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BIOCHEMICAL MARKERS OF STALLION SPERM QUALITY
(review)

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Abstract

Seminal plasma is a multicomponent fluid that serves as a vehicle for delivering spermatozoa to the oocyte. This fluid transports male gametes and provides their protection and nutrition during further movement in the female genital tract (T.R. Talluri et al., 2017). Thereby, understanding the effect of the components of seminal plasma on reproductive cells, as well as the search for markers of cryo-resistance and sperm fertility is undoubtedly interesting for researchers. Because stallion sperm lifespan, capacitation capacity, and fertility vary widely between individuals, it is important for horse breeding to investigate the factors that influence these parameters. The purpose of our review is to analyze current publications on the study of biochemical markers that characterize sperm quality and to consider methods for determining reactive oxygen species and oxidative stress products in spermatozoa and seminal plasma. Metabolites of seminal plasma, enzymes activity in it, indicators of oxidative stress and antioxidant defense system can serve as biochemical markers of sperm quality (S. Pesch et al., 2006). To ensure motility, spermatozoa need a large amount of ATP. Monosaccharides and organic acids such as lactate, pyruvate, citrate, and succinate are good energy substrates for these cells. This gives rise to interest in them as markers of fertility (C.R. Darr et al., 2016; E.B. Menezes et al., 2019; M.F. Lay et al., 2001). The concentration of nitric oxide (II) metabolites is another promising indicator for assessing the quality of stallions sperm, since it plays an important role in the regulation of sperm motility and capacitation and the fertilization process (M.B. Herrero et al., 2000; P.T. Goud et al., 2008; F. Francavilla et al., 2000). Among enzymes of seminal plasma, lactate dehydrogenase, alanine and aspartate aminosferases, γ-glutamyl transpeptidase are of interest. While semen analysis is considered the gold standard for diagnosing male fertility, it cannot detect the molecular abnormalities that are responsible for unexplained cases of male infertility. Currently, oxidative stress is considered one of the main causes of such phenomena. It damages sperm proteins, lipids and DNA, which in turn leads to poor embryo implantation and a decrease in pregnancy rates. In this review, the main producers of free radicals in sperm and the antioxidant defense system of male gametes as well as methods for the determination of reactive oxygen species and end products of oxidative stress in spermatozoa and seminal plasma are considered (A. Agarwal et al., 2003; S. Bisht et al., 2017; H. Sies, 2018). Based on the literature data, it was concluded that biochemical markers, such as seminal plasma metabolites, enzyme activity in it, and indicators of oxidative stress, have significant potential for characterizing stallion sperm quality.

Keywords: stallions, fertility, spermatozoa, seminal plasma, oxidative stress, enzymes, metabolites

Sperm consists of spermatozoa suspended in a liquid medium called seminal plasma which is secreted by the epididymis and accessory gonads before and during ejaculation. Seminal plasma is a complex fluid that serves as a vehicle for the delivery of ejaculated sperm on the way from the testes to their target, the oocyte. Seminal plasma not only transports spermatozoa, but also provides them with protection and nutrition during further movement in the female reproductive tract [1]. It consists of various biochemical components

such as proteins (including antioxidant and intracellular enzymes), metabolites, mineral elements that are important for the functioning of sperm [2]. These parameters have been recommended as markers of sperm quality because they indicate function and characterize sperm damage [3].

The anatomy of the accessory sex glands, the composition of their secretions, and the chemical composition of seminal plasma differ not only between animal species, but also between individuals within a breed [3]. Since the lifespan of spermatozoa, their ability to capacitate and the fertility of stallions varies from one individual to another, for a comprehensive assessment of the reproductive status of sires, it is necessary to study the factors influencing these indicators.

The purpose of our review is to analyze current publications on the study of biochemical markers characterizing the quality of stallion sperm, and to consider methods for determining reactive oxygen species and oxidative stress products in sperm and seminal plasma.

Sperm plasma metabolites. Of significant interest is the study of seminal plasma metabolites as markers of stallion fertility. Determination of metabolite concentrations is quite simple and can provide information about animal fertility and disease. Seminal plasma contains a large number of organic compounds. Thus, a study of the metabolome of bull sperm plasma using gas chromatography with mass spectrometric detection revealed 63 metabolites [4]. Of the low-molecular organic compounds, it contained the most fructose, citric, lactic, phosphoric acids and urea. At the same time, stallion sperm has a number of features, one of which is a low concentration of fructose and low fructolytic activity of sperm under anaerobic conditions [5].

