APPLICATION OF HERBAL EXTRACTS AS ANTIOXIDANT AGENTS IN RAM SEMEN – A REVIEW

Tsveta Georgieva

University of Forestry, Faculty of Veterinary Medicine, Sofia, Bulgaria
E-mail: c.georgieva@ltu.bg
ORCID: 0000-0003-0749-5707 T.G.
(Submitted: 11 September 2024; Accepted: 10 October 2024; Published 25 November 2024)

ABSTRACT

Semen preservation is an important tool in livestock reproduction, allowing for the widespread use of high-quality genetics in artificial insemination programs. However, the process of semen preservation often leads to oxidative stress, a major cause of sperm damage, including lipid peroxidation, DNA fragmentation, and reduced motility. In response to this challenge, the use of antioxidants has been explored to mitigate oxidative damage in semen. Recently, there has been growing interest in the application of herbal extracts as natural antioxidant agents in ram semen preservation. This review explores the potential benefits and mechanisms of action of herbal extracts as antioxidants in ram semen, highlighting their role in improving semen quality and fertility.

Key words: oxidative stress, herbal extract, antioxidant, sperm.

Introduction

Artificial insemination (AI) can undoubtedly be considered the oldest and most widely used assisted reproductive technique (ART) in animal husbandry. The three factors determining the widespread application are: a relatively easy to perform technique, economically advantageous and with a high success rate at the end of the insemination campaign (Faigl *et al.,* 2012). Fresh or frozen semen can be used for AI in small ruminants (Tsakmakidis, 2010). Immediately after receiving the ejaculate, it is diluted with a medium that has similar characteristics to sperm – a sperm extender. The purpose of dilution with a sperm extender is to protect and preserve the spermatozoa from temperature shock after leaving the breeder's body and to keep them alive for as long as possible under different temperature regimes (Gundogan *et al.*, 2011). New combinations of saline solutions, different carbohydrates and antibiotics are constantly being tried. In the last decade, the use of natural ingredients such as non-antibiotic growth stimulants, phytobiotics, which negatively affect the growth of pathogenic bacteria, entered medicine and reproduction. The secondary metabolites of plants, the micro- and macroelements contained in them, vitamins and provitamins have a high antioxidant capacity and can potentiate the action of the body's enzyme systems for the neutralization of free radicals, be catalysts in the reaction of pairing free electrons or directly capture unpaired electrons. The addition of plant antioxidants reduces the levels of oxidative stress by neutralizing free radicals, stabilizes the cell membrane and helps to prolong the survival of spermatozoa in an *in vitro* environment.

What is oxidative stress?

Oxidative stress (OS) is defined as an imbalance in the redox state of the cell, caused either by too high levels of oxidants, or conversely, by too little amount of antioxidants. When excessive amounts of reactive oxygen species (ROS) are produced or antioxidant activity is reduced, the balance between oxidation and reduction is disturbed, causing oxidative stress (Kowalczyk, 2022). Reactive forms of oxygen are free radicals, particles with one or more unpaired electrons and are capable of independent existence (Halliwell and Gutteridge, 2007). Of greatest importance to the cell are superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (-OH). Already at the moment of obtaining the ejaculate through the method of the artificial vagina, the spermatozoa are subjected to oxidative stress. Ejaculate storage outside the reproductive system, dilution, different storage temperature regimes are causes of physical and chemical cell damage (Yoshida *et al.*, 2003; Gallardo, 2007). Mammalian spermatozoa contain a high percentage of unsaturated fatty acids in their plasma membrane and are susceptible to the adverse effects of ROS (Anger *et al.*, 2003). In comparison to other domestic mammals, ram spermatozoa have a higher proportion of unsaturated fatty acids in their cell membranes and a lower content of cholesterol and phospholipids. This composition makes them particularly susceptible to the harmful effects of reactive oxygen species (ROS) (Holt, 200). Because of its composition, the ram sperm membrane is prone to lipid peroxidation, which disrupts gamete and acrosome structure and function (Alvarez and Storey, 1993). Besides structural defects of the cell membrane, oxidative stress reduces sperm motility (Aitken, 1984, Maxwell and Watson, 1996). At the same time, spermatozoa contain very low levels of enzymatic antioxidants, which are insufficient to protect sperm from high levels of ROS, and the cytoplasm contains small concentrations of the enzyme capable of neutralizing them. Imbalance between oxidants and antioxidants in sperm leads to metabolic, functional and morphological disorders of gametes and can be the main cause of infertility (Fraczek et Kurpisz, 2007). OS is caused by the disruption of the pro-oxidant defense of the cell and the generation of a large amount of reactive oxygen species. ROS in turn alters energy metabolism, motility, viability and DNA integrity in sperm (Armstrong *et al.*, 1999, Krzyzosiak *et al.*, 2000, Baumber *et al.*, 2002). Sperm DNA integrity can be irreversibly compromised, due to increased susceptibility to OS (Simoes *et al.*, 2013). In addition, ROS are involved in gamete chromatin condensation, regulating the number of reproductive cells by inducing apoptosis or proliferation of spermatogonia (Aitken, 1999). The large intracellular amount of ROS can directly induce sperm apoptosis (Kothari *et al.*, 2010). Oxidative stress results from a constant imbalance between the generation of ROS and the ability of the intracellular antioxidant system to deactivate them. Mitochondria are the organelles in which oxidative processes are controlled, therefore mitochondrial dysfunction or damage induces OS. Intramitochondrial or extramitochondrial accumulation of ROS under oxidative stress leads to lipid peroxidation and glycoxidation reactions, which ultimately increases the endogenous production of reactive aldehydes and their derivatives and is detrimental to the cell (Boutros et Ray, 2023). Sperm with abnormal morphology (mainly with cytoplasmic debris indicating their immaturity and reduced fertility potential) produce higher amounts of ROS than sperm with normal structure (Gomez *et al.,* 1996; Aziz *et al.,* 2004). There is also a difference in the amount of ROS produced by sperm at different stages of maturation. ROS themselves and their end products such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (-OH) are toxic to spermatozoa (Agarwal *et al.,* 2003), they provoke the lipid peroxidation reaction, which is considered for one of the most important causes of reduced sperm reproductive capacity in mammals (Aitken, 1995). Lipid peroxidation is an inevitable process during the manipulations of the seminal fluid – dilution, exposure to light and different temperature regimes. The LP reaction can also be catalyzed by transition metal ions, most commonly iron(II) cation; Fe2+, iron(III) cation; Fe3+ (Stohs et Bagchi, 1995).

