Role of oxidative stress in normal and abnormal function of equine spermatozoa

Papel do estresse oxidativo na função normal e anormal do espermatozóide equino

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Abstract

Oxidative stress plays an important role in both the normal and abnormal function of equine spermatozoa. At low-levels of generation of reactive oxygen species (ROS), there are important effects on sperm function, particularly capacitation. However, higher concentrations of ROS can lead to damage to chromatin, membranes and proteins of spermatozoa. An understanding of the generation and removal of ROS along with their effects on normal sperm physiology and pathophysiology is important to studies related to preservation of equine sperm and to subfertility in the stallion.

Keywords: Equine, sperm, oxidative stress, antioxidant, enzyme scavenger.
***Palavras-chave**: equino,semen, estresse oxidativo.*

Introduction

Oxidative stress has long been known as an important process in mammalian spermatozoa having been part of the original description of oxidative damage to mammalian cells (MacLeod, 1943). Although reactive oxygen species (ROS) may form as a normal consequence of oxidative metabolism, specific generating mechanisms within particular cell types, such leukocytes, may also result in formation of ROS. From an abundance of literature, it is evident that low-level generation of ROS plays an important role in normal sperm function and that elevated ROS concentrations resulting from either an imbalance in the production or degradation of ROS may adversely affect sperm. During sperm storage, the effects of oxidative stress may be even more important in situations where much of seminal plasma is removed from a semen sample because much of the antioxidant capacity in semen resides with seminal plasma, and not in sperm themselves which have relatively little cytoplasm.

Generation of ROS

Two potential mechanisms appear to account for generation of ROS by equine sperm. A sperm-specific NADPH oxidase (NOX5) present in the plasma membrane of the sperm head may be responsible for low-level ROS production whereas electron leakage from sperm mitochondria appear to produce ROS attributed to sperm metabolism (Sabeur and Ball, 2006, 2007). Although the superoxide anion ($O_2^{\cdot-}$) is the primary ROS generated by equine sperm, this molecule rapidly dismutates to form hydrogen peroxide (H_2O_2) (Ball *et al*., 2001b; Burnaugh *et al*., 2007). Hydrogen peroxide, in turn, is the ROS which accounts for the major cytotoxic effect in sperm (Baumber *et al*., 2000). Production of ROS is increased in the presence of cryodamaged, dead or morphologically abnormal sperm, and sperm with residual cytoplasm or abnormal midpieces appear to produce greater quantities of ROS (Ball *et al*., 2001b). Under these conditions, generation of ROS is principally driven by electron leakage from the mitochondrial electron transport chain with subsequent reduction of molecular oxygen to form the superoxide anion (Sabeur and Ball, 2006).

Subsequent to cryopreservation, damage to sperm mitochondria likely results in generation of superoxide which contributes to oxidative damage to frozen-thawed equine sperm. Morphological evaluation of sperm after cryopreservation frequently reveals morphologic changes in the sperm midpiece that is characterized by moderate to marked swelling of the mitochondria suggesting that sperm mitochondria are a significant site of cryodamage with uncoupling of normal oxidative metabolism, generation of ROS and induction of degenerative processes such as apoptosis (Brum *et al*., 2008).

In human semen, contaminating leukocytes are an important source of ROS (Aitken *et al*., 1994). We examined the influence of neutrophil (PMN) addition to equine sperm to assess the relative effect of PMNs on equine sperm in vitro (Baumber *et al*., 2002). Equine sperm were separated from seminal plasma by density-gradient centrifugation and isolated equine PMNs were purified from whole blood. The addition of PMNs activated with phorbol ester increased hydrogen peroxide generation, and significantly decreased sperm motility (Baumber *et al*., 2002). However, this effect was only noted when the ratio of sperm:PMNs was 5:1 or 2.5:1, which suggests that relatively large contamination of equine semen with PMNs was required to induce notable adverse effects on equine sperm motility.