Sperm motility is one of the most important properties determining male fertility. The movement of sperm along the female genital tract occurs independently and against the movement of the fluid. A significant amount of energy is required to ensure mobility. In this regard, sperm use different methods for producing ATP, the glycolysis in the cytoplasm (including through the involvement of fructose) and oxidative phosphorylation in mitochondria. It is believed that pyruvate is an important substrate for the production of energy by male reproductive cells, including stallions, under aerobic conditions [6]. In addition to oxidative decarboxylation of pyruvate and the Krebs cycle, β-oxidation of fatty acids may occur in sperm as a source of reduced coenzymes for the respiratory chain [7]. There is also evidence that sperm mitochondria contain a lactate-oxidizing complex, which allows them to actively utilize lactate as a source of pyruvate under aerobic conditions [7, 8]. Consequently, the content of lactic acid in seminal plasma is an important indicator of sperm energy metabolism.

Citrate, another essential component of seminal plasma, enters the spermoplasm mainly with the secretion of the prostate gland. Despite the presence of mitochondria in spermatozoa, it is believed that the use of citrate in the tricarboxylic acid cycle does not have a significant effect on its concentration in the sperm plasma, since carbohydrates are the main source of energy for sperm movement. The main function of citrate is considered to be the binding of spermoplasm cations and maintaining osmotic balance [8]. There is evidence that the concentration of citrate in the sperm plasma reflects the androgen content in mammals [9].

Succinate is also of interest as an energy substrate for sperm. One recent study demonstrated that succinic acid is a good substrate for oxidation in mitochondria during sperm capacitation, providing an increase in their proton potential [10]. The authors suggest that succinate is transported into mitochondria using a dicarboxylate transporter.

Research on nitric oxide metabolites can provide information about fertility. NO is involved in many physiological processes, including the regulation of reproductive function [11]. It is important for spermatogenesis, penile erection, folliculogenesis, and ovulation, among other things [12]. In sperm, NO plays an important role in the regulation of spermatozoa motility and capacitation [13]. However, the effect of NO on mammalian sperm is dose-dependent. Low amounts of NO are beneficial, while high amounts appear to be harmful [14]. Spermatozoa are capable of producing NO, and its synthesis is critical for motility, capacitation, and fertilization [15-17]. NO has been shown to stimulate human sperm motility through activation of soluble guanylate cyclase, subsequent cGMP synthesis, and activation of cGMP-dependent protein kinases [18]. Information about the role of the NO system in stallion reproduction is rather limited [19]. However, immunohistochemical studies have demonstrated the presence of all three isoforms of nitric oxide synthase NOS (eNOS, nNOS, and iNOS) in the stallion reproductive system, with their expression varying between different cell types [20]. In stallions, there is a positive correlation between NO production, on the one hand, and the motility and speed of sperm after thawing, on the other [21]. In old individuals, the concentration of NO metabolites in the seminal plasma is lower than in young individuals [21].

Seminal plasma enzymes. Assessment of enzyme activity in seminal plasma can be recommended as a biological marker of seminal fluid quality, since their content characterizes the function and reflects the integrity of spermatozoa [22, 23]. Changes in the activity of seminal plasma enzymes must be interpreted, taking into account where the enzyme is localized and how it enters the spermoplasm, whether it is actively secreted or appears in it due to a violation of the integrity and increased permeability of membranes. Thus, alkaline phosphatase is mainly derived from the testes and epididymis and can be used as a marker to differentiate azoospermia or oligospermia from ejaculatory failure in clinical cases [24]. In addition, this enzyme is secreted from the plasma membrane of spermatozoa [25].

Aspartate aminotransferase (AsAT) and alanine aminotransferase (AlAT) are intracellular enzymes found in the cytoplasmic droplets of sperm [26]. Increased activity of AsAT and AlAT in the spermoplasm of stallions may be associated with damage to the spermatozoa membrane [23]. Lactate dehydrogenase (LDH) is found in the cytosol, mitochondria, and plasma membrane of spermatozoa [27]. The enzyme gamma-glutamyl transpeptidase (GGT) is known to be located in the external region of spermatozoa [28]. GGT is an enzyme involved in the gamma-glutamyl cycle, which transports amino acids across membranes. In cells, the enzyme is present not only in the cytoplasmic membrane, but also in lysosomes and cytoplasm.

In a study by S. Pesch et al. [23], LDH activity was significantly correlated with motility, live-to-dead sperm ratio of stallions, and sperm pathology. The authors concluded that LDH can be considered the most prognostically significant enzyme for determining sperm quality.

Indicators of oxidative stress and fertility of stallions. Although semen analysis is considered the gold standard for diagnosing male fertility, it cannot detect abnormalities at the molecular level that are responsible for unexplained cases of infertility [29, 30]. Currently, oxidative stress (OS) is considered one of the main causes of unexplained cases of male infertility [31]. OS leads to damage to proteins, lipids and DNA of sperm, which, in turn, causes poor embryo implantation and a decrease in pregnancy rates. In addition, it also affects the health of the offspring and can cause mutations

in the germline, leading to severe pathologies, polygenic disorders, and even cancer [32]).

