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Review

Reactive oxygen species can alter physiological parameters of sperm; the future of macromolecules in boar semen dilutions

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Abstract. Studies have indicated that excessive production of reactive oxygen species (ROS) can affect cellular signalling pathways. In reproductive health, the biotechnological manipulation of sperm used for human and animal reproduction can induce thermal and biochemical changes that increase ROS generation, causing effects such as decreased motility and a reduced fertilization potential. To prolong sperm viability, these effects can be partially offset through the use of seminal fluid diluents. Such diluents are classified into two main groups: those used for short-term preservation (1-3 days) and those used for long-term preservation (more than 4 days). The advantages of using long-term diluents include the ability to transport semen over long distances, which allows diagnostic testing to be performed, for example, by applying PCR techniques, before the semen is used. However, it has been reported that despite extending the lifespan of sperm, these dilution methods do not significantly improve its fertilization capacity. ROS modulators can produce a protective effect on sperm cell membranes, which benefits semen used for artificial insemination or subjected to storage (chilled or cryopreserved). Therefore, it is important to identify pharmacological properties over time as well as the concentration dependency of diluents; to estimate the oxidative stress status of a sample in the presence of a diluent by measuring ROS levels in seminal plasma, DNA oxidation, lipid peroxidation and mitochondrial enzymatic activity at different temperatures and storage times; and to examine the reducing effects of macromolecules under induced oxidative stress conditions on the physiological properties of sperm.
Key Words: porcine sperm, viability, reactive oxygen species.

Introduction.

Sperm physiology. The initial stages of the egg and sperm interaction are primarily dependent on male gamete activation. Thus, after semen is deposited in the female tract, the sperm undergo a process known as "capacitation", which prepares the sperm for the acrosome reaction (AR) (Darszon et al 2007). The mature sperm undergo several changes in the female tract. These changes have been defined as a sequence of complex processes that occur in parallel in the flagellum (hyperactivation) and the head (preparation for AR) of the sperm. Reorganization of the plasma membrane also occurs in this context as a result of the removal of cholesterol, hyperpolarization of the membrane potential and increases in intracellular calcium ($Ca^{2+}i$) and pH levels. To reach and fertilize the egg, the sperm must pass through four stages: 1) becoming motile for the first time, 2) developing hyperactivated motility to travel inside the female tract and reach the oocyte, 3) undergoing chemotaxis to find and approach the egg and 4) executing the acrosome reaction via exocytosis with the purpose of releasing hydrolytic enzymes that cleave the glycoproteins of the zona pellucida (ZP), thereby allowing penetration by the sperm.

Sperm viability. Fresh semen that has been ejaculated and collected (the sperm-rich fraction) undergoes mechanical stress during processing and handling in the laboratory due to transfer or the preparation of rapid dilutions. Temperature variation poses an additional stress, as a difference of $\pm$ 1°C can have negative effects on the quality of semen. Sperm handling procedures involve a number of cellular alterations that can

generate a premature state of sperm capacitation (Maxwell & Johnson 1997). The capacitation process leads to the acrosome reaction and reduces the lifetime of sperm. Therefore, stabilizing sperm that are undergoing technological treatments is necessary for subsequent successful fertilization. Such stabilization extends the fertile life of sperm (Vazquez et al 1996), thereby increasing the fertilization capacity. A premature state of capacitation is mainly attributed to the degree of spermatozoa dilution during different biotechnological removal treatments, which result in the depletion of adsorbed proteins and other compounds present in the seminal plasma that are necessary to maintain sperm viability. Research has shown that the addition of adequate amounts of seminal plasma can contribute to sperm membrane stability and protect sperm that are subjected to cryopreservation processes (Ollero et al 1997), high dilutions (Centurion et al 2003; Maxwell & Johnson 1999) or separation processes utilizing flow cytometry (Larsen et al 2004).

