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ORIGINAL PAPER

The Oxidative Stress, Cryoconservation and Fertility. Aspects of Molecular Oxidation and its Effects on the Reproductive Functions off Ram

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Abstract
The adverse effects of reactive oxygen species on the reproductive function in mammals have been studied for approximately three decades in humans, their connection to infertility being well described in the specialized literature.
The objectives of this study were to increase the degree of understanding of the production mechanisms of reactive oxygen species and of the way in which these induce physiological and molecular alterations in the ram sperm cell, as well as the connection between these alterations and the reproduction physiology in the mammalian sperm cell.

Introduction

The assisted reproduction techniques (ART), especially artificial insemination and *in vitro* fertilization have been used successfully over the recent years to treat human infertility and to optimize the efficiency of animal reproduction.
Artificial insemination offers numerous genetic and economic advantages for the ovine production, being the most secure method for the introduction in the flock of superior genes from individuals lacking specific diseases and with the purpose of increasing the number of products. The method also ensures reproduction outside the natural mating season (September-January), allowing the production of an adequate quantity of milk all year round.
In sheep, the reproduction biotechnologies, which also involve artificial insemination, developed rapidly after the optimization of the methods for the production and preservation of the seminal material and for the estrus synchronization in females. These technologies must take into account the specificity of the ovine species compared to other animal species. The intracervical insemination with fresh semen leads to adequate conception rates, but it is limited by the rather small number of doses that can be obtained at the same time from a male. Moreover, in order to obtain maximum fertility, the insemination must take place within ten hours maximum from the collection of the seminal material, which makes it difficult to apply this technique in large flocks or located at great distances from the collection center. Another specificity

of sheep is that the semen is inseminated vaginally because the transcervical insemination is difficult to achieve due to the female anatomy of the cervix. An alternative is the intrauterine insemination with preserved seminal material. However, the costs of this technique do not permit its routine application in large flocks.

The preserved seminal material was used in order to maximize the genetic benefits offered by the artificial insemination, but unlike bovines, the frozen ram semen has a rather reduced fertility. Despite the considerable progress of the recent years, cryopreservation leads to the decrease by minimum 20% of sperm cell motility, which means that these techniques still exercise considerable stress on the sperm cells. In order to obtain an acceptable fertility, it is necessary to develop an optimal method of preservation of the seminal material, refrigerated or frozen, which should be integrated easily and rapidly in the insemination methodologies.

The processing methods involved in the assisted reproduction techniques, as well as the dilution and preservation of the seminal material, can lead to the setting in of oxidative stress. Studies accomplished on human, bull and dog semen demonstrated that during the freezing-thawing process there is a supra-production of reactive oxygen species. Moreover, it was reported that the freezing-thawing process is also responsible for the decrease in the level of cellular antioxidants, such as reduced glutathione or superoxide dismutase. Even though considerable progress was accomplished in this domain, the preservation procedures are still insufficiently perfected and there is still to study the way in which the harmful effects of the reactive oxygen species intervene during certain physiological processes.

1. The conservation of the seminal material

The preservation of the genetic resources has a great importance, considering the implications in numerous domains, such as agriculture, biotechnologies, or clinical medicine. The conservation of the germinal cells also has a major contribution in biodiversity conservation. In zootechny, the preservation of the seminal material from valuable animal breeds, as well as from rare breeds or endangered ones can be used to organize gene banks, genetically improve the domestic species and exchange germplasm as frozen semen at international level. Also, cryoconservation is very important in aquaculture and also in the field of biotechnology for the preservation of the germplasm from transgenic animals.

The purpose of preserving sperm cells is to prolong the fertilization capacity. The short-term conservation (liquid conservation through refrigeration) involves the reduction of sperm cells metabolism by the reduction of the storage temperature, while the long-term conservation (freezing) involves the suppression of metabolism at temperatures below 0^{o} Celsius. But even though the fertilization capacity is prolonged by the storing in liquid or frozen form, this process

inevitably reduces the percentage of motile sperm cells and leads to degenerative modifications of the membrane integrity, which finally leads to the decrease of fertility after artificial insemination (Zamfirescu, 1994; Donovan, 2004).
The first reports regarding semen preservation date back to 1776, when Spallanzani discovered that frog, stallion and human sperm cells become immotile after cooling in snow for 30 minutes, but become motile again after warming (quoted in Maxwell and Salamon, 1993). After this discovery, several other studies were accomplished over the past century in order to determine the optimal cooling rate, the composition of the thinner and the temperature at which the sperm cells of domestic animals and humans are preserved.