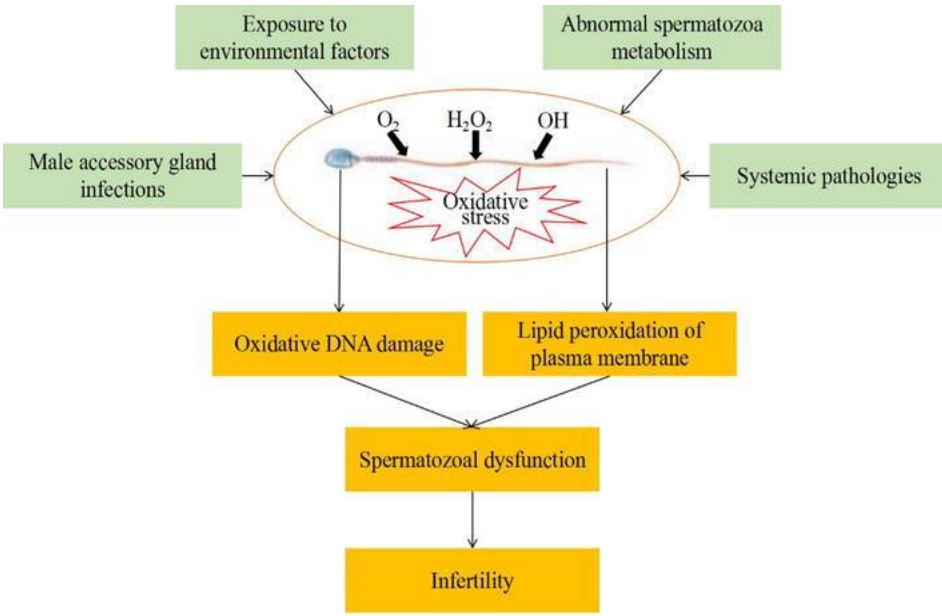

Figure 1: Factors contributing to oxidative stress-induced male infertility, (Fang et Zhong, 2020)

Antioxidant protection of sperm

The main system of antioxidant enzymes in sperm is called the enzyme triad and includes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX).

Glutathione peroxidases (GPx1-5) are cytoplasmic enzymes that catalyze the reduction of hydrogen peroxide (H_2O_2) to water (H_{2O}) and oxygen (O_2), as well as catalyze the reduction of peroxide radicals to alcohols and oxygen using glutathione (GSH) as electron donor (Fanucchi, 2014). The active molecule contains selenium in the form of selenocysteine. In sperm, GPx1-5 is mainly found in the mitochondrial matrix (Peeker *et al.,* 1997), but a nuclear form has also been found, which has a protective effect on sperm DNA against OS damage and is actively involved in the process of chromatin condensation (Pfeifer *et al.*, 2001). Furthermore, GPX has also been identified in seminal plasma, indicating that it likely originates from the prostate gland, where it adds an additional layer of antioxidant defense during sperm ejaculation and transport. (Yeung *et al.,* 1998; Walczak-Jedrzejowska *et al.,* 2013; Kowalczyk, 2022).

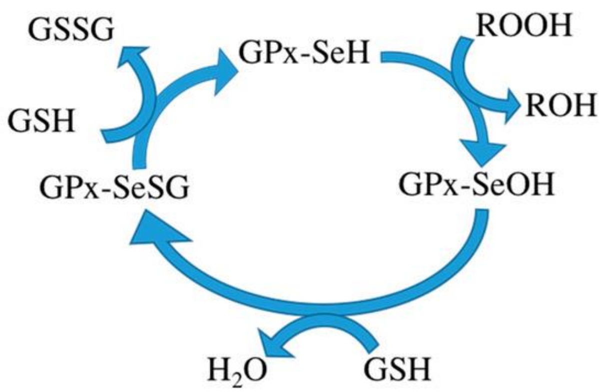

Figure 2: The mechanism of the GPx, (Huang *et al.*, 2017)

Catalase (CAT) is an iron-containing porphyrin enzyme classified as an oxyreductase. It plays a crucial role in converting hydrogen peroxide (H_2O_2) into oxygen (O_2) and water (H_2O), a process that avoids the production of harmful free radicals (Shengshui *et al.*, 2008). By facilitating this reaction, catalase enhances the cell's resistance to oxidative stress (Harris, 1992). Notably, a single molecule of catalase can decompose millions of hydrogen peroxide molecules into water and oxygen every second (Goodsell, 2004). This enzyme is frequently employed by cells to efficiently and swiftly neutralize hydrogen peroxide into less reactive substances, namely oxygen gas and water (Gaetani *et al.,* 1996).

