled semen?" Journal of Equine Veterinary Science. 2014; 34(11-12): 1307-13122014

3. Giaretta E., Estrada E., Bucci D., Spinaci M., Rodríguez-Gil J.E., Yeste M., "Combining reduced GSH and AA has supplementary beneficial effects on boar sperm cryotolerance" Theriogenology. 2015; 83(3):399-407.

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Research Article

Effects of Resveratrol on Vitrified Porcine Oocytes

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Vitrified MII porcine oocytes are characterized by reduced developmental competence, associated with the activation of the apoptotic pathway. Resveratrol (R), a polyphenolic compound present in several vegetal sources, has been reported to exert, among all its other biological effects, an antiapoptotic one. The aim of this study was to determine the effects of R (2 μM) on the apoptotic status of porcine oocytes vitrified by Cryotop method, evaluating phosphatidylserine (PS) exteriorization and caspases activation. R was added during IVM (A); 2 h postwarming incubation (B); vitrification/warming and 2 h postwarming incubation (C); all previous phases (D). Data on PS exteriorization showed, in each treated group, a significantly higher ($P < 0.05$) percentage of live nonapoptotic oocytes as compared with CTR; moreover, the percentage of live apoptotic oocytes was significantly ($P < 0.05$) lower in all R-treated groups relative to CTR. The results on caspase activation showed a tendency to an increase of viable oocytes with inactive caspases in B, C, and D, while a significant ($P < 0.05$) increase in A compared to CTR was recorded. These data demonstrate that R supplementation in various phases of IVM and vitrification/warming procedure can modulate the apoptotic process, improving the resistance of porcine oocytes to cryopreservation-induced damage.

1. Introduction

Vitrification of oocytes is the most recent cryopreservation methodology, used in different species such as human [1], bovine [2], goat, and swine [3].

Currently, several studies have been carried out to improve the efficacy of cryopreservation protocols, validating different cryoprotectant solutions, incubation times, oocytes containers, and other many conditions [4].

Recent progresses in the vitrification technique are attested by the high number of born after cryopreservation of human [5], mouse [6], cat [7], and bovine [8] oocytes, but no piglets have been obtained from cryopreserved swine oocytes so far.

Compared with other domestic species, the high intracellular lipid content [9] and the wide cell volume make porcine oocytes more susceptible to storage at low temperature, with a consequent decrease of oocytes survival rate and apoptotic progression after thawing [10, 11].

Moreover, the survival and development of unfertilized vitrified porcine oocytes are significantly lower than those of fertilized vitrified ones [12, 13]. During the vitrification/warming process many oocyte ultrastructures, such as mitochondria, smooth endoplasmatic reticulum, meiotic spindle, and plasma membrane, show considerable damages that contribute to reduce the developmental potential of oocytes after fertilization [14, 15].

In addition, oocytes that survive cryopreservation significantly reduce their glutathione (GSH) content and accumulate reactive oxygen species (ROS) [16].

ROS, such as superoxide anions (O^-_2), hydroxyl radicals (OH^-), and H_2O_2, are generated during intermediate steps of oxygen reduction; their heap, also associated with the glutathione efflux, is one of the main factors which are effective in inducing the apoptotic activation, characterized by biochemical events that result in specific morphological changes including cell shrinkage and progressive DNA and cell membrane damage, ultimately leading to cell death. Signals to death receptors (extrinsic apoptotic pathway) or to mitochondria (intrinsic apoptotic pathway) concur in the activation of caspases, a family of cysteine proteases with similar aminoacid sequences, structure, and specificity that promote morphological and biochemical cell changes, typical of apoptosis [17, 18]. Finally, phosphatidylserine (PS), that is normally confined to the inner plasma membrane leaflet,

after apoptotic stimuli is externalized and subsequently recognized by specific PS receptors of macrophages and other phagocytic cells, inducing apoptotic cells engulfment [19–21].

Therefore, one of the current challenges to reproductive cryobiologists is to prevent oocytes degeneration in order to maintain their developmental competences.

To preserve the antioxidant defence system in oocyte, specific substances that play antioxidant roles, such as ascorbate [22], epigallocatechin-3-gallate [23], β-mercaptoethanol [13], anthocyanin [24], and trans-ε-viniferin [25], were added during in vitro oocytes/embryo culture and storage.

Recently, treatment of porcine oocytes with 2 μM Resveratrol during IVM [26] reduces the intracellular level of ROS and increase GSH concentration in matured oocytes, resulting in increase of blastocyst development after parthenogenetic activation (PA) and in vitro fertilization (IVF).

Resveratrol, through its simultaneous activity on multiple molecular targets, is effective in modulating different cell pathways and, depending on its concentration, and its effect may be reversed [27]. Resveratrol has been reported to act as antioxidant because of its ability to decrease mitochondria ROS production, scavenge superoxide radicals, inhibit lipid peroxidation, and regulate the expression of antioxidant cofactors and enzymes [28].

On thise basis we decided to add 2 μM Resveratrol in maturation, vitrification, and postvitrification media in order to determine its effect on apoptotic process and oocytes viability.

For this purpose the externalization of phosphatidylserine using Annexin V (Annexin V/Hoechst 33342/PI) and the caspase activation through FITC-VAD-FMK staining (FITC-VAD-FMK/Hoechst 33342/PI) were evaluated.

2. Materials and Methods

Unless otherwise specified, all the reagents were purchased from Sigma-Aldrich (Milan, Italy). Cryotops (Kitazato, Fuji, Japan) were obtained from BioCare Europe (Roma, Italy).

2.1. In Vitro Maturation (IVM) of Cumulus-Oocyte Complexes (COCs). Ovaries were collected at a local abattoir and transported to the lab within 2 h in a thermos filled with physiological saline at 30–35°C. COCs from follicles 3–6 mm in diameter were aspirated using a 18-gauge needle attached to a 10 mL disposable syringe. Under a stereomicroscope, intact COCs were selected and transferred into a petri dish (35 mm, Nunclon, Denmark) prefilled with 2 mL of modified PBS supplemented with 0.4% BSA. Only COCs with complete and dense cumulus oophorus were used. After three washes in NCSU 37 [29] supplemented with 5.0 mg/mL insulin, 0.57 mM cysteine, 10 ng/mL epidermal growth factor, 50 μM β-mercaptoethanol, and 10% porcine follicular fluid (IVM medium), groups of 50 COCs were transferred to a 4-well multidish (Nunclon) containing 500 μL of the same medium per well and cultured at 39°C in a humidified atmosphere of 5% CO_2 and 7% O_2 in N_2. During the first 22 h of maturation, the IVM medium was supplemented with 1.0 mM db-cAMP, 10 IU/mL eCG (Folligon, Intervet, Boxmeer, The Netherlands), and 10 IU/mL hCG (Corulon, Intervet). After culturing for 22 h, COCs were transferred to fresh maturation medium and cultured for a further 24 h period [29].

2.2. Oocyte Vitrification with Cryotops and Warming. The protocol of vitrification with Cryotop carrier and solution has been described by Kuwayama et al. [30, 31]. Briefly, denuded oocytes ($N = 5$) were transferred into equilibration solution (ES) consisting of 7.5% ethylene glycol (EG) and 7.5% dimethylsulfoxide (DMSO) in Hepes-buffered Ham's F10 (HF10; Gibco, Invitrogen, Monza, Italy) and 20% fetal calf serum (FCS; Gibco) at 39°C for 5–15 min. Thereafter, oocytes were transferred into 20 μL drops of vitrification solution (VS) consisting of 15% EG, 15% DMSO, and 0.5 M sucrose dissolved in HF10 and 20% FCS. After incubation for 20–30 s, oocytes were loaded on Cryotop and plunged into liquid nitrogen (LN2). The entire process, from exposure in VS to plunging into LN2, was completed within 45–60 s. Vitrified oocytes were warmed by submerging vitrification devices directly into 39°C thawing solution (1.0 M sucrose dissolved in HF10 and 20% FCS) for 1 min, and then they were transferred to a dilution solution (0.5 M sucrose dissolved in HF10 and 20% FCS) for 3 min. Subsequently, oocytes were washed twice for 5 min in washing solutions (HF10 supplemented with 20% FCS) before being transferred in IVM medium for 2 h.

2.3. Annexin V Staining of Phosphatidylserine Residues. The Annexin V binding assay was employed to detect phosphatidlserine (PS) externalization on the plasma membrane. Annexin V, a member of the phospholipid-binding annexin family, binds most efficiently to PS, which is externalized on the outer plasma membrane of cells exposed to apoptotic stimuli.

Oocytes were washed three times with Dulbecco's phosphate-buffered saline (DPBS) and transferred to 100 μL of binding buffer (Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit, Molecular Probes, Eugene, USA) with 5 μL of Alexa Fluor Annexin V, 1 μL of propidium iodide (PI) (100 μg/mL), and 0.2 μL of 5 mg/mL Hoechst 33342 (Ho) for 20 min at 39°C in the dark. After incubation, the oocytes were washed three times in binding buffer and then mounted on glass slides which were examined with an Eclipse E 600 (Nikon Europe BV, Badhoevedorp, The Netherlands) epifluorescence microscope equipped with a digital camera.

Oocytes were classified as follows:

(i) live nonapoptotic oocytes with Ho-positive nuclei and no annexin staining (A−/PI−) (Figure 1(a)),

(ii) live apoptotic oocytes with Ho-positive nuclei and annexin-positive signal on the membrane (A+/PI−) (Figure 1(b)),

(iii) necrotic oocytes which showed PI-positive red nuclei, indicative of membrane damage with or without annexin staining on the membrane (PI+) (Figure 1(c)).

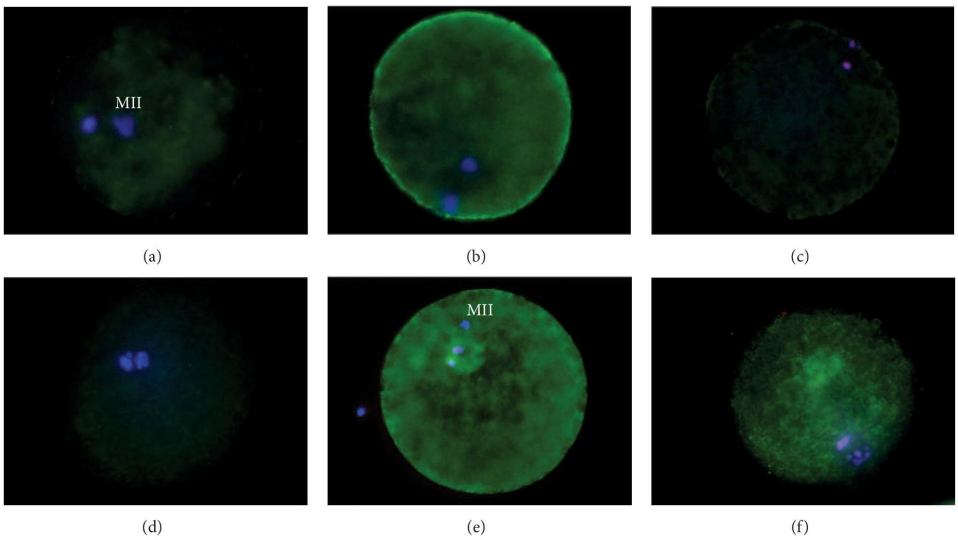

Figure 1: ((a)–(c)) Fluorescent micrographs of oocytes after combined staining with Annexin V/Hoechst 33342 (Ho)/PI. (a) Live non apoptotic oocytes with Ho positive nuclei and no annexin staining (A−/PI−); (b) Live apoptotic oocytes with Ho positive nuclei and annexin positive signal on the membrane (A+/PI−); (c) Dead oocyte which showed PI positive red nuclei (PI+). ((d)–(f)) Representative fluorescent micrographs of pig oocytes after FITC-VAD-FMK, Ho and PI staining. (d) Live non apoptotic oocytes with Ho positive nuclei and no FITC-VAD-FMK staining (VAD−/PI−); (e) Live apoptotic oocyte with the cytoplasm stained in green by FITC-VAD-FMK (VAD+/PI−); (f) Dead oocyte with the metaphase plate stained in red by PI (PI+).