1.1. Liquid conservation of sperm cells

The storing of "refrigerated" semen has been extensively studied since the beginning of the 1900s. The experiments were mostly focused on the effect of temperature decrease on the physiology of sperm cells. The first studies date back to 1934, when Milovanov (quoted in Maxwell and Salamon, 1993) discovered that a percentage of the sperm cells that are rapidly cooled to temperatures close to 0^o Celsius become irreversibly immotile. The phenomenon was initially called "shock temperature" and it is now known as "cold shock". Subsequently, the optimal cooling rate was determined for the ram and bull sperm cells (10°C/h; Maxwell and Salamon, 1993) and it was established that the addition of certain lipids from egg yolk or milk and of other lipoproteins and phospholipids in the storage thinners protects the sperm cells against the irreversible effects of cold shock. These discoveries were paramount for the subsequent studies regarding the obtaining of certain appropriate thinners for the preservation of ram sperm cells at low temperatures (Maxwell, 1993; Paulenz, 2001).

The temperature for the short-term conservation (by refrigeration) of sperm cells

The objective of storing sperm cells at subambient temperatures (21^oC) is to reduce their metabolism for the purpose of prolonging viability. It was found that a large variety of temperatures from the interval 0-15^oC ensure viability and fertility in the ram sperm cells. The initial studies reported that the temperatures between 10 and 15^oC would be optimal. However, the subsequent experiments showed that the interval 0-5^oC maintains the viability of the sperm cells for a longer time. It is recommended that the semen preserved at 5^oC should be used within a maximum of 24 hours from collection for intracervical artificial insemination and within a maximum of 6 days for intrauterine insemination (Salamon, 1979). The use of *in vitro* fertilization systems (IVF) for ram sperm cells preserved for 7 days at 5°C leads to high fertilization rates. Even after 14 days of storage there can be *in vitro* fertilization. Since the semen stored at 15°C has a shorter lifespan compared to the one kept at 5°C, it is recommended that it

should be used for artificial insemination within 6-12 hours from collection (Maxwell, 1993; Zamfirescu, 1994).

1.2. The preservation of sperm cells by freezing

Due to the relatively short span of sperm cell fertility when preserved as liquid, there has been considerable progress in the studies regarding preservation at temperatures below 0°C. The first reports regarding the success of sperm cells freezing dates back to 1937 when Bernstein and Petropavlovsky (quoted by Salamon and Maxwell, 2000) froze rabbit, Guinea pig, ram, boar, bull and stallion sperm cells in media cu 9.2% glycerol at -21°C. Initially, the methods and thinners used for the freezing of bull sperm cells were used for ram as well, but unsuccessfully. Positive results were obtained after the development of freezing techniques as solid on blocks of ice at -80-95°C and of freezing techniques in plastic vials, where vapors of liquid nitrogen were used to freeze at -190°C (Salamon, 1971; Visser and Salamon, 1974).

Events that occur during the cryopreservation processes

Cryopreservation involves several steps such as dilution of the seminal material, cooling, freezing and thawing. Each of these steps can cause the deterioration of the sperm cells by the alteration of the normal functions and of their fertilization potential. The main causes for sperm cell deterioration during cryopreservation are:

1. cold shock (Watson, 2000)
2. osmotic shock (Watson, 2000)
3. oxidative stress (Aitken, 2001; Sikka, 2001; Agarwal, 2003)

The cooling process that occurs before freezing, from body temperature to nearly water freezing point (5 ^{o}C), determines the installation of a type of stress known as "cold shock" (Watson, 2000; Luvoni, 2006). During the cooling process, the lipids in the sperm cell membrane, which do not bond covalently to the membrane proteins, suffer phase transitions from liquid to gel, which also result in the modification of the function of the membrane proteins (Parks, 1992).

