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INFLUENCE OF EXOGENOUS APPLIED ACTIVE OXYGEN SPECIES ON THE REPRODUCTIVE INDICATORS IN MALE ANIMALS AND POSSIBILITIES FOR ENHANCEMENT OF FECUNDATE ABILITY OF SPERMATOZOA IN ARTIFICIAL INSEMINATION OF SHEEP

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Abstract

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Certain interest in the field of reproductivity is focused on the research work about the role of the active oxygen species and in particular – their impact on the fertility of the gametes in the last 10-15 years. There are mainly surveys on oxidative stress that showed its negative impact on the gametes. It was proved that over generating of AOS in gametes leads to pathological changes and dysfunction of spermatozoa. On the other hand in certain concentrations AOS can be biologically effective and even important. For example AOS can regulate the activity of antioxidant enzymes. They also have the role of oxygen sensors; regulate cell adhesion and the production of antibodies in leucocytes etc. It was shown that hydrogen peroxide is essential for many chemical reactions in the organism. It is part from the normal metabolism in the human body. Together with superoxide anion hydrogen peroxide takes part in the regulation of spermatozoa capacitating and tyrosine phosphorylation of proteins.

In spermatology exogenous hydrogen peroxide (H_2O_2) is used mainly for generation of extreme in vitro conditions for examining different aspects of spermatozoon viability. Notwithstanding, in the research works on the spermatozoa the degradation function of the exogenous H_2O_2 is predominantly highlighted, there are also other surveys which point out its positive effect. It was proved that the relatively low concentrations of the exogenous H_2O_2 have a wholesome effect on the spermatozoa capacitating whereas the high concentrations – inhibit that process. Therefore correct dosage of exogenous H_2O_2 can be the basis of its positive action. Our experiments showed that as a result of *per oral* treatment of male mice with H_2O_2, under certain specific circumstances, the level of testosterone in the blood serum increases considerably (in times).

Key words: active oxygen species, hydrogen peroxide, spermatozoa, reproduction, testosterone

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Active Oxygen Species and Their Impact on the Reproductivity of the Mammals

Active oxygen species (AOS) is a broad term used to describe the free radicals. A free radical is every atom or molecule which has one non-bound electron (Halliwel and Gutteridge, 1999). Due to this property, the free radicals, according to their quantity, can have a regulatory or toxic biological effect. The main source of AOS in the cells is the mitochondria. According to Skulachov (1999) about 98% of the entire amount of oxygen getting into the cells is used to oxidize the substrata, respectively – formation of ATP (adenosine-5'-triphosphate) and giving off heat. Only 2% from the oxygen is used in the reactions in forming AOS. According to the author along with the enhancing entry of oxygen in the cells or the breaking of the electronic-transport chains in the mitochondria the quantity of AOS can exceptionally increase. In stress moments the generation of AOS is extremely high.

In the last 10-15 years, in the sphere of reproductivity, a certain interest is focused on the research work based on the role of the active oxygen species and in particular – their impact on the fertility of the gametes. There are mainly surveys on oxidative stress and its negative impact on the gametes. It was proven that in the case of the spermatozoa, the extreme generating of AOS can lead to pathology, connected with the impaired spermatozoon function (Aitken et al., 1994; Cummins et al., 1994). Spermatozoa membranes, which contain high level of poly-unsaturated fat acids, are the first damaged by AOS cell compartment (Zalata et al., 1998). That attack can lead to uncontrollable chain reactions that can cause damage to the cell membrane: a radical can take one hydrogen atom out of poly-unsaturated fat acid forming conjugated diene, which after being rearranged readily connects with molecular oxygen that leads to the formation of lipid peroxide radical (process of initiation). The latter one, in its turn, can take one hydrogen atom from other poly-unsaturated fat acid (process of spreading) as it produces lipid hydroperoxide and new lipid radical, which can repeat the chain of events. As a consequence of the process of lipid peroxidation the plasma membrane of the spermatozoon loses its fluidic character and integrity which is manifested with the lack of the gametes motion ability (Jones et al., 1979; Alvares et al., 1987; Aitken et al., 1993), and also absence of acrosome reaction (Cummins et al., 1991) and non-accomplishment of spermatozoon-ovocyte fusion (Aitken et al., 1985; Aitken et al., 1993a; Aitken et al., 1994; Zouari et al., 1993). The lack of the fluidic character of the membrane has direct effect on fusogenicity which explains the inability of the peroxidized cells to show normal levels of sperm-ovocyte fusion (Aitken et al., 1993a). It is claimed that AOS can function as signal molecules for apoptosis initiation (Aitken et al., 1989).

