Pferdeheilkunde 22 (2006) 2 (März/April) 212-217

Reactive oxygen species and their influence on stallion semen fertility – a review

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Summary

Losses in motility and fertilizing ability of stored semen can at least in part be attributed to lipid peroxidation of the sperm plasma membrane. Physiologically, mitochondrial respiration is the main source of reactive oxygen species (ROS). In processed semen, ROS originate from contaminating leucocytes and from spermatozoa with residual cytoplasm. In addition, normal spermatozoa produce ROS as a result of their flagellar activity. At low concentrations, ROS have positive biological effects and regulate physiological sperm functions. Mammalian sperm cell membranes have a specific lipid composition with a high content of polyunsaturated fatty acids, making them particularly susceptible to damage by ROS. Peroxidation increases membrane permeability and decreases metabolic activity of sperm cells. To control the effects of ROS, semen contains antioxidants. Enzymatic antioxidants are glutathione peroxidase, superoxide dismutase and catalase. Antioxidants have been substituted in semen through the diet or by adding antioxidants to semen extender. However, as the loss of sperm motility during cooled-storage is not only an effect of plasma membrane dysfunction but also of mitochondrial membrane dysfunction, addition of antioxidants to semen during cooled-storage may have only limited effects.

Keywords: reproduction, stallion, cooled semen, peroxidation

Einfluss freier Sauerstoffradikale auf die Fertilität von Hengstsamen – eine Übersicht

Motilitätsverluste und eine reduzierte Befruchtungsfähigkeit von gekühlt gelagertem Samen sind unter anderem auf eine vermehrte Peroxidation von Lipiden in der Spermienmembran zurückzuführen. Die mitochondriale Atmungskette ist unter physiologischen Bedingungen die Hauptquelle für Sauerstoffradikale (reactive oxygen species, ROS). ROS in aufbereitetem, gelagerten Samen stammen vor allem von kontaminierenden Leucozyten und von Spermien mit Zytoplasmatropfen. Normale Spermien produzieren ROS infolge ihrer Bewegungsaktivität. In geringen Konzentrationen haben ROS positive biologische Effekte und sind an der Regulation physiologischer Spermienfunktionen beteiligt. Spermien weisen eine spezifische Lipidzusammensetzung mit einem hohen Gehalt an mehrfach ungesättigten Fettsäuren auf. Dies macht die Spermienmembran besonders empfindlich für ROS-induzierte Peroxidationsvorgänge. Peroxidation erhöht die Membranpermeabilität und reduziert Stoffwechselvorgänge in den Spermien. Einer Schädigung der Spermien durch ROS wirken verschiedene Antioxidantien im Samen entgegen (u.a. Glutathionperoxidase, Superoxiddismutase und Katalase). Experimentell ist versucht worden, die antioxidative Kapazität im Samen durch Zusatz von Antioxidantien zum Samenverdünner sowie durch Fütterung von Antioxidantien zu erhöhen. Da Verluste der Spermienqualität während der gekühlten Lagerung nicht nur auf Schädigung der Spermienmembran, sondern auch auf Schädigung mitochondrialer Membranen zurückzuführen ist, hat der Zusatz von Antioxidantien zum Samen während der gekühlten Lagerung jedoch nur begrenzt positive Effekte.

Schlüsselwörter: Reproduktion, Hengst, Samenkonservierung, Peroxidation

Introduction

The use of cooled semen is a routine practice in modern horse reproduction. The widespread use of AI with cooled transported semen has accelerated genetic progress by making selected stallions available outside the region where the stallion is located. When semen transport from the collection centre to the place where the mare is to be inseminated can be organized within 24 hours, cooled semen is preferred to frozen semen because of its better fertilizing capacity. Furthermore, AI with semen from stallions of controlled genital health status has markedly reduced the risk of sexual transmissible diseases for the mare.

Despite its obvious advantages, AI does not always lead to satisfying pregnancy results. Reasons range from fertility problems in the mare, low semen quality and inadequate treatment of the ejaculate to wrong timing of insemination. However, even if these apparent critical points are avoided, semen from certain stallions rapidly looses motility and viability during cooled storage. Although overall losses in motility and fertilizing ability of stored semen can at least in part be attributed to lipid peroxidation of the sperm plasma membrane (*Aitken* 1994, *Storey* 1997), the reasons why individual stallions have a low fertility when used via cooled or frozen-thawed semen are currently not well understood (*Brinsko* et al. 2000, *Battelier* et al. 2001). It has been suggested that cholesterol content of the sperm plasma membrane may affect suitability of semen from individual males for cooled-storage (*Cross* 1998, for review see *Aurich* 2005). In men, a relation between reduced fertility and production of reactive oxygen species (ROS) in

semen has been demonstrated. Less information is available on ROS production and male fertility in animals.