2.4. Assessment of Activated Caspases. The activation of caspases was detected through FITC-VAD-FMK (Molecular Probes, Leiden, The Netherlands); VAD-FMK is a cell permeable caspase inhibitor that covalently binds activated caspases, conjugated to FITC. Oocytes were washed twice with DPBS and then incubated with 500 μL of DPBS containing 1 μM of FITC-VAD-FMK and 0.2 μL of 5 mg/mL Ho for 30 min at 39°C. During the final 5 min of VAD-FMK/oocyte incubation, 3 μL of a solution 1 mg/mL of propidium iodide were added to detect dead oocytes. Oocytes were then washed twice for 5 min with DPBS and mounted on glass slides. Samples were assessed by fluorescence microscopy. Stained oocytes were classified in three groups:

(i) viable oocytes without active caspases (VAD−/PI−)(Figure 1(d)),

(ii) viable oocytes with FITC-VAD-FMK positivity, indicative of caspase activation (VAD+/PI−) (Figure 1(e)),

(iii) dead oocytes (PI+) (Figure 1(f)).

2.5. Experimental Design. All oocytes were submitted to IVM, vitrification, and warming procedures and were evaluated after incubation for 2 h into maturation medium.

Oocytes were divided into the following 5 experimental groups:

(CRT) without Resveratrol addition;

(A) 2 μM Resveratrol supplementation during IVM;

(B) 2 μM Resveratrol supplementation during the postwarming incubation for 2 h;

(C) 2 μM Resveratrol supplementation during vitrification/warming and 2 h post-warming;

(D) 2 μM Resveratrol supplementation in all previous steps.

2.6. Statistical Analysis. Each experiment was repeated at least 3 times. All statistical analyses were performed using R version 2.15.2. [32]. Chi square test was performed and the level of significance was set at $P < 0.05$.

3. Results

3.1. Experiment 1: Detection of Apoptosis by Annexin V Labeling. The addition of R, in all treated groups, induced a percentage of live nonapoptotic oocytes (A−PI−) significantly higher ($P < 0.05$) than nontreated control (Table 1). Moreover, in all groups supplemented with R the percentage of live apoptotic oocytes (A+PI−) was significantly ($P < 0.05$) lower than in CTR group. Finally, R supplementation in B, C, and D groups significantly ($P < 0.05$) reduced the percentage of dead oocytes (PI+) compared to CTR.

3.2. Experiment 2: Assessment of Activated Caspases. The results on caspases activation showed a tendency to an increase of viable oocytes with inactive caspases (VAD−PI−)

Table 1: Effect of Resveratrol supplementation on exteriorization of phosphatidylserine, assayed by Annexin V/Hoechst 33342/PI staining, in vitrified oocytes. Data are presented as mean percentage.

Group	*N* oocytes	% viable oocytes (A− PI−)	% apoptotic oocytes (A+ PI−)	% dead oocytes (PI+)
CTR	310	66.1[a]	28.4[a]	5.5[a]
A	150	78.7[b]	15.3[b]	6[a]
B	124	83.1[b]	16.9[b]	0[b]
C	122	80.3[b]	18.9[b]	0.8[b]
D	114	81.6[b]	17.6[b]	0.9[b]

Different superscripts within the same column indicate significant differences ($P < 0.05$).
CTR: control group; (A) 2 μM R supplementation during IVM; (B) 2 μM R supplementation during the postwarming incubation for 2 h; (C) 2 μM R supplementation during vitrification/warming and 2 h after warming; (D) 2 μM supplementation in all previous steps.

Table 2: Effect of Resveratrol on caspase activation in vitrified oocytes, as assayed by FITC-VAD-FMK/Hoechst 33342/PI staining. Data are presented as mean percentage.

Group	*N* oocytes	% live oocytes (PI−)		% dead (PI+)
		VAD−	VAD+	
CTR	165	81,82[a]	10,30[a]	7,88[a]
A	84	91,67[b]	7,14[a]	1,19[b]
B	76	86,84[ab]	9,21[a]	3,95[ab]
C	75	84,00[ab]	13,33[a]	2,67[ab]
D	91	89,01[ab]	7,69[a]	3,30[ab]

Different superscripts within the same column indicate significant differences ($P < 0.05$).
CTR: control group; (A) 2 μM R supplementation during IVM; (B) 2 μM R supplementation during the postwarming incubation for 2 h; (C) 2 μM R supplementation during vitrification/warming and 2 h after warming; (D) 2 μM supplementation in all previous steps.

in B, C, and D groups, while a significant ($P < 0.05$) increase in A group compared to CTR was recorded.

No significant variations were observed in the percentage of live oocytes with active caspases (VAD+/PI−), while a significant reduction of dead oocytes (PI+) was detected in A group compared to CTR (Table 2).

4. Discussion

While ascorbate [22] and β-mercaptoethanol [13] have been already assayed in vitrification-warming solutions, in mouse embryos and porcine oocytes, respectively, this study, for the first time, tested Resveratrol in the improvement of oocyte cryopreservation. Ascorbate (0.1 mmol/l) has been demonstrated to reduce the levels of hydrogen peroxide in mouse embryos, increasing the inner cell mass when added in slow-freezing or vitrification solutions; β-mercaptoethanol (50 μmol/l) decreased ROS activity but did not improve viability and fertilization ability of vitrified-warmed MII oocyte, while significantly increased blastocyst formation ability of porcine oocytes vitrified after in vitro fertilization.

Our results demonstrate that 2 μM R in IVM and vitrification-warming phases increases oocytes viability, modulating the apoptotic process. Annexin V labeling showed a significant increase in live nonapoptotic oocytes and a parallel reduction of live apoptotic oocytes in all groups added with R as compared with CTR groups.

FITC-VAD-FMK staining evidenced a tendency to an increase of viable oocytes with inactive caspases in all R-treated groups compared with CTR; a significant difference was recorded only between A and CTR groups. However, the percentage of viable oocytes with active caspases was not affected by R treatment.

Several recent studies demonstrated that PS-mediated phagocytosis can occur without caspases activation [33–35]. Thus, we can hypothesize that the different trends in viability and apoptosis observed in annexin V and FITC-VAD-FMK assays may depend on an involvement of caspases and PS externalization in the whole apoptotic process, with a prevalent action of R in one of these two events.

Our results show that R mainly influences PS exteriorization, rather than caspases activation. This R effect can be related to GSH increase, observed by Kwak et al. [26] after the addition of the same R concentration to IVM solution. This hypothesis is supported also by He et al. [36], who demonstrated that inhibition of GSH efflux had no effect on the activation of caspases 3, 8, and 9, but decreased the translocation of PS. Cellular GSH homeostasis plays a crucial role in radical scavenging activity [18] and in cytoplasmatic maturation of porcine oocyte [37–39]. Somfai et al. [16] reported that low intracellular GSH levels and high H_2O_2 concentration in vitrified porcine oocytes, besides other ultrastructural cryodamages, increase the sensitivity to oxidative stress at the beginning of embryo culture, reducing porcine oocytes development and subsequent male pronucleus formation.

In addition, several studies demonstrated the ability of R to scavenge ROS [40, 41] and to modulate intracellular GSH depletion or synthesis, in relation to its concentration [42–44]

Therefore we can suppose that R could be able to maintain GSH homeostasis, with a subsequent inhibition of PS externalization.

Our results on R effect on caspase activation, which is not as evident as PS externalization in vitrified oocytes assayed after 2 h incubation after thawing, agree well with those by Vallorani et al. [11], who observed that caspase activation seems to be a reversible phenomenon. That work showed a significant reduction of vitrified live oocytes presenting a faint FITC-VAD-FMK staining after 2 h of postwarming incubation, compared to those observed immediately after warming; moreover, no significant differences were detected, in the same time lapse, in the number of apoptotic oocytes (A+P−) as assayed by Annexin V staining. Taken together, our and those results seem to suggest that 2 h of postwarming incubation may be beneficial in modulating the caspase cascade that can be further reduced by R addition, in a significant manner when added in IVM solutions.

A previous study [45] demonstrated that in neuronal cells the externalization of PS occurs during the late phase of apoptosis while caspase activation begins in the early one.

Therefore, we can hypothesize that while caspase activation can be arrested after 2 h post warming incubation, PS externalization may be a tardive and irreversible apoptotic event that could be avoided by R addition in one or more steps of the IVM and vitrification procedure.

In our study we did not observe any algebraic sum of positive effects of Resveratrol when added during vitrification/warming and 2 h of culture after warming (C group) or in the whole IVM-vitrified-warming-postwarming process (D group). Resveratrol could be immediately oxidized, modulating the GSH redox balance and thus increasing the oocyte-reducing power. Therefore, it seems that it might be employed either in IVM, in vitrified-warming, or 2 h post warming solutions, in order to prevent, inhibit, or repair cryoinjury damages.

5. Conclusions

In conclusion, the present results confirm the occurrence of vitrification-induced oocyte injuries, as previously reported, and suggest to improve vitrification protocols by Resveratrol addition.

Supplementation with 2 μM Resveratrol in IVM, vitrification-warming, or 2 h postwarming solutions could improve and optimize the quality and the resistance of IVM porcine oocytes to cryopreservation, modulating cell apoptotic process.

Other polyphenolic compounds could help in minimizing cryodamage and in optimizing current Cryotop vitrification method, improving the success of applications in female gamete preservation.

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2nd PAPER

Title:

IS RESVERATROL EFFECTIVE IN PROTECTING STALLION COOLED SEMEN?

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Abstract

The aim of this work was to evaluate the effect of resveratrol (RSV) during stallion liquid storage of stallion sperm for 24 h at either 10°C or 4°C. The antioxidant Resveratrol was added in order to reduce the oxidative damage that occurs during cold storage. Aliquots of 2 ml of diluted semen were stored either at 4 or at 10°C under anaerobic conditions, in absence (CTR group) or presence of RSV at different concentrations (10, 20, 40, 80 µM). Sperm quality parameters were assessed at 0 h and after 24 h of storage. Resveratrol treatment did not affect sperm quality parameters at 0 h. At 24 h storage a significant ($p < 0.01$) decrease of sperm quality was observed independently from RSV supplementation and storage temperature. A significant decrease of viable spermatozoa with high mitochondrial membrane

potential (SYBR+/PI-/JC-1+) was evident at 24 h storage in 40 µM and 80 µM RSV group, compared with CTR group. Moreover a decline of total motility (TM) in 80 µM RSV group compared with the control group and a decrease of progressive motility (PM) and average path velocity (VAP) in 80 µM RSV group when compared with control and RSV 20 µM groups were observed. In conclusion, our findings demonstrate that RSV supplementation does not enhance sperm quality of stallion semen after 24h of storage. Moreover, 40 and 80 µM RSV concentrations could damage sperm functional status, probably acting as pro-oxidant. Finally, although 24 h storage significantly affected the majority of sperm quality parameters, no significant differences were found in groups maintained at 4 or 10° C, suggesting that stallion semen could be equally preserved at these different temperatures.

1. Introduction

The use of cooled-shipped semen has become a routine method in the equine industry in order to extend the life expectancy of the spermatozoa that are transported to breed mares for artificial insemination [1]. Several key factors affect the lifespan of spermatozoa such as collection protocols, chemical composition of semen extenders, centrifugation, cooling rate and different storage temperatures [2,3,4]. During the last few years, new insights into cooled-semen technology have been made and opened possibilities to improve semen quality and fertilizing ability. Indeed, many cellular injuries appear during the cooling process, such as the disruption of membrane lipids, resulting in damage to mitochondria and loss of integrity of plasma and acrosomal membranes. These stresses, collectively called “cold shock”, result in an irreversible loss of motility, viability, and fertilizing capacity of sperm [5]. The generation of reactive oxygen species (ROS), which occurs during low temperature storage, is one of the causes of “cold shock” damage. The oxidative stress, caused by abnormal ROS production, leads to damage to membrane phospholipids, chromatin and proteins [6,7]. The susceptibility of spermatozoa to oxidative damage is attributed to the high concentration of unsaturated fatty acids in membrane phospholipids, the limited antioxidant capacity as well as their ability to generate ROS[8,9].