Oxidative stress is an imbalance between oxidants and antioxidants in favor of oxidants, leading to disruption of redox signaling and/or molecular damage [33]. Cell damage in OS occurs mainly due to the action of reactive oxygen species (ROS), which are represented by free radicals, the compounds that have unpaired electrons. These are hydroxyl radical ($OH^{\bullet}$), superoxide anion radical ($O_2^{\bullet -}$), peroxide radicals ($RO_2^{\bullet -}$), as well as non-radical molecules with oxidizing properties, e.g., the singlet oxygen, hydrogen peroxide (H_2O_2), hypochlorous acid (HOCL), lipid peroxides (LOOH), ozone (O_3) [34]. In addition to ROS, active forms of nitrogen, the nitric oxide (NO) and peroxynitrite ($ONOO^-$) play an important role in molecular damage during OS, although in some cases the name nitrosative stress is used for this process.

Of the ROS, superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) are the most important for spermatozoa [35]. These compounds are sequentially formed during the reduction of oxygen in the respiratory chain of mitochondria:

$+e^- + e^- + 2H^+ + e^- + H^+ + e^- + H^+,$

$O_2 \rightarrow O_2^{\bullet -} \rightarrow H_2O_2 \rightarrow H_2O + OH^{\bullet} \rightarrow 2H_2O.$

Superoxide and hydroxyl radicals are unstable compounds with half-lives of milli- and nanoseconds, respectively, causing these radicals to react at the site of their formation [36]. Hydrogen peroxide can be a source of formation of hydroxyl radical, the most reactive ROS:

$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^{\bullet} + Fe^{3+}$ (Fenton reaction);

$O_2^{\bullet -} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + O_2$ (Haber-Weiss reaction).

At physiological concentrations, ROS are involved in capacitation by stimulating the synthesis of adenylate cyclase [37], in the acrosomal reaction by regulating the activity of protein kinases [27], and in sperm-oocyte fusion by inhibiting protein tyrosine phosphatase and thus maintaining phospholipase A2 activity [38].

The two most important sources of ROS for sperm are leukocytes and immature sperm [39]. Leukocytes, especially neutrophils and macrophages, are closely associated with excessive ROS production leading to male gamete dysfunction [32]. They produce approximately 1000 times more ROS than sperm. They play a significant role in genital tract infections, inflammation, and cellular defense mechanisms [40, 41]. One recent study found a positive correlation between leukocyte counts, ROS, and the number of sperm with fragmented DNA, and a negative correlation between leukocyte counts, sperm concentration, and spermatozoa_motility [42].

Stallion spermatozoa are called “professional” producers of ROS (mainly superoxide and H_2O_2) due to their high mitochondrial activity. They are characterized by a complex system for controlling redox homeostasis [43]. Since ROS damage the cellular structures near which they are formed, the study of ROS production in sperm is of significant interest. Mitochondria and the cytoplasmic membrane are most important for the production of ROS in sperm. The appearance of immature sperm, especially with excess residual cytoplasm, as well as various sperm abnormalities (residual cytoplasm or cytoplasmic droplets) are associated with excessive ROS production [44, 45]]. Typically, such sperm are removed by Sertoli cells during spermatogenesis. Excessive ROS formation in cytoplasmic droplets is associated with the presence of the enzyme glucose-6-phosphate dehydrogenase, which produces NADPH, which is then oxidized by two oxidoreductases located in the cytoplasmic and mitochondrial membranes, respectively [46].

Antioxidant protection of sperm. The specific cellular structure of sperm and their plasma membrane, the large number of mitochondria, the small volume of the cytoplasm and the low amount of antioxidants in it make male gametes quite vulnerable to damage by free radicals [47]. Enzymatic and non-enzymatic antioxidants are present in sperm. Enzymatic antioxidants are proteins that neutralize excess ROS and prevent damage to cellular structure. These include superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase [48]. Currently, these enzymes are considered as possible markers of cryotolerance in stallion spermatozoa [49, 50].