Superoxide dismutase (SOD) enzymes play a crucial role in redox reactions, where they simultaneously oxidize and reduce superoxide radicals (O2−), producing either oxygen (O2) or hydrogen peroxide (H2O2) (Badal, 2017). In this dismutation process, a single particle acts as both the oxidizing and reducing agent. There are both intra- and extracellular forms of SOD. Intracellularly, two primary forms have been identified: copper-zinc SOD (Cu, ZnSOD or SOD-1), predominantly found in the cytoplasm and utilizing copper and zinc in its active site, and manganese SOD (MnSOD or SOD-2), which is located in the mitochondrial matrix and uses manganese. Given that mitochondria consume over 90% of cellular oxygen, they are particularly susceptible to oxidative stress, and MnSOD is critical for neutralizing reactive oxygen species (ROS) at their source. The extracellular form, SOD-3, is found in the extracellular matrix and, like SOD-1, has zinc and copper in its active site rather than manganese (Fraczek et Kurpisz, 2005; Galecka *et al.*, 2008). In sperm plasma, SOD is highly active, with approximately 75% of the activity attributed to SOD-1 and the remaining 25% to SOD-3. Both isoforms are synthesized in the prostate and become activated when mixed with sperm during ejaculation (Peeker *et al.,* 1997). All SOD isoforms require a metal cofactor (Cu, Zn, or Mn) to facilitate their redox activity.

Non-enzymatic antioxidants

Non-enzymatic antioxidants are also called synthetic antioxidants (Agarwal *et al.,* 2005). It is about some trace elements (Se, Zn, Cu) and vitamins (Vit A; Vit E; Vit C).

Selenium (Se)

Selenium plays a crucial role in normal mammalian spermatogenesis, primarily through two key selenoproteins: phospholipid hydroperoxide glutathione peroxidase (PHGPx/GPx4) and selenoprotein P. PHGPx/GPx4, the predominant selenoprotein, influences germ cells within the convoluted seminiferous tubules of the testes. It has various functions and serves as the main connection between selenium levels and sperm quality (Boitani et Puglisi, 2008). A deficiency in selenium results in the degeneration of the seminiferous epithelium, decreased testicular size, altered testicular consistency, and disrupted spermatogenesis (Camejo *et al.,* 2011).

Zinc (Zn)

Zinc is present in high concentrations in seminal fluid and plays a crucial role in various aspects of sperm metabolism. It acts as a key anti-inflammatory agent and is involved in redox reactions to neutralize free radicals (Fallah *et al.*, 2018). Zinc is essential for sperm function, influencing lipid interactions and stabilizing the sperm membrane (Chia *et al.*, 2000). Additionally, it plays a regulatory role in the acrosome reaction, facilitating the release of acrosin and hyaluronidase, both of which are critical for fertilization and implantation (Eggert-Kruse *et al.,* 2002)

Copper (Cu)

Copper (Cu) is an essential trace element necessary for the proper development of living organisms. Due to its redox potential, copper acts as a cofactor for numerous enzymes that facilitate key cellular processes (Herman *et al.,* 2020). However, copper is highly reactive, and in its free form, it can generate large amounts of free radicals, leading to protein and DNA damage. To prevent this, organisms have developed precise mechanisms to regulate copper levels in cells. Copper-dependent enzymes, including ceruloplasmin, superoxide dismutase SOD1 and SOD3, metallothioneins, and cytochrome c oxidase, are involved in all stages of gametogenesis (Ogórek *et al.*, 2017).

Manganese (Mn)

Manganese plays a role in the metabolism of carbohydrates, amino acids, and cholesterol, with various manganese-dependent enzymes facilitating these processes. Manganese superoxide dismutase (MnSOD) is a key antioxidant enzyme located in the mitochondria. It addresses superoxide radicals – reactive oxygen species generated during ATP production – by converting them into hydrogen peroxide. This hydrogen peroxide can then be further reduced to water by other antioxidant enzymes (Candas and Li, 2014)

Vitamin A

Vitamin A is a natural antioxidant that promotes the regeneration of epithelial cells and plays a crucial role in regulating spermatogenesis by acting on receptors in the epithelium of the seminiferous tubules. It controls the differentiation of germ layer epithelial cells and can even initiate a spermatogenic wave on its own (Kim et Akmal, 1996). While it is commonly used as a dietary supplement, it also has significant local effects. A deficiency in vitamin A can lead to impaired spermatogenesis and decreased sperm motility (Hogarth et Griswold, 2010).

Vitamin E

Vitamin E, when used as a dietary supplement, is crucial for male reproductive performance (Zubair, 2017; Tufarelli et Laudadio, 2016). It has been found to enhance sperm quality in both birds and mammals, increasing ejaculate volume and sperm motility (Umesiobi, 2012). As a key component of antioxidant therapy, vitamin E markedly improves sperm volume, concentration, production, and motility, while reducing the number of abnormal and dead sperm (Yousef *et al.*, 2003).