Generation of reactive oxygen species

Reactive oxygen species are short-lived reactive chemical intermediates containing one or more electrons with unpaired spin (*Sanocka* and *Kurpisz* 2004). As free radicals they are highly reactive and oxidize lipids, amino acids and carbohydrates and can cause DNA damage. Therefore, ROS have been suggested as an aetiological factor in a variety of diseases (*Rowley* et al. 1984, *Andorn* et al. 1990, *White* et al. 1994).

Generation of ROS occurs during normal cell metabolism. Physiologically, mitochondrial respiration is the main source of superoxide anion radicals. During reduction of oxygen to water by cytochrome C oxidase, ROS can leak into the cell (*Sanocka* and *Kurpisz* 2004). As shown in humans, spermatozoa generate superoxide anions (*Aitken* and *Clarkson* 1987, *Alvarez* et al. 1987). Because of a low reactivity and short half-life this molecule is not particularly harmful and dismutates spontaneously or under the influence of intracellular superoxide dismutase to hydrogen peroxide. However, by reacting with other molecules, it can transform these targets into more toxic radicals (*Alvarez* and *Storey* 1984, *Halliwell* and *Gutteridge* 1989). Hydrogen peroxide is relatively stable, has a higher oxidant potential than superoxide anion and permeates biological membranes (*DeLamirande* et al. 1997). Small amounts of iron, which is nearly ubiquituous in biological fluids, catalyses the formation of hydroxyl radicals from H_2O_2. This ROS has toxic effects on many cell components, but a short half-life.

In processed semen, ROS originate mainly from contaminating leucocytes (*Aitken* et al. 1994, *DeLamirande* and *Gagnon* 1994, *Plante* et al. 1994, *Whittington* and *Ford* 1999) and from spermatozoa with excess residual cytoplasm (*Aitken* and *Baker* 2004, *Brouwers* et al. 2005). In humans, granulocytes in semen originate from the epididymis, prostate (*Simbini* et al. 1998) but also the seminal vesicles (*Gonzales* et al. 1992).

Spermatozoa with residual cytoplasm, indicating insufficient maturation, produce significantly more ROS than normal spermatozoa (*Gomez* et al. 1996, *Aitken* and *Baker* 2004, *Brouwers* et al. 2005). Also spermatozoa with other morphological deformities such as tail defects have an increased ROS production (*Aziz* et al. 2004). Spermatozoa in different stages of maturation produce different amounts of ROS (*Gil-Guzman* et al. 2001). This may be related to a higher activity of enzymes such as glucose-6-phosphate-dehydrogenase involved in ROS production (*Gomez* et al. 1996, *Aitken* et al. 1997, *Esfandiari* et al. 2003, *Aziz* et al. 2004). Sertoli cell function may be affected by ROS producing leucocytes, leading to disturbances in spermatogenesis and deformed spermatozoa (*Henkel* et al. 2005). However, it remains unclear to what degree leucocytes exert their effects on spermatozoa during spermatogenesis, epididymal maturation or after ejaculation.

In addition, normal spermatozoa produce ROS as a result of their flagellar activity (*Gavella* and *Lipovac* 1992). Ram spermatozoa specifically produce ROS using an amino acid oxidase that generates hydrogen peroxide and thus suppresses sperm motility (*Upreti* et al. 1992). Centrifugation of semen and removal of 90% of seminal plasma has beneficial effects on sperm viability during cooled storage (*Pickett* et al. 1975) but centrifugation does also increase the amount of ROS in semen (*DeJager* et al. 1996, *Parinaud* et al. 1997).

