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# INFLUENCE OF POLYPHENOLIC COMPOUNDS OF GREEN WALNUT EXTRACT ON SPERMOGRAM INDICES OF BREEDING RABBITS

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***Abstract***

*Lately, the research and study of oxidative stress on the functioning of the male reproductive system has intensified. Free radical oxidation processes play a very important role in the functioning of each living cell, on the one hand, this is a necessary stage of various metabolic processes, but on the other hand, the processes of free radical formation intensify, which can cause various pathological changes in the cells and tissues of living organisms. In living systems there are mechanisms for generating active forms of oxygen, as well as biological systems for protecting intact cells from the influence of active forms of oxygen (ROS). In the norm between these systems there is an equilibrium, which provides a normal functioning of subcellular structures and organs as a whole, but this balance very often can be disturbed in the direction of non-compensation of ROS generation and the appearance of oxidative stress. This is a state of the living cell, in which the discoordination of ROS formation processes and the functioning of the antioxidant protection system takes place. At the same time, under normal conditions, the modified macromolecules undergo regeneration or are destroyed. In the processes of oxidative stress the recovery regimes are insufficient, and as a result damaged molecules accumulate in the body (Поварова et al., 2015). It is currently considered that the balance between the ROS generation system and the antioxidant protection system after their elimination have a decisive significance in regulating the functioning of cells. A significant imbalance of the antioxidant-prooxidant system can cause inhibition of the fertility properties of ejaculate. According to the results of the literature, at present the following main interdependent causes of dysregulation of male fertile function in the process of development of oxidative stress are highlighted. First of all, a decrease in the mobility of male reproductive cells is observed, which occurs due to a decrease in the elasticity of the membrane of reproductive cells, and therefore a decrease in the mobility of the flagellum (Галимова et al., 2016). The involvement of oxidative stress in the pathogenesis of male infertility has predetermined the study and research of the effectiveness of various antioxidants in regulating and proceeding metabolic processes in the process of spermatogenesis.*

***Key words****: mobility, reproductive sex cells, ROS, stress.*

## INTRODUCTION

ROS are formed as necessary by-products during normal inter- and intracellular signaling enzymatic reactions. Mammalian spermatozoa represent a growing list that exhibit the ability to generate ROS when incubated under aerobic conditions, such as hydrogen peroxide ($H_2O_2$), superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$) and hypochlorite ($ClO^-$). Due to their highly reactive nature, ROS can easily combine with other molecules, directly causing oxidation that can lead to structural and functional changes and lead to cell damage (Guérin et al., 2001).

If previously for a very long time the oxidative stress was considered the result of ROS overproduction, currently it qualifies as a result of redox - deregulation. Reversible oxidation of thiols in cysteine remnants of key proteins acts as an on/off switch, which controls the function of male reproductive cells. But if dysregulation occurs, these remnants are irreversibly oxidized, which leads to incorrect functioning and finally to the death of male reproductive cells (Micic et al., 2019).

In low concentrations ROS have a positive effect: they influence on prostoglandin metabolism, endothelial function, participate in gene regulation and cell growth, in intracellular signaling processes and in other types of signaling transduction, as well as play an important role in the regulation of antimicrobial protection (Ефремов et al., 2017).

Although a significant negative correlation has been found between ROS and IVF fertilization rate, however, controlled ROS generation has

been shown to be essential for capacity development and hyperactivation (Lamirande et al., 1993), these being the two spermatozoa processes that are necessary to ensure fertilization. *In vivo* physiological concentrations of ROS are involved in ensuring membrane fluidity, maintaining fertilization capacity and acrosomal reaction of spermatozoa (Bucak et al., 2010). Maintaining an adequate level of ROS is therefore essential for proper sperm functionality. ROS causes adverse effects on the plasma membrane of spermatozoa, DNA and physiological processes, thereby affecting the quality of spermatozoa.

The main source of ROS in physiological conditions is mitochondrial respiration. Mitochondrial dysfunction can deepen electron loss and thus increase ROS levels to toxic levels, deregulating redox homeostasis. It is assumed that ROS in fairly high concentrations are capable of causing destructive peroxidation by the formation of reactive aldehydes.