ROS scavengers in seminal plasma

Because of the relatively small cytoplasmic volume of the mature spermatozoon, most of the antioxidant scavengers present in semen reside in seminal plasma. The primary scavengers in seminal plasma include catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx). There is a wide species variation in the activity of these scavengers in seminal plasma. In equine seminal plasma, the activities of catalase was 98.7 ± 29.2 U/mg protein, the activity of SOD was 29.15 ± 6.64 U/ mg protein and the activity of GPx was 0.87 ± 0.06 μM NADPH oxidized/min/mg protein (Ball *et al*., 2000; Baumber and Ball, 2005). Most of the catalase activity appeared to originate from the prostate gland; whereas SOD was derived primarily from the prostate gland and ampulla and, GPx was present in highest activity in testis and in the cauda epididymis (Baumber and Ball, 2005). The activity of both catalase and superoxide dismutase in equine seminal plasma was relatively high compared to other species, and there was significant variation between stallions in the activities of these scavengers. Based upon these observations, semen processing which entails the removal of seminal plasma may increase the susceptibility of equine sperm to oxidative damage due to the removal of these enzyme scavengers.

A number of other components of seminal plasma provide antioxidant activity and help prevent oxidative damage to sperm. Low-molecular weight factors such as lactate, urate, taurine, hypotaurine, pyruvate, ascorbic acid, tocopherol, ergothioniene and albumin are present in seminal plasma and are capable of removing certain reactive oxygen species (Mann *et al*., 1963; Alvarez and Storey, 1983; Kovalski *et al*., 1992; Halliwell and Gutteridge, 1999). There is little research in equine semen to examine the importance of these low molecular weight antioxidants; however, one study suggested that these components may constitute most of the antioxidant capacity of semen (Thiele *et al*., 1995).

Effects of ROS on normal equine sperm function

As noted above, low-level ROS generation by equine sperm is stimulated by calcium ions, and it appears that a membrane-associated NADPH oxidase, NOX5, is responsible for the production of the superoxide anion as part of cell signaling events in equine sperm. Generation of superoxide anion, in turn, is important in induction of capacitation which is associated with a increase in tyrosine phosphorylation (Baumber *et al*., 2003b; Burnaugh et al., 2007). During the cryopreservation of equine sperm, we hypothesize that the low-level generation of ROS may lead to capacitation-like processes which contribute to shortened sperm longevity after insemination. This observation is supported by the observation that cryopreserved sperm have an increased intracellular calcium concentration, an increased generation of ROS, and a reduced antioxidant capacity because of removal of seminal plasma. Studies in our laboratory support the observation that "cryocapacitation" of sperm results in similar but not identical changes in sperm membrane as detected during capaciation *in vitro* (Thomas *et al*., 2006).

Cytopathic effects of ROS on equine sperm

Increased generation of ROS by equine sperm may occur in the presence of large numbers of morphologically abnormal or damaged sperm in a sample which may adversely affect the remaining viable sperm due to oxidative stress. Although superoxide anion appears to be the primary product generated by sperm, our research demonstrates that the less polar hydrogen peroxide is the most important ROS resulting in damage to equine sperm (Baumber *et al*., 2000). Generation of ROS with the xanthine / xanthine oxidase (X-XO) system results in production of superoxide anion which rapidly dismutates to H_2O_2. The increased H_2O_2 results in a decrease in sperm motility with no detectable decrease in viability, acrosomal integrity, or mitochondrial membrane potential (Baumber *et al*., 2000). The addition catalase (which catabolizes H_2O_2), but not superoxide dismutase (SOD), which catabolizes the superoxide anion, maintained normal motility secondary to this induced oxidative stress. Based upon these studies, sperm motility appears to be a sensitive indicator of oxidative stress and may be one of the first parameters affected during oxidative stress.

DNA damage is another well-known cytopathic effect of ROS. In equine sperm, exposure to increasing concentrations of ROS generated by X-XO resulted in a dose-dependent increase in DNA damage as detected by the Comet Assay (Baumber *et al*., 2003a). This DNA damage was blocked in the presence of catalase or reduced glutathione (GSH) but not in the presence of SOD, which indicates that H_2O_2 was the major ROS responsible for DNA damage in these cells (Baumber *et al*., 2003a). Interestingly, damage to sperm DNA appears to be initiated at levels of oxidative stress comparable to those previously shown to affect sperm motility. Aitken has proposed that DNA damage may be initiated at relatively low levels of oxidative stress which might be consistent with induction of capacitation without change in the motility of human sperm (Aitken *et al*., 1998). Therefore, low-levels of oxidative stress may allow sperm with DNA damage to fertilize the oocyte. Sperm have limited to no

ability to repair DNA damage, and studies from other species indicate that although fertilization may occur, the rate of subsequent embryonic development is reduced and the rate of early embryonic death is increased in situations in which fertilization is initiated by DNA-damaged sperm (Ahmadi and Ng, 1999; Morris *et al*., 2002). If this is also true in equine sperm, some of the damage to equine sperm DNA may be present in motile sperm which do not evidence alteration in standard parameters evaluated as part of routine semen analysis.

