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Antioxidant supplementation and purification of semen for improved artificial insemination in livestock species

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Abstract: Improving sire reproductive performance in artificial insemination (AI) programs and maximizing the use of sires with valuable genomes are high on the list of priorities for livestock breeders in the United States and worldwide. While fixed-time AI and accurate estrous detection have already ameliorated the reproductive management of cows and sows, substantial gains remain to be made in the selection and utilization of male animals for field AI. In addition to better, more objective evaluation of breeding soundness and semen quality, numbers of spermatozoa per AI dose could be reduced and numbers of AI doses per collection could be increased without sacrificing conception rates if the fittest spermatozoa can be purified from raw semen and protected from adverse effects of dying and decaying spermatozoa present in both the fresh and the extended semen. The present article reviews recent progress in methodology for semen purification, including nanopurification and semen/extender supplementation with antioxidants.

Key words: Sperm, semen, fertility, antioxidant, purification, nanotechnology

1. Introduction

Improving reproductive performance in livestock artificial insemination (AI) programs and maximizing the use of sires with valuable genomes are high on the list of priorities for livestock breeders in the United States and worldwide. Substantial progress has been achieved in the optimization of female reproductive performance through better estrous detection and management of female reproductive functions (1–3). While modest additional gains can be made by further improving female fertility (4), the largest gains depend on improving the reproductive management of sires (5). Such progress can be made through improvement of semen collection, analysis, and cryopreservation, incorporating new biomarker-based andrological evaluations and state-of-the art instrumentation for automated, objective, and high-throughput semen analysis and processing (6,7).

Defective spermatozoa dilute the total number of fertilization-competent spermatozoa in AI semen doses, affect the functioning of normal spermatozoa in the same ejaculate (8,9), and release harmful decay products such as reactive oxygen species (ROS) (10). Therefore, gains in sperm viability and a reduction of the minimum necessary sperm number per AI dose could be achieved by the supplementation of collected semen with antioxidants/ROS scavengers and by the development of simple, rapid, and inexpensive techniques for the removal of defective spermatozoa and cellular debris from semen. With this mindset, we review the current knowledge on semen supplementation and purification in livestock species, and we provide a general rationale for the implementation of such technologies in animal production.

2. ROS production by defective and decaying spermatozoa

Semen storage is associated with cold shock and atmospheric oxygen (11–13) that lead to higher production of ROS and an imbalance between free radicals and the antioxidant system of extended semen. Oxygen radicals at the physiological concentration have a positive effect, for example, on the intracellular signaling involved in the processes of cell proliferation, differentiation, and migration, as well as in sperm capacitation, hyperactivation, and sperm–oocyte fusion (14,15). Substantial evidence exists that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capability (16,17). Low levels of ROS have been shown to be essential for fertilization, acrosome reaction/acrosomal

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exocytosis, and sperm motility (18,19). However, excessive production of ROS might have a cytotoxic effect through the production of free radicals that affect functional characteristics of the spermatozoa, such as reducing sperm motility (20), inactivating glycolytic enzymes, and damaging the acrosomal membranes (21), which would render the spermatozoa unable to fertilize an oocyte (22).

ROS-induced sperm damage is mediated by oxidative attack of bis-allylic methylene groups on sperm phospholipid-bound polyunsaturated fatty acids, leading to lipid peroxidation (23). Boar spermatozoa have high polyunsaturated fatty acid content, so they are very susceptible to lipid peroxidation (24,25) and low antioxidant capacity of seminal plasma (24,26). The effects of lipid peroxidation include irreversible loss of motility, inhibition of respiration, leakage of intracellular enzymes, damage to sperm DNA (27), or deficiencies in penetration of the oocyte zona pellucida and sperm–oocyte fusion (28). Semen represents a complex redox system that combines the antioxidant potential of seminal plasma and spermatozoa with the prooxidant potential of spermatozoa through the production of ROS. Enzymatic antioxidant defense mechanisms in seminal plasma and spermatozoa include superoxide dismutase, glutathione reductase, glutathione peroxidase, and catalase. Nonenzymatic antioxidants include reduced glutathione (GSH), urate, ascorbic acid, vitamin E, taurine, hypotaurine, carotenoids, and ubiquinones. The interplay of antioxidant and prooxidant mechanisms in semen determines the overall rate of lipid peroxidation in spermatozoa (29).

ROS generation in living organisms may be caused by several mechanisms, including ionizing radiation (30,31), bioactivation of xenobiotics (32), inflammatory cells (33), increased cellular metabolism (34), decompartmentalization of transition metal ions (35), activation of oxidases and oxygenases (36), and loss of antioxidant capacity (14,37).