According to Aitken and Clarkson (1987a, b), the hypothetical mechanism of the loss of the spermatozoon function under oxidative stress is connected with the extreme production of active oxygen species. There are some speculations whether they come from the spermatozoa in the sperm or come as a result of the phagocytosis process that is carried out by the leucocytes infiltrating the sperm (Ford, 2004).

The research works carried out by Iwasaki and Gagnon (1992) show that the impaired spermatozoa are the most probable source of AOS. The higher levels of AOS correlate with the reduced number of versatile spermatozoa, and vice versa – the higher level of spermatozoon versatility has been observed in samples of less number of AOS.

It was found that the connection between the leucocytospermia and AOS, correlates with enhanced hemokin (IL-8) and reduced SOD activity of the sperm (Rajasekaran et al., 1995). That means that the enhanced oxidative stress in leucocytospermia is caused by defective AOS-scavenging system, which on its turn has been modulated by specific pro-inflammatory cytokines.

According to Ford (2004) AOS can regulate the expression of the antioxidant enzymes. They act as oxygen sensors which regulate the cellular adhesion and the productivity of antigens from the leucocytes. They also have pro-apoptozis effect and enhance the influence of the epidermal- and thrombocyte-stimu-

lating growing factors; they facilitate the initiation of the motility by stimulating cAMP-synthesis and the protein phosphorylation processes in the sperm. Lecterc et al. (1997) prove that the increase of the intercellular Ca^{2+} concentration at the time of the capacitation leads to increase of AMP, which stimulates the productivity of superoxide radical ($O_2^{\cdot-}$), which on its turn provokes the increase of the phosphorylation process by tyrosine.

Theoretically, the cell damage of the spermatozoa comes as a result of a broken balance between the formation of active oxygen species and AOS-scavenging factors (Aitken, 1999). Antioxidants, which are present in the sperm plasma, compensate the lack of cytoplasmatic enzymes in the spermatozoa (Tramer, 1998). The contact of the sperm plasma with the spermatozoa at the time of the ejaculation protects the spermatozoa against lipid peroxidation when they pass through the female sexual tract (Cheu et al., 2002).

Antioxidants Are Quenchers of Active Oxygen Species

AOS detoxification potential of the gonads and the seminal fluid is normally sustained by adequate levels of antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (Storey, 1997) as well as glutathione and ascorbate (Woodall and Ames, 1997).

It is known that superoxide dismutase, catalase and glutathione peroxidase are the enzymes that neutralize AOS but each one of them has its own target: SOD – reduces superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2). Hydrogen peroxide on its turn is neutralized to water by means of catalase (no donor of electrons) or glutathione peroxidase (with electron donor – glutathione). The catalase catalyses the oxidation of the hydrogen peroxide to water and molecular oxygen, the glutathione peroxidase – to water and oxidized glutathione (GSSG). On other hand catalase can block spermatozoa acrosome reaction (which can be accomplished spontaneous or in the presence of lysophosphatidylcholine) in hamsters semen, (Rivlin et al., 2004). The fact that slower adding of catalase reduces its inhibiting action assumes that catalase effect is rather on the process of capacitation than on the mechanism inducing acrosomic reaction. Cummins et al. (1991) proves that catalase can block the acrosome reaction and the sperm-ovocyte fusion when adding human spermatozoa stimulated with calcium ionophor A 23187, progesterone and recombinant zona pellucida 3 protein (ZP3). Consequently, in that case, the hydrogen peroxide can improve the functional competence of the mammalian spermatozoa.

Hydrogen Peroxide (H_2O_2) – Some Biological Functions

Biological effect of the unigenous H_2O_2

The hydrogen peroxide belongs to the group of compounds which do not have a free electron in their external electronic layer as superoxide radical ($O_2^{\cdot-}$) , the singlet oxygen ($^1O_2$), organic hydroperoxides ($OH\cdot$). But the energy between the paired electrons in the last electronic layer of H_2O_2 is too high and in when spontaneous dissociation occurs a free electron can be loosen.