Sperm motility. After ejaculation, once the sperm mixes with the seminal plasma, which exhibits a pH <7.0, there is an alkalizing effect on the sperm cytoplasm (Hamamah & Gatti 1998), in turn inducing sperm motility. Another important factor in the activation of sperm motility is an increase in cAMP levels, which has been reported to activate protein kinase A (PKA) and cause axoneme phosphorylation (Harrison & Gadella 2005). Nevertheless, even though sperm may be motile, the fertilization capacity can be minimal but is reversed when the sperm undergo capacitation (Chang 1951). Once the sperm enter the oviduct, they go through a process of hyperactivation, which is characterized by high amplitude, asymmetrical beating pattern of the sperm tail (flagellum). These movements are associated with an increase in speed, a decrease in linearity and an increase in the amplitude of lateral head movement and the whipping of the flagellum, all of which differ from what is observed in isolated ejaculated sperm (Suarez 2008; Suarez & Pacey 2006). Various physiological stimuli, such as $Ca^{2+}i$, cAMP, bicarbonate and metabolic substrates, have been used for the initiation or maintenance of hyperactivation motility in vitro. In hamster sperm, $Ca^{2+}i$ can be added to the medium to maintain hyperactivation (Yanagimachi 1994), and treatment of boar sperm with the $Ca^{2+}i$ ionophore induces increased motility (Suarez & Dai 1992). Additionally, studies on the levels of cytoplasmic Ca^{2+} in the flagellum of hyperactivated hamster sperm showed significantly higher levels of motility (Suarez et al 1993). In a similar context, the involvement of intracellular stores of Ca^{2+} in the base of the bull sperm flagellum was demonstrated using antibodies against inositol 1,4,5-triphosphate (IP3) receptors, which normally release this cation from intracellular stores. Furthermore, pharmacological activation of these stores induces sperm cell activation (Ho & Suarez 2001).

Additionally, electrophysiology assays have shown the presence of voltage-dependent calcium channels that are important for sperm capacitation (Darszon et al 2005; Wennemuth et al 2000). Among these channels, sperm calcium channel channels (CatSper) have been shown to be important for activation phenomena and for the hyperactivation of sperm motility (Qi et al 2007; Xia et al 2007). These channels are composed of 4 components (CatSper1-4) (Quill et al 2001; Ren et al 2001) and are characterized by six transmembrane domains (6TM1) that are calcium-permeable and have so far only been described in murine and human sperm (Arnoult et al 1999; Wennemuth et al 2000). However, the presence of voltage-dependent Ca^{2+} (Cav) channels associated with CatSper has been demonstrated in both human and murine spermatozoa. These channels may be important for modulating the function of Cav channels and thereby regulate sperm function (Zhang et al 2006). However, there are no articles in literature showing the macromolecules membrane-stabilizing effect on the activation of sperm physiology, especially in intracellular calcium levels.

Sperm capacitation. Capacitation is a process that occurs in sperm consisting of a series of changes that occur before fertilization takes place in the female reproductive tract. Capacitation requires communication between the sperm and the environment while the sperm travels to the site of fertilization and concludes with the interaction of the sperm and the ZP, which initiates signalling leading to the AR (Breitbart 2003; Rossato et al 2001). The AR allows sperm to penetrate the physical barriers imposed by the oocyte and, thus, enables the delivery of genomic contents.

During capacitation, the sperm cell undergoes a series of biochemical and biophysical changes at the level of the membrane, the cytoplasm and the nucleus, which results in changes in motility patterns that enable the sperm to reach the oocyte and perform the AR prior to the fertilization process (Anderson & Killian 1994). Thus, the displacement of factors decapacitates the sperm membrane, like antioxidants, metal ion and peptides, increases the removal of cholesterol from the membrane and increases $Ca^{2+}i$ levels through the activation of Cav channels and CatSper, which leads to an increase in cAMP levels and phosphorylation of tyrosine residues (Aitken & McLaughlin 2007; Pons-Rejraji et al 2011).