Osmotic shock intervenes at the temperature drop from -5 ^{o}C to -15 ^{o}C and at thawing. If the sperm cells are cooled below the freezing point, the water from the freezing thinner will freeze, leaving the sperm cells suspended in a highly concentrated solution. Thus, an osmotic gradient is created between the inside and the outside of the sperm cell and the water will exit the cell causing its dehydration. If the sperm cells are cooled rapidly, the intracellular water will not be able to exit the cell fast enough and thus intracellular ice crystals will form. The intracellular crystallization has harmful effects on the viability of sperm cells (Pukazhenthi, 1999). If the sperm cells are cooled slowly, the water continues to exit the cell until the cell is dehydrated. The optimal cooling rate is between 10-80°C/min. By adding glycerol (0.5M), the optimal cooling rate for ram sperm

cells can be increased to 50-60°C/min (Duncan and Watson, 1992). During thawing, the sperm cells are exposed to the same processes as during freezing, but in a reverse order and with less control (Hammerstedt, 1990). It was demonstrated that the ram sperm cell is highly sensitive to osmolarity change, similar to the human one (Curry, 1994), cat one (Curry, 1994) or stallion one (Ball, 2001).
Oxidative stress (Aitken, 2001; Sikka, 2001; Agarwal, 2003) is added to osmotic and cold shock. The recent experiments have shown even a causality relationship between the osmotic and oxidative stress, and conditions of hyper or hypo-osmolarity lead to the increase of the production of superoxide anions (Burnaugh at al., 2010). Several studies have shown that oxidative stress induces deteriorations of the membrane and of the DNA in the human sperm cell (Aitken, 1999), stallion sperm cell (Baumber, 2003), ram sperm cell (Peris, 2007) or bull sperm cell (Bilodeau, 2002; Nair, 2006).

1.3. Extender used in the sperm cell cryopreservation technology

The survival of the sperm cells in the seminal plasma is limited to a few hours, which renders necessary the dilution of the sperm cells suspension into a solution capable of preserving the fertilization capacity of sperm cells during *in vitro* storage at low temperatures. For liquid preservation, the semen de congelação da solução, o qual é determinado como amust be diluted in a nutritive medium which should support its survival in conditions of reduced metabolic activity, when both precocious capacitation and cellular death can occur. The purpose of the storage by refrigeration of the seminal material in farm animals is to reduce the degree of endogenous protein phosphorylation in order to prevent precocious capacitation (Vishwanath, R, 2000). The role of a thinner used in freezing is to inhibit metabolism and provide a medium that should preserve the membrane integrity and functions (Watson, 1995).
The thinners used are either natural (whole cow milk, powder milk, skimmed milk or UHT pasteurized milk) or synthetic. The synthetic thinners must contain a buffer substance (tris, citrate, phosphate, Tes, Hepes), an energy source (fructose, glucose, mannose), lipids for protection against cold shock (from egg yolk, milk), cryoprotective substances and antibiotics to prevent the development of contaminating microorganisms (Maxwell and Salamon, 1993).
The buffer substances have an important role because toxic catabolites result from the sperm cells metabolism and they contribute to the increase in lactic acid concentration in the extracellular environment, which becomes acid and affects the cell viability. The sperm cells has an optimal pH of around 7, thus the dilution media must be buffered between 6.9 and 7.1 (Salamon and Maxwell, 2000).
Egg yolk is the most efficient protection agent of the sperm cells against cold shock. It was demonstrated that it improves the sperm functions and maintains sperm fertility after preservation by refrigeration or freezing (Watson, 1981; Moussa et al., 2002). Egg yolk has a lipoprotein fraction with low high density

(LDL) which interacts with the cell surface during cryopreservation, protecting thus the plasmatic membrane (Moussa et al., 2002). This way the lipid particles prevent the tearing of the membrane during the cooling process and help to stabilize the structure of the double lipid layer (Vishwanath, 2000; Aurich, 1997). Also, the egg yolk lipoproteins interact with the proteins present in the seminal plasma, preventing thus their attachment to the plasmatic membrane, a process which leads to the loss of the membrane lipids (Bergeron, 2004). Moreover, the egg yolk is a rich source of vitamin E, contributing thus to the increase of the antioxidant power (Alvarez, 1993).