The axosome and associated dense fibers of the middle part of the spermatozoa are covered by mitochondria, which generate energy from intracellular ATP depletion deposits. Excess ROS affects motility and the ability to fertilize. The mechanisms of spermatozoa damage (Villaverde et al., 2019) can also be caused by many chemicals, such as polymer catalysts, phthalates, etc. Many of these substances intensify oxidative stress and therefore deregulate spermatogenesis (Menezo et al., 2014).

Under unfavorable conditions, the reproductive sex cells use their own apoptotic pathway, which involves the formation of mitochondrial ROS, loss of mitochondrial membrane potential, activation of capsase, influence of phosphatidylserine. High levels of ROS in spermatozoa cause oxidation of lipids, proteins and DNA, which lead to lipid peroxidation, oxidation of protein base structures and enzymes, and can cause mutations as a result of DNA oxidation (Micic et al., 2019). The presence of a high level of ROS in the seminal fluid can lead to negative changes in ejaculate parameters and a decrease in the indices of pregnancy, both by natural means and by artificial insemination methods (Ефремов et al., 2017).

DNA damage as a result of oxidative stress directly influences the quality of embryos (Micic et al., 2019).

As a result of the high level of oxidative stress, the amount of damaged DNA in male reproductive cells becomes higher, which can cause male infertility, miscarriages can occur and periodic pregnancy loss.

Spermatozoa are sensitive to oxidative stress due to insufficient level of antioxidant protection, small volume and limitation of cytoplasmic space, which does not allow to hold a set of protective enzymes (Ефремов et al., 2017). In addition, there is a lack of a single mechanism for detecting and restoring DNA damage (Bisht et al., 2017).

It should be noted that the lipids of spermatozoa membranes are very sensitive to oxidative stress, because they contain a significant amount of polyunsaturated fatty acids, phospholipids and stearins (Aitken et al., 2016). Given that the lipid components of the spermatozoa membranes participate in the regulation of their maturation, in the process of spermatogenesis, capacitation, acrosomal reaction and finally merging with the egg, it is clearly visible that lipid peroxidation can disrupt the functions listed above (Ефремов et al., 2017).

The vulnerability of spermatozoa to oxidative damage is further deepened, because they actively generate ROS, to stimulate the increase of tyrosine phosphorylation, which is related with their mobility (Aitken et al., 2014).

The excessive increase in the concentration of lipid peroxidation products is accompanied by loss of sperm mobility, which may limit the survival period in the female genital pathways (Bisht et al., 2017; Nowicka-Bauer et al., 2020). At the same time, the life span of spermatozoa (the moment of complete loss of motility) is closely correlates with their level of superoxide dismutase activity (Nowicka-Bauer et al., 2020). At the same time, it is necessary to take into account the fact that a significantly increased amount of ROS is generated by the leukocytes in the semen.

Increased ROS along with decreased antioxidant defenses lead to redox imbalance, reduced sperm mobility and damage to sperm DNA. Spermatozoa are very sensitive to the harmful effects of ROS due to the large

amounts of unsaturated fatty acids that are found in their cell membranes. Reactive oxygen species promote peroxidation. The sequence of events involves lipid peroxidation, loss of membrane integrity with increased permeability, reduced mobility of spermatozoa, structural DNA damage, and apoptosis (Sanocka-Maciejewska et al., 2005). There were several intrinsic and extrinsic factors associated with increased oxidative stress in the male reproductive system.

At the physiological level, ROS are associated with the development of spermatozoa fertilization properties, favoring chromatin compaction in maturing spermatozoa, mobility, chemotaxis, spermatozoa fertilization capacity, acrosomal reaction and oocyte interaction (Du Plessis et al., 2015). Excessive ROS production is a major cause of sperm injury. Indeed, due to the high amount of membrane unsaturated fatty acids and the lack of cytoplasmic antioxidant enzymes, spermatozoa are very sensitive to oxidation (Agarwal et al., 2017), with consequent negative effects on the quality/functioning of spermatozoa (Aitken et al., 2016).

In addition to the physiological role of ROS, excessive ROS generation and oxidative stress appear to be associated with harmful effects on spermatozoa, leading to changes in morphological and dynamic cellular properties and, ultimately, to a lower fertilization capacity.