SOD (EC 1.15.1.1) catalyzes the reaction $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$. The main task of superoxide dismutases is the removal of superoxide anion radicals. Superoxide is constantly formed during cell life as the first intermediate product in O2 reduction reactions. Its main producer is the mitochondrial respiratory chain [51]. SOD is a critical enzyme for the bioavailability of NO by preventing its reaction with superoxide and conversion to peroxynitrite [52]. In boar seminal plasma, extracellular SOD is detected, containing Cu and Zn as cofactors [53]. The results of the study by M. Kowalowka et al. [53] show that in boar, extracellular SOD serves as a seminal plasma antioxidant enzyme that plays an important physiological role in counteracting oxidative stress in sperm. It has been demonstrated that in stallions whose ejaculates have good freezing ability, SOD activity is higher than in the group with low cryoresistance, which is associated with the preservation of the integrity of the acrosomal membrane [49, 50].

Catalase (EC 1.11.1.6) is an enzyme that catalyzes the disproportionation reaction of hydrogen peroxide $2H_2O_2 \rightarrow H_2O + O_2$. This heme protein is also capable of oxidizing low molecular weight alcohols and nitrites in the presence of hydrogen peroxide. The presence of catalase in sperm has been demonstrated in sheep and cattle. It potentially plays a role in the aging process and OS control in cells [54]. Along with SOD and glutathione peroxidase, catalase serves as the main endogenous antioxidant enzyme in the seminal plasma of stallions [55].

Glutathione peroxidases (EC 1.11.1.9 and EC 1.11.1.12) catalyze the reduction of H2O2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione: $2GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O$. Some glutathione peroxidase isoenzymes contain selenium as a cofactor, which is linked to cysteine in their active site [56]. The level of reduced glutathione inside cells is maintained thanks to glutathione reductase, which reduces glutathione, and GGT, which transports glutathione into the cell.

Non-enzymatic antioxidants include vitamin C, vitamin E, zinc, selenium, taurine, hypotaurine, and glutathione [34]. Many of them exhibit their properties by being cofactors (selenium for glutathione peroxidase, zinc for SOD) or coenzymes (glutathione for glutathione peroxidase) of antioxidant enzymes. Vitamin E is able to react directly with peroxide radicals, sequentially oxidizing into a fairly stable tocopheryl radical, and then into tocopheryl quinone, interrupting the chain reactions of free radical oxidation. A large number of studies are currently devoted to studying the effect of externally added antioxidants on sperm quality and safety [34, 54, 55].

Methods for determining ROS and final OS products in spermatozoa and seminal plasma. To study OS, it is of interest to determine the generation of both ROS and end products of free radical oxidation.

One of the fairly stable products of lipid peroxidation is malondialdehyde, which is usually determined by reaction with thiobarbituric acid [57]. In addition to malondialdehyde, some other compounds also react; together they

are called thiobarbituric acid reactive substances (TBARS). There is evidence that TBARS content is increased in frozen but not in chilled bull semen [58]. However, there were no differences in malondialdehyde concentrations in fresh and frozen semen in healthy men [59].

Oxidation of proteins under the influence of free radicals leads to the formation of carbonyl groups, fragmentation and aggregation of protein molecules. These modifications can affect the functions of proteins. Carbonyl derivatives of amino acid residues in proteins are stable end products of protein oxidation, which makes their determination an informative method for studying the oxidative modification of proteins [60]. They can be assessed quantitatively by reaction with 2,4-dinitrophenylhydrazine. The resulting 2,4-dinitrophenylhydrazones have a specific absorption spectrum in the visible and ultraviolet parts of the spectrum [61].

A chemiluminescence assay can be used to directly measure sperm ROS generation: special reagents interact with the oxidation end products, resulting in an electrical signal that can be measured as photons per minute using a luminometer [62]. This assay is effective for measuring both intracellular and extracellular ROS.

Another test for measuring intracellular sperm ROS is flow cytometry using fluorescent and chemiluminescent probes [63]. The most common technique is with 2′-7′-dichlorodihydrofluorescein diacetate, which allows direct assessment of the redox status of the cell [64]. Another simple and cost-effective test for measuring ROS using a light microscope is the nitroblue tetrazolium reaction. The principle of the method is based on the transformation of nitroblue tetrazolium into the blue pigment diformazan upon interaction with superoxide produced by sperm or leukocytes [62].

Thus, at present, studying the effects of seminal plasma components on germ cells, as well as the search for markers of cryostability and fertility of stallion sperm are of undoubted interest. Seminal plasma contains a large number of components (metabolites, enzymes, antioxidants) that affect the structural integrity, progressive motility and fertilizing ability of sperm. Among the metabolites, attention is focused on energy substrates, thr pyruvate, lactate, citrate, succinate. Stallion sperm are very vulnerable to damaging factors during cryopreservation, in particular to oxidative stress due to the significant content of polyunsaturated fatty acids in the phospholipids of the plasma membrane. In this regard, methods for studying the redox status are promising for assessing ejaculates: determination of substances that react with thiobarbituric acid, products of oxidative modification of proteins, NO metabolites, chemiluminescent analysis, flow cytometry. Active attempts are being made to detect markers of resistance of stallion sperm to freezing and subsequent thawing among antioxidant enzymes, i.e., SOD, catalase, and glutathione peroxidase. Biochemical markers, such as seminal plasma metabolite concentrations, seminal plasma enzyme activity, and indicators of oxidative stress, have significant potential for characterizing the quality and cryostability of stallion sperm.