Biological effects of reactive oxygen species on spermatozoa

At low concentrations, ROS have positive biological effects and act selectively on prostanoid metabolism, gene regulation, cellular growth, signal transduction pathways and participate in the regulation of vasotonus and antimicrobial defense. Low ROS levels also regulate physiological sperm functions (*DeLamirande* et al. 1997, *Sanocka* and *Kurpisz* 2004). Small amounts of free radicals in human semen stimulate sperm capacitation, hyperactivation, acrosome reaction and sperm oocyte fusion (*Saran* and *Bors* 1989, *DeLamirande* and *Gagnon* 1993a, *Griveau* and *LeLannou* 1997, *DeLamirande* et al. 1998). An increase in extracellular superoxide anions is essential for capacitation (*DeLamirande*

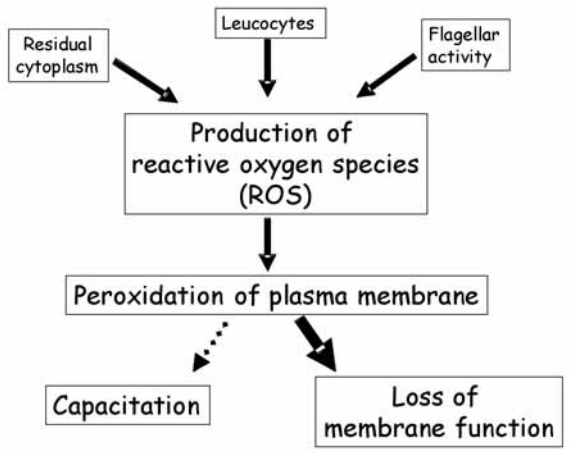

Fig 1 Source and effects of reactive oxygen species in semen
Herkunft und Wirkung freier Sauerstoffradikale im Samen

and *Gagnon* 1995) and low concentrations of hydrogen peroxide stimulate capacitation and hyperactivation (*Bauskin* et al. 1991, *Griveau* et al. 1994). Reactive oxygen species also play a role in the acrosome reaction and in zona pellucida binding (*Aitken* et al. 1989 and 1995). Conversely, the block of ROS production with catalase inhibits hyperactivation and acrosome reaction (*Griveau* et al. 1994). All these processes are redox-regulated and mild oxidative conditions are needed for spermatozoa to reach their full fertilization capacity. Because the effects of ROS added to semen (e.g. induction of the acrosome reaction) last longer than the actual presence of ROS in the sample, it has been suggested that ROS only initialize a cascade leading finally to fertilization (*Bize* et al. 1991, *DeLamirande* and *Gagnon* 1993a and b). Some authors suggest that a specific enzyme in the sperm membrane is activated during capacitation (*DeLamirande* and *Gagnon* 1993b, *Aitken* et al. 1995, *DeLamirande* and *Gagnon* 1995). The acrosome reaction involves tyrosine phosphorylation of specific proteins (*Naz* et al. 1991, *Tesarik* et al. 1993) and tyrosine kinases and phosphatases are redox regulated (*Bauskin* et al. 1991, *Hecht* and *Zick* 1992)

Negative effects of reactive oxygen species on spermatozoa

Spermatozoa are highly specialised cells for the transport of paternal DNA to the oozyte. Mammalian sperm cell membranes have a specific lipid composition with a high content of polyunsaturated fatty acids, plasmalogens (ether-linked lipids) and sphingomyelins. Like most biological membranes, they have an asymmetrical arrangement of lipids within the lipid bilayer. Lipid composition of the sperm plasma membrane is different from somatic cells with an increased content of phospholipids, sterols, satured and polyunsaturated fatty acids. Amongst the sterols, the efflux of cholesterol from the sperm plasma membrane plays an important role for capacitation (*Cross* 1998). Composition of the plasma membrane changes from epididymal maturation to penetration of the oozyte, with plasmalogenes becoming the major phospholipids (*Aveldano* et al. 1992).

Conditions in the female genital tract are primarily anaerobic, reducing the potential damage to spermatozoa by ROS (*Foote* et al. 2002). Their special structure and high amount of polyunsaturated fatty acids (PUFA) makes mammalian spermatozoa in processed semen particularly susceptible to damage by ROS (*Kodama* et al. 1996). Peroxidation of PUFA in sperm cell membranes is an autocatalytic, self propagating reaction, resulting in the loss of membrane functionality and integrity. Peroxidation can be divided into the steps of initiation, propagation and termination. Initiation of peroxidation is the abstraction of a hydrogen atom from an unsaturated fatty acid. Propagation is defined as formation of a lipid alkyl radical followed by its rapid reaction with oxygen to a lipid peroxyl radical. Peroxyl radicals ignite a chain reaction in which the intermediate product lipid hydroperoxide is formed. In the presence of iron ions lipid hydroperoxides desintegrate into alkoxy radicals or peroxyl radicals. At termination of the chain reaction all oxygen and hydrogen species are used off and the peroxyl radical reacts to a stable product.