Spermatozoa are particularly sensitive to ROS-induced oxidation due to the presence in their plasma membrane of high levels of polyunsaturated fatty acids such as docosahexaenoic acid which contains six double bonds per molecule (Aitken et al., 2016). Indeed, ROS mediate the extraction of hydrogen from the hydrocarbon side chain of a fatty acid, yielding to a carbon-centered lipid radical (L$^{\bullet}$) whose interaction with oxygen produces a lipid peroxyl radical (LOO$^{\bullet}$), capable of reacting with an adjacent radical. As a result of internal molecular rearrangements, conjugated dienes and hydroperoxides are generated (Yoshida et al., 2015).

LPO products can also react with proteins, DNA and phospholipids, generating end products involved in cell dysfunction. In particular, the interaction of LPO products with amino residues can lead to protein oxidation, affecting the structural and functional characteristics of the protein (Niki, 2014). In this context, it has been observed that LPO products like 4-hydroxy-2-nonenal (4-HNE) are able to propagate ROS generation by interacting with proteins of the mitochondrial electron transport chain of spermatozoa (Aitken et al., 2016).

Lipid peroxidation is strictly associated with alteration of membrane fluidity and permeability, inhibition of membrane-bound enzymes and receptors, and activation of the apoptotic cascade, supporting the involvement of oxidative stress in abnormalities of spermatozoa mobility and morphology. Among LPO products, 4HNE appears to be highly responsible for the cytotoxic effects on the cell membrane of spermatozoa, inducing loss of membrane integrity, changes in mobility, and compromising sperm-oocyte interactions. It has been observed that the effects mediated by 4HNE depend on several factors: the state of cellular differentiation, the amount of substrates for 4HNE attack and antioxidant defense systems (Nowicka-Bauer et al., 2020).

It is traditionally accepted that nucleic acids are another crucial target of oxidative stress. Both nuclear and mitochondrial DNA are vulnerable to the attack of hydroxyl radicals ($^{\bullet}$OH), which leads to the formation of several biomarkers of oxidative stress. ($^{\bullet}$OH) can react with guanine to produce 8-hydroxy-2'-deoxyguanosine (8-OH-G), an important marker of DNA oxidative damage, detectable in several biological samples (Burton et al., 2011).

The lack of adequate antioxidant systems makes spermatozoa highly susceptible to DNA oxidation. The oxidation of sperm DNA is also due to the lack of complete strategies for DNA repair in spermatozoa. Indeed, if 8-oxoguanine glycosylase (OGG1) is able to remove the 8-OHdG residue from DNA by producing an abasic site, the spermatozoa do not possess any base excision repair system for insertion of a new base (Aitken et al., 2014).

Several studies have indicated that the generation of ROS is associated with DNA fragmentation and chromatin deficient packaging, promoting apoptosis with relevant consequences on spermatozoa count (Aitken et al., 2014).

## MATERIALS AND METHODS

The study was conducted on 20 breeding rabbits, which were administered "per os" in a dose of 1 ml of green walnut hydroalcoholic extract with a total polyphenol content of 548.37 mg/100 g gallic acid equivalent (GAE). The total content of polyphenolic compounds was determined by the Folin-Ciocalteu method. The method consists in determining the content of total polyphenols from plant sources by measuring the optical density of an extract, which by complexation with Folin-Ciocalteu reagent determines the absorption at wavelength 750 nm, using UV-Vis spectrophotometer. For the study was used the spectrophotometer "ПЭ-5400 УФ", with a spectral range from 190 to 1000 nm.

The animals were divided into two groups: the experimental group and the control group, with 10 animals in each group. The extract was administered for two cycles of spermatogenesis. Spermogram indices, the ability to fertilize of reproductive material and biochemical indices of blood serum were studied.

## RESULTS AND DISCUSSIONS

In Table 1 are presented some physiological indices of the spermogram of breeding rabbits.