REFERENCES

1. Talluri T.R., Mal G., Ravi S.K. Biochemical components of seminal plasma and their correlation to the fresh seminal characteristics in Marwari stallions and Poitou jacks. *Veterenary World*, 2017, 10(2): 214-220 (doi: 10.14202/vetworld.2017.214-220).
2. Juyena N.S., Stelletta C. Seminal plasma: an essential attribute to spermatozoa. *Journal of Andrology*, 2012, 33(4): 536-551 (doi: 10.2164/jandrol.110.012583).
3. Tvrda E., Sikeli P., Lukacova J., Massanyi P., Lukac N. Mineral nutrients and male fertility. *Journal of Microbiology, Biotechnology and Food Sciences*, 2013, 3(1): 1-14.
4. Velho A.L.C., Menezes E., Dinh T., Kaya A., Topper E., Moura A.A., Memili E. Metabolomic

markers of fertility in bull seminal plasma. *PLoS ONE*, 2018, 13(4): e0195279 (doi: 10.1371/journal.pone.0195279).
5. Mann T. Biochemistry of stallion semen. *Journal of reproduction and fertility. Supplement*, 1975, 23: 47-52.
6. Darr C.R., Varner D.D., Teague S., Cortopassi G.A., Datta S., Meyers S.A. Lactate and pyruvate are major sources of energy for stallion sperm with dose effects on mitochondrial function, motility, and ROS production. *Biology of Reproduction*, 2016, 95(2): 1-11 (doi: 10.1095/biolreprod.116.140707).
7. Menezes E.B., Velho A.L.C., Santos F., Dinh T., Kaya A., Topper E., Moura A.A., Memili E. Uncovering sperm metabolome to discover biomarkers for bull fertility. *BMC Genomics*, 2019, 20(1): 714 (doi: 10.1186/s12864-019-6074-6).
8. Lay M.F., Richardson M.E., Boone W.R., Bodine A.B., Thurston R.J. Seminal plasma and IVF potential. Biochemical constituents of seminal plasma of males from in vitro fertilization couples. *Journal of Assisted Reproduction and Genetics*, 2001, 18(3): 144-150 (doi: 10.1023/a:1009420306173).
9. Mann T. The Biochemistry of semen and of the male reproductive tract. *Medical Journal of Australia*, 1965, 1(18): 652-653 (doi: 10.5694/j.1326-5377.1965.tb72030.x).
10. Paventi G., Lessard C., Bailey J.L., Passarella S. In boar sperm capacitation L-lactate and succinate, but not pyruvate and citrate, contribute to the mitochondrial membrane potential increase as monitored via safranine O fluorescence. *Biochemical and Biophysical Research Communications*, 2015, 462(3): 257-262 (doi: 10.1016/j.bbrc.2015.04.128).
11. Herrero M.B., Gagnon C. Nitric oxide: a novel mediator of sperm function. *Journal of Andrology*, 2001, 22: 349-356.
12. Roselli M., Keller P.J., Dubey R.K. Role of nitric oxide in the biology physiology and pathophysiology of reproduction. *Human Reproduction Update*, 1998, 4(1): 3-24 (doi: 10.1093/humupd/4.1.3).
13. Balercia G., Moretti S., Vignini A., Magagnini M., Mantero F., Boscaro M., Riccardo-Lamonica G., Mazzanti L. Role of nitric oxide concentrations on human sperm motility. *Journal of Andrology*, 2004, 25(2): 245-249 (doi: 10.1002/j.1939-4640.2004.tb02784.x).