Several antioxidants have been used in extender medium in order to reduce oxidative damage: ascorbic acid and catalase [10], pyruvate and xanthurenic acid [11], melatonin [12], superoxide dismutase [13], butylatedhydroxytoluene[14] and quercetin [15]. RSV is a natural

grape-derived polyphenolicphytoalexin that possesses pleiotropic effects including anticancer, anti-aging, anti-inflammatory and anti-oxidant ones [16,17]. RSV effectively scavenges superoxide and peroxide radicals generated from enzymatic and non-enzymatic systems, and it affords protection against damage caused by ROS in somatic cells [18]. RSV may have reversed effects depending on both dose and species. The antioxidant power of RSV on male gametes has already been tested during conservation of ram, human and boar semen. According to [19] treating ram semen (during cooling at 5°C for 24 h or incubating the samples at 37°C for 2h) with 15 or 20 µM RSV significantly inhibits lipid peroxidation (LPO); moreover 15 µM RSV prolongs the conservation period of semen, maintaining motility and slowing down the appearance of acrosomal damage. Recent studies in human demonstrated a reduction of LPO in semen cryopreserved with 0.1, 1 and 10 mM RSV and in fresh semen incubated for 1h at 37°C with 15 µM RSV [20,21]. Moreover, according to Collodel et.al. [21]progressive sperm motility increased in fresh semen incubated for 1h at 37°C with 30, 15 and 6 µM RSV while treatment with 100 µM RSV led to a loss in viability in human spermatozoa as well as in rat spermatocytes. In the boar various doses of RSV (10, 33, 66, 100 µM) supplemented during semen preservation at 17°C for 7 days did not improve sperm quality parameters [22].

Based on this information, the main aim of this work was to test the effects of supplementation of RSV during stallion semen conservation for 24 h at 4° or 10°C, temperatures able to maintain good stallion semen fertility [23], and to determine its effects on the main parameters of semen quality: motility, viability and acrosome reaction, mitochondrial membrane potential and sperm DNA integrity.

2.Materials and methods

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Milan, Italy).All experimental procedures were carried out according to DL 116/92, implementation of the 86/609/EEC, regarding the protection of animals used for experimental or other scientific purposes. Furthermore, the protocol was approved by the University of Bologna Care Committee and the Italian Ministry of Health.

2.1. Experimental design

Three fresh ejaculates were collected from two Standardbred stallions of proven fertility, individually housed at the National Institute of Artificial Insemination, University of Bologna, Italy, using a Missouri artificial vagina with an inline filter (Nasco, Fort Atkinson, WI, USA). Ejaculates were immediately evaluated for volume, concentration (NucleoCounter SP 100, Chemometec, Denmark) and motility (see below: Evaluation of motility)and sent to the laboratory within 10 min at 20-25 °C.

Semen was diluted to a final concentration of $30x10^6$/mL spermatozoa in Kenney's extender [24] and divided in aliquots of 2 ml filling up 2ml Eppendorfs in order to keep out the presence of air and to guarantee anaerobic conditions. Semen samples were placed in a water bath at room temperature, supplemented with RSV at different concentrations (0, 10, 20, 40, 80 µM), then storedin two different refrigerators set to 4°C or 10°C.Sperm quality parameters were assessed immediately after RSV supplementation(0 h) and after 24 h of storage at 4° or10° C.

*2.2. Evaluation of viability*Twenty-five µl of semen were incubated with 2 µL of a 300 µM solution of propidium iodide (PI), and 2 µL of a 10 µM solution of SYBR green-14, obtained from the live/dead sperm viability kit (Molecular Probes, Inc., Eugene, OR, USA) for 5 min at 37 °C in the dark. Ten µL of the stained suspensions were placed on clean microscope slides, carefully overlaid with coverslips, and at least 200 spermatozoa per sample were observed under a Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoeverdop, The Netherlands). Spermatozoa stained with SYBR-14 but not with PI were considered as viable (SYBR-14- PI-). Spermatozoa both SYBR-14+ and PI+ and those SYBR-14−/PI+ were considered with damaged membranes or dead.

2.3 Evaluation of acrosome reaction

The occurrence of an acrosome reaction was evaluated using FITC-conjugated agglutinin derived from *Pisumsativum* (FITC-PSA) staining. Briefly, the spermatozoa, after washing twice with PBS, were fixed and permeabilized for at least 30 min at −20°C in 95% ethanol, dried into microscope slides, incubated with FITC-PSA solution (1 mg PSA-FITC/10 ml H_2O) for 15 min in the dark and then observed using the above described microscope. The presence of a green

acrosomal fluorescence was considered indicative of an intact acrosome, whereas a partial or total absenceof fluorescence was considered to indicate acrosome disruptionor acrosome reaction (RA).

2.4 Evaluation of mitochondrial activity

For each sample, an aliquot (25µl) of semen was incubated with 2 µl of a 300 µM propidium iodide (PI) stock solution, 2 µl of a 10 µM SYBR green-14 stock solution, both obtained from the live/dead sperm viability kit (Molecular Probes, Inc.), and 2 µl of a 150 µM JC-1 solution for 20 min at 37°C in the dark. Ten microliters of the sperm suspension were then placed on a slide and at least 200 spermatozoa per sample were scored using the above described microscope. Spermatozoa stained with SYBR green-14 and not stained with PI were considered as viable. Spermatozoa SYBR positive and PI positive and those SYBR negative / PI positive were considered as cells with non-intact membrane or dead. JC-1 monomers emit a green fluorescence in mitochondria with low membrane potential, while emitting a bright red-orange fluorescence in case of multimer formation (J-aggregates) in mitochondria with high membrane potential. When an orange fluorescence was present in the mid piece, live spermatozoa were considered to have functional active mitochondria (SYBR+/PI-/JC-1+).

2.5. Evaluation of motility

Motility was measured using a computer-assisted sperm analysis system (CASA, Hamilton Thorne, IVOS Ver. 12, standard equine set up). Sperm motility endpoints assessed were: percent of total motile spermatozoa(TM), percent of progressive spermatozoa (PM), percent rapid (RAP) and slow (SLOW) motility, curvilinear velocity (VCL), straight-line velocity (VSL), mean velocity (VAP) and straightness coefficient (STR). The setting parameters of the program were the following: frames per second 60, number of frames 45, path velocity 30 microns/sec, straightness 50.

2.6 Evaluation of DNA damage

An aliquot of semen at 0 and 24h storage at either 4°C or 10°C in absence or presence of different Resveratrol concentrations (10, 20,40, 80 µM) was snapfrozen in liquid nitrogen, stored at - 80 °C until analysis and retrospectively analyzed after thawing for susceptibility of

spermatozoa to DNA denaturation using the sperm chromatin structure assay(SCSA)[25,26,27]. Individual samples were thawed in a water bath at 35°C for 15–30sec. The samples, tubes and reagents were kept on ice during the following processing. An aliquot of 10µl of semen was mixed with 180–195µl of a TRIS buffer (1mM disodium EDTA, 0.01MTris–HCl, 0.15M NaCl in 500ml deionized water, pH 7.4) to a final volume of 200µl. Four hundred microliters of acid-detergent solution (0.08MHCl, 0.1% Triton X-100 (Sigma–Aldrich), 0.15M NaCl) were immediately added and after 30sec the solution was quenched with 1.2ml of an Acridine Orange (AO) solution (0.1M citric acid monohydrate, 0.2M Na2HPO4, 0.15M NaCl, 1mM disodium EDTA, 4µg/ml AO (Polysciences, Inc., Warrington, PA, USA), pH 6). The samples were immediately placed into the flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA) and allowed to equilibrate for 30s before data acquisition. Sample volume varied to accommodate a flow rate of 100–200cells/sec Five thousand events were accumulated per sample. Flow cytometer settings were calibrated using spermatozoa from a known fertile stallion. Using this control sample, settings were adjusted so that mean green fluorescence was at 500 channels (FL-1@500) and mean red fluorescence at 150 channels (FL-3@150). Data were recorded and stored in List Mode and SCSA values were calculated using WinList software (Verity Software House, Topsham, ME, USA). Quantification of DNA denaturation in each spermatozoon was determined by the term alpha-t (αt), the ratio of red/(red+green fluorescence) for each individual spermatozoon analyzed. Alpha-t (αt) describes the relationship between the amounts of green (double-stranded DNA) and red (single-stranded DNA) fluorescence. Endpoints included the percentage of Cells Outside the Main Population (%COMP-αt), Mean-αt and the standard deviation-αt. The COMP-αt was determined by selecting those spermatozoa located to the right of the control main population, and represents a percentage of the total number of spermatozoa with denatured DNA.

2.7. Statistical analysis

Data were analyzed by R version 3.0.3[28]. After checking for normal distribution, data were analyzed by an ANOVA test followed by the Tukey*post hoc* test for multiple comparison. Data are expressed as mean percentage ± standard deviation. In all statistical analyses, the minimal level of significance was set at $p<0.05$ unless otherwise specified.

3. Results

Resveratrol treatment did not affect sperm quality parameters at 0 h, while some negative effects were evident at 24 h storage independent of storage temperature. As shown in Table 1 no differences were registered between 4°C and 10°C storage. For this reason data obtained at 24h at 4°C and 10°C in each RSV treated group were pooled in Table 2. The differences evidenced by ANOVA at 24 h between treatments were a reduction of viable cells with active mitochondria in RSV 40 and 80 µM groups compared to CTR group, a decline of TM in 80 µM RSV group when compared with CTR group and a decrease of PM and VAP in 80 µM RSV group when compared with CTR and RSV 20 µM groups.

Storage time significantly affected sperm quality parameters as well as sperm motility. A decrease of viable cells, viable cells with active mitochondria, total motility percentage, progressive motility percentage, VAP was evident with a concomitant increase in acrosome reacted cells. No effect of storage time was registered regarding chromatin integrity and VCL.

4. Discussion

The present study evaluated the effect of RSV during stallion sperm liquid storage for 24 h at either 10°C or 4°C. Our results show that RSV addition was not effective in preventing the decrease of stallion sperm quality and motility parameters after refrigeration. The treatment with RSV at 10, 20, 40 and 80 µM concentrations did not enhance semen quality after 24 h of storage at 4°C or 10°C. Moreover, 40 and 80 µM RSV exerted a negative effect on mitochondrial activity, while RSV 80 µM treatment negatively affected several motility parameters (TM, PM, VAP). A significant decline in viability, acrosome integrity, mitochondrial membrane potential, TM, PM and VAP appeared after 24 h storage, irrespective of storage temperature, thus confirming horse semen sensitivity to refrigeration process[28,29]. Instead, in accordance with Aurich et.al.[30], who did not observe a significant decline of chromatin quality after 24 h of storage at 5°C, no significant changes in SCSA were observed in our work. The lack of a protective effect of RSV on sperm quality parameters after refrigeration fully agrees with a previous study by Ball et.al. [32], who showed that addition of various water and lipid soluble antioxidants did not improve the motility during liquid semen storage at 5°C. Moreover Kankofer et al. [33] and Pagl et al. [34] demonstrated that, during cold-storage,

there was no increase of lipid peroxidation and the antioxidant capacity of semen could be maintained by the interaction between seminal extender and seminal plasma. Thus, the natural antioxidant systems of whole semen appear to be sufficient to prevent ROS production during the first 24 h of storage. However, despite the peroxidative damage to cell membrane lipids is unlikely the predominant cause, significant losses in sperm viability, motility and mitochondrial activity are recorded after sperm cooling. In fact, a study demonstrated that during refrigeration stallion semen undergoes an apoptotic process [35]. In that study a high percentage of stored spermatozoa showed active caspases 3 and 7 and phosphatidyl serine exteriorization, without any change in lipid peroxidation. Moreover, a significant correlation between the loss of motility and the increase of apoptotic cells was recorded. A modification of mitochondrial membrane potential, an altered membrane permeability and a decline of motility may represent refrigeration-induced apoptotic-like changes, that could not be inhibited by antioxidant addition. The causes of cold-induced apoptosis need to be better investigated in further studies.

As already demonstrated, RSV often displays hormesis-like biphasic dose responses. While at low doses it can improve cell function, at high doses it can acts as a pro-apoptotic compound, leading to cell death[36]. In our work 80µM RSV at 24h storage reduced both TM, if compared with CTR, and PM and VAP, if compared with CTR and RSV 20µM. Moreover, mitochondrial activity showed a decrease in JC1+ live cells due to treatment with 40 and 80 µM RSV at 24 h storing, independent of temperature. In addition, we checked whether this effect was due to cell death or to a decreased mitochondrial activity. It seems that there was an effective loss of mitochondrial activity, as the total number of JC1 positive cells decreased in a similar manner as that observed for JC1+ live cells. Thus, these concentrations of RSV could probably induce an oxidative and/or pro-apoptotic damage in stallion semen. Indeed, several studies have shown that mitochondria play an important role in the regulation of cell death and represent one of the primary target of cellular damage. After pro-apoptotic stimuli, opening of membrane pores, with consequent decrease of mitochondrial membrane potential ($\Delta\Psi_m$), causes the release of mitochondrial apoptotic factors in the cytoplasm, which induce the cascade-apoptotic process [37,38].