During storage of equine sperm, there is also a measurable increase in DNA damage as detected by the comet assay with both cooled (Linfor and Meyers, 2002) and frozen storage (Baumber *et al*., 2003a). Unfortunately, the addition of antioxidants (α-tocopherol, reduced glutathione, ascorbic acid) or enzyme scavengers (catalase, superoxide dismutase) to cryopreservation extenders did not reduce the level of DNA fragmentation subsequent to freezing and thawing of equine sperm cells (Baumber *et al*., 2005). Interestingly, the addition of SOD to cryopreservation extender significantly increased DNA fragmentation, suggesting again that H_2O_2 is the primary ROS resulting in DNA damage in equine sperm, in this case due to the conversion of $O_2^{-\cdot}$ to H_2O_2.

Effect of ROS on sperm membrane damage

Sperm membranes, including those of the horse, are characterized by a high concentration of polyunsaturated fatty acids (Parks and Lynch, 1992) which are susceptible to peroxidative damage (Aitken, 1995). Neither H_2O_2 nor $O_2^{-\cdot}$ are energetic enough to initiate lipid peroxidation on their own, and a transition metal catalyst is required to cause the chain reaction leading to lipid peroxidation (Ball and Vo, 2002). The presence of a transition metal catalyst such as Fe^{++} can initiate lipid peroxidation which then results in the formation of lipid peroxides and the formation of cytotoxic malondialdehyde as well as the more potent 4-hydroxynonenol (Aitken, 1995). The resultant change in the sperm membrane with lipid peroxidation alters membrane fluidity which can affect the ability of sperm membranes to fuse during acrosomal exocytosis.

Although lipid peroxidation is well characterized for mammalian sperm, equine spermatozoa appear relatively more resistant to membrane peroxidation than sperm of other domestic animals (Baumber *et al*., 2000; Neild *et al*., 2005). Cryopreservation of equine sperm, however, increased lipid peroxidation particularly over the region of the sperm midpiece (Neild *et al*., 2005). Storage of liquid semen at 5^{o}C for 24 to 48 hours also resulted in a detectable increase in lipid peroxidation in equine sperm (Ball and Vo, 2002; Raphael *et al*., 2008). The addition of α-tocopherol significantly reduced lipid peroxidation in equine sperm exposed to ferrous promoters, and the presence of Fe^{++} during cooled storage of equine sperm significantly increased lipid peroxidation and decreased sperm motility (Ball and Vo, 2002). The vitamin E analog, tocopherol succinate, is more water soluble and appears to load more readily into mitochondria than native vitamin E. Experimentally, tocopherol succinate was superior to tocopherol in preventing lipid peroxidation of equine sperm; however, the succinate ester of tocopherol suppressed motility of equine sperm to a greater extent than did α-tocopherol which limits its practical application (Almeida and Ball, 2005).

We also evaluated the effect of addition of the ROS scavenger, catalase, as well as lipid and water-soluble antioxidants on the maintenance of equine sperm motility during cooled storage (Ball *et al*., 2001a). The addition of catalase to nonfat skim milk extenders did not improve maintenance of motility during 72-hr storage at 5^{o}C. Several lipid-soluble antioxidants were evaluated including butylated hydroxytoluene (BHT), α-tocopherol, or the synthetic antioxidant, Tempo. BHT significantly reduced progressive motility during storage, and there were no positive treatment effects of either α-tocopherol or Tempo on maintenance of motility. In a final experiment, water-soluble antioxidants were evaluated including the vitamin E analog, Trolox, Vitamin C, and bovine serum albumin, none of which had a positive effect on maintenance of sperm motility during cooled storage. Aurich *et al*. (1997) observed a positive effect of addition of ascorbic acid on preservation of membrane integrity of cooled equine sperm; however, there was no effect of addition of catalase under similar conditions. In conclusion, the addition of catalase or a variety of lipid- or water-soluble antioxidants did not improve the maintenance of motility during short-term cooled storage of equine sperm in the presence of skim-milk based extenders.