Vitamin C

Vitamin C (ascorbic acid) is a water-soluble vitamin that is found in concentrations about ten times higher in seminal plasma compared to blood serum (Fraga *et al.*, 1991). As a strong antioxidant, vitamin C enhances spermatogenesis and prevents sperm agglutination by boosting motility and guiding sperm movement along the correct path (Geva *et al.*, 1996; Glenville, 2008). It serves as a key scavenger of various reactive oxygen species (ROS) and its elevated levels in seminal plasma, relative to blood plasma, highlight its significant local effectiveness (Lewis *et al.*, 1997).

Phytogenic extracts with potential in reproduction

Plants and fungi exhibit significantly greater metabolic diversity compared to other organisms, with up to 1 million types of metabolites reported in plant species (Dixon *et al.*, 2003; Rai *et al.*, 2017; Fang *et al.*, 2019). Each plant species contains more than 5,000 metabolites (Fernie *et al.*, 2004). While their basic primary metabolism is similar to that of non-plant species, plants and fungi have the unique ability to produce a wide array of specialized (or secondary) compounds, which make up most of their biologically active metabolites (Alseekh et Fernie, 2018). Various in vitro methods are available for both qualitative and quantitative assessment of the antioxidant activity of these metabolites (Chaves *et al.*, 2020). The initial step in evaluating the antioxidant activity of a plant extract involves selecting the appropriate method (Abramovič *et al.*, 2017). A current trend in medicine is to replace synthetic antioxidants with natural ones derived from plant extracts or isolated plant-based products (Rajurkar et Hande, 2011). A major similarity between plant extracts is that they all contain polyphenols, unsaturated and saturated fatty acids, saponins, tannins, alkaloids. The similarity in their antioxidant composition justifies their similar action.

Polyphenols (phenols) are secondary plant metabolites and can be divided into three subgroups: flavonoids, phenolic acids and lignans. In their chemical composition, they contain one or more aromatic rings with one or more hydroxyl groups. **Flavonoids** are the first subclass of polyphenols and mainly include flavones, flavonols, flavanols and other derivatives (Manach *et al.*, 2004). **Phenolic acids** are the second subgroup of polyphenols, as the main representative and the most active is caffeic acid (Haslam et Cai, 1994), the main active agent in chicory. **Lignans** are phenylalanine-derived polyphenols found in flaxseed and other grains (Saleem *et al.*, 2005). Polyphenols act as antioxidants in vitro by scavenging reactive oxygen species and chelating redox-active transition metal ions. They may also function indirectly as antioxidants by 1) inhibiting redox-sensitive transcription factors; 2) inhibition of "pro-oxidant" enzymes that can easily be oxidized; 3) potentiation of antioxidant enzymes, such as glutathione S-transferases and superoxide dismutases (Frei et Higdon, 2003). Tannin compounds are a class of polyphenolic biomolecules widely distributed in many plant species. They have a protective role and regulate plant growth (Ferrell et Thorington, 2006).

Fatty acids are carboxylic acids with an unbranched chain of an even number of carbon atoms. They can be saturated and unsaturated fatty acids (Moss *et al.,* 1997). Both groups are major components of lipids (up to 70% of their composition) in some species of microalgae (Chen, 2012), but in most organisms they do not occur in their own form, but exist as three main classes of esters: triglycerides, phospholipids and cholesterol esters. Fatty acids are both important nutritional sources of energy for the body and important structural components for the cells within it (Smith, 1994). Linolenic, palmitic, stearic and oleic fatty acids are the most abundant fatty acids in plants (Tvrzicka, 2011).

Coumarin is an aromatic organic compound. It belongs to the chemical class of benzopyrones and is considered a lactone. Lactones are cyclic carboxylic esters of fatty acids. They contribute to the aroma of fruits and vegetables (Fahlbusch, 2007).

Saponins are a subclass of terpenoids, the largest class of plant extracts. They are classified as triterpene glycosides, spirostanol glycosides and steroid alkaloid glycosides (Furuya, 1988). The amphipathic nature of saponins gives them activity as surfactants with the potential ability to interact with cell membrane components such as cholesterol and phospholipids, making saponins useful for the development of cosmetics and drugs (Lorent *et al.,* 2014). Saponins have also been used as adjuvants in vaccine development (Sun *et al.,* 2009). In plants, saponins can serve as antioxidant agents, scavenging free radicals formed during the life cycle of pathogenic bacteria and fungi (Foerster, 2006).

Alkaloids are a class of basic, naturally occurring organic compounds that contain at least one nitrogen atom. They are produced by a wide variety of organisms: bacteria, fungi, plants and animals. Alkaloids have a wide range of pharmacological actions, the most significant of which is their antioxidant action, expressed in different directions of bioactivity. Examples are alkaloids with antimalarial (eg quinine) properties, antiasthmatic (ephedrine), anticancer (eg homoharringtonine) (Kittakoop et al, 2014), analgesic (eg morphine), antibacterial (eg chelerythrine) (Cushnie *et al.,* 2014).