14. Vignini A., Nanetti L., Buldreghini E., Moroni C., Ricciardo Lamonina G., Mantero F., Boscaro M., Mazzanti L., Balercia G. The production of peroxynitrite by human spermatozoa may affect sperm motility through the formation of protein nitrotyrosine. *Fertility and Sterility*, 2006, 85(4): 947-953 (doi: 10.1016/j.fertnstert.2005.09.027).
15. Herrero M.B., Chatterjee S., Lefièvre L., de Lamirande E., Gagnon C. Nitric oxide interacts with the cAMP pathway to modulate capacitation of human spermatozoa. *Free Radical Biology and Medicine*, 2000, 29(6): 522-536 (doi: 10.1016/s0891-5849(00)00339-7).
16. Goud P.T., Goud A.P., Diamond M.P., Gonik M.P., Abu-Soud H. Nitric oxide extends the oocyte temporal window for optimal fertilization. *Free Radical Biology and Medicine*, 2008, 45(4): 453-459 doi: (doi: 10.1016/j.freeradbiomed.2008.04.035).
17. Francavilla F., Santucci R., Macerola B., Ruvolo G., Romano R. Nitric oxide synthase inhibition in human sperm affects sperm-oocyte fusion but not zona pellucida binding. *Biology of Reproduction*, 2000, 63(2): 425-429 (doi: 10.1095/biolreprod63.2.425).
18. Miraglia E., De Angelis F., Gazzano E., Hassanpour H., Bertagna A., Aldieri E., Revelli A., Ghigo D. Nitric oxide stimulates human sperm motility via activation of the cyclic GMP/protein kinase G signaling pathway. *Reproduction*, 2011, 141(1): 47-54 (doi: 10.1530/REP-10-0151).
19. Khan F.A., Sholtz E.L., Chenier T.S. The nitric oxide system in equine reproduction: surrent status and future directions. *Journal of Equine Veterinary Science*, 2015, 35(6): 481-487 (doi: 10.1016/j.jevs.2015.02.009).
20. Ha T.Y., Kim H.S., Shin T. Expression of constitutive endothelial, neuronal and inducible nitric oxide synthase in the testis and epididymis of horse. *Journal of Veterinary Medical Science*, 2004, 66(4): 351-356 (doi: 10.1292/jvms.66.351).
21. Ortega Ferrusola C., González Fernández L., Macías García B., Salazar-Sandoval C., Morillo Rodríguez A., Rodríguez Martinez H., Tapia J.A., Peña F.J. Effect of cryopreservation on nitric oxide production by stallion spermatozoa. *Biology of Reproduction*, 2009, 81: 1106-1111 (doi: 10.1095/biolreprod.109.078220).
22. Eghbali M., Alavi-Shoushtari S.M., Asri-Rezaei S., Ansari M.H.K. Effects of the seminal plasm iron and lead content on semen quality of water buffalo (*Bubalus bubalis*) bulls. *Veterinary Research Forum*, 2010, 1(3): 142-148.
23. Pesch S., Bergmann M., Bostedt H. Determination of some enzymes and macro and microelements in stallion seminal plasma and their correlations to semen quality. *Theriogenology*, 2006, 66(2): 307-313 (doi: 10.1016/j.theriogenology.2005.11.015).
24. El-Bishbishy H.A., Aly H.A.A., El-Shafey M. Lipoic acid mitigates bisphenol A-induced testicular mitochondrial toxicity in rats. *Environmental Health*, 2013, 29(10): 875-887 (doi: 10.1177/0748233712446728).
25. Bucci D., Isani G., Giaretta E., Spinaci M., Tamanini C., Ferlizza E., Galeati G. Alkaline phosphatase in boar sperm function. *Andrology*, 2014, 2(1): 100-106 (doi: 10.1111/j.2047-2927.2013.00159.x).