A number of investigators have examined the addition of antioxidants to cryopreserved equine sperm without demonstrating clear cut positive effects on post-thaw parameters or fertility. In our laboratory, the addition of the enzyme scavengers, catalase or SOD, or low molecular weight antioxidants such as reduced glutathione, ascorbic acid or α-tocopherol, did not decrease DNA fragmentation, or increase mitochondrial membrane potential, viability or motility of frozen equine sperm after thawing (Baumber *et al*., 2005). In contrast, Aguero *et al*. (1995) reported a positive effect of addition of α-tocopherol to cryopreserved equine sperm. In cattle, (Foote *et al*., 2002) evaluated multiple antioxidants and combinations of antioxidants for addition to both liquid and frozen bovine sperm, and concluded that antioxidants were generally not beneficial. Interestingly, there were interactions between extender type (skim-milk based vs. egg yolk based extenders) and the addition of antioxidants. These authors suggest that the addition of casein and other milk proteins in extenders may provide abundant ROS scavenging capability to many extender formulations and may, therefore, obviate the need for addition of lipid or water-soluble antioxidants to semen extenders containing milk products (Foote *et al*., 2002). As noted above, this notion may also apply to equine semen as evidenced by the lack of positive effect of addition of antioxidants in many studies utilizing skim-milk based extenders. There may also

be important differences between different species as reports in other species suggests that addition of some enzyme scavengers, BHT, GSH, and tocopherol or its analogs had a positive effect on post-thaw parameters of boar sperm (Grossfeld *et al*., 2008).

Although the literature concerning the addition of vitamin E (α-tocopherol) to semen extenders appears to have at best variable response on maintenance of sperm function and fertility, a number of studies suggest that dietary supplementation of vitamin E may positively impact semen quality and maintenance of sperm during storage. Dietary supplementation with vitamin E reduced lipid peroxidation of sperm membranes in both turkeys and in chickens (reviewed by Breque *et al*., 2003). Furthermore, the addition of organic selenium to the di*et al*so increases activity of selenium-dependent glutathione peroxidase in seminal plasma (Breque *et al*., 2003). Surprisingly, supplementation of the female with dietary vitamin E and organic selenium also appeared to improve fertility, perhaps through effects on sperm storage in the oviduct (Breque *et al*., 2003). Dietary levels of vitamin E and selenium In boars affected the percentage of motile, morphologically normal sperm present in the ejaculate as well as the fertilization rate in gilts mated (Marin-Guzman *et al*., 1997). A recent report in the stallion demonstrated an improved maintenance of sperm motility during cooled semen storage after the dietary addition of 3000 IU Vitamin E per day for 14 weeks (Gee *et al*., 2008). Another study demonstrated favorable effects of the dietary addition of a rice-oil supplement to stallions with increases in sperm concentration, motility and total antioxidant capacity of semen noted during treatment with this supplement (Arlas *et al*., 2008). These studies suggest that dietary addition of antioxidants should be explored further as a means of altering oxidative stress in equine semen that might be associated with cooled or frozen storage of semen or possibly associated with reduced fertility.

Summary and Conclusions

Oxidative stress has been associated with perturbation of normal sperm function, including damage to chromatin, proteins, and membrane lipids, but it is important to consider that low-level generation of ROS appears to have an important role in intracellular signaling events in sperm. Seminal plasma is a rich source of enzyme scavengers and low molecular-weight antioxidants whereas the sperm cell has limited antioxidant capacity related to its small cytoplasmic volume. Many features of semen processing, including removal of seminal plasma, centrifugation, cooling, and rewarming probably contribute to oxidative damage to sperm during storage. Unfortunately, existing studies regarding the addition of antioxidants to equine sperm during storage do not demonstrate clear cut positive effects, and more research needs to be conducted to assess these treatments. Dietary addition of both vitamin E and selenium in the stallion may have positive effects on semen and again, more research should be conducted to address this possibility.

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