Conclusion

The application of herbal extracts as antioxidant agents in ram semen is a promising area of research aimed at enhancing semen quality, viability, and fertility during storage or artificial insemination. Antioxidants are crucial in protecting sperm cells from oxidative stress, which can lead to lipid peroxidation, DNA damage, and reduced motility. Various herbal extracts, rich in bioactive compounds such as flavonoids, phenolics, and vitamins, have demonstrated antioxidant properties and have been explored for their potential to improve semen preservation outcomes.

Acknowledgement

The study has grant by the Project: NIS-B 1288/19.10.2023. Topic of the project: “Research of the effect of herb extracts on the quality indicators of ram semen, during different reproductive periods“. Project manager: Assos.Prof. Kalin Yordanov Hristov, PhD.

References

1. Abramovič H., Grobin B., Poklar Ulrih N., Cigić B., (2017). *The methodology applied in DPPH, ABTS and Folin–Ciocalteau assays has a large influence on the determined antioxidant potential.*

Acta Chim. Slov.; 64:491–499. doi: 10.17344/acsi.2017.3408.

2. Agarwal A, Gupta S, Sharma RK. (2005). *Role of oxidative stress in female reproduction.* Reprod Biol Endocrinol. Jul 14;3:28. doi: 10.1186/1477–7827–3–28. PMID: 16018814; PMCID: PMC1215514.
3. Agarwal, A., Saleh, R.A., Bedaiwy, M.A., (2003). *Role of reactive oxygen species in the pathophysiology of human reproduction,* Fertil Steril, 79, 829–843.
4. Aitken, R.J. (1984*). Pathophysiology of human spermatozoa*. Curr. Opin. Obstet. Gyn., 6, 128–135.
5. Aitken, R.J., (1995). *Free radicals, lipid peroxidation and sperm function* Reprod Fertil Dev, 7, 659–668.
6. Aitken, RJ., (1999). *The human spermatozoon–a cell in crisis?* J Reprod Fertil. 1999;115:1–7. The Amoroso Lecture.
7. Alseekh, S. and Fernie, A.R., (2018). *Metabolomics 20 years on: what have we learned and what hurdles remain?* Plant J, 94: 933–942. doi.org/10.1111/tpj.13950.
8. Alvarez, J., Storey, B., (1993). *Evidence that Membrane Stress Contributes More than Lipid Peroxidation to Sublethal Cryodamage in Cryopreserved Human Sperm: Glycerol and Other Polyols as Sole Cryoprotectant*. doi.org/10.1002/j.1939–4640.1993.tb00383.x,
9. Anger, J.T., B. R. Gilbert, M. Goldstein, (2003). *Cryopreservation of sperm: Indications, methods and results.* The Journal of Urology, 170: 1079 – 1084.
10. Armstrong, J.S., Rajasekaran, M., Chamulitrat, W., Gatti, P., Hellstrom, W.J., Sikka, S.C., (1999). *Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism.* Free Radic. Biol. Med., 26, 869–880.
11. Aziz, N, Saleh, RA, Sharma, RK, *et al.*, (2004). *Novel association between sperm reactive oxygen species production, sperm morphological defects, and the spermsperm deformity index*. Fertil Steril.;81:349–54.
12. Badal, S., (2017). *Chapter 16 – Plant Metabolites and More Treating Various Ailments: Natural Products Treating* Diseases, Pharmacognosy, Academic Press, Pages 337–361, ISBN 9780128021040, doi.org/10.1016/B978–0–12–802104–0.00016–0.
13. Baumber, J., Vo, A., Sabeur, K., Ball, B.A., (2002). *Generation of reactive oxygen species by equine neutrophils and their effect on motilityofequine spermatozoa.* Theriogenology, 57, 1025–1033.
14. Boitani C, Puglisi R., (2008). *Selenium, a key element in spermatogenesis and male fertility*. Adv Exp Med Biol.;636:65–73. doi: 10.1007/978–0–387–09597–4_4. PMID: 19856162.
15. Boutros, M., Ray, S. (2023). *Oxidative stress,* Reference Module in Biomedical Sciences, Elsevier, ISBN 9780128012383, doi.org/10.1016/B978–0–12–824315–2.00223–2.
16. Camejo, M. I., L. Abdala, G. Vivas–Acevedo, R. Lozano–Hernandez, M. Angeli–Greaves, E. D. Greaves, (2011). *Selenium, copper and zinc in seminal plasma of men with varicocele, relationship with seminal parameters*. Biol Trace Elem Res., 143 :1247 254.
17. Candas, D. and Li, J. J., (2014). *MnSOD in Oxidative Stress Response–Potential Regulation via Mitochondrial Protein Influx.* Antioxidants & Redox Signaling 20:10, 1599–1617.
18. Chaves N, Santiago A, Alías JC. (2020). *Quantification of the Antioxidant Activity of Plant Extracts: Analysis of Sensitivity and Hierarchization Based on the Method Used.* Antioxidants (Basel). Jan 15;9(1):76. doi: 10.3390/antiox9010076. PMID: 31952329; PMCID: PMC7023273.
19. Chen, L., (2012). *Biodiesel production from algae oil high in free fatty acids by two–step catalytic conversion*. Bioresource Technology. 111: 208–214. doi:10.1016/j.biortech.2012.02.033.
20. Chia SE, Ong CN, Chua LH, Ho LM, Tay SK., (2000). *Comparison of zinc concentrations in blood*

and seminal plasma and the various sperm parameters between fertile and infertile men. J Androl.; 21(1):53–7.