26. Katila T. In vitro evaluation of frozen-thawed stallion semen: a review. *Acta Veterinaria Scandinavica*, 2001, 42(2): 199-217 (doi: 10.1186/1751-0147-42-199).
27. O'Flaherty C., Breininger E., Beorlegui N., Beconi M.T. Acrosome reaction of bovine spermatozoa: role of reactive oxygen species and lactate dehydrogenase C4. *Biochimica and Biophysica Acta — General Subjects*, 2005, 1726(1): 96-101 (doi: 10.1016/j.bbagen.2005.07.012).
28. Agarwal Y.P., Vanha-Perttula T. Glutation, L-glutamic acid and γ-glutamil transpeptidase in the bull reproductive tissues. *International Journal of Andrology*, 1988, 11(2): 123-131 (doi: 10.1111/j.1365-2605.1988.tb00988.x).
29. Pizzol D., Ferlin A., Garolla A., Lenzi A., Bertoldo A., Foresta C. Genetic and molecular diagnostics of male infertility in the clinical practice. *Frontiers in Bioscience-Landmark*, 2014, 19: 291-303 (doi: 10.2741/4208).
30. Ramya T., Misro M.M., Sinha D., Nandan D. Sperm function and seminal oxidative stress as tools to identify sperm pathologies in infertile men. *Fertility and Sterility*, 2010, 93(1): 297-300 (doi: 10.1016/j.fertnstert.2009.05.074).
31. Agarwal A., Saleh R.A., Bedaiwy M.A. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertility and Sterility*, 2003, 79(4): 829-843 (doi: 10.1016/s0015-0282(02)04948-8).
32. Bisht S., Faiq M., Tolahunase M., Dada R. Oxidative stress and male infertility. *Nature Reviews Urology*, 2017, 14(8): 470-485 (doi: 10.1038/nrurol.2017.69).
33. Sies H. On the history of oxidative stress: Concept and some aspects of current development. *Current Opinion in Toxicology*, 2018, 7(2): 122-126 (doi: 10.1016/j.cotox.2018.01.002).
34. Bansal A.K., Bilaspuri G.S. Impacts of oxidative stress and antioxidants on semen functions. *Veterinary Medicine International*, 2010, 2011: 686137 (doi: 10.4061/2011/686137).
35. Kumar N., Singh A.K. Reactive oxygen species in seminal plasma as a cause of male infertility. *Journal of Gynecology Obstetrics and Human Reproduction*, 2018, 47: 565-572 (doi: 10.1016/j.jogoh.2018.06.008).
36. Miranda-Vilela A.L., Alves P.C., Akimoto A.K., Pereira L.C., Nazare Klautau-Guimaraes M.D., Grisolia C.K. The effect of hydrogen peroxide-induced oxidative stress on leukocytes depends on age and physical training in healthy human subjects carrying the same genotypes of antioxidant enzymes' gene polymorphisms. *American Journal of Human Biology*, 2010, 22(6): 807-812 (doi: 10.1002/ajhb.21086).
37. Breitbart H. Intracellular calcium regulation in sperm capacitation and acrosomal reaction. *Molecular and Cellular Endocrinology*, 2002, 187(2): 139-144 (doi: 10.1016/s0303-7207(01)00704-3).
38. Khosrowbeygi A., Zarghami N. Fatty acid composition of human spermatozoa and seminal plasma levels of oxidative stress biomarkers in subfertile males. *Prostaglandins, Leukotrienes & Essential Fatty Acids*, 2007, 77(2): 117-121 (doi: 10.1016/j.plefa.2007.08.003).
39. Garrido N., Meseguer M., Simon C., Pellicer A., Remohi J. Pro-oxidative and antioxidative imbalance in human semen and its relation with male fertility. *Asian Journal of Andrology*, 2004, 6(1): 59-65.
40. Henkel R.R. Leukocytes and oxidative stress: dilemma for sperm function and male fertility. *Asian Journal of Andrology*, 2011, 13(1): 43-52 (doi: 10.1038/aja.2010.76).
41. Saleh R.A., Agarwal A., Kandirali E., Sharma R.K., Thomas A.J., Nada E.A, Evenson D.P., Alvarez J.G. Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa. *Fertility and Sterility*, 2002, 78(6): 1215-1224 (doi: 10.1016/s0015-0282(02)04237-1).
42. Lobascio A.M., De Felici M., Anibaldi M., Greco P., Minasi M.G., Greco E. Involvement of seminal leukocytes, reactive oxygen species, and sperm mitochondrial membrane potential in the DNA damage of the human spermatozoa. *Andrology*, 2015, 3(2): 265-270 (doi: 10.1111/andr.302).
43. Peña F.J., O'Flaherty C., Ortiz Rodríguez J.M., Martín Cano F.E., Gaitskell-Phillips G.L., Gil M.C., Ortega Ferrusola C. Redox regulation and oxidative stress: the particular case of the stallion spermatozoa. *Antioxidants*, 2019, 8(11): 567 (doi: 10.3390/antiox8110567).
44. Keating J., Grundy C.E., Fivey P.S., Elliott M., Robinson J. Investigation of the association between the presence of cytoplasmic residues on the human sperm midpiece and defective sperm function. *Journal of Reproduction and Fertility*, 1997, 110(1): 71-77 (doi: 10.1530/jrf.0.1100071).
45. Zini A., Defreitas G., Freeman M., Hechter S., Jarvi K. Varicocele is associated with abnormal retention of cytoplasmic droplets by human spermatozoa. *Fertility and Sterility*, 2000, 74(3): 461-464 (doi: 10.1016/s0015-0282(00)00703-2).
46. Sabeti P., Pourmasumi S., Rahiminia T., Akyash F., Talebi A.R. Etiologies of sperm oxidative stress. *International Journal of Reproductive Biomedicine*, 2016, 14(4): 231-240.
47. Bollwein H., Fuchs I., Koess C. Interrelationship between plasma membrane integrity, mitochondrial membrane potential and DNA fragmentation in cryopreserved bovine spermatozoa. *Reproduction in Domestic Animals*, 2008, 43(2): 189-195 (doi: 10.1111/j.1439-0531.2007.00876.x).
48. Agarwal A., Gupta S., Sharma R.K. Role of oxidative stress in female reproduction. *Reproductive Biology and Endocrinology*, 2005, 3: 28 (doi: 10.1186/1477-7827-3-28).
49. Catalán J., Yánez-Ortiz I., Tvarijonaviciute A., González-Aróstegui L.G., Rubio C.P., Barranco I., Yeste M., Miró J. Seminal plasma antioxidants are related to sperm cryotolerance in the horse. *Antioxidants*, 2022, 11(7): 1279 (doi: 10.3390/antiox11071279).