Our results also revealed no differences in parameters evaluated between the two different temperatures after 24 h of storage. Vidament et al.[23] obtained similar pregnancy rate with stallion semen stored for 22h at either 4°C or 10°C. However a different effect of temperature

was observed when semen was refrigerated for a longer time. Apoptotic features and a decrease in fertility rates after 48h of refrigeration were higher in 15°C samples [39,40].

In conclusion, while 40 and 80µM RSV exert a negative effect on the quality of extended equine semen during storage at 4°C and 10°C, the supplementation with lower doses (10 and 20µM) did not improve the sperm parameters assessed in this study. Thus, it cannot be excluded that lower or similar RSV doses could improve other sperm characteristics such as oxidative or apoptotic-related parameters. Moreover RSV activity should be examined after a refrigeration longer than 24 h, in which exogenous ROS have been demonstrated to increase DNA fragmentation and to induce lipid peroxidation in stallion sperm[41,42].

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Parameters	Treatment	0h	4° 24h	10° 24h
Viability	CTR	77.3±6.6	66.4±5.4	73.0±3.5
	RSV10	84.1±4.3	71.8±1.5	71.6±3.3
	RSV20	78.6±3.2	67.6±5.1	66.5±11.0
	RSV40	72.5±12.7	67.6±4.6	68.3±7.7
	RSV80	75.2±1.8	67.3±1.1	63.7±2.2
DNA damaged cells				
%	CTR	26.2±1.3	32.6±2.7	30.7±3.6
	RSV10	31.1±2.6	32.6±5.4	32.7±7.2
	RSV20	31.2±4.6	30.5±5.2	32.7±6.8
	RSV40	28.2±1.4	32.2±1.1	30.0±2.5
	RSV80	27.3±2.3	32.1±5.3	31.0±5.3
RA %	CTR	15.0±5.9	23.2±9.5	20.7±6.6
	RSV10	14.3±5.6	27.4±4.5	35.0±10.9
	RSV20	17.7±6.9	22.5±8.4	22.8±8.8
	RSV40	17.1±7.1	28.5±11.1	34.1±4.7
	RSV80	16.6±8.8	23.4±8.5	31.7±3.6
JC-1+ (%)	CTR	62.1±4.0	55.5±4.7	56.8±5.4
	RSV10	67.4±2.0	51.9±8.1	47.3±10.2
	RSV20	63.6±8.2	57.4±4.3	47.7±5.7
	RSV40	67.4±2.1 ^{a}	50.4±6.0 ^{b}	45.8±1.5 ^{b}
	RSV80	62.3±6.8	45.7±6.3	48.1±7.7
SYBR+/PI-/JC-1+				
(%)	CTR	59.7±4.1	50.3±6.6	53.8±4.5 $_{A}$
	RSV10	66.3±1.4	45.5±10.4	43.1±8.1 $_{AB}$
	RSV20	61.2±4.8	49.9±7.5	44.8±6.4 $_{AB}$
	RSV40	64.0±2.9 ^{a}	39.7±12.8 ^{b}	39.9±5.1 $_{B}$ ^{b}
	RSV80	58.1±8.1	37.1±10.4	37.7±1.9 $_{B}$

TM %	CTR	60.0±5.6	53.7±6.1	53.5±4.5
	RSV10	60.0±6.2	52.3±7.8	49.7±7.6
	RSV20	57.5±10.0	52.2±5.6	51.2±6.0
	RSV40	60.0±7.3	51.5±6.4	50.5±5.7
	RSV80	57.0±10.8	46.0±3.0	41.7±9.4
PM %	CTR	43.5±3.3^{a}	32.5±2.6 $_{A}^{ab}$	29.7±2.2 ^{b}
	RSV10	42.7±7.0 ^{a}	27.7±4.1$_{AB}^{b}$	29.0±6.9 ^{ab}
	RSV20	40.2±9.7	31.0±2.2 $_{AB}$	27.5±2.5
	RSV40	40.5±7.1^{a}	27.5±2.1$_{AB}^{b}$	26.7±4.8 ^{b}
	RSV80	36.0±6.9	25.0±2.6 $_{B}$	21.3±4.7
VAP	CTR	128.6±6.0	117.5±4.4	116.5±4.5
	RSV10	127.1±5.1	115.8±6.7	114.0±5.3
	RSV20	125.6±6.7	117.7±7.9	113.7±8.2
	RSV40	124.0±8.2	116.0±8.2	109.5±6.8
	RSV80	115.0±6.2 ^{a}	108.3±2.1 ^{b}	101.3±8.3 ^{b}
VCL	CTR	263.7±13.7	263.7±19.6	264.7±14.6
	RSV10	270.0±15.2	260.3±24.4	265.7±22.8
	RSV20	262.4±20.2	268.0±26.5	264.7±25.8
	RSV40	260.8±19.4	268.7±23.5	256.0±18.6
	RSV80	244.7±16.2	257.3±8.4	240.3±21.1

Table 1. Quality parameters of stallion spermatozoa at 0 h and after 24 h of storage at 4°C and 10°C in presence of 10, 20, 40, 80 µM resveratrol (RSV10, RSV20.RSVO40, RSV80, respectively). Values of viability, DNA damaged cells, reacted spermatozoa (RA), viable spermatozoa with functional active mitochondria (SYBR+/PI-/JC-1+) and motility parameters (TM, PM, VAP, VCL) are expressed as mean ± standard deviation. Different capital letters within a column and different small letters within a row indicate significant ($p<0.05$) differences.

Parameters	Treatment	0h	24h
Viability	CTR	77.3±6.6[1]	69.7±5.5[2]
	RSV10	84.1±4.3[1]	71.8±2.3[2]
	RSV20	78.6±3.2[1]	67.1±8.0[2]
	RSV40	72.5±12.7[1]	67.9±5.9[2]
	RSV80	75.2±1.8[1]	65.5±2.5[2]
DNA damaged cells %	CTR	26.2±1.4	31.7±3.0
	RSV10	31.1±2.6	32.6±5.2
	RSV20	31.2±4.6	31.6±5.6
	RSV40	28.2±1.4	31.1±2.1
	RSV80	27.3±2.3	31.6±4.8
RA %	CTR	15.0±5.9	21.9±7.4
	RSV10	14.3±5.6	31.2±8.1
	RSV20	17.7±6.9	22.7±7.7
	RSV40	17.0±7.1	31.9±7.1
	RSV80	16.6±8.8	27.5±7.4
JC-1$^+$ (%)	CTR	62.1±4.0	56.1±4.7
	RSV10	67.4±2.0[1]	49.6±8.6[2]
	RSV20	63.6±8.2	52.6±7
	RSV40	67.4±2.1[1]	48.1±4.7[2]
	RSV80	62.3±6.8[1]	46.9±6.5[2]
SYBR$^+$/PI$^-$/JC-1$^+$ (%)	CTR	59.7±4.1[1]	52.1±5.5 $_a$ [2]
	RSV10	66.3±1.4[1]	44.3±8.5$_{ab}$ [2]
	RSV20	61.2±4.8[1]	47.3±7.0$_{ab}$ [2]
	RSV40	64.0±2.9 [1]	39.8±9.0$_b$ [2]
	RSV80	58.1±8.1[1]	37.4±6.7$_b$ [2]
TM %	CTR	60.0±5.6[1]	53.6±5.0$_a$ [2]
	RSV10	60.0±6.2[1]	51.0±7.0$_{ab}$ [2]
	RSV20	57.5±10.0[1]	51.8±5.4$_{ab}$ [2]
	RSV40	60.0±7.3[1]	51.0±5.7$_{ab}$ [2]
	RSV80	57.0±10.8[1]	43.8±6.7$_b$ [2]

PM %	CTR	58.9±7.3 [1]	31.1±2.7$_a$ [2]
	RSV10	43.5±3.3 [1]	28.3±5.1$_{ab}$ [2]
	RSV20	42.7±7.0 [1]	29.3±2.9$_a$ [2]
	RSV40	40.2±9.7[1]	27.1±3.4$_{ab}$ [2]
	RSV80	40.5±7.1[1]	23.2±4.0$_b$ [2]
VAP	CTR	128.6±6.0[1]	117.0±4.2$_a$ [2]
	RSV10	127.1±5.1[1]	114.9±5.5$_{ab}$ [2]
	RSV20	125.6±6.7[1]	115.8±7.8$_a$ [2]
	RSV40	124.0±8.2[1]	112.8±7.8$_{ab}$ [2]
	RSV80	115.0±6.2[1]	104.8±6.7 $_b$[2]
VCL	CTR	263.7±13.7	264.3±16.0
	RSV10	270.0±15.2	263.0±21.3
	RSV20	262.4±20.2	266.4±24.3
	RSV40	260.8±19.4	262.4±20.7
	RSV80	244.7±16.2	248.8±17.1

Table 2. Quality parameters of stallion spermatozoa at 0 h and 24 h liquid storage in presence of 10, 20, 40, 80 µM resveratrol (RSV10, RSV20.RSVO40, RSV80, respectively). As no difference was recorded at 24h between 4°C and 10°C in each Resveratrol treated group, the values were pooled.

Values of viability, DNA damaged cells, reacted spermatozoa (RA), viable spermatozoa with functional active mitochondria (SYBR+/PI-/JC-1+) and motility parameters (TM, PM, VAP, VCL) are expressed as mean ± standard deviation. Different letters within a column and different numbers within a row indicate significant ($p<0.05$) differences.

3rd PAPER

Title

COMBINING REDUCED GLUTATHIONE AND ASCORBIC ACID HAS SUPPLEMENTARY BENEFICIAL EFFECTS ON BOAR SPERM CRYOTOLERANCE

Running title

GSH, ascorbic acid and boar sperm cryopreservation

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Abstract

The main aim of this work was to evaluate how supplementing freezing and thawing media with reduced glutathione (GSH) and/or L-acid ascorbic (AC) affected the quality parameters of frozen-thawed boar spermatozoa. With this purpose, semen samples of 12 ejaculates coming from 12 boars were used. Each ejaculate was split into seven aliquots to which 5 mM GSH and 100 µM AC were added separately or together at two different steps of freeze-thawing. Various sperm parameters (levels of free-cysteine residues in sperm nucleoproteins, sperm viability, acrosome-membrane integrity, intracellular peroxide and superoxide levels (ROS), and total and progressive motility) were evaluated before freezing and 30 and 240 min after thawing. Both GSH and AC significantly improved boar sperm cryotolerance when they were separately added to freezing and thawing media. However, the highest improvement was recorded when both freezing and thawing media were supplemented with 5 mM GSH plus 100 µM AC. This improvement was observed in sperm viability and acrosome integrity, sperm motility and nucleoprotein structure. Although ROS levels were not much increased by freeze-thawing procedures, the addition of GSH and AC to both freezing and thawing extenders significantly decreased intracellular peroxide levels and had no impact on superoxide levels. According to our results, we can conclude that supplementation of freezing and thawing media with both GSH and AC has a combined, beneficial effect on frozen-thawed boar sperm that is greater than that obtained with the separate addition of either GSH or AC.

Keywords: Sperm cryopreservation, boar semen, sperm survival, ascorbic acid, reduced glutathione

1. Introduction

Currently, cryopreservation is the most efficient method for long-term sperm storage in boars and in other mammalian species [1]. In addition, the use of frozen-thawed boar semen may improve biosafety and could minimise adverse effects of a sudden outbreak of a contagious illness or a natural disaster. Furthermore, successful cryopreservation of boar semen is necessary to produce an effective gene banking [2]. Despite these potential advantages, cryopreserved semen is not routinely used in the pig industry and this is largely due to cell

cryodamage that occurs during freezing and thawing procedures. Such a cryodamage ultimately leads to a reduction in the sperm fertilizing ability [3,4].