21. Cushnie, TP., Cushnie, B., Lamb, AJ., (2014). *Alkaloids: An overview of their antibacterial, antibiotic–enhancing and antivirulence activities*. Int J Antimicrob Agents. 44 (5): 377–386. doi:10.1016/j.ijantimicag.2014.06.001. PMID 25130096.
22. Dixon, A.R., Strack, D., (2003). *Phytochemistry meets genome analysis, and beyond.* Phytochemistry, Volume 62, Issue 6, Pages 815–816, ISSN 0031–9422, doi.org/10.1016/S0031–9422(02)00712–4.
23. Eggert–Kruse W, Zwick EM, Batschulat K, Rohr G, Armbruster FP, Petzoldt D, *et al.*, (2002). *Are zinc levels in seminal plasma associated with seminal leukocytes and other determinants of semen quality?* Fertil Steril.; 77(2):260–9.
24. Fahlbusch, K. G., (2007), *"Flavors and Fragrances"*, Ullmann's Encyclopedia of Industrial Chemistry (7th ed.), Wiley, pp. 74–78.
25. Faigl, V., Vass, N., Jávor, A., Kulcsár, M., Solti, L., Amiridis, G., & Cseh, S. (2012). *Artificial insemination of small ruminants — A review*. Acta Veterinaria Hungarica, 60(1), 115–129. doi.org/10.1556/avet.2012.010.
26. Fallah A, Mohammad–Hasani A, Colagar AH., (2018). *Zinc is an Essential Element for Male Fertility: A Review of Zn Roles in Men's Health, Germination, Sperm Quality, and Fertilization*. J Reprod Infertil. Apr–Jun;19(2):69–81. PMID: 30009140; PMCID: PMC6010824.
27. Fang Y, Zhong R., (2020). *Effects of Oxidative Stress on Spermatozoa and Male Infertility.* Free Radical Medicine and Biology. IntechOpen; Available from: dx.doi.org/10.5772/intechopen.86585.
28. Fang, C., Fernie, R. A., Luo, J., (2019). *Exploring the Diversity of Plant Metabolism*, Trends in Plant Science, Volume 24, Issue 1, Pages 83–98, ISSN 1360–1385, doi.org/10.1016/j.tplants.2018.09.006.
29. Fanucchi, M.V., (2014). *Chapter 11 – Development of Antioxidant and Xenobiotic Metabolizing Enzyme Systems*, The Lung (Second Edition), Academic Press, 223–231, ISBN 9780127999418, doi.org/10.1016/B978–0–12–799941–8.00011–0.
30. Fernie A.R., Trethewey R.N., Krotzky A.J., Willmitzer L., (2004). *Metabolite profiling: From diagnostics to systems biology*, Nature Reviews Molecular Cell Biology, 5 (9), 763 – 769, doi: 10.1038/nrm1451.
31. Ferrell, K. E., Thorington, R. W., (2006). *Squirrels: the animal answer guide.* Baltimore: Johns Hopkins University Press. 91. ISBN 978–0–8018–8402–3.
32. Foerster, H., (2006). *MetaCyc Pathway: saponin biosynthesis I.* Archived from the original on 15 September 2019. Retrieved 23 February 2009.
33. Fraczek, M., Kurpisz, M., (2005). *The redox system in human semen and peroxidative damage of spermatozoa.* Postepy Higieny i Medycyny Doswiadczalnej;59:523–34.
34. Fraczek, M., Kurpisz, M., (2007). *Inflammatory mediators exert toxic effects of oxidative stress on human spermatozoa.* J Androl.;28:325–33.
35. Fraga, C.G., P. A. Motchnik, M. K. Shigenaga, H. J. Helbock, R. A., Jacob, B. N. Ames, (1991). *Ascorbic acid protects against endogenous oxidative DNA damage in human sperm.* Proc Natl Acad Sci USA., 88:11003–11006.
36. Frei B., Higdon J.V., (2003). *Antioxidant activity of tea polyphenols in vivo: evidence from animal studies*. J Nutr. Oct;133(10):3275S–84S. doi: 10.1093/jn/133.10.3275S. PMID: 14519826.