The addition of cryoprotectors in the sperm thinners has beneficial effects in the cryopreservation process. The selection of a certain cryoprotector for a particular species of animals depends on the permeability of the sperm cell membrane to it and on the possible toxic effects. In general, the concentration of cryoprotector in the dilution environment varies between 4 and 6%, leading to a reduction of the freezing point to approximately -30°C. Recent studies have demonstrated that in higher concentrations, these are toxic (Petrunkina, 2007).

The cryoprotectors normally used are glycerol and dimethyl sulfoxide (DMSO). Glycerol is the most known cryoprotector, being considered essential for the long-term preservation of mammalian sperm cells. The cryoprotective effects of glycerol on the sperm cells were discovered by Polge in 1949 and were attributed especially to its property of binding water molecules (Salomon and Maxwell, 1995), but also to its ability to prevent phase transitions during cooling by increasing the water permeability and the fluidity of the sperm cells plasmatic membranes (Holt, 2000). Also, glycerol prevents the formation of intracellular ice crystals, reduces osmotic stress by replacing water needed to maintain the cellular volume and reduces the water freezing point. This is why the use of glycerol to preserve sperm cells by freezing is very spread, recent studies demonstrating that glycerol has the most efficient cryoprotective effect for the preservation of mammalian semen (Hammerstedt, 1990). For ram semen, glycerol is also the most used cryoprotector (Salomon and Maxwell, 2000).

1.4. The ultrastructure of the sperm cell

1.4.1. Semen

Semen represents a complex product of the male genital apparatus, being made up of sperm cells (produced in the seminiferous tubules of the testicle) and seminal plasma. After passing through the epididymis, the mature sperm cells reach the "deferent canal" where they are mixed with the secretions of the adjunct glands located along the male genital tract. The sperm cells (strongly concentrated in the epididymis) will be diluted approximately ten times with this mixture of secretions during ejaculation, resulting the semen. The seminal plasma represents

the transporting fluid for the sperm cell from the male to the female, as well as the energy and nutrients source (Zamfirescu, 2003).
The composition of the seminal plasma varies among species, but there are also variations among individuals of the same species in what regards the molecular composition, but also its volume. The seminal plasma contains high levels of nucleases, nucleotidases and lysosomal enzymes, such as proteinases, phosphatases, glycosidases and hyaluronidases. Also, plasma has the role to protect sperm cells against the attack of free radicals produced by sperm cells and leukocytes by means of the non-enzymatic antioxidants (taurine, hypotaurine, α-tocopherol, ascorbic acid, glutathione, uric acid or pyruvate) and enzymatic antioxidants (catalase, superoxide dismutase) (Silva, 2006).

1.4.2. The sperm cell

The observations of the recent years with the help of the electronic microscope established precisely the ultrastructure of the sperm cell. From the morphological point of view, the sperm cell is made up of head, neck and tail. The plasmatic membrane surrounds the sperm cell entirely and it is characterized by specific regions which differ in the content of glycoproteins and lipids.
The ram sperm cells have a total length of 70-80μm, the size of the various components being given in Table 1.

Table 1. The size of the various component parts of the ram sperm cell (Pesch, 2006)

	Length (μm)	**Width (μm)**
Head	8.2	4.25
Middle piece	14.0	0.8
Main piece	45.0	0.5

The head is slightly flattened and ovoid (as seen on the flattened side). Seen on the edge, it has a pear-shaped contour with the base of the head thicker and the anterior side gradually thinned. The head is made up of acrosome and nucleus and it is surrounded by the plasmatic membrane. The acrosome contains hydrolytic enzymes such as the acrosin, hyaluronidase and other hydrolases and esterases. These enzymes are essential for the lysis of the pellucid zone and penetration of the corona radiata of the oocytes. These are set free during the acrosomal reaction when the external acrosomal membrane coagulates with the plasmatic membrane. The acrosome forms a muff-type structure which covers two thirds of the sperm cell head. The acrosome is made up of an internal and an external membrane which surround a dense, amorphous electron zone, with fine grain. It can be divided into three different segments:

a) the anterior edge, which is the apical segment
b) the main segment

c) the caudal portion, which is the equatorial segment

The nucleus contains a condensed chromatin which bears the genetic information. Only in the neck area there is a small portion with uncondensed chromatin where transcription, translation and protein biosynthesis are possible. The products resulted from the protein biosynthesis are released through the pores of the nuclear membrane only in the neck area (Aurich, 2005).