Every ejaculate is contaminated with potential secretors of ROS. In the male reproductive system, ROS can be produced by leukocytes or from the spermatozoa themselves. Most semen specimens contain variable numbers of leukocytes, with neutrophils noted as the predominant type (38,39). Activated neutrophils generate and release ROS in high concentrations to exert cytotoxic reactions against nearby cells and pathogens (40). Leukocytospermia has been associated with decreased sperm concentration, motility, and morphology, as well as decreased hyperactivation and defective fertilization (41). This relationship is not definitive. Several other studies have not found any evidence of an association between leukocytospermia and abnormal sperm characteristics (38). While the significance of leukocytospermia in the fertilizing potential of an individual sample remains difficult to quantify, leukocytospermia can be considered a marker of urological or systemic inflammation and possible sperm dysfunction (42). Spermatozoa also have been noted to generate ROS independently of leukocytes (43) and the ability to generate ROS depends on the maturation status of the spermatozoa.

Lipid peroxidation is an important pathophysiological process occurring in numerous diseases and stress conditions and results in a series of degenerative processes affecting the organization and function of various cellular components (44). The lipid peroxidation process caused by ROS such as H_2O_2 is the limiting factor of the lifespan of mammalian spermatozoa. Many studies have investigated the possible effect of this process on the loss of sperm functional characteristics (45–47).

3. Effect of antioxidant supplementation on sperm viability and fertilizing potential

Antioxidants, in general, are compounds that dispose of, scavenge, and suppress the formation of ROS and lipid peroxidation. Among the well-known biological antioxidants, GSH, glutathione peroxidase, catalase, and superoxide dismutase (SOD) have significant roles as suppressors or scavengers of free radicals. Hence, the application of ROS scavengers is likely to improve sperm function (48–50). Due to the small cytoplasmic component, the antioxidant capacity of spermatozoa is limited during prolonged storage at temperatures above 0 °C. In addition, prolonged fresh semen storage decreased GSH content of boar (29) and bull spermatozoa (51,52) and reduced SOD value by 50% in bull spermatozoa (51).

3.1. Glutathione/GSH

Glutathione (GSH/gamma-glutamyl-cysteinyl-glycine) is a tripeptide of glutamic acid, cysteine, and glycine ubiquitously distributed in living cells. It plays an important role in the intracellular defense against oxidative stress (23). Its cysteine subunit provides and exposes a sulfhydryl group that directly scavenges free radicals. Once oxidized, the glutathione disulfide is regenerated/reduced by glutathione reductase, using NADPH as the cofactor, to complete the cycle. High concentrations (1000 μg/g tissue) are found especially in the testis of rats (53) and the reproductive tract fluids and epididymal spermatozoa of bulls (54). Numerous studies have been published on the beneficial effect, or lack thereof, of semen supplementation with GSH in livestock species. For example, the supplementation of bull semen with GSH improved sperm characteristics and in vitro fertilization rates (55), but appeared to have a limited beneficial influence on field fertility after AI (56).

3.2. Resveratrol and other grape wine flavonoids

Resveratrol is a natural polyphenol found mainly in red grapes and red wine that plays an important role as a

lipid soluble antioxidant, acting as an effective scavenger of superoxide (O_2^-), hydroxyl (OH), and metal-induced radicals (57–60). Resveratrol also exhibits a protective effect against ethanol-induced lipid peroxidation in testes (61) and lipid peroxidation induced by *tert*-butyl hydroperoxide in spermatozoa, protecting sperm chromatin and membranes (57) and preventing DNA damage caused by ROS (62). Resveratrol inhibited lipid peroxidation of ram spermatozoa most effectively when applied at low concentrations (15 μg/10^9 spermatozoa), as demonstrated by the TBARS test (59). Resveratrol inhibited the BαP-diol-epoxide adduct formation in sperm DNA, and apoptotic and necrotic cell death (62).

Other flavonoid substances with antioxidant action have been described. With regard to semen supplementation, quercetin is a member of a family of flavonoid polyphenols with more intense antioxidant activity than vitamins C and E, as well as lesser toxicity. Phenolic compounds act as free radical scavengers and, at times, as metal chelators, acting both in the initiation step as well as in the propagation of oxidative process (63). Quercetin improved stallion sperm motility and acrosome integrity, and markedly enhanced the zona binding ability of spermatozoa while also reducing DNA fragmentation in cryopreserved spermatozoa (64).