To improve cryopreservation technology for boar semen, many studies have focused on trying to understand the mechanisms underlying cryodamage [5,3,6]. From these reports, the most evident damage from freeze-thawing procedures has been understood to affect plasma and acrosome membranes, mitochondrial mid-piece and axonema [7]. More recently, boar sperm cryopreservation has also been demonstrated to alter the nucleoprotein-DNA structural interaction through disrupting disulphide bridges between cysteines in sperm nucleoproteins [8,9,10,11].

In order to minimise sperm cryodamage, different antioxidants, such as α-tocopherol [12], reduced glutathione [13,14,10,11], superoxide dismutase and catalase [15], have been added to freezing extenders. Following this, positive effects have been reported for post-thaw sperm survival and motility and for sperm fertilizing ability both *in vitro* and in *vivo* [11,14]. In the case of reduced glutathione (GSH), which plays a relevant role for the maintenance of intracellular redox balance [16], its beneficial effects on post-thaw sperm function and survival have been reported to depend on the intrinsic ejaculate freezability, since poor freezability ejaculates require a higher GSH concentration than good freezability ones [17]. Ascorbic acid (AC) is another antioxidant that has shown positive effects during vitrification-warming of porcine embryos [18,19], and supplementing freezing media with an AC-derivative, ascorbic acid 2-O-α-glucoside (AA-2G), has been seen to improve sperm motility and survival, and reduce lipid peroxidation and DNA damage in Okinawan native pig [20].

While the separate addition of GSH and AC to freezing and/or thawing media has been reported to be beneficial for boar sperm survival after thawing, no previous work has either compared the effects of these two antioxidants in the same study, or combined them to evaluate whether the extent of their beneficial effects increases. Against this background, the present study sought to determine the effects of supplementing freezing and/or thawing media with GSH and/or AC (i.e. alone or combined) on several parameters of boar frozen-thawed spermatozoa, including sperm membrane integrity, motility, intracellular peroxide and superoxide levels, and integrity of nucleoprotein structure. A total of six treatment combinations plus a control were evaluated, and results show that boar sperm survival at post-thawing is increased when GSH and AC are added together to both freezing and thawing media.

2. Materials and Methods

The experimental protocol was designed following the guidelines established by the Animal Welfare Directive of the Regional Government of Catalonia (Spain), the Ethics Commission of the Autonomous University of Barcelona (Bellaterra, Spain), and the Spanish welfare and protection standards in swine (R.D. 1392/2012).

2.1. Seminal samples

In total, twelve ejaculates collected from twelve healthy Pietrain boars, 2-3 years of age, were used in this study. These animals were housed in climate-controlled buildings (Servicios Genéticos Porcinos, S.L.; Roda de Ter, Spain), fed with an adjusted diet (2.3 Kg·day^{-1}) and provided with water *ad libitum.*

Sperm-rich fractions were collected manually twice per week using the gloved hand method, diluted 1:1 (v:v) with a commercial extender (Androstar Plus®, Minitüb Ibérica SL; Tarragona, Spain), and cooled down to 16ºC-17ºC. An aliquot (extended semen) from each of these ejaculates was taken before starting the cryopreservation process to evaluate all sperm quality parameters.

2.2. Cryopreservation and thawing of sperm samples

Semen samples were cryopreserved using the Westendorf method [21] as adapted by [10]. Briefly, all ejaculates were cryopreserved 24h after extraction and storage at 17ºC [6]. At that time, the ejaculates were centrifuged at 17ºC and 600×*g* for 5 min. Pellets were subsequently recovered and diluted to 1.5×10^9 spermatozoa·mL^{-1} (using a Makler counting chamber; Sefi-Medical Instruments; Haifa, Israel) in a freezing medium containing lactose and egg yolk (LEY). Spermatozoa were next cooled down to 5ºC for 90 min, and subsequently diluted to 1×10^9 spermatozoa·mL^{-1} in a second medium (LEYGO) containing LEY plus 2% glycerol and 0.5% Orvus es Paste (Equex STM; Nova Chemical Sales Inc.; Scituate, MA, USA). Afterwards, sperm samples were packed in 0.5-mL labelled plastic straws (Minitub Ibérica, SL; Tarragona, Spain) distinguishing between the different treatments (i.e. control plus six experimental treatments). The straws were then transferred to a programmable freezer (Icecube14S-B; Minitub Ibérica, SL). The freezing programme (SY-LAB software; Minitub Ibérica, SL; Spain) consisted of 313 sec of cooling at the following rates: -6ºC·min^{-1} from 5ºC to -5ºC (100 sec), -39.82 ºC·min^{-1} from -5ºC to -80ºC (113 sec), maintained for 30 sec at -80ºC, and cooled at

−60ºC·min^{-1} from −80ºC to −150ºC (70 sec). The straws were finally plunged into liquid N_2 (−196 ºC) for further storage.

After at least two weeks of storage at -196ºC, four straws per ejaculate and treatment were taken, thawed at 37ºC for 20 sec and immediately diluted with three volumes of warmed thawing medium (Androstar Plus®) at 37ºC, at a final dilution of 1/4 [22]. When appropriate, thawing medium was supplemented with 5 mM GSH and/or 100 µM AC.

2.3 Experimental design

During the sperm cryopreservation process, each ejaculate was divided into the following 7 experimental groups: (FT – C) without any antioxidant addition; (GSH) with 5 mM GSH added to LEY and LEYGO cryopreservation extenders; (AC) with 200 µM AC added to LEY and LEYGO cryopreservation extenders; (GSH+AC) with 5 mM GSH and 100 µM AC added to LEY and LEYGO cryopreservation extenders; (AC/AC) with 100 µM AC added to LEY and LEYGO cryopreservation extenders and thawing medium; (GSH/GSH) with 5 mM GSH added to LEY and LEYGO cryopreservation extenders and thawing medium; and (GSH+AC/GSH+AC) with 5 mM GSH and 100 µM AC supplementation added to LEY and LEYGO cryopreservation extenders and thawing medium. These combinations of treatments were set up after a series of preliminary experiments which showed the main harmful effects on boar sperm were caused during cooling and freezing steps.

All sperm parameters (sperm membrane integrity, intracellular peroxide and superoxide levels, sperm motility and amounts of free-cysteine residues in sperm nucleoproteins) were evaluated at 30 and 240 min after thawing at 37ºC, following other previous studies from our group [10,6,11].

2.4. Flow cytometric analyses

Information about flow cytometry analyses is given according to the recommendations of the International Society for Advancement of Cytometry (ISAC) [23]. These analyses were conducted to sperm membrane and acrosome integrity, and intracellular peroxide and superoxide levels. In each case, the sperm concentration in each treatment was previously adjusted to 1×10^6 spermatozoa·mL^{-1} in a final volume of 0.5 mL, and spermatozoa were then stained with the appropriate combinations of fluorochromes, following the protocols described below (i.e. SYBR-14/PI, PNA-FITC/PI, H_2DFCDA/PI, HE/YO-PRO®-1 or PI after hypotonic treatment to correct raw data).

Samples were evaluated through a Cell Laboratory QuantaSC™ cytometer (Beckman Coulter; Fullerton, California, USA). This instrument was equipped with two light sources: an arch-discharge lamp and an argon ion laser (488 nm) set at a power of 22 mW, but in our case, only the single-line visible light from the argon laser was used to perform the analyses. Cell diameter/volume was directly measured with the Cell Lab Quanta™ SC cytometer employing the Coulter principle for volume assessment, which is based on changes in electrical resistance produced by non-conductive particles suspended in an electrolyte solution. This system, thus, has forward scatter (FS) replaced by electronic volume (EV). Furthermore, the EV channel was calibrated using 10-μm Flow-Check fluorospheres (Beckman Coulter) by positioning this size bead in channel 200 on the volume scale.

Two different optical filters were used: a) FL-1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, Band Pass filter: 525 nm, detection width 505-545 nm; and b) FL-3 (red fluorescence): Long Pass filter: 670/30 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to particular staining methods. FL-1 was used to detect green fluorescence from SYBR14, PNA-FITC, YO-PRO®-1 and H_2DFCDA, whereas FL-3 was used to detect red fluorescence from HE and PI.

Sheath flow rate was set at 4.17 μl min^{-1} in all analyses, and EV and side scatter (SS) were recorded in a linear mode (in EE *vs.* SS dot plots) for at least 10,000 events per replicate. The analyser threshold was adjusted on the EV channel to exclude subcellular debris (particle diameter<7 μm) and cell aggregates (particle diameter >12 μm). Therefore, the sperm-specific events were positively gated on the basis of EV and SS distributions, whereas the others were gated out. In some protocols, as described below, compensation was used to minimise spill-over of green fluorescence into the red channel.

Information on the events was collected in List-mode Data files (.LMD), and these generated files were then analysed using Cell Lab Quanta®SC MPL Analysis Software (version 1.0; Beckman Coulter) to quantify dot-plot sperm populations (FL-1 *vs.* FL-3) and to analyse the cytometric histograms. In PNA-FITC/PI, H_2DFCDA/PI and HE/YO-PRO®-1 assessments, data obtained from flow cytometry experiments were corrected according to the procedure described by [24]. Each assessment for each sample and parameter was repeated three times in independent tubes prior to calculating the corresponding mean ± standard error of the mean (SEM).

Unless otherwise stated, all flurochromes used for these analyses were purchased from Molecular Probes® (Invitrogen; Eugene, Oregon, USA) and diluted with dimethyl sulfoxide (DMSO; Sigma) following manufacturer instructions.

2.4.1. Sperm membrane integrity

Sperm viability was assessed using the LIVE/DEAD® Sperm Viability Kit (SYBR-14/ PI), according to the protocol described by [25]. Sperm samples were incubated at 37.5ºC for 10 min with SYBR-14 at a final concentration of 100 nM, and then with PI at a final concentration of 12 µM for 5 min and at the same temperature. FL-1 was used for measuring SYBR-14 fluorescence, while PI fluorescence was detected through FL-3. After this assessment, three sperm populations were identified: i. viable green-stained spermatozoa (SYBR-14$^+$/PI$^-$); ii. non-viable red-stained spermatozoa (SYBR-14$^-$/PI$^+$) and, iii. non-viable spermatozoa that were stained both green and red (SYBR-14$^+$/PI$^+$). Non-sperm particles (debris) were found in the SYBR-14$^-$/PI$^-$ quadrant.

Single-stained samples were used for setting the electronic volume (EV) gain, FL-1 and FL-3 PMT-voltages and for compensation of SYBR-14 spill over into the PI/FL-3 channel (2.45%).

Plasma membrane integrity was also evaluated through PNA-FITC/PI. Spermatozoa were stained with the lectin from *Arachis hypogaea* (peanut agglutinin, PNA) conjugated with fluorescein isothiocyanate (FITC) and counterstained with PI, following the protocol described in [26]. With this purpose, spermatozoa were stained with PNA-FITC (final concentration: 2.5 µg·mL^{-1}) and PI (final concentration: 12 µM) and incubated at 37.5ºC for 10 min. PNA-FITC fluorescence was collected through FL-1 and PI fluorescence was detected through FL-3. As spermatozoa were not previously permeabilised, they were identified and placed in one of the four following populations [6]: i.) spermatozoa with intact plasma membrane (PNA-FITC$^-$/PI$^-$); ii.) spermatozoa with damaged plasma membrane that presented

a non-fully intact outer acrosome membrane (PNA-FITC$^+$/PI$^+$); iiii.) spermatozoa with damaged plasma membrane and lost outer acrosome membrane (PNA-FITC$^-$/PI$^+$); and iv.) spermatozoa with damaged outer acrosome membrane (PNA-FITC$^+$/PI$^-$).

Unstained and single-stained samples were used for setting the electronic volume (EV) gain, FL-1 and FL-3 PMT-voltages and for compensation of PNA-FITC spill over into the PI channel (2.45%).

2.4.2. Assessment of intracellular ROS levels

Intracellular peroxide (H_2O_2) and superoxide ($O_2^{-\bullet}$) levels were determined using two different oxidation-sensitive fluorescent probes: 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and hydroethidine (HE). Following a procedure modified from [27], a simultaneous differentiation of viable from non-viable spermatozoa was performed by co-staining the spermatozoa either with PI or with YO-PRO®-1.

In the case of peroxides, spermatozoa were stained with H_2DCFDA at a final concentration of 200 µM and PI at a final concentration of 12 µM, and incubated at 25ºC for 60 min in the dark. H_2DCFDA is a stable cell-permeable non-fluorescent probe that is intracellularly de-esterified and becomes highly fluorescent 2',7'-dichlorofluorescein (DCF$^+$) upon oxidation [27]. This DCF$^+$ fluorescence was collected through FL-1, whereas PI fluorescence was detected through FL-3. Unstained and single-stained samples were used for setting the electronic volume (EV) gain, FL-1 and FL-3 PMT-voltages and data were not compensated.