The nuclear membrane and the internal acrosomal membrane form the nuclear casing.

The neck has a length of approximately 1 μm and makes the connection between head and tail. It is the most fragile area, which is why brutal manipulations can easily separate the head of the sperm cell from the tail.

The tail of the sperm cell is made up of three regions: the middle piece, the main piece and the terminal piece.

The middle piece is approximately 5 μm long and contains an elongated mitochondrion. Its aspect is that of a spiral filament which surrounds the axonemal complex. This is where the enzymes involved in energy production by the conversion of fructose into ATP are located. The mitochondrion provides energy (ATP) which is mainly used for motility.

The main piece: the axial core of this region is made up of 20 fibrils (two of which are central) that represent the main contractible elements. The contractible fibrils are covered with a fibrous layer.

The terminal piece is short and has no fibrous layer, thus the fibers are free and detached one from the other (Zamfirescu, 2003).

The sperm cells can display deviations from the normal structure, some anomalies. The morphological anomalies of the sperm cells can be categorized as:

-primary anomalies, determined by spermiogenesis disturbances, occur in the seminiferous tubules and consist in the morphological and structural alteration of the sperm cell head.

-secondary anomalies, which occur in the epididymis or are effects of the action of thermal, physical or chemical factors during ejaculation and manipulation of the semen; they especially target the alteration of the sperm cell tail.

The rams with normal fecundity do not exceed 14% anomaly incidence.

1.4.3. Seminal plasma

Seminal plasma is a pH buffer medium, with a role in the maintenance of osmolarity and nutrient provision. Seminal plasma has a slightly acidic pH, around 6.8, with variations between 5.9 and 7.3. The osmotic pressure is similar to the one of blood and of saline solution (around 300-310 mOsm/l).

The volume of semen, in ram, is 1.5 ml on average (with variations between 0.7 and 3 ml).

The color of semen is milky-white, with creamy aspect.

The thickness is appreciated by looking at semen in the collection glass immediately after harvesting, in the direction of a light source. In the ram semen, "wave" movements of the sperm cells can be seen, and they characterize a very good quality seminal material.
The concentration is 1-3 billion sperm cells/ml, being the most concentrated semen in farm animal males, namely 3-5 times more concentrated than bull semen and ten times more concentrated than boar and stallion semen. The concentration variation limits are between 0.5 and 6 billion sperm cells/ml (Zamfirescu, 2004).

2. Reactive oxygen species, antioxidants and the semen roles

Molecular oxidation affects the normal cell functions and may lead to cell degeneration or even cell death. The free radicals represent the major factor involved in this oxidation and they can attack and alter or inactivate the biological activity of proteins, lipids and nucleic acids, which are essential for the cell functions. In order to eliminate the harmful effects of oxidation, it is necessary to understand the mode of operation of the various free radicals involved in the processes of cell homeostasis and the way in which it can come to pathology. The biology of free radicals has implications in several aspects of modern life, from food industry, where the prevention of oxidation is crucial in food preservation, to the medical sciences such as neurology, cardiology and, more recently, reproduction medicine. The studies done on human seminal material demonstrated that molecular oxidation has an important role in the occurrence of infertility, especially when the reproductive tissue or the gametes are stored or manipulated *in vitro*.

2.1. Overview of the reactive oxygen species

Free radicals can be classified into two major groups: reactive oxygen species (ROS) and reactive nitrogen species (RNS).
The group of free radicals derivatives of oxygen and of their precursors forms the reactive oxygen species. This category includes primary free radicals, chemical species characterized by the presence of an unpaired electron and which are derived from oxygen by the reduction of an electron (the superoxide radical, the hydroxyl radical), but also non-radical species such as singlet oxygen, hydrogen peroxide, and hypochlorous acid, which can be precursors of free radicals. Their reactivity is highly variable, depending on the nature of the radical. Their reactivity can be quantified by the value of the halving time ($\mathbf{t_{1/2}}$, the reduction time for the halving of the number of reactive radicals) at 37^{o}C (body temperature). A reduced halving time corresponds to an increased reactivity (Silva, 2006).