It was found that H_2O_2 is essential in carrying out many chemical reactions in the human body. It is part of the normal metabolism. The hydrogen peroxide is an effective, in vivo, oxidant in clinical states connected with chronically enhanced oxidative stress (Farr, 1987; Altman, 1995). It is a pro-coagulant under certain conditions and anticoagulant under other (Polgar and Taylor, 1980). It posses pro-inflammatory effect in some cases and anti-inflammatory in some other cases (Agrawal and Harper, 1982). It induces certain genes and suppresses others (McFaul, 1986; Yamaja Setty, et al., 1984). Hydrogen peroxide is a critically important secondary mediator in many metabolite pathways (Ward et al., 1985; Xu et al., 1998; Chen et al., 2001; Steiner, et al., 1984; Boyington et al., 2000). In some cases it has pro-cancer effect, in others – anticancer. H_2O_2 has many roles in the enzyme dynamics of the body, it induces certain enzyme systems and damages the function of others (Gorren et al., 1986; Yamaja Setty, et al., 1984; Polgar and Taylor, 1980; Del Maestro et al., 1980; Babior et al., 1973). For ex-

ample: 1. Hydrogen peroxide inactivates xanthinee oxidase by the means of reactions forming hydroxyl radicals; 2. It inhibits enzymes by oxidation of the susceptible thiol groups, for instance these in the active center of the enzyme glucose-3-phosphate dehydrogenase. In that way it decreses the level of ATP-dependent synthesis of many proteins; 3. Inhibits the enzyme glyceraldehydes phosphate dehydrogenase (GAPDH) providing a mechanism through which H_2O_2 regulates pathophysiology of the endothelium; 4. Hydrogen peroxide regulates the activity of critical energy enzymes like K-Na ATP-ase (sodium-potassium ATP-ase) (Del Maestro et al., 1980); 5. Activates potential antioxidant enzymes, including GPx (Babior et al., 1973).

Other essential metabolic aspects of H_2O_2 are:

- Moving of hexose monophosphate shunt (Hothersall et al., 1982);
- Regulation of mitochondrial enzyme routes (Eto et al., 1999; DesMarais, 2000);
- Synthesis of prostaglandin (Zoschke and Staite, 1987);
- Metabolic pathways of bioamins, including those of norepinephrine, dopamine and serotonin (Heikkila and Cohen, 1971);
- Synthesis pathways of progesterone and estrogen (Agrawal and Harper, 1982).

Biological effect of the endogenous H_2O_2 in spermatozoa

The mammalian spermatozoa have the ability to synthesize H_2O_2 from of O_2 (Tosic and Walton, 1946). Early investigations on H_2O_2 generation in mammal spermatozoids demonstrate direct cytotoxic effect of this molecule on the gametes (Jones et al., 1979). Those investigations were the first indication that semen plasma is powerful source of antioxidants and antioxidant enzymes which protect the spermatozoids form oxidative stress (Jones et al., 1979). That conclusion becomes apparent in the experiments which include the treatment of human spermatozoa with oxidants formed via the system xanthine-xanthine oxidase. Xanthine oxidase catalyses monovalent and bivalent reduction of molecular oxygen (O_2), as a result superoxide radical ($O_2^{\cdot -}$), and nitrogen peroxide are formed and oxidation of the substrate xanthine to uric acid proceeds. The reactive oxygen species, generated from this system, induce dramatic decrease in the mobility of the spermatozoa that is connected with the decrease of the levels of the intercellular ATP and the stimulation of lipid peroxidation (De Lamirande and Gagnon, 1992a, 1992b; Aitken et al., 1993a). With the presence of transitional metals like iron and copper, H_2O_2 and O_2^- can interact in Haber-Weiss reaction as a result of which destructive hydroxylate radical is generated:

$$O_2^{\cdot -} + H_2O_2 \xrightarrow[\text{Fe or Cu}]{} OH^- + OH^- + O_2$$

The hydroxyl radical is considered to be an extremely powerful initiator of lipid peroxidation (Halliwell and Gutteridge, 1999). Here it is important to mention that the superoxide radical reduces the metal ions and after dismutation hydrogen peroxide is produced; when the level of the superoxide radical increases, the concentration of H_2O_2 and OH- augments as well (Chen and Kao, 1999).

The later surveys on the hydrogen peroxide are focused on its role in the process of capacitation of the spermatozoa and the acrosome reaction. It was found out that along with the superoxide anion, H_2O_2 takes part in the regulation of the process of spermatozoon capatitaion and tyrosine phosphorylation of proteins (Aitken et al., 1996; De Lamirande et al., 1997; Leclerc et al., 1997).

The participation of H_2O_2 in actin-polymerization of bull sperm was studied by Brene et al. (2003). The effect of H_2O_2 on the increase of the level of intercellular Ca^{2+}, and of the fertilization potential of mice sperm (Cohen et al., 1998) was also evaluated.

Other profound research works were carried out focusing on the origin and formation of the testicular hydrogen peroxide. The testicular macrophages are able to produce superoxide anion (Wei et al., 1988). Superoxide anions can interact and produce the anion of H_2O_2 and oxygen (McCord et al., 1977) and if there are any protons available H_2O_2 may also be produced (Johnston, 1981).