50. Papas M., Catalán, J., Fernandez-Fuertes B., Arroyo L., Bassols A., Miró J., Yeste M. Specific activity of superoxide dismutase in stallion seminal plasma is related to sperm cryotolerance. *Antioxidants,* 2019, 8(11): 539 (doi: 10.3390/antiox8110539).
51. Miriyala S., Holley A.K., St Clair D.K. Mitochondrial superoxide dismutase — signals of distinction. *Anti-Cancer Agents in Medicinal Chemistry*, 2011, 11(2): 181-190 (doi: 10.2174/187152011795255920).
52. Obal D., Dai S., Keith R., Dimova N., Kingery J., Zheng Y-T., Zweier J., Velayutham M., Prabhu S.D., Li Q., Conklin D., Yang D., Bhatnagar A., Bolli R., Rokosh G. Cardiomyocyte-restricted overexpression of extracellular superoxide dismutase increases nitric oxide bioavailability and reduces infarct size after ischemia/reperfusion. *Basic Research in Cardiology*, 2012, 107(6): 305 (doi: 10.1007/s00395-012-0305-1).
53. Kowalowka M., Wysocki P., Fraser L., Strzezek J. Extracellular superoxide dismutase of boar seminal plasma. *Reproduction in Domestic Animals*, 2008, 43(4): 490-496 (doi: 10.1111/j.1439-0531.2007.00943.x).
54. Bucak M.N., Ateşşahin A., Varişli O., Yüce A., Tekin N., Akçay A. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen. Microscopic and oxidative stress parameters after freeze-thawing process. *Theriogenology*, 2007, 67(5): 1060-1067 (doi: 10.1016/j.theriogenology.2006.12.004).
55. Del Prete C., Stout T., Montagnaro S., Pagnini U., Uccello M., Florio P., Ciani F., Tafuri S., Palumbo V., Pasolini M.P., Cocchia N., Henning H. Combined addition of superoxide dismutase, catalase and glutathione peroxidase improves quality of cooled stored stallion semen. *Animal Reproduction Science*, 2019, 210: 106195 (doi: 10.1016/j.anireprosci.2019.106195).
56. Margis R., Dunand C., Teixeira F.K., Margis-Pinheiro M. Glutathione peroxidase family — an evolutionary overview. *The FEBS Journal*, 2008, 275(15): 3959-3970 (doi: 10.1111/j.1742-4658.2008.06542.x).
57. Sanocka D., Kurpisz M. Reactive oxygen species and sperm cells. *Reproductive Biology and Endocrinology*, 2004, 2: 12-26 (doi: 10.1186/1477-7827-2-12).
58. Chatterjee S., Gagnon C. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. *Molecular Reproduction and Development*, 2001, 59(4): 451-458 (doi: 10.1002/mrd.1052).
59. Wang Y., Sharma R.K., Agarwal A. Effect of cryopreservation and sperm concentration on lipid peroxidation in human semen. *Urology*, 1997, 50(3): 409-413 (doi: 10.1016/S0090-4295(97)00219-7).
60. Dalle-Donne I., Rossi R., Giustarini D., Milzani A., Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta*, 2003, 329(1-2): 23-38 (doi: 10.1016/S0009-8981(03)00003-2).
61. Dubinina E.E., Burmistrov S.O., Khodov D.A., Porotov I.G. *Voprosy meditsinskoy khimii*, 1995, 41(1): 24-26 (in Russ.).
62. Agarwal A., Majzoub A. Laboratory tests for oxidative stress. *Indian Journal of Urology*, 2017, 33(3): 199-206 (doi: 10.4103/iju.IJU_9_17).
63. Allamaneni S.S., Agarwal A., Nallella K.P., Sharma R.K., Thomas Jr. A.J., Sikka S.C. Characterization of oxidative stress status by evaluation of reactive oxygen species levels in whole semen and isolated spermatozoa. *Fertility and Sterility*, 2005, 83(3): 800-803 (doi: 10.1016/j.fertnstert.2004.05.106).
64. Eruslanov E., Kusmartsev S. Identification of ROS using oxidized DCFDA and flow-cytometry. *Methods in Molecular Biology*, 2010, 594: 57-72 (doi: 10.1007/978-1-60761-411-1_4).