1. The superoxide radical ($O_2^{-\cdot}$) ($t_{1/2}$=1x 10^{-6} s)

The superoxide radical is the first intermediary product in the process of molecular oxygen reduction to water in the respiratory chain. It is relatively inert and cannot cross the cell membrane. During phagocytosis, it is generated on the route of the NADPH oxidase.

$$O_2 + e^- \longrightarrow O_2^{-\cdot}$$

$$NADPH + 2O_2 \xrightarrow{\text{NADPH oxidaza}} NADP^+ + H^+ + 2O_2^{-\cdot}$$

2. The hydroxyl radical ($HO^{\cdot}$) ($t_{1/2}$=1x 10^{-9} s)

It is the most active oxygen species. It reacts with great speed with absolutely any organic molecule (carbohydrates, amino acids, lipids, nucleic acids, organic acids) and may lead to the formation of crossed covalent bonds or to the propagation of free radicals to a wide range of biomolecules (Baumber at al., 2003). In general, it is the result of the oxidation of heavy metal ions (Fe^{2+} or Cu^+) by the hydrogen peroxide (the Fenton reaction). The oxidized iron (Fe^{3+}) can subsequently catalyze the reaction between the superoxide and the hydrogen peroxide (the Haber-Weiss reaction), leading to the production of several hydroxyl radicals.

The Fenton reaction:

$$H_2O_2 + 1\ e^- (Fe^{2+}/Cu^+) \longrightarrow Fe^{3+} / Cu^{2+} + HO^{\cdot} + HO^-$$

The Haber-Weiss reaction

$$O_2^{-\cdot} + H_2O_2 \longrightarrow O_2 + H_2O + HO^-$$

3. The alkoxyl radicals ($RO^{\cdot}$) and peroxyl radicals ($ROO^{\cdot}$) ($t_{1/2}$=1x10^{-6}s, $t_{1/2}$=1x 10^{-2} s respectively)

They are formed during the break up of certain sublayers such as lipids and proteins (Silva, 2006).

4. The singlet oxygen ($^1O_2$) $t_{1/2}$=1x 10^{-6} s

It is generated in the reactions of oxidoreduction, break up or radiolysis. It has an electrophile character, reacting with many organic compounds such as polyunsaturated fatty acids, cholesterol, and oleins with which it forms hydroperoxids, or with organic compounds containing sulfur or nitrogen atoms, producing oxides. Among antioxidants, α-tocopherol, carotenoids and bilirubin have the capacity to "extinguish" oxygen as singlet (Niţu et al., 2002).

5. Hydrogen peroxide (H_2O_2)

H_2O_2 is formed rapidly by the dismutation reaction of superoxide radical $O_2^{-\cdot}$, a reaction catalyzed by the superoxide dismutase (SOD).

$$2\ O_2^{-\cdot} + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

The reduction of the hydroperoxyl radicals can lead to the spontaneous formation of H_2O_2 (Alvarez and Storey, 1989; by Lamirande and Gagnon, 1995a).

$$HO_2^{\cdot} + e^- + H^+ \rightarrow H_2O_2$$

Same as O_2, the hydrogen peroxide is a weak oxidant agent, acting especially on the unsaturated compounds and on tryptophan. Among all the reactive oxygen species, H_2O_2 is the most stable and easy to measure. It is a non-electrically charged molecule for which the membrane is permeable, as it can diffuse through the mitochondrial or nuclear membrane.

6. The hypochlorous acid (HOCl)

As a response to infectious agents, the activated neutrophils produce hypochlorous acid (Klebanoff, 2005). The reaction is catalyzed by the myeloperoxidase (MPO).

$$H_2O_2 + Cl^- \xrightarrow{MPO} HOCl + OH^-$$

(the formation of the hypochlorous acid)

The $O_2^{-\cdot}$ radicals react with the hypochlorous acid and form hydroxyl radicals, which are much more reactive. The hypochlorous acid is toxic to young cells and can also initiate chlorination reactions of proteins and lipids .