In the case of superoxides, samples were stained with HE (final concentration: 4 µM) and YO-PRO®-1 (final concentration: 25 nM) and incubated at 25ºC for 40 min in the dark [27]. Hydroethidine is freely permeable to cells and is oxidised by $O_2^{-\bullet}$ to ethidium (E$^+$) and other products. Fluorescence of ethidium (E$^+$) was detected through FL-3, and that of YO-PRO®-1 was collected through FL-1. Data were not compensated.

Data are expressed as means ± SEM of percentages of viable spermatozoa with high intracellular H_2O_2 levels (high DCF $^+$ fluorescence) and of viable spermatozoa with high $O_2^{-\bullet}$ levels (high ethidium fluorescence; E$^+$).

2.4.3. Correction of Data: Identification of non-DNA containing particles

The percentage of non-DNA-containing particles (alien particles) was determined since in some flow cytometry assessments, especially when working with cryopreserved

spermatozoa, there may be an overestimation of sperm particles. Indeed, alien particles such as cytoplasmic droplets, cell debris, or diluent components (such as egg yolk), will often show EV/ SS characteristics similar to those of spermatozoa and cannot thus be excluded via light scatter [24]. For this reason, 5 µL of each sperm sample was diluted with 895 µL of milliQ®-distilled water. Samples were then stained with PI at a final concentration of 12 µM and incubated at 37.5ºC for 3 min, according to the procedure described in [24]. Percentages of alien particles (*f*) were used to correct the percentages of non-stained spermatozoa (q_1) in each sample and treatment after PNA-FITC/PI, H_2DFCDA/PI and HE/YO-PRO®-1 assays, according to the following formula:, where q_1' was the percentage of non-stained spermatozoa after correction.

2.5. Sperm motility

Sperm-motility analysis was performed through a commercial CASA system (Integrated Sperm Analysis System V1.0, Proiser; Valencia, Spain). This system is based on the analysis of 25 consecutive digitalised photographic images obtained from a single field at a magnification of 100× in a negative phase-contrast field (Olympus BX41 microscope; Olympus 10x 0.30 PLAN objective lens). These 25 consecutive photographs were taken in a time lapse of 1 sec, which implied a velocity of image capturing of one photograph every 40 10^{-3} s. Five to six separate fields were taken for each replicate, and three replicates were evaluated per sample.
For each assessment, 15 µL of sperm sample were placed in a Makler counting chamber (Sefi-Medical Instruments), and total and progressive motility together with other kinetic parameters were recorded [28]. Total motility was defined as the percentage of spermatozoa that showed a VAP>10 µm·s^{-1}, whereas progressive motility was defined as the percentage of spermatozoa that showed a VAP>45 µm·s^{-1}.

2.6. Evaluation of free-cysteine residues in sperm nucleoproteins before and after freeze-thawing

The determination of free cysteine radicals in sperm head proteins as an indirect measure of disrupted disulphide bridges within nucleoproteins was carried out following the protocol adapted to boar spermatozoa and described by Flores et al. [9]. Briefly, samples were centrifuged at 600 ×*g* and 17°C for 20 min and resuspended in an ice-cold 50 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 1% (v:v) Nonidet, 0.5% (w:v) sodium deoxycolate, 1 mM benzamidine, 10 µg·mL^{-1} leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM

Na_2VO_4. Spermatozoa were subsequently homogenised through sonication (Ikasonic U50 sonicator, Ika® Labortechnick; Staufen, Germany). Obtained homogenates were centrifuged at 850 ×*g* and 4°C for 20 min. Both the resultant supernatants and the upper layer of the pellet were discarded and pellets were resuspended in 300 µL of Tris buffer. The purity of this separation was determined by observation under a phase-contrast microscope (Zeiss Primo Star, Karl Zeiss; Jena, Germany) at 400× magnifications (Zeiss Plan-Achromat 40×/0.65; Karl Zeiss). Such purity was described as the percentage of loose heads in comparison with the presence of whole, non-fractioned sperm and separated tails in the sample. In all cases, the mean purity percentage was higher than 95% of loose heads in comparison with other sperm presentations, such as intact spermatozoa or cells with different types of tail rupture without separating the heads from their respective mid-pieces.

The levels of free cysteine radicals in sperm nucleoproteins were determined in the samples obtained by using the 2,2'-dithiodipyridine technique (2,2'-dipyridyl disulphide; Sigma-Aldrich®) as described in [29]. With this purpose, the 10-µL aliquots of resuspended, isolated sperm heads obtained as described above were added to 990 µL of an aqueous solution of 0.4 mM 2,2'-dithiodipyridine. Ten-µL aliquots of cysteine standards from 0.1 to 5 mM (Sigma-Aldrich®) were also added to 990 µL of 0.4 mM 2,2'-dithiodipyridine for evaluation. In all cases, mixtures were incubated at 37°C for 60 min, and levels of free cysteine radicals were finally determined through spectrophotometric analysis at a wavelength of 343 nm. The results obtained were normalised through a parallel determination of the total protein content of samples by the Bradford method [30], using a commercial kit (Quick Start™ Bradford Protein Assay; BioRad; Hercules, California, USA). Three replicates per sample and treatment were evaluated, and the corresponding mean ± SEM was calculated.

2.7. Statistical analyses

Data were analysed using a statistical package (IBM® SPSS® 21.0 for Windows; IBM corp.; Chicago, Illinois), and are presented as mean ± SEM. First, all data (x) were tested for normality and homoscedasticity using the Shapiro-Wilk and Levene tests. When necessary, data were transformed using arcsine square root (arcsin $\sqrt{x}$), prior to run a general linear model for repeated measures (mixed factorial design), where the random effects-factor was the boar, the fixed-effects factor was the treatment (i.e. FT-C, GSH, AC, GSH/GSH, AC/AC, and GSH+AC/GSH+AC) and the intrasubject factor was the post-thawing incubation time (i.e. 30

min or 240 min). In all cases, each sperm parameter was the dependent variable, and multiple post-hoc comparisons were calculated using a *t*-test with Bonferroni adjustment.
In all statistical analyses, the minimal level of significance was set at $P<0.05$.

3. Results

Table 1 shows all sperm parameters evaluated before starting freeze-thawing procedures (i.e. extended semen). As the main aim of this work was evaluating how the addition of GSH and/or AC to freezing and thawing extenders, the following tables and subsections are mainly focused on data from 30 min and 240 min post-thawing in the seven experimental groups (control plus six treatments groups). In any case, cryopreservation significantly ($P<0.05$) decreased most sperm quality parameters, as seen at both 30 min and 240 min post-thawing (Tables 2 to 5).

3.1. Effects of supplementing freezing and thawing media with GSH and/or AC on sperm membrane integrity

As shown in Table 2, sperm viability at 30 min post-thawing was significantly ($P<0.05$) increased by AC when both freezing and thawing media were supplemented, but not when AC was only added to freezing media (i.e. AC). At 30 min post-thawing, GSH supplementation in freezing and, to a greater extent, in both freezing and thawing media, significantly ($P<0.05$) increased the percentage of viable spermatozoa when compared to control group.
At 240 min post-thawing, significant ($P<0.05$) differences between control, AC and GSH treatments were observed, with a progressive increase in sperm viability from control to AC and GSH groups. In addition, a significant ($P<0.05$) decrease in sperm viability from 30 to 240 min was observed in all treatments, except when both freezing and thawing media were supplemented with GSH and AC. In fact, the percentage of $SYBR14^+/PI^-$ spermatozoa when the two antioxidants were added together to both freezing and thawing media was significantly ($P<0.05$) higher than in control and the other five treatments.
As stated, sperm membrane integrity was also evaluated through PNA-FITC/PI test. At 30 min post-thawing, percentages of viable spermatozoa without damage in their acrosome membranes ($PNA\text{-}FITC^-/PI^-$ spermatozoa) were significantly ($P<0.05$) lower in control than in all the other treatments except that only contained AC in the freezing medium (i.e. AC). Additionally, after 240 min post-thawing, all treatment groups significantly ($P<0.05$) differed

from the control in the percentage of PNA-FITC$^-$/PI$^-$ spermatozoa. Notwithstanding, the percentage of PNA-FITC$^-$/PI$^-$ spermatozoa when both freezing and thawing media were supplemented with a combination of GSH and AC was significantly (P<0.05) higher than in the other treatments.

3.2. Effects of supplementing freezing and thawing media with GSH and/or AC on intracellular ROS levels

At 30 min post-thawing, percentages of viable spermatozoa with high peroxide levels (DCF$^+$/PI$^-$) were significantly (P<0.05) higher in control group than in the other treatments. In contrast, after 240 min post-thawing, only the treatment that combined GSH with AC both in freezing and thawing media presented a significantly (P<0.05) lower percentage of DCF$^+$/PI$^-$ spermatozoa compared to the other groups (Table 3). In addition, it is worth noting that peroxide levels were seen to decrease significantly (P<0.05) in all treatments except GSH-GSH and GSH+AC/Control from 30 to 240 min post-thawing.

Regarding superoxides, the percentages of viable spermatozoa with high superoxide levels (E$^+$/YO-PRO-1$^-$) did not differ significantly (P>0.05) before and after cryopreservation. In addition, no differences (P>0.05) between experimental groups (i.e. controls and treatments) were observed either at 30 or 240 min post-thawing.

3.3. Effects of supplementing freezing and thawing media with GSH and/or AC on sperm motility

Percentages of total sperm motility at 30 min post-thawing were significantly (P<0.05) higher when freezing and thawing media contained GSH or GSH combined with AC. At 240 min post-thawing, not only those treatments containing GSH (i.e. GSH and GSH+AC) presented a significantly (P<0.05) higher percentage of total motile spermatozoa but also those that were only supplemented with AC (Table 4). In addition, supplementing freezing and thawing media with GSH+AC counteracted the significant (P<0.05) decrease of total sperm motility that occurred from 30 to 240 min post-thawing in the other experimental groups.

Regarding progressive sperm motility (Table 4), all treatments presented a significantly (P<0.05) higher percentage of progressive motile spermatozoa than control, after both 30 and 240 min post-thawing. Moreover, the two experimental groups that contained GSH+AC in freezing and in both freezing and thawing media presented significantly (P<0.05) higher

percentages of progressive motile spermatozoa than control and the other treatments after 240 min post-thawing.

3.4. Effects of supplementing freezing and thawing media with GSH and/or AC on integrity of nucleoprotein structure

Both GSH and AC supplementation counteracted the increase in free cysteine radicals in sperm nucleoproteins due to freeze-thawing protocols both after 30 and 240 min post-thawing (Table 5). Thus, after 30 min post-thawing levels of free cysteine radicals in sperm nucleoproteins were significantly (P<0.05) lower in GSH treatment than in AC, whereas the combination of GSH and AC did not significantly differ from GSH treatments.

After 240 min post-thawing, the treatment containing GSH+AC in both freezing and thawing media presented a significantly (P<0.05) lower amount of free cysteine radicals in sperm nucleoproteins than the other treatments, and did not significantly differ from the level observed at 30 min post-thawing (Table 5). In addition, treatments that included GSH presented a significantly (P<0.05) lower level of free cysteine radicals than control and treatments that only contained AC (Table 5).

4. Discussion

Our data clearly indicate that both GSH and AC have a combined improving effect on boar sperm cryotolerance, as results of sperm viability, motility and free cysteine residues levels demonstrate. This positive combination with a maximal effect at 5 mM GSH plus 100 µM AC is highlighted when comparing the results obtained in the presence of either GSH or AC alone with those obtained when both effectors are combined. Thus, while GSH and AC alone also improve sperm quality with regard to the control, the extent of such improvement is not as high as when GSH plus AC are added. This finding is a very important outcome if one takes into account that despite sperm cryopreservation offering many benefits to the swine industry, it is not regularly used for artificial insemination because of its lower sperm quality and fertilizing ability [31].