37. Furuya, T., (1988). *CHAPTER 12 – Saponins (Ginseng Saponins)*, Phytochemicals in Plant Cell Cultures, Academic Press, Pages 213–234.
38. Gaetani GF, Ferraris AM, Rolfo M, Mangerini R, Arena S, Kirkman HN, (1996). *Predominant role of catalase in the disposal of hydrogen peroxide within human erythrocyte.* Blood. 87 (4): 1595–1599. doi:10.1182/blood. V87.4.1595.bloodjournal8741595. PMID 8608252.
39. Galecka, E, Jacewicz, R, Mrowicka, M, Florkowski, A, Galecki, P., (2008). *Enzymy antyoksydacyjne – budowa, właściwości, funkcje [Antioxidative enzymes – structure, properties, functions*. Pol Merkur Lekarski;25:266–8.
40. Gallardo, M., 2007. *Evaluation of antioxidant system in normal semen.* Rev Invest Clin., 59 (1):42–47.
41. Geva E., Bartoov B., Zabludovsky N., Lessing, J. B., Lerna–Geva L., Amit A, (1996). *The effect of antioxidant treatment on human spermatozoa and fertilization rate in an invitro fertilization program*. Ferti Steril; 66(3):430–434.
42. Glenville M., (2008). *The nutritional approach to male factor infertility*. Dragons Tale;18:4–5.
43. Gomez, E, Buckingham, DW, Brindle, J, Lanzafame, F, Irvine, DS, Aitken, RJ., (1996). *Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function*. J Androl.;17:276–87.
44. Goodsell, D.S., (2004). *Catalase. Molecule of the Month*. RCSB Protein Data Bank. Retrieved 2016–08–23.
45. Gundogan, M., Avdatek, F., YENI, D., (2011). *Effect of extenders on motility, morphology and osmotic resistance parameters of ram sperm during liquid storage* Revue Méd. Vét., 162, 11, 546–551.
46. Halliwell, B. and Gutteridge, J.M.C. (2007) *Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death.* In: Halliwell, B., Gutteridge, J.M.C., eds., Free Radicals in Biology and Medicine, 4rd Edition, Oxford University Press, New York, 187–267.
47. Harris, E.D., (1992). *Regulation of antioxidant enzymes*. The FASEB J.6, 2675 – 2683.
48. Haslam E, Cai Y., (1994). "*Plant polyphenols (vegetable tannins): gallic acid metabolism"*. Natural Product Reports. 11 (1): 41–66. doi:10.1039/NP9941100041. PMID 15206456.
49. Herman S, Lipiński P, Ogórek M, Starzyński R, Grzmil P, Bednarz A, Lenartowicz M., (2020). *Molecular Regulation of Copper Homeostasis in the Male Gonad during the Process of Spermatogenesis*. Int J Mol Sci. Nov 28;21(23):9053. doi: 10.3390/ijms21239053. PMID: 33260507; PMCID: PMC7730223.
50. Hogarth CA, Griswold MD., (2010). *The key role of vitamin A in spermatogenesis*. J Clin Invest. 2010 Apr;120(4):956–62. doi: 10.1172/JCI41303. Epub 2010 Apr 1. PMID: 20364093; PMCID: PMC2846058.
51. Holt, W. 2000. *Fundamental aspects of sperm cryobiology: the importance of species and individual differences*. Theriogenology, 53:47– 58.
52. Holt, W. V., (2000). *Basic aspects of frozen storage semen*. Anim. Reprod Sci 62: 3–22.
53. Huang, Y.; Ge, D.–Y.; Zong, H.; Yin, J.–X.; Qu, X.–N.; Lv, S.–W., (2017). *Active Site Mimicry of Glutathione Peroxidase by Glutathione Imprinted Selenium–Containing Trypsin. Catalysts* 2017, *7*, 282. doi.org/10.3390/catal7100282, ISBN 9780127150055, doi.org/10.1016/B978–0–12–715005–5.50019–4.

54. Kim, K.H., Akmal, K.M. (1996). *Role of Vitamin A in Male Germ–Cell Development. In: Desjardins, C. (eds) Cellular and Molecular Regulation of Testicular Cells*. Serono Symposia USA Norwell, Massachusetts. Springer, New York, NY, doi.org/10.1007/978–1–4612–2374–0_7.
55. Kittakoop, P., Mahidol, C., Ruchirawat, S., (2014). *Alkaloids as important scaffolds in therapeutic drugs for the treatments of cancer, tuberculosis, and smoking cessation*. Curr Top Med Chem. 14 (2): 239–252. doi:10.2174/1568026613666131216105049. PMID 24359196.
56. Kothari, S., A. Thompson, A. Agarwal, S. du Plessis, (2010). *Free radicals: their beneficial and detrimental effects on sperm function.* Indian J. Exp. Biol., 48 (5): 425–435.
57. Kowalczyk A., 2022. *The Role of the Natural Antioxidant Mechanism in Sperm Cells*. Reprod Sci. 2022 May; 29(5):1387–1394. doi: 10.1007/s43032–021–00795–w. Epub 2021 Nov 29. PMID: 34845666; PMCID: PMC9005387.
58. Kowalczyk, A., (2022). *The Role of the Natural Antioxidant Mechanism in Sperm Cells.* Reprod. Sci. 29, 1387–1394. doi.org/10.1007/s43032–021–00795–w.
59. Krzyzosiak, J., Evenson, D., Pitt, C., Jost, L., Molan, P., Vishwanath, R., (2000). *Changes in susceptibility of bovine sperm to insitu DNA denaturation, during prolonged incubation at ambient temperature under conditions of exposure to reactive oxygen species and nuclease inhibitor*. Reprod. Fertil. Dev., 12, 251–261.
60. Lewis, S.E., Sterling, E.S., Young, I.S., Thompson W., (1997). *Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men*. Fertil Steril, 67, 142–147.
61. Lorent, J. H., Quetin–Leclercq, J., Mingeot–Leclercq, M.P., (2014). *The amphiphilic nature of saponins and their effects on artificial and biological membranes and potential consequences for red blood and cancer cells*. Organic and Biomolecular Chemistry. Royal Society of Chemistry. 12 (44): 8803–8822. doi:10.1039/c4ob01652a.
62. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L., (2004). *"Polyphenols: food sources and bioavailability".* The American Journal of Clinical Nutrition. 79 (5): 727–747. doi:10.1093/ajcn/79.5.727. PMID 15113710.
63. Maxwell, W.C., Watson, P.F. (1996). *Recent progress in the preservation of ram semen*.Anim. Reprod. Sci., 42 (1996), pp. 55–65
64. Moss, G. P., Smith, P. A. S., Tavernier, D., (1997). *IUPAC Compendium of Chemical Terminology*. Pure and Applied Chemistry. International Union of Pure and Applied Chemistry. 67 (8–9): 1307–1375. doi:10.1351/pac199567081307.
65. Ogórek M, Gąsior Ł, Pierzchała O, Daszkiewicz R, Lenartowicz M., (2017). *Role of copper in the process of spermatogenesis.* Postepy Hig Med Dosw (Online). Aug 9;71(0):663–683. doi: 10.5604/01.3001.0010.3846. PMID: 28791960.
66. Peeker R., Abramsson L., Marklund SL., (1997). *Superoxide dismutase isoenzymes in human seminal plasma and spermatozoa.* Mol Hum Reprod; 3:1061–6.
67. Pfeifer, H, Conrad, M, Roethlein, D, Kyriakopoulos, A, Brielmeier, M, Bronkamm, GW, *et al.*, (2001). *Identification of a specific sperm nucleinucleus selenoenzyme necessary for protamine thiol cross–linking during sperm maturation*. FASEB J.; 15:1236–8.
68. Rai, A., Saito, K. and Yamazaki, M., 2017. *Integrated omics analysis of specialized metabolism in medicinal plants*. Plant J, 90: 764–787. doi.org/10.1111/tpj.13485.
69. Rajurkar NS, Hande SM., (2011). *Estimation of phytochemical content and antioxidant activity of some selected traditional Indian medicinal plants*. Indian J Pharm Sci. Mar;73(2):146–51. doi: 10.4103/0250–474x.91574. PMID: 22303056; PMCID: PMC3267297.