$$R\text{-}NH_2 + HOCl \rightarrow RNHCl + H_2O$$

2.2. The production and degradation of the reactive oxygen species in the seminal material

The first reports regarding oxidative stress and its involvement in male infertility date back to 1943, when the andrologist John MacLeod demonstrated that human sperm cells maintain motility in aerobic conditions, in the presence of catalase (quoted by Baker and Aitken, 2005). His hypothesis that human sperm cells are vulnerable to oxidative stress induced by reactive oxygen species such as hydrogen peroxide, was later confirmed by numerous other studies (Sikka et at.,

1996; Sharma and Agarwal, 1996; Sikka, 2001). At seminal level, oxidative stress sets as a result of an unbalance between the generation of ROS and their elimination. In physiological conditions, free radicals are permanently synthesized in small quantities. Lacking afflictions, there is a balance between the synthesis of free radicals and the antioxidant defense systems of the organism. Oxidative stress sets in when this balance is altered, whether by an excess of pro-oxidative structures or by a deficit of antioxidant agents, (Aitken, 1994; Favier, 2003). The sperm cells are even more susceptible to modifications caused by oxidative stress because their plasmatic membrane contains large quantities of polyunsaturated fatty acids (Alvarez and Storey, 1993), while their cytoplasm contains low amounts of antioxidant enzymes (Jones et al., 1979; Aitken and Fisher, 1994; Sharma and Agarwal, 1996). Moreover, the intracellular antioxidant enzymes cannot protect the plasmatic membrane which surrounds the acrosome and the flagellum, as these are protected only by the antioxidant defense in the seminal plasma (Iwasaki and Gagnon 1992; Zini at al., 1993).

There are two main sources for the generation of reactive oxygen species in the semen: the leukocytes and the sperm cells that are immature or have anomalies (Garrido et al., 2004). The leukocytospermic semen displays a larger percentage of DNA deteriorations and a larger number of immature germinal cells, which might be due to alterations during the regulation of the sperm maturation (Alvarez et al., 2002). Neutrophils and macrophages are especially associated with an excessive production of ROS (Agarwal and Prabakaran, 2005). The presence of leukocytes is also associated with a low content of α-tocopherol in the seminal plasma and, in most cases; it is also associated with a reduced seminal volume and affected motility and morphology (Therond et al., 1996).

The main generation places for ROS in the sperm cell are the mitochondrion and the plasmatic membrane. Quantitatively, the mitochondrion (the NADH regions dehydrogenase and ubiquinona) is the major production location for the superoxide radical ($O_2^{-\bullet}$) and for the hydrogen peroxide (H_2O_2) (Cadenas and Davies, 2000). The sperm cells are very rich in mitochondria because they constantly need an energy supply to maintain their motility (Agarwal et al., 2005a). At the level of the plasmatic membrane, the ROS generation occurs in the route of the NADPH-oxidase (Agarwal et al., 2003).

In general, sperm cells produce ROS when spermiogenesis defects occur (cytoplasmic drops), with a positive correlation between immature sperm cells and ROS production (Zini et al., 1993; Gomez et al., 1996; Gil-Guzman el al., 2001; Said et al., 2004). The immature sperm cells seem to be more susceptible to oxidative deteriorations induced by NADPH (Richer and Ford, 2001; Said et al., 2005), and materialized as a lower number of sperm cells, affected motility and morphological anomalies. During spermatogenesis there occurs a reduction of the cytoplasm content in order to allow the sperm cell to regain its elongated shape.

The teratozoospermal immature sperm cells are often characterized by the presence of cytoplasmic residues at the middle piece. Moreover, the immature sperm cells with cytoplasmic drops have high levels of cytosolic enzymes (lactic acid dehydrogenase, creatine phosphokinase (CK), glucose-6-phosphate dehydrogenase), the activity of these enzymes being correlated to sperm dysfunctions (Griveau and Le Lannou,1997). The enzyme glucose-6-phosphate dehydrogenase controls the rate of glucose flow and the intracellular production of NADPH through the hexose-monophosphates shunt. NADPH will fuel the enzymatic route of NADPH oxidase (located at the level of the spermatic membrane) for the generation of ROS (Fisher and Aitken, 1997; Said et al., 2005). As a result, these immature sperm cells will produce high amounts of ROS, compared to normal sperm cells (Tremellen, 2008). If we divide the total ROS production into the one produced by sperm cells (intrinsic) and the one produced by leukocytes (extrinsic), it was observed that the extrinsic rate is about 1000 times higher. Even though both routes for the production of ROS correlate negatively with the DNA integrity, the relationship is significantly stronger for the intrinsic production. This is why the intrinsic ROS production is considered an important variable in what regards the fertility potential of the semen (Tremellen, 2008).