A positive effect of GSH and/or AC supplementation on nucleoprotein structure integrity was seen in all treatments, when compared to control group. In fact, at 30 min and especially at 240 min post-thawing, the levels of free cysteine residues in sperm nucleoproteins were lower in AC and GSH treatments than in control group. It is important to underline the

beneficial effect observed at 240min post-thawing because at this time point, as demonstrated by other studies [8,9,10], the destabilisation of nucleoprotein structure leads to chromatin decondensation and seems to underline the subsequent DNA fragmentation [32]. In fact, according to a recent study [11], levels of free cysteine radicals in sperm nucleoproteins are significantly correlated with percentages of spermatozoa with fragmented DNA at 240 min post-thawing. Our data suggest that the positive, combined effects of both GSH and AC are due to a combination of two separate protective actions. The first one would be the direct protective effect that GSH has on the disulphide bonds established between sperm nucleoproteins [33]. The other effect would be a purely antioxidant action exerted by both GSH and AC that would also contribute to protect the nucleoprotein structure. Furthermore, the combined effects of GSH and AC could prevent or delay the DNA fragmentation induced after the destabilisation of the nucleoprotein structure caused by freeze-thawing [10]. In addition, the GSH+AC combination could also enhance sperm quality parameters in poor freezability boar ejaculates, as these have been reported to be less resistant than good freezability ejaculates to cryopreservation procedures, not only in terms of sperm motility and viability, but also in their nucleoprotein structure integrity [17].

Regarding ROS production, our results support previous works that highlighted the low generation rate of peroxides and superoxides by boar sperm during freeze-thawing procedures, especially when compared with other species [6,10,27,34]. In fact, our data indicated that viable spermatozoa with high levels of H_2O_2 and $O_2^{\bullet -}$ at 30 and 240 min post-thawing were less than 4% in all treatments, thereby suggesting that ROS have a marginal role in explaining boar sperm cryodamage. In spite of this, while all treatments presented a significantly lower percentage of DCF^+/PI^- spermatozoa when compared to negative control at 30 min post-thawing, levels of intracellular $O_2^{\bullet -}$ were not influenced by AC and/or GSH treatments. In the case of superoxides, however, it is worth mentioning that while HE is currently considered as one of the best methods to evaluate intracellular $O_2^{\bullet -}$ levels, it is understood that other molecules different from superoxides can oxidize HE into E^+ [35]. Even in the absence of superoxides, other one electron oxidants can convert HE into dimeric products, such as HE–E+ or E^+–E^+ that may hamper a proper evaluation of intracellular superoxide levels [36] and could ultimately lead to misinterpretation.

Previous studies have demonstrated that 5 mM GSH significantly reduces intracellular peroxide levels in frozen-thawed boar sperm when added to freezing or thawing media [13,14,17], whereas 100 µM AC supplementation to oocyte culture and vitrification media also

decreases significantly relative peroxide levels [19]. The positive effects of GSH and/or AC supplementation to freezing and/or thawing media in the decrease of H_2O_2 intracellular levels could be related to a reduction of the oxidizing agents, as freeze-thawing procedures have been demonstrated to increase peroxides in boar spermatozoa [34]. Glutathione, in its reduced state (GSH), donates electrons to unstable molecules, such as ROS, while AC acts both as electron donor and enzymatic cofactor [37,38], thereby equilibrating the intracellular redox balance [16] and protecting cell membranes [39]. Therefore, whilst ROS production seems not to be the principal cause of cryodamage in boar sperm, the addition of GSH and AC to cryopreservation media improves the physiological balance between ROS production and antioxidant spermatozoa defences. Moreover, the centrifugation of fresh spermatozoa before freeze-thawing protocols may cause a loss of seminal plasma components, which play a key role in protecting sperm cells against ROS-mediated attack [40]. In fact, because of the limited capacity of the antioxidant system present in mammalian spermatozoa, the scavenging capacity of seminal plasma appears to be fundamental to provide the protection against free superoxide and peroxide radicals [38,39].

Besides the presence of antioxidant proteins and enzymes in seminal plasma, low molecular weight substances, such as GSH and AC, constitute the antioxidant defence system of boar seminal plasma [42]. Supporting this, a recent study characterized the activity of enzymatic and non-enzymatic antioxidants in the cauda epididymal spermatozoa and epididymal fluid and demonstrated an increase of antioxidant capacity in post-dialysis seminal plasma [43]. Furthermore, it has been demonstrated that the removal of dialyzable seminal plasma components and the prolonged contact time between sperm and seminal plasma that occurs during dialysis procedure enhance sperm quality at post-thawing [44]. In addition, the maintenance of boar spermatozoa in the whole seminal plasma, rather than the simple use of the rich fraction prior to freeze-thawing procedures, has been reported to reduce the cryo-induced DNA fragmentation in frozen-thawed semen [45]. Thus, albeit the mechanisms responsible for cryodamage in boar spermatozoa may be multiple, seminal plasma seems to play a key role while counteracting cryodamage. However, removing seminal plasma is a necessary step during freeze-thawing procedures. In this scenario, exogenous GSH and AC added to freezing and thawing solutions could substitute some endogenous components of seminal plasma.

Another question regards the mechanism/s by which sperm cryoprotection is increased when GSH and AC are combined. According to a recent study, supplementing *in vitro* maturation

medium (IVM) for boar oocytes with 50 µg·mL^{-1} ascorbic acid exerts a strong antioxidant effect through decreasing intracellular ROS content and increasing GSH levels [46]. Therefore, from our results, we could hypothesize that AC increases the intracellular GSH content in frozen-thawed boar spermatozoa, thereby resulting in an additional beneficial effect when compared to single GSH supplementation. Supporting this, intracellular GSH content has been seen to decrease after boar sperm cryopreservation [47].

In conclusion, the present study confirms the improvement of frozen-thawed boar sperm quality when GSH and AC are separately added to freezing and thawing media. However, results are even better when these two antioxidants are combined and added to both freezing and thawing media. Thus, the combined addition of AC and GSH to freezing and thawing boar sperm extenders is suggested in boar sperm cryopreservation.

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	Marker	**Extended semen**
Sperm membrane integrity	Viable spermatozoa ($SYBR14^+/PI^-$)	90.5 ± 3.9
	Viable spermatozoa with no damages in acrosome membrane ($PNA\text{-}FITC^-/PI^-$)	91.7 ± 3.7
Intracellular ROS levels	% Viable spermatozoa with high peroxide levels (DCF^+/PI^-)	2.0 ± 0.2
	% Viable spermatozoa with high superoxide levels ($E^+/YO\text{-}PRO\text{-}1^-$)	3.5 ± 0.3
Sperm motility	% Total motile spermatozoa	88.6 ± 4.7
	% Progressive motile spermatozoa	68.5 ± 3.7
Integrity of nucleoprotein structure	Free cysteine radicals in sperm nucleoproteins ($nm\ Cys \cdot \mu g\ protein^{-1}$)	2.9 ± 0.2

Table 1 Values of sperm parameters in extended semen samples used in the present study. Data (*n*=12 independent experiments) are expressed as means ± standard error of means (SEM).

Freezing media (LEY, LEYGO)	**Thawing medium**	Viable spermatozoa ($SYBR14^+/PI^-$)		Viable spermatozoa with no damages in acrosome membrane ($PNA\text{-}FITC^-/PI^-$)	
		30 min	*240 min*	*30 min*	*240 min*
Control	Control	53.8 ± 2.6^{a}	$33.5 \pm 1.4^{a,*}$	51.4 ± 2.5^{a}	$31.6 \pm 1.6^{a,*}$
AC	Control	53.4 ± 2.5^{a}	$39.7 \pm 1.6^{b,*}$	50.6 ± 2.4^{a}	$39.8 \pm 1.9^{b,*}$
AC	AC	60.4 ± 2.8^{b}	$41.0 \pm 1.8^{b,*}$	$58.2 \pm 2.8^{b,c}$	$41.9 \pm 2.4^{b,*}$
GSH	Control	61.7 ± 3.0^{b}	$50.2 \pm 2.5^{c,*}$	$59.7 \pm 2.6^{b,c}$	$45.1 \pm 2.2^{b,c,*}$
GSH	GSH	$69.1 \pm 3.3^{c,d}$	$52.5 \pm 2.4^{c,*}$	$64.5 \pm 3.1^{c,d}$	$50.7 \pm 2.6^{c,d,*}$
GSH+AC	Control	$65.4 \pm 3.0^{b,c}$	$53.7 \pm 2.5^{c,*}$	$63.3 \pm 3.2^{c,d}$	$52.3 \pm 2.6^{d,*}$
GSH+AC	GSH+AC	72.4 ± 3.4^{d}	61.6 ± 3.1^{d}	67.2 ± 3.6^{d}	$60.9 \pm 3.2^{e,*}$

Table 2 Integrity of sperm membrane evaluated through SYBR14/PI and PNA-FITC/PI tests after freeze-thawing with different supplemented freezing and thawing media. Data (*n*=12 independent experiments) are expressed as means ± standard error of means (SEM). Different letters mean significant differences between rows within the same column (i.e. the same post-thawing incubation time), whereas asterisks mean significant differences between columns (i.e. post-incubation times, 30 min *vs.* 240 min) for a given combination of treatments (i.e. a given row).

Freezing media (LEY, LEYGO)	**Thawing medium**	Viable spermatozoa with high peroxide levels (DCF^+/PI^-)		Viable spermatozoa with high superoxide levels (E^+/YO-PRO-1^-)	
		30 min	*240 min*	*30 min*	*240 min*
Control	Control	3.8 ± 0.4^{a}	$2.2 \pm 0.2^{a,*}$	3.6 ± 0.4^{a}	3.8 ± 0.4^{a}
AC	Control	2.9 ± 0.3^{b}	$2.0 \pm 0.2^{a,*}$	3.3 ± 0.3^{a}	3.5 ± 0.3^{a}
AC	AC	2.7 ± 0.4^{b}	$1.9 \pm 0.2^{a,*}$	3.5 ± 0.4^{a}	3.6 ± 0.4^{a}
GSH	Control	2.4 ± 0.3^{b}	$1.7 \pm 0.2^{a,*}$	3.2 ± 0.3^{a}	3.5 ± 0.3^{a}
GSH	GSH	2.6 ± 0.3^{b}	2.0 ± 0.2^{a}	3.0 ± 0.3^{a}	3.3 ± 0.3^{a}
GSH+AC	Control	2.5 ± 0.3^{b}	1.9 ± 0.2^{a}	3.1 ± 0.3^{a}	3.5 ± 0.3^{a}
GSH+AC	GSH+AC	2.4 ± 0.3^{b}	$1.3 \pm 0.1^{b,*}$	3.2 ± 0.3^{a}	3.4 ± 0.4^{a}

Table 3 Viable spermatozoa with high intracellular peroxide (DCF^+/PI^-) and superoxide levels (E^+/YO-PRO-1^-)after freeze-thawing with different supplemented freezing and thawing media. Data (*n*=12 independent experiments) are expressed as means ± standard error of means (SEM). Different letters mean significant differences between rows within the same column (i.e. the same post-thawing incubation time), whereas asterisks mean significant differences between columns (i.e. post-incubation times, 30 min *vs.* 240 min) for a given combination of treatments (i.e. a given row).

Freezing media (LEY, LEYGO)	**Thawing medium**	Total sperm motility (%)		Progressive sperm motility (%)	
		30 min	*240 min*	*30 min*	*240 min*
Control	Control	50.8 ± 3.1[a]	32.9 ± 2.0[a, *]	30.4 ± 1.8[a]	16.5 ± 0.9[a, *]
AC	Control	53.5 ± 3.6[a]	39.1 ± 2.1[b, *]	39.6 ± 2.3[b]	21.5 ± 1.2[b, *]
AC	AC	57.6 ± 3.2[a, b]	42.3 ± 2.4[b, *]	44.2 ± 2.6[b]	31.4 ± 1.8[c, *]
GSH	Control	60.2 ± 3.7[b, c]	48.3 ± 2.8[c, *]	50.1 ± 2.9[b, c]	34.6 ± 2.1[c, d,*]
GSH	GSH	67.4 ± 4.2[c, d]	53.8 ± 3.3[c, d, *]	53.2 ± 2.8[c]	38.4 ± 2.2[d, *]
GSH+AC	Control	66.5 ± 3.8[c, d]	55.4 ± 3.5[d, e, *]	52.9 ± 2.7[c]	45.4 ± 2.6[e, *]
GSH+AC	GSH+AC	70.5 ± 4.0[d]	63.8 ± 3.7[e]	55.3 ± 2.9[c]	47.1 ± 2.6[e, *]

Table 4 Total and progressive sperm motility after freeze-thawing with different supplemented freezing and thawing media. Data (*n*=12 independent experiments) are expressed as means ± standard error of means (SEM). Different letters mean significant differences between rows within the same column (i.e. the same post-thawing incubation time), whereas asterisks mean significant differences between columns (i.e. post-incubation times, 30 min *vs.* 240 min) for a given combination of treatments (i.e. a given row).