Extrinsic ROS from leucocytes as well as intrinsic ROS from spermatozoa also induce DNA fragmentation (*Lopes* et al. 1998, *Irvine* et al. 2000). The source of ROS clearly influences their effects. Intrinsic ROS production is highly correlated with DNA fragmentation. From the extrinsically produced ROS only H_2O_2 but not superoxid and hydroxyl radicals are membrane permeable. Thus, in biological systems, only H_2O_2 can damage DNA while other extrinsic ROS will cause mainly lipid peroxidation of cell membranes (*Henkel* et al. 2005). Leucocyte-derived extrinsic ROS production is positively correlated with the sperm cells own, intrinsic ROS production, indicating that damaged spermatozoa produce more ROS than intact ones (*Saleh* et al. 2002).

In humans, it has been demonstrated by measuring mitochondrial membrane potential (MMP) that ROS also damage mitochondria. Men with abnormal semen parameters have decreased MMPs and high ROS concentrations in semen. Damaged mitochondria may also play a role in apoptosis. Compared with normal men, infertile semen donors had significantly higher levels of ROS, cytochrome C and caspase 3 and 9. Cytochrome C is a marker for mitochondrial integrity because it leaks from damaged mitochondria into seminal plasma. Caspases are proteases that promote apoptosis. Release of these proteins from mitochondria due to oxidative stress is likely to accelerate apoptosis (*Wang* et al. 2003a and b).

Peroxidation increases membrane permeability and thus decreases metabolic activity of sperm cells due to permeation of enzymes, substrates nucleotide cofactors and ATP (*Storey* 1997). Therefore, loss of motility is not only related to lipid peroxidation of the plasma membrane, but also to a decrease in energy supply by the mitochondria due to ATP depletion (*DeLamirande* and *Gagnon* 1992, *Ruiz-Pesini* et al. 1998).

Antioxidative defence systems in semen

To control the negative effects of ROS, mammalian ejaculates contain intra and extracellular antioxidants of enzymatic and non enzymatic origin. Enzymatic antioxidants are glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT). SOD specifically scavenges superoxide radicals and converts them to hydrogen peroxide and oxygen which in turn are broken down into water by CAT and GSH-Px. Superoxide radicals, supposed to be the primary species generated by spermatozoa, are neutralized by SOD to H_2O_2, which also damages spermatozoa and is neutralized by CAT and GSH-Px. Non enzymatic, low molecular weight antioxidants are α-tocopherol, ß-carotene, ascorbate, urate, transition-metal chelators, transferrin, lactoferrin and caeruloplasmin (*Sanocka* and *Kurpisz* 2004).

All glutathione peroxidases reduce hydrogen peroxide and alkyl hydroperoxides at the expense of glutathione with different specificity. Amongst about 30 mammalian selenoproteins the most known are cytosolic GSH-Px (cGSH-Px), phospholipids hydroperoxide GSH-Px (PHGSH-Px), plasma GSH-Px (pGSH-Px) and gastrointestinal GSH-Px (GIGSH-Px). The enzymes cGSH-Px, pGSH-Px and GIGSH-Px are homotetramers and PHGSH-Px is a monomer with a molecular size smaller than the subunits of the other glutathione peroxidases (*Brigelius-Flohe* 1999). All seleno-dependent peroxidases require selenium for their biosynthesis and activity but respond differently to selenium deficiency. The most stable isoform is GIGSH-Px followed by PHGSH-Px and pGSH-Px and cGSH-Px. The extracellular isoenzyme pGSH-Px regulates hydroperoxide turnover in blood plasma but also in milk and extracellular fluid of the intestine, lung and in amniotic fluid. Cytosolic GSH-Px is thought to counteract hydroperoxide-modulate apoptosis (*Kayanoki* et al. 1996) or eicosanoid metabolism (*Weitzel* and *Wendel* 1993) within cells. Its expression in the epithelium of the gastrointestinal tract makes GIGSH-Px a major antioxidant of the intestinal epithelium (*Esworthy* et al. 1998) and a defense against ingested lipid hydroperoxides (*Chu* et al. 1993).

The enzyme PHGSH-Px is the major peroxidase in the testes (*Brigelius-Flohe* 1999). It is distributed between the cytosol and subcellular organelles and exists in high amounts in mitochondrial membranes and nuclei (*Godeas* et al. 1994 and 1996). The isoform specifically reduces phospholipid hydroperoxides into their corresponding alcohols thus interrupting the cascade of radical formation (*Ursini* et al. 1982). PHGSH-Px is also involved in sperm maturation and differentiation. By oxidizing SH groups of protamines from epididymal

sperm in the presence of hydroperoxides, PHGSH-Px participates in the condensation of chromatin (*Godeas* et al. 1997). A positive correlation exists between seminal plasma selenium and sperm density, sperm number, motility and viability in humans (*Xu* et al. 2003).