Biological effect of the exogenous H_2O_2

In nature H_2O_2 is derived from ozone. The ozone consists of 3 oxygen atoms (O_3). The ozone comes as a result of the ultra-violet rays from the sun which split a molecule of atmosphere oxygen (O_2) into two separate unstable oxygen atoms. They combine with other molecules and form O_3. Ozone molecule is unstable. It rapidly releases an oxygen atom which along with the falling rain water turns into H_2O_2.

The hydrogen peroxide is colorless liquid, heavier than water. It is derived from the plant cells and plays manifold and important roles in the cell communication and energy systems of the plants (Moskova, 2007). It is commonly found in small quantities in the rain water and snow. It can be found in greater quantities in the natural springs, most of which are of healing character. It is not known how H_2O_2 fulfils its effects, yet. There are certain data on the successful application of the exogenous H_2O_2 (per oral or intravenous) as a therapeutical compound. It was found out that hydrogen peroxide improves the arterial, venous and lymph circulation, and acts as vasodilator (Ali and Ali, 1997; Rubanyi and Vanhoutte, 1986; Yamaja Setty et al., 1984).

Biological effect of the exogenous H_2O_2 in spermatozoa

In spermatology, the exogenous H_2O_2, is used mainly in creating extreme *in vitro* conditions for examining different aspects of spermatozoon viability. H_2O_2 is applicable in tests which indirectly examine, in vitro, the sensitivity of the spermatozoa to oxidative stress (Misro et al., 2004). In certain dosage H_2O_2 can be used to cause oxidative stress in bull sperm in order to evaluate the preventive effect of glutathione and other thiols on the motility of frozen spermatozoa (Bilodeau et al., 2001), as it is considered that the incubation of human spermatozoa under aerobic conditions generates larger quantities of lipid peroxide, which has negative effect on the mobility of gametes (Engel et al., 1999). Other authors (Stocco et al., 1993) are trying to establish if they treat the interstitial cells of Leydig with H_2O_2 that will cause inhibition of the steroid productivity. They found out that the hydrogen peroxide inhibits the steroid genesis in Leydig tumor cells as first inhibiting the activity of 3α-hydroxysteroid dehydrogenase, following by other effects like inhibition of protein synthesis and transfer of cholesterol to side chain process of its decomposition.

Despite the fact that most research works are focused on the destructive function of the exogenous H_2O_2 on spermatozoa, there are studies that point its positive effect. It was found that application of relatively low levels of exogenous H_2O_2 can have positive effect on the capacitation of the spermatozoa, whereas the higher hydrogen peroxide concentration inhibits that process (Rivlin et al., 2004). Therefore correct dosage of exogenous H_2O_2 can be the basis of its positive action.The influence of H_2O_2 on the process of spermatogenetic capacitation as well as its use as therapeutic agent make us carry out some preliminary research works connected with the effect of H_2O_2 on certain reproductive indicators of the animals.

The conducted experiments present some unique preliminary results concerning the testosterone level in the mice blood serum and the versatility of ram spermatozoa. (Vaisberg et al., in press). As a result of the per oral treatment of male mice with H_2O_2, under certain specific circumstances, the level of that hormone in the blood serum increases considerably (in times) (Table1).

Table 1
Testosterone concentration in mice blood serum

Testosterone, nmol/l			
№	Control	№	Treated
1	1.58	5	5.04
2	0.38	6	8.22
3	0.10	7	9.80
4	0.68	8	12.54
x±Sx	0.685 ± 0.32*	x±Sx	8.90 ± 1.56*
*P<0.001			

In the process of incubation of diluted sperm, at 39^0C and in the presence of H_2O_2 during certain moment of their incubation the spermatozoa motility increased significantly (Table 2)

Table 2
Spermatozoon motility (%) incubated with H_2O_2 at 39^{0}C for 5 hours (n 13)

Variant	Hour of incubation		
	1st	3rd	5th
Control	78.07 ± 1.44	73.46 ± 2.11*	63.07 ± 4.14
Treated	76.92 ± 1.55	81.15 ± 1.89*	66.92 ± 4.76

Those preliminary results are considered to be an objective reason for further more careful studying the effects of hydrogen peroxide. In conclusion, the extremely limited and one-sided (concerning only the destructive function) information about the effect of the exogenous H_2O_2 on the mammals reproductivity on one side, and our preliminary original results – on the other side, as well as some specialized data about the therapeutically effect of H_2O_2, make us carry out more careful tests of the probable stimulating effect of the exogenous hydrogen peroxide on animals organism, respectively on their reproductive system so that the biotechnologies focusing on obtaining sperm of good quality and high fecundate potential could be further developed.

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