The sperm handling procedures applied in artificial insemination (AI) involve a series of alterations that may result in a state of premature sperm capacitation (Maxwell & Johnson 1997). The capacitation process leads to the AR and causes a decrease in the lifespan of sperm. A premature state of capacitation can be induced through creating sperm dilutions, which are a component of various treatments and potentially lead to the removal of adsorbed proteins and other compounds present in the seminal plasma that are necessary to maintain sperm viability. AI has been established as a common practice for human and animal reproduction, and developing molecules that are capable of stabilizing the sperm membrane to extend the lifetime of sperm is therefore important.

Effects of reactive oxygen species (ROS) on sperm physiology. ROS are molecules derived from cell metabolism processes and include superoxide anions (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), nitric oxide (NO) and peroxynitrite (ONOO) (Rhoades et al 1990). These species cause cell injury through reacting with membrane phospholipids, by oxidizing sulfhydryl groups in enzymes and other proteins or via DNA fragmentation. Additionally, O^{2-} and H_2O_2 can diffuse through membranes and cause widespread damage (Rhoades et al 1990). The pig sperm lipid membrane is highly susceptible to damage caused by ROS, which can affect changes in sperm capacitation, the AR and fusion to the ZP of the oocyte during fertilization. Boar spermatozoa are highly susceptible to ROS, but the involvement of ROS in damage and/or capacitation is unclear. Exposing boar sperm to an ROS-generating system for 30 min was shown to rapidly increase hydrogen peroxide (H_2O_2) levels and lipid peroxidation in all sperm as well as to increase PLA in dead sperm, but did not affect intracellular O^{-2}; additionally, sperm viability remained high, but sperm became immotile in this experiment (Awda et al 2009). In the middle section of sperm, ROS alter mitochondrial processes that generate the energy required to propel the sperm flagella and, thus, reduce sperm motility (Awda et al 2009). Loss of sperm function can occur due to the presence of high levels of ROS after ejaculation. Kirchhoff (1998) and Agarwal et al (2006) indicated that sperm can produce and export ROS to the extracellular environment, with most of these ROS being generated by mitochondria as the product of monovalent reduction of molecular oxygen during oxidative phosphorylation (Agarwal et al 2006; Kirchhoff 1998). Moreover, Venereo (2002); Cerolini et al (2001) and Sellés (2003) suggested that the high content of unsaturated fatty acids in sperm plasma membranes and the small volume of cytoplasm facilitates the production of ROS (Sellés et al 2003; Venereo Gutierrez, 2002), which impairs the function of sperm and can result in decreased motility and vitality as well as a reduction in sperm and oocyte fusion (Cerolini et al 2001). These observations are in agreement with the findings of Agarwal et al (2006), who demonstrated that ROS-induced peroxidation of polyunsaturated fatty acids esterifies the phospholipids of sperm membranes, leading to permeabilization of the plasma membrane and acrosome and to a loss of sperm viability, motility and fertilization capacity (Agarwal et al 2006). Proteins are also targets of ROS, but less so than lipids because of the slow progress of the reactions involved. It has been suggested that the oxidation of amino acid residues can cause breakage of the peptide bonds between proteins and protein aggregates. Oxidative damage to proteins may result in the loss of enzyme catalytic activity, damage to the integrity of structural proteins or disruption of the regulation of metabolic pathways (Sanocka & Kurpisz 2004). DNA, particularly mitochondrial DNA because of its location, is also a target of attack by ROS and is exposed to a high, constant flow of ROS from the respiratory chain; mitochondrial DNA also lacks histones in its structure, which decreases

its stability (Kadirvel et al 2009; Koshikawa et al 2009). We explain these effects in Figure 1. One way to modulate the impact of increased ROS levels on the physiology and lifespan of sperm is to use membrane stabilizers that help to maintain membrane structure and prevent ionic imbalances that could lead to premature capacitation and eventual spermatic damage.