3.3. Plant extracts

Curcumin is a bright yellow compound found in turmeric, which is derived from the rhizomes of the plant *Curcuma longa*, a perennial herb of the family Zingiberaceae (65). Curcumin is a lipophilic polyphenol that is insoluble in water (66). Curcumin has been shown to scavenge free radicals (67). Under in vitro conditions, curcumin significantly inhibited the generation of ROS, such as superoxide anions and H_2O_2, and nitrite radical generation by activated macrophages, which plays an important role in inflammation (68). Curcumin lowers the production of ROS in vivo (69). The antioxidant mechanism of curcumin is attributed to its unique conjugated structure, which includes 2 methoxylated phenols and an enol form of diketone; the structure of curcumin shows typical radical-trapping ability as a chain-breaking antioxidant (70). Supplementation of fresh bull semen with curcumin significantly increased the sperm content of GSH after thawing (71). Administration of curcumin to male rodents challenged by a reproductive toxicant appeared to have a protective effect toward testicular function and fertility (72,73).

3.4. Berry and plant extracts/phytochemicals

Food substances consumed by humans and feed components provided to livestock can have therapeutic, nutritional, or toxic effects on the body. When administered in their crude form, certain food/feed substances can help cure specific ailments. Plants have been used for therapeutic purposes since the dawn of human history, but thus far, little is known about the possible beneficial effects of plant extracts and berry juices on male fertility, testicular function, and sperm quality. Juniper berries are used as a spice, particularly in European cuisine, and they are the only spice derived from conifers. The berries are used in northern European and particularly Scandinavian cuisine to impart a sharp, clear flavor to meat dishes (74). Juniper berries and berry extract are used in folk medicine for different purposes in different countries. In Turkey, juniper tar, leaves, and fruits are used widely to heal wounds, abdominal pain and stomach disorders, gynecological diseases, and calcinosis in joints, as well as against fungal infections and kidney inflammation and to pass kidney stones (75–77).

Another folk medicinal plant is *Xylopia aethiopica*, commonly known as African Guinea pepper, which has a preservative effect; the fruit extract has been shown to be an antimicrobial agent with antiinflammatory and antipyretic action. *X. aethiopica* has been found to contain some phytochemicals, which exhibit a wide range of biological effects as a consequence of their antioxidant properties (78).

Grape seed extract is a natural extract from the seed of *Vitis vinifera*. It is a rich source of one of the most beneficial groups of plant flavonoids, proanthocyanidin oligomers, as well as vitamins, minerals, and other phytochemicals. These naturally occurring phytochemicals, like flavonoids, exert many health-promoting effects (79), including the ability to increase intracellular vitamin C, decrease capillary permeability and fragility, and scavenge oxidants and free radicals (80). The activity of proanthocyanidin oligomers is approximately 50 times greater than that of vitamin C and vitamin E in terms of antioxidant action. Phytochemicals as antioxidants play vital roles in human health (81) and could find use as semen additives and/or fertility supplements to livestock feed (82). One example of such phytochemical use is maca (*Lepidium meyenii*) root extract, a supposed aphrodisiac grown in high altitudes of the Andean region of South America. Maca root powders or alcoholic extracts appeared to improve semen characteristics in men (83) and testicular function in male rats (84).

3.5. Bee honey/propolis compounds

Propolis has been used as a folk medicine from ancient times. It is an adhesive, dark yellow to brown balsam. Propolis is the generic name for the resinous substance collected by honeybees from various plant sources (85) that has a wide range of biological activities including antibacterial, antiviral, antiinflammatory, and antioxidative properties (86). Activities of propolis are based on its rich contents of flavonoids, phenolic acids, and terpenoids. In fact, propolis could contain more than 300 components,

including phenolic aldehydes, polyphenols, sesquiterpene quinines, coumarins, steroids, amino acids, and inorganic compounds (87). Among them, phenolic compounds such as flavonoids are thought to be primarily responsible for the biological activity of propolis. Current knowledge indicates that propolis protects the reproductive system from toxicity; in particular, flavonoids and phenolic compounds have an antioxidant activity and show protective effects against aluminum chloride, which caused testicular dysfunction, deterioration in semen quality, and lower testosterone concentrations (88). Studies in various mammals reported that propolis significantly increased testosterone, body weight, relative weight of the testis, relative weight of the epididymis, percentage of motile and morphologically normal spermatozoa, and content of seminal plasma enzymes, and it decreased concentrations of free radicals and lactate dehydrogenase in rabbits (89). Propolis decreased the percentages of dead and abnormal spermatozoa and increased testosterone in rats (90). Propolis was also reported to inhibit the generation of superoxide anions and to reverse the consumption of GSH, which is synthesized in the liver and has a free-radical–scavenging activity (91).