70. Saleem, M., Hyoung, K., Muhammad Shaiq, A., Yong Sup, L., (2005). *An update on bioactive plant lignans*. Natural Product Reports. 22 (6): 696–716. doi:10.1039/B514045P. PMID 16311631.

71. Simoes, R, Feitosa, WB, Siqueira, AFP, Nichi, M, Paula–Lopes, FF, Marques, MG, Assumpcao, MEO, (2013). *Influence of bovine sperm DNA fragmentation and oxidative stress on early embryo in vitro development outcome*. Reproduction.;146(5):433–41.doi.org/10.1530/rep–13–0123.

72. Smith, S., (1994). *The Animal Fatty Acid Synthase: One Gene, One Polypeptide, Seven Enzymes.* The FASEB Journal. 8 (15): 1248–1259. doi:10.1096/fasebj.8.15.8001737.

73. Stohs, S.J., Bagchi, D. (1995). *Oxidative mechanisms in the toxicity of metal ions* Free Radic Biol Med, 18, 321–336.

74. Sun, Hong–Xiang; Xie, Yong; Ye, Yi–Ping, (2009). *Advances in saponin–based adjuvants*. Vaccine. 27 (12): 1787–1796. doi:10.1016/j.vaccine.2009.01.091. ISSN 0264–410X. PMID 19208455.

75. Tsakmakidis. I.A., (2010). *Ram semen evaluation: development and efficiency of modern techniques small Rumin*. Res., 92 (2010), 126–130.

76. Tufarelli, V., Laudadio, V., (2016). *Antioxidant activity of vitamin E and its role in avian reproduction* J. Exp. Biol. Agric. Sci., 4 (3 Suppl.), 266–272.

77. Tvrzicka, E., Kremmyda, L.–S., Stankova, B., & Zak, A. (2011). *Fatty acids as biocompounds: their role in human metabolism, health and disease – a review*. Part 1: classification, dietary sources and biological functions. Biomedical Papers, 155(2). doi.org/10.5507/bp.2011.038.

78. Umesiobi, D.O., (2012). *The effect of vitamin E supplementation on the libido and reproductive capacity of Large White boars* S. Afr. J. Anim. Sci., 42, 559–563.

79. Walczak–Jedrzejowska R, Wolski JK, Slowikowska–Hilczer J., (2013). *The role of oxidative stress and antioxidants in male fertility*. Cent European J Urol. 2013;66(1):60–7. doi: 10.5173/ceju.2013.01.art19. Epub 2013 Apr 26. PMID: 24578993; PMCID: PMC3921845.

80. Yeung, CH, Cooper, TG, De Geyter, M., De Geyter, C, Rolf, C, Kamischke, A, Nieschlag, E., (1998). *Studies on the origin of redox enzymes in seminal plasma and their relationship with results of in–vitro fertilization*. Mol Hum Reprod.; 4:835–9.

81. Yoshida, K., T. Yamasaki, M. Yoshiike, S. Takano, I. Sato, T. Iwamoto, (2003). *Quantification of seminal plasma motility inhibitor/semenogelin in human seminal plasma*. Journal of Andrology, 24: 878–884.

82. Yousef, M.I., Abdallah, G.A., Kamel, K.I., (2003). *Effect of ascorbic acid and vitamin E supplementation on semen quality and biochemical parameters of male rabbits* Anim. Reprod. Sci., 76 (2003), 99–111.

83. Zubair, M., (2017). *Effects of dietary vitamin E on male reproductive system.* Asian Pacific J. Reprod., 6, 145.