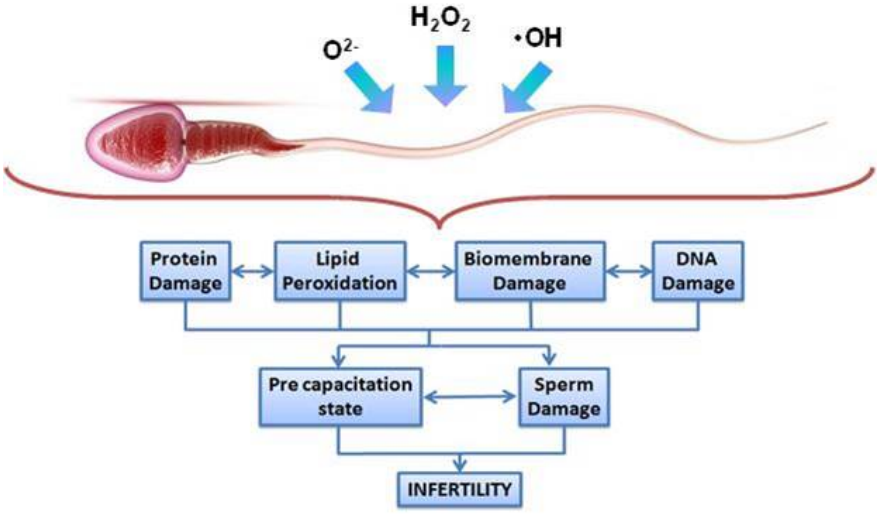

Figure 1. Effect of ROS on sperm physiology. The increase in extracellular ROS levels after ejaculation induces several changes in sperm physiology, including protein damage, lipid peroxidation, membrane and DNA damage, a premature capacitation state, sperm damage and a decrease in the sperm lifespan.

Effects of temperature changes on boar sperm. The distinctive lipid membrane composition of boar spermatozoa render them highly sensitive to cold shock (Pursel 1979), which alters sperm viability. Thus, when the temperature is reduced, the lateral movements of the phospholipids that constitute the membrane are also reduced, and lipid phase separations occur. This cold shock scenario is associated with irreversible damage to membrane proteins, which alters sperm function and viability (Simpson et al 1987). This susceptibility to cold shock means that in practice, boar semen samples must be kept at 15-20°C because a drop in the storage temperature limits their viability (Guthrie & Welch 2005). Preservation at these moderately low temperatures in turn limits the storage capacity of the samples because cellular metabolism cannot be reduced, nor can microbiological conditions be controlled with the same effectiveness at lower temperatures (5°C).

Types of diluents for semen preservation. Preserving semen through refrigeration (15°C or 5°C) or freezing is carried out to prolong the viability of male gametes for hours, days or indefinitely, because at room temperature, spermatozoa exhibit lower survival rates, mainly due to the depletion of energy reserves. The dilution of ejaculates aims to increase the available volume while maintaining the sperm concentration at adequate levels to serve the greatest number of females. The degree of dilution depends as much on the volume required for insemination (which varies according to the type of insemination, whether vaginal, cervical or intrauterine) as on the concentration of motile spermatozoa to be used for insemination. Regardless of the type of insemination, the number of motile sperm is correlated with fertility (Evans et al 1980).

Diluents are classified into two main groups: those utilized for short-term preservation (1-3 days or less) and those used for long-term preservation (over 4 days). The former are mainly employed in systems for the distribution of semen doses that are delivered within a close range (typical of European or American systems where the production of semen doses at the same farm is common practice). Long-term preservation is usually applied in the US or Norway, when there is a great distance

between the location of semen production and the final destination. The advantages of long-term diluents include the ability to transport samples over long distances, which allows diagnostic (Medrano et al 2005; Roca et al 2006) testing to be performed on semen before it is used. Such testing may be conducted using PCR (polymerase chain reaction) techniques, potentially aimed at the detection of viruses or a complete analysis of semen quality. This attribute allows better work organization in semen collection centres and greatly facilitates the distribution of these samples to breeding farms.

However, as discussed above, sperm are particularly sensitive to ROS levels, and these levels have been observed to increase immediately after ejaculate collection. Therefore, it is important to develop molecular compounds that are capable of stabilizing sperm membranes against increased reactive oxygen species levels and can be added to diluents that are currently in use.