Table 1. Physiological indices of spermogram of breeding rabbits

| Group | Volume, ml | Concentration, mlrd/ml | Mobility, points | Survival, hours |
|---|---|---|---|---|
| Control | 0.66±0.13 | 0.18±0.02 | 7.8±0.43 | 13.9±0.41 |
| Experimental | 1.2±0.16 | 0.23±0.08 | 8.5±0.37 | 21.3±0.29 |

As follows, from the results obtained and presented in Table 1 is observed a sharp difference in the studied indices. The volume of reproductive material obtained from breeding rabbits in the experimental group increases almost twice, the concentration of reproductive cells increases by 0.05 billion/ml, mobility increases by 0.7 points and survival at room temperature by 7.4 hours.

In the following study was investigated the fertility rate of female rabbits. In this experiment were included 60 female rabbits, which were divided into two groups, of 30 animals in each group. The experimental group was artificially inseminated with reproductive material received from the rabbits which were administered the green walnut extract, and the control group was also inseminated with reproductive material obtained from the rabbits which did not receive a supplement. The research results are presented in Table 2.

Table 2. Fertility rate of artificially inseminated rabbits

| Group | Number of parturients, who gave birth | Number of offspring received, in average from a female rabbit | Duration of gestation, days |
|---|---|---|---|
| Control | 22 | 6.5±0.5 | 28-32 |
| Experimental | 29 | 8.6±0.4 | 28-30 |

Thus, as can be seen, the rabbits from the control group were fertilized in an amount of 73.3%, and the rabbits from the experimental group were fertilized in an amount of 96.6%. It is also observed the increase in the number of offspring and the shortening of the gestation period in the rabbits from the experimental group.

Another investigation included the study of some biochemical indices in the blood serum of breeding rabbits. The research results are presented in Table 3.

Table 3. Biochemical indices in the blood serum of breeding rabbits

| Group | GST, nM/L | Phosphorus, mM/L | Mg, mM/L | Total protein, g/L |
|---|---|---|---|---|
| Control | 16.53±0.15 | 1.47±0.14 | 0.760±0.11 | 71.2±1.21 |
| Experimental | 22.1±0.32 | 1.7±0.18 | 0.840±0.18 | 74.7±1.46 |

Thus, as we can see, the polyphenols in green walnuts have an essential influence on the quantitative changes of glutamate-S-transferase (GST), which proves its positive action on the fecundity of male reproductive cells of breeding rabbits and the essential protective effect in oxidative stress.

Phosphorus is an essential trace element that is one of the main constituents of the body's cells. Phosphorus is a participant in many metabolic processes. Phosphorus compounds play an important role in energy metabolism (ATP and creatine phosphate are energy accumulators that provide many processes in the body with energy). This trace element is also part of nucleic acids.

Magnesium is also an element that participates in the processes of energy synthesis, is an active participant in the synthesis of proteins, the secretion of parathyroid hormone, forms complexes with nucleic acids, phospholipids of cell membranes, stabilizing their fluidity and permeability. By regulating the cholesterol content in the blood, it improves blood circulation in the body's parenchymal tissues, including the testes. Magnesium is the second most concentrated intracellular cation, it is part of 300 enzymes.

Proteins, in turn, serve as a building material for all cells and tissues of the body. It is from proteins that enzymes, many hormones and antibodies are built. In addition, they perform the function of carriers of hormones, vitamins, minerals, fat-like substances and other components of metabolism in the blood, and also provide their transport to cells. Proteins are also responsible for maintaining the correct acid-base balance (pH). Finally, it is a source of energy. Proteins having these various functions and actively participating in the metabolic processes in the body have an essential influence on the functioning of the male reproductive system and on the quality of the reproductive material and finally influencing the quantity and quality of the offspring obtained.

## CONCLUSIONS

It is necessary to evaluate the oxidative stress and to use antioxidants to regulate the activity of the reproductive system.

It is very important to determine the dose and duration of antioxidants for blocking ROS, to determine which antioxidants could be used to regulate the activity of the reproductive system and prevent male infertility.

It is also necessary to establish at what level of functioning of the reproductive system it is necessary to administer antioxidants.

Simultaneously with advanced technologies for artificial insemination, the need arises to combine different antioxidants to preserve male reproductive material at hypothermic temperatures, which optimally maintain the functioning of male reproductive cells in order to achieve a higher fecundity.

Early administration of antioxidants to farm animals increases the quantitative and qualitative indices of male reproductive material and therefore increases the efficiency of ova fecundity.

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