2.3. The toxicity of reactive oxygen species

Until recently, it was considered that ROS have only a toxic action on the sperm cells. However, there are studies that showed that, in order for them to be capable of fertilization, sperm cells require small amounts of ROS, these being essential for the acrosome reaction, capacitation, hyperactivation and fusion with the oocyte (Griveau and Le Lannou, 1997; Agarwal et al., 2004, 2008).

The oxidative stress caused by an excessive generation of ROS is one of the main factors involved in the pathogenesis of male infertility (Gil-Guzman et al., 2001; Agarwal et al., 2008). In high quantities, the superoxide, peroxynitrite, hydroxyl radicals, and the hydrogen peroxide, by interaction with the membrane lipids, proteins, and nuclear and mitochondrial DNA, affect motility, viability and the functions of the sperm cell (Iwasaki and Gagnon, 1992; Sikka, 2004).

The mechanisms by which sperm cells are affected by ROS are multiple. The modification of cell membrane permeability affects the quality and number of sperm cells that reach the oocyte in the female reproductive tract, or affects the fertilization process by preventing the initiation of the fusion process between sperm cell and oocyte (Agarwal el al., 2003). Finally, ROS can affect embryo development and fetus health by the modification of the sperm DNA. Free radicals play an important role in the apoptosis process as well, by the activation of the caspases (Martinez et al., 2009). In normal conditions, abnormal sperm cells are eliminated from the semen by apoptosis, but in the case of excessive

ROS production, more sperm cells are affected, leading to the decrease of their number and implicitly to the lowering of fertility (Agarwal el al., 2005).

2.3.1. Cellular sub-layers of reactive oxygen species

2.3.1.1. The lipids are considered the macromolecules most susceptible to being attacked by ROS. They are present in the plasmatic membrane of the sperm cell as polyunsaturated fatty acids (fatty acids that contain more than two double bonds). Their distribution and orientation in the structure of the plasmatic membrane offer it the fluidity needed by the sperm cell to support normal physiological functions (Bell et al., 1993).
The ROS attack on these fatty acids leads to a cascade of chain chemical reactions called lipid peroxidation. Generally, for any type of cell, the most harmful effect of lipid peroxidation is the disturbance of the structure and function of the plasmatic membrane. Lipid peroxidation leads to the modification of membrane fluidity, the loss of membrane integrity and even the irreversible loss of sperm cells motility (Storey, 1997). The elimination of the metabolites resulted from lipid peroxidation leads to the increased activity of glutathione peroxidase and to the reduction of the NADH concentration and glutathione reduced. The reduction of the NADH concentration will subsequently affect the cell homeostasis of Ca^{2+} (Alvarez and Storey, 1989; Storey, 1997). The alteration of the intracellular balance of calcium affects the motility of the sperm cell (Gupta et al., 2005).
The peroxidation process (the self-oxidation of LH lipids with formation of LOOH hydroperoxides) is represented by the following reactions:

$$\mathbf{LH + R^{\cdot} \rightarrow L^{\cdot}}$$

$$\mathbf{L^{\cdot} + O_2 \rightarrow LOO^{\cdot}}$$

$$\mathbf{LOO^{\cdot} + LH \rightarrow L^{\cdot} + LOOH}$$

where R is an initiation radical (e.g. $HO^{\cdot}$), while $LOO^{\cdot}$ is a lipid peroxyl radical.
The first step is the removal (normally by a $HO^{\cdot}$ radical, but also by other radicals) of a hydrogen atom from a methylene group ($-CH_2-$) adjacent to a double bond from the given fatty acid. The result is an unpaired electron for a carbon atom which will be stabilized by a molecular rearrangement, and the result will be a conjugated diene (alternative double-simple-double bonds). This will react with the oxygen forming a peroxyl radical which will extract a hydrogen atom from another lipid molecule (initiating a