3.6. Vitamins and other antioxidants

Vitamin E is a major chain-breaking antioxidant and one of the primary components of the antioxidant system in sperm membranes, in which it appears to have a dose-dependent effect (92). It plays a vital role in protecting cell membranes from oxidative damage, trapping and scavenging all 3 types of free radicals, namely superoxide, H_2O_2, and hydroxyl radicals (18), generated during the conversion of lipid hydroperoxides in the peroxidative chain reaction (93,94). Feeding boars supplemental vitamin E has been shown to enhance the ejaculate volume and sperm concentration (26), and the addition of vitamin E to the storage diluent/semen extender increased sperm resistance to lipid peroxidation (24). During cryopreservation, vitamin E supplementation had a positive effect on sperm motility, mitochondrial membrane potential, and membrane integrity, depending on the fraction of ejaculate (24,95,96).

Vitamin C is a water-soluble chain-breaking antioxidant, which has the capacity to scavenge oxygen radicals in the aqueous phase. It neutralizes hydroxyl, superoxide, and hydrogen peroxide radicals and prevents sperm agglutination (18). It also prevents lipid peroxidation, recycles oxidized vitamin E (tocopheryl quinine), and protects against DNA damage induced by H_2O_2 radicals (97). The incorporation of both vitamin C and vitamin E into semen extenders improved the motility and viability of bull and boar spermatozoa (95,98).

Coenzyme Q-10 or ubiquinone is a nonenzymatic antioxidant and energy-promoting agent, a membrane stabilizer, and a regulator of mitochondrial permeability transition pores (99). In spermatozoa, Coenzyme Q-10 is concentrated in the mitochondria of the midpiece, in which it recycles vitamin E and prevents its prooxidant activity (100). Energy-dependent processes in the spermatozoa rely on the availability of coenzyme Q-10 (101). Coenzyme Q-10 in seminal fluid shows a direct correlation with semen characteristics (102). Coenzyme Q-10 has 2 forms: reduced (ubiquinol) and oxidized (ubiquinone). A strong correlation among sperm count, motility, and ubiquinol-10 content in seminal fluid has been reported (103). In a recent study, exogenous administration of coenzyme Q-10 was effective for improving sperm kinetic features in patients with idiopathic asthenozoospermia (104).

Albumins are known to improve sperm motility and plasma membrane integrity, and to protect acrosomes from temperature shock during the freeze-thawing of ram semen (105). Albumins may also promote sperm survival in the reproductive tract of the cow prior to fertilization (106). Furthermore, it was reported that albumins improve fertility and increase the catalase antioxidant activity following the freeze-thawing of bull semen (107).

Substances other than antioxidants may contribute to the maintenance of sperm oxidative homeostasis. The prime function of such compounds is not to combat the production or action of ROS; however, their presence may decrease the risk of oxidative stress development. Uric acid (108), bilirubin (109), and taurine or hypotaurine (110) are the best-known representatives of this group.

4. Semen purification methods for livestock species

Alone or combined with the aforementioned semen/extender supplements, the purification of intact, morphologically normal, viable, and motile spermatozoa offers a promising approach to improvement of AI semen doses in production agriculture. Methods of semen purification are based on sperm mobility in overlaid culture medium (sperm swim-up), gradient centrifugation (Percoll, PureSperm), centrifugation through a filtering matrix (glass wool filtration), magnetic bead separation [magnetic-activated cell sorting (MACS) technique], and nanoparticle separation (semen nanopurification). Semen sexing could be considered a purification technique because it removes aneuploid spermatozoa, but due to the extensiveness of the literature, this technique will not be discussed here. While most methods discussed in this section improve sperm quality and fertilization in vitro, some are not suitable for routine use in animal production due to cost, labor intensity, or expensive or complicated equipment.

4.1. Sperm swim-up

This common technique for separation of motile spermatozoa is based on their ability to migrate against

gravitational force when overlaid by a layer of serum-containing culture medium in a test tube (111). Historically, swim-up has been applied to improve sperm quality and to remove viruses, bacteria, cellular debris, and somatic cells from human semen, as well as to make attempts at separating X and Y chromosome-bearing spermatozoa. Characteristics of bull spermatozoa separated by swim-up correlated with field fertility of corresponding ejaculates in AI (112). While the volume scale of the swim-up procedure may not be practical for bulk-processing of semen for AI, it has found good use in bovine IVF protocols (113).