Superoxide dismutases are metalloproteins, divided into 3 groups depending on the ion in their active center. SOD containing Cu/Zn is found mainly in the cytosol, Mn SOD is found in mitochondria and Fe SOD exists mainly in procaryota. An extracellular form of Cu/Zn SOD was detected in extracellular matrix and fluids of eucaryota as well. Superoxide dismutase has a high affinity for heparin. It dismutates the superoxide radical into peroxide and molecular oxygen. Hydrogen peroxide, which is able to cross cell membranes, must be removed by either catalase or GSH-Px. In rat testis, cytosolic, mitochondrial as well as extracellular SOD were detected (*Bauche* et al. 1993). In humans, nearly all SOD activity in sperm is from the cytosolic isoenzyme (*McCord* and *Fridovich* 1969).

Semen catalase has been studied in humans (*Jeulin* et al. 1989) but also stallions (*Ball* et al. 2000, *Koskinen* et al. 2002). CAT is responsible for dismutation of H_2O_2 to O_2 and H_2O. Activity of CAT has been analysed in testes, accessory sexual glands and cauda epididymal fluid of stallions (*Ball* et al. 2000). The major source are prostatic secretions and at least part of CAT activity in spermatozoa represents adsorbed molecules from prostatic fluid. *Koskinen* et al. (2002) determined CAT activity in different fractions of stallion ejaculates and found the lowest activity in pre-ejaculatory fluid.

Addition of antioxidants to semen and semen extenders

Antioxidants have been substituted in semen either through the diet or by adding antioxidants to semen extender before dilution and storage of semen. Oral intake of vitamin C and E prevented Pb-associated sperm ROS generation in Pb-exposed rats, increased epididymal sperm motility and enhanced the capacity of spermatozoa to penetrate oocytes in vitro (*Hsu* et al. 1998). Oral treatment of asthenozoospermic men with vitamin E decreased lipid peroxidation in semen and improved sperm motility (*Suleiman* et al. 1996). In birds, where prolonged storage of spermatozoa occurs in specialised sites of the female genital tract, maintenance of the fertilizing ability of spermatozoa mainly seems to depend on the presence of efficient antioxidative systems (*Brèque* et al. 2003).

Also direct addition of antioxidants to semen before storage has protective effects on sperm function. Positive effects of added antioxidants, however, depend on the individual extender and antioxidant used. Addition of ascorbic acid to skim milk extender increases the percentage of membrane-intact spermatozoa (*Aurich* et al. 1997) while addition of pyruvate increased the percentage of motile spermatozoa (*Bruemmer* et al. 2002) during storage at 5°C and also fertility. Similar effects have been found in humans (*Parinaud* et al. 1997) and in bulls (*Foote* et al. 2002) after addition of different antioxidants to semen. GSH-Px added to ram semen had positive effects on sperm motility and acrosome integrity during cooled storage (*Maxwell* and *Stojanov* 1996). In humans, antioxidants like glutathione or N-acetylcysteine have been suggested to protect against the damaging effects of leukocyte-derived ROS on sperm motility and may be of clinical value in assisted reproduction procedures (*Baker* et al. 1996). *Griveau* et al. (1994) investigated the effect of the addition of dithriothreitol, reduced glutathione (GSH), CAT and SOD to semen. Dithriothreitol and SOD increased hyperactivation and acrosome reaction while GSH improved the acrosome reaction, indicating a potential therapeutic use in men.

A positive effect of milk-based extenders alone, i.e. without added antioxidants, on the antioxidative activity in diluted stallion semen could be demonstrated recently (*Kankofer* et al. 2005). Activity of GSH-Px, SOD and CAT was increased after dilution of semen with a milk-based extender. The same effect was seen when seminal plasma alone, but not when spermatozoa separated from seminal plasma were diluted with semen extender. This suggests interactions between seminal plasma and extender resulting in an increase in antioxidative capacity. The protective effects of many routinely used semen extenders may be related at least in part to an increase in antioxidative capacity after dilution of semen.

However, as the loss of sperm motility during cooled-storage is not only an effect of plasma membrane dysfunction but also of mitochondrial membrane dysfunction (*DeLamirande* and *Gagnon* 1992, *Ruiz-Pesini* et al. 1998), addition of antioxidants to semen during cooled-storage may only have limited effects.

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