Membrane stabilizers. It has been reported that peptides containing an RGD sequence can induce a transient effect on $Ca^{2+}i$ (Viets et al 2001). The importance of the regions surrounding the RGD sequence in enhancing the ability of the cations to facilitate the RGD-integrin interaction and subsequent biological activity has also been confirmed (Zhu et al 2000). These sequences have been described as being similar to those obtained from venomous animals, corresponding to the linear array of amino acids found in neurotoxins. In contrast, in our laboratory, we have obtained an analogue consisting of 12 amino acids from a larger peptide, BbKi (265 aa), which displays a non-linear RGD sequence with a loop between the RG-D amino acids. This analogue was produced from a protein obtained from the seeds of *Bauhinia bauhiniodes*, a plant of Amazonian origin from Brazil, and is not a neurotoxin. This analogue's association constant for the molecular recognition of membrane integrins is equivalent, but it exhibits a different dissociation constant and trypsin-like protease activity against acrosomes. Furthermore, this activity has been shown to occur in mammalian sperm acrosomes (Honda et al 2002). Our laboratory has determined the activity of this peptide in HEK-293 cells (Fernandez et al 2013) and achieved inhibition of the extracellular ROS generated through the manipulation of ejaculated sperm. Thus, the protective action of this peptide on the plasma membrane can be utilized in biotechnological applications, specifically for porcine sperm that are used in cervical or uterine artificial insemination because the diluents have not been able to extend the life of this type of sperm for subsequent IA treatments. The classic model activation of sperm, is using albumin in the solution, but is describe a enhancer effect in guinea pig samples (Bhattacharyya 1992) and in these not have protection effect. In stallion the albumin are used for activation of the sperm and compared with another macromolecules like polyvinylalcohol (PVA), for increasing the process of penetration, but not preserving the samples, but the authors describe increases in the viability when using the PVA in the diluent (Choi et al 2003) In bovine samples, when in solution are present macromolecules, the sperm are protected in function, motility and capacitation (Anderson & Killian 1994) and in culture of oviduct increased the viability. Moreover, a report of Lignin-derived macromolecules (LDMs) describe antifertility effect, in particular the ligno-sulfonic acid (LSA), altered the penetration of the sperm in oocytes, but not change the motility and in special the viability (Tollner et al 2002), these evidence suggested effect of macromolecules in diluent solution, changes the motility and the capacitation, but at the same time showed effect in the viability of the sperm cells. Nevertheless, the use of macromolecules for preserving samples or activation of the sperm is not completely elucidated in the boar model, but the use of boar sperm-specific macromolecules more could be important for the industry and improve the cryopreservation protocols used.

Concluding remarks. Handling procedures result in alterations in sperm that can induce premature sperm capacitation (Maxwell & Johnson 1997). This process leads to the acrosome reaction and decreases the lifespan of sperm. Loss of fertilization capacity is caused by the presence of high levels of ROS generated after ejaculation. Kirchhoff (1998) and Alvarez (2006) demonstrated that sperm ROS, most of which are produced by mitochondria, are exported to the extracellular environment. Moreover, Venereo

(2002), Cerolini et al (2001) and Sellés (2003) suggested that the high content of unsaturated fatty acids in the sperm plasma membrane and the small volume of cytoplasm facilitates the production of ROS and decreases the ability of sperm to fuse with the egg; this notion is also supported by Castillo et al (2001) and Alvarez (2006).

Semen samples are obtained through mechanical handling, and it is necessary to dilute the obtained biological material (semen concentrate) to amplify the amount of spermatic material available for application to females. This process is affected by factors such as pollutants, infectious agents, alterations in pH levels and osmolarity, all of which can induce oxidative stress and affect the fertilization capacity of sperm. The use of semen extenders has not alleviated the problems associated with refrigerated and/or cryopreserved sperm conservation under short-, medium- or long-term storage conditions for AI. Our laboratory has developed a 12-aa peptide analogue (for use as a dilution medium additive for IA) that displays a protective effect on sperm membranes and increases sperm performance and efficiency.

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