General Discussion and Conclusions

Our studies suggest that antioxidant addition during gamete cryopreservation can improve the quality of spermatozoa and oocyte. However the addition of antioxidants can fail to induce a beneficial effect or even cause a cell damage. In fact RESV supplementation, at tested concentrations, did not improve quality of stallion cooled semen and, furthermore, exerted a negative effect at high doses.

In the first study we demonstrated that 2µM RESV addition during the different phases of IVM and vitrification – warming procedures modulated the apoptotic process, enhancing the quality of devitrified oocytes. We assayed the effect of RESV on caspases activation and phosphatidilserine (PS) exteriorization, two important events of the cellular apoptotic process. Our results demonstrated that 2µM RESV when supplemented on IVM medium significantly increased the percentage of viable oocytes with inactive caspases if compared with CTR group. In parallel, the same RESV concentration significantly decrease the percentage of oocytes with PS exteriorization in all experimental groups treated with the polyphenol, if compared with the control one. Indeed according to our data, RESV mainly influences PS externalization rather than caspases activation. In agreement with other studies (Somfai et al., 2007; Kwak et al., 2012), it was hypothesized that RESV, at this concentration, is able to induce an intracellular GSH increase, counteracting the GSH depletion that occurs during oocyte vitrification and thus restoring cellular redox balance. This hypothesis is supported also by He et al. (2003), who demonstrated that inhibition of GSH efflux from cells had no effect on the activation of caspases, but decreased the translocation of PS. Therefore we can suppose that RESV addition could be able to maintain GSH homeostasis, with a consequent inhibition of PS externalization. Moreover, it is important to underline that, while caspase activation was demonstrated to be a reversible phenomenon (Vallorani et al., 2012), PS translocation from the inner to the external plasma membrane leaflet is a tardive and irreversible apoptotic event, which induce apoptotic cell engulfment from phagocytic cells (Adayev et al., 1998). Taken together, these results suggest that RESV addition in one or more steps of IVM and vitrification procedure could avoid the apoptotic process of PS externalization, increasing the percentage of viable vitrified oocytes.

In the second study RESV was not effective in preventing the decrease of stallion sperm quality parameters after refrigeration. In fact, the treatment with RESV at 10, 20, 40, 80 µM did not enhance semen quality after 24h storage at 4 and 10°C under anaerobic conditions. Moreover 40 µM and 80 µM RESV exerted a negative effect on mitochondrial activity, while 80µM RESV negatively affected several motility parameters (TM, PM and VAP). Therefore, at these doses, RESV could probably induce an oxidative and/or proapoptotic damage in stallion

semen. Moreover our results revealed no differences in parameters evaluated between two different temperatures (4 and 10°C) after 24h of storage under anaerobic conditions. Thus, it cannot be excluded that low doses RESV (10 and 20 uM or lower) could improve sperm quality parameters after a refrigeration longer than 24h, in which exogenous ROS have been demonstrated to increase DNA fragmentation and induce LPO in stallion semen (Baumber et al., 2003; Ortega Ferrusola et al., 2009). The opposite effects that RESV can exert at different concentrations are still debated. It was demonstrated that it can exert pro-apoptotic or anti-apoptotic effects depending on its concentration and on different cells and species investigated. Moreover is still unclear the mechanism by which RESV acts as antioxidant or pro-oxidant molecule. In fact it was recently demonstrated that increasing doses of RESV (25-100μM) can exert pro-apoptotic effect in murine adipocytes (Chen et al., 2015). On the contrary, Zou et al. study (2014) reported the inhibitory effect of RESV against trophoblast oxidative stress and apoptosis at 100 μM concentration, demonstrating antioxidant and anti-apoptotic effects of the polyphenol also at high doses. Biphasic proprieties of RESV were also demonstrated in androgen-sensitive prostate cancer cells and in endothelial cells: it exerts anti-apoptotic activity at low doses (~nM - 5 μM), whereas it shows a pro-apoptotic activity at high doses (15 μM-100 μM) (Signorelli and Ghidoni, 2005; In et al., 2006). About gametes, a recent study demonstrated that 15 μg/mL RESV was able to prevent the deleterious effects produced by oxidative damage on mouse spermatozoa, resulting in the maintenance of fertility (Mojica-Villegas et al., 2014). Moreover, Takeo et al., (2014) demonstrated that 20μM RESV added on IVM medium of bovine oocytes significantly increased the ATP content and the mitochondrial membrane potential of matured oocytes and improved the ratio of normal fertilization and the total cell number of blastocysts. Therefore according to the cell types and the phytoalexin concentration, RESV can exhibit different proprieties. As specifically regards the antioxidant propriety, RESV it is known to be a scavenger of superoxide, hydroxyl radicals, and peroxynitrite (Pervaiz and Holme, 2009) and to stabilize ROS, chelating Fe++ and Cu++ ions. Therefore RESV, as the other antioxidant molecules, acting as redox (reduction–oxidation) agent, might become a pro-oxidant under special conditions. Moreover it is able to maintain the concentration of intracellular antioxidant enzymes such as catalase, glutathione reductase, glutathione peroxidase and superoxide dismutase (SOD) (de la Lastra and Villegas, 2007). In addition this polyphenol seems to play a role in oxidative stress modulation by upregulating antioxidant genes with consequent increase of intracellular antioxidant enzymes (Cat and MnSOD) (Inglés et al., 2014) and down-regulating various inflammatory biomarkers, such as TNF and IL-6. Indeed, according to recent studies, RESV seems to play an important

role in the activation of sirtuins (SIRTS) (proteins involved in the transcription, apoptosis, and energetic cell regulation) and/or AMPK signaling pathway (Saldanha et al., 2013). As demonstrated by Ma et al. (2015) Sirt1 activity is required for proper mitochondrial distribution and activity during pig oocyte maturation, which therefore might affect fertilization and subsequent early embryo development.

Therefore our results, in agreement with those by other authors, suggest that RESV can exert pro-apoptotic or anti-apoptotic actions, depending on the gamete studied and the doses used, in a species-specific manner.

Furthermore, although apoptotic-like changes occur after semen and oocyte storage, the causes of cold-induced apoptosis need to be better investigated. In fact, it is still not clear if the oxidative stress is the principal cause of cold injury. On one hand, it has been demonstrated that ROS levels increase after vitrification and warming procedure on swine and bovine oocytes, and antioxidant addition to vitrification solutions prevents ROS formation (Gupta et al., 2010; Zhao et al., 2011). On the other hand, as regard semen storage, recent studies reported that ROS levels could play a different role according to the species. Johannisson et al. study (2014) detected hydrogen peroxide and superoxide by dichlorodihydrofluorescein-diacetate (H2DCFDA) and hydroethidine (HE) respectively, on stallion cooled semen, in order to determine their relationship with semen quality parameters (sperm morphology, motility, viability and chromatin integrity). The results of that study reported a large variation in superoxide and hydrogen peroxide production among stallions and also among ejaculates for certain individual stallions. The study demonstrated that endogenous hydrogen peroxide in the stored samples at 6°C for 24h , might have been detrimental to sperm motility, while there were no significant correlations between mean values for live superoxide-positive spermatozoa and progressive motility. In addition, live hydrogen peroxide-negative spermatozoa and chromatin damage were negatively correlated, indicating that low levels of hydrogen peroxide were correlated with good chromatin integrity.

Another recent interestingly study reported that stallion fertility is positively correlated with oxidative phosphorylation. In fact, according to this study semen motility parameters (TM, PM,VCL, VAP) and fertility were positive correlated with ROS production and lipid peroxidation in live cells (assessed after 24h storage at room temperature), suggesting that ROS production, within subclinical levels, could be the result of the intense mitochondrial activity (Gibb et al., 2014). Therefore, in stallion spermatozoa ROS may be some of the factors

influencing fertility and combinations of ROS variables may be included in a future model for prediction of the fertility of a semen sample.

In addition, it is important to highlight that the energy metabolism of stallion spermatozoa depends mostly on mitochondrial ATP production, unlike other species, such as boar and human, in which spermatozoa metabolism is highly dependent on glycolysis (Marin et al., 2003; du Plessis et al., 2014). Therefore, in the equine species mitochondrial activity and consequent sperm motility could depend on aerobic or anaerobic storage conditions, thereby influencing endogen ROS production. If aerobic conditions may lead to intense mitochondrial activity and consequent ROS increase, anaerobic storage may reduce cellular energy production, with consequent cellular damage. Furthermore, although the higher antioxidant activity of stallion semen was already demonstrated to be enough to counteract semen storage injuries (Kankofer et al., 2005), further studies are necessary to investigate if mitochondria-targeted antioxidants may be the solution to reduce the downstream effects of ROS on sperm functionality. Different parameters, such as aerobic and anaerobic conditions, time and temperature storage, individual and ejaculates differences, have to be taken into account for successful formulation of equine semen extenders.

In the matter of boar semen our study demonstrated that antioxidants addition on cryopreserved semen can counteract quality depletion of thawed semen. Five millimolar GSH and 200µM AA reduced nucleoprotein damage especially after 240 min post thawing and improved semen viability and motility. It is important to underline the beneficial effect observed at 240 minutes after thawing because at this time point, as reported by other studies (Flores et al., 2008, 2011), the destabilization of nucleoprotein structure leads to chromatin decondensation and seems to underline the subsequent DNA fragmentation. Our data suggest that the positive, combined effects of both GSH and AA are due to a combination of two separate protective actions. The first one would be the direct protective effect that GSH has on the disulfide bonds established between sperm nucleoproteins (Chatterjee et al., 2001). The other effect would be a purely antioxidant action exerted by both GSH and AA that would also contribute to protect the nucleoprotein structure. As regards ROS generation, while intracellular superoxide (•O2–) levels were not influenced by AA and GSH treatments, at 30min after thawing spermatozoa with high peroxide (H_2O_2) levels were significantly higher in the control group than in the other treatments, while at 240min post-thawing only the treatment that combined GSH with AA both in freezing and thawing media presented a significantly lower percentage of spermatozoa with high peroxide levels compared with the other groups. GSH, in its reduced state, is able to modulate intracellular ROS concentration,

donating electrons to unstable molecules, such as ROS, whereas AA acts both as electron donor and enzymatic cofactor (Padh, 1991; Belin et al., 2010), thereby equilibrating the intracellular redox balance and protecting cell membranes. From our results, we could hypothesize that AA increases the intracellular GSH content in frozen-thawed boar spermatozoa, thereby resulting in an additional beneficial effect when compared with single GSH supplementation. Supporting this hypothesis, a decrease of GSH content has been observed after boar sperm cryopreservation (Gadea et al., 2004).

In agreement with Guthrie et al. study (2012), our results showed a low generation rate of peroxides and superoxides (less than 4% in all treatments) on boar sperm during freezing-thawing procedures. Similar results were obtained by Yeste et al. (2013), who reported that good and bad freezability of boar ejaculates differs in the integrity of nucleoprotein structure but not in ROS levels. These results agree with those previously reported by Gomez et al. (2013). In contrast, a similar study conducted on horse semen demonstrated that good and poor freezability of stallion ejaculates differs in their reactive oxygen species levels after cryopreservation, but not in the damage extent on sperm nucleus. Conversely a similar study conducted in stallions reported significant differences in spermatozoa containing high ROS levels when stallion good freezability ejaculates (GFE) and poor freezability ejaculates (PFE) were compared (Yeste et al., 2014). For these reasons, the works of Yeste et al. (2013, 2014) suggested that the endogenous ROS defence systems could be challenged in a different extent during cooling storage and cryopreservation.

In conclusion, our studies suggest that antioxidant supplementations could enhance gamete storage technology, but further studies are necessary to elucidate the impact of oxidative stress on gamete storage of different species. The reasons of the reduced gamete quality after storage have to be better investigated in order to discriminate the different causes of cellular damage, according to the different species and gamete considered, in order to find the best approach for successful cryopreservation.

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