4.2. Gradient separation

Gradient-based separation techniques are based on the lower density and reduced ability of defective spermatozoa to pass through a layer of colloid during centrifugation (114). The most commonly used Percoll gradient is a colloidal solution of 15–30 nm silica particles coated with polyvinylpyrrolidone (115). Percoll was introduced in infertility clinics in the early 1980s (116) and was concomitantly or subsequently adapted for livestock species (117–121), becoming a favorite technique for sperm preparation prior to IVF (122). Meanwhile, optimized sperm gradient media such as PureSperm and OptiPrep replaced Percoll in human IVF after it was withdrawn from human use by the manufacturer (123). A common alternative to Percoll in livestock species is Ficoll, an aqueous solution of a hydrophilic polysaccharide (124). Similar to Percoll, motile spermatozoa can be recovered by Ficoll wash, which also can be used for sperm inactivation and seminal plasma removal (117,120).

4.3. Glass wool and other filtration methods

Clusterin (CLU) from seminal plasma and epididymal fluid binds to defective bull spermatozoa, and the percentage of CLU-positive spermatozoa appears to correlate negatively with estimated conception rates of artificially inseminated cattle (125). Clusterin-positive frozen-thawed bull spermatozoa are retained in glass wool during glass wool-Sephadex (GWS) filtration, reportedly reducing the content of defective spermatozoa from 14% to 1% (126). Glass wool alone outperformed Sephadex and Percoll gradient separation techniques in embryo cleavage and blastocyst development rates after bovine IVF (127). Improved sperm quality was reported after glass wool filtration in bulls (128). The GWS filtration of stallion spermatozoa improved both sperm quality (129) and mare pregnancy rates after deep-horn insemination with low sperm numbers (130). Leukosorb filters also removed defective spermatozoa from stallion semen with an efficiency comparable to GWS (131). Compared to other microspin chromatography column separation methods, the use of glass wool yielded the highest sperm motility in stallions (132). Characteristics reflective of sperm apoptosis were ameliorated by glass wool filtration of canine spermatozoa (133). The glass wool filtration technique was originally proposed for human spermatozoa (134) and later validated for use in human assisted reproductive therapy (ART), alone or in conjunction with annexin V-based cell sorting (135), which will be discussed below. Glass wool filtration removes human spermatozoa with DNA and acrosomal damage (136,137).

4.4. Magnetic beads

MACS is based on the affinity of proapoptotic protein annexin V to the externalized phosphatidylserine residues on the reorganized plasma membrane of apoptotic, necrotic, or otherwise membrane-damaged spermatozoa (138,139). Consequently, magnetic beads conjugated with annexin V bind to spermatozoa with damaged plasma membranes, allowing for their depletion by a magnet (139). This method is most commonly used in human ART, where annexin V beads and glass wool or annexin V-coated petri dishes can be used for sperm preselection for IVF or intracytoplasmic sperm injection [as reviewed by Said et al. (140)]. Within livestock species, MACS was successfully applied to increase kindling rates in rabbits (141) and to improve motility characteristics after thawing in bull spermatozoa (142).

4.5. Nanopurification

Nanotechnology refers to material manipulation on a scale of <100 nm. Recent applications of nanotechnology in animal production include nanovaccines, veterinary diagnostic tests, nanoground feed additives, milk enriched with calcium nanoparticles, and lateral flow devices for male fertility testing (143). Efforts in our laboratory have been focusing on the development of nanotechnology for semen purification, based on the presence of unique surface determinants in defective spermatozoa. Such determinants are targets for iron-oxide nanoparticles coated with antibodies (protein determinants) or lectins (glycan determinants). We have been focusing largely on ubiquitin, a proteolytic chaperone protein of epididymal origin found on defective sperm surfaces and on lectins PNS and PSA. These lectins have a high affinity to sperm-acrosomal glycans exposed in defective spermatozoa by mechanical damage, cryodamage, premature capacitation, and spontaneous acrosomal exocytosis, or due to aberrant acrosomal biogenesis during spermatid differentiation in the testis.

Compared to filtration/centrifugation-based techniques, semen nanopurification can be used on a whole ejaculate volume scale, requires no equipment other than a simple magnet bar and tubes, eliminates sperm damage by centrifugal force, and can be incorporated entirely in the workflow of semen collection and cryopreservation.

5. Conclusion

Recent realizations that antioxidants can improve semen quality will undoubtedly lead to innovation in AI instrumentation and supplies. The ROS scavenging

antioxidants could be provided in feed, added directly to semen extender, or added to insemination catheters. Inexpensive, rapid, and efficient semen purification techniques such as nanopurification have the potential to reduce the minimum necessary number of spermatozoa per AI dose needed to achieve pregnancy and may find an application in the rescue of low-quality semen collections from males with highly valuable genomes/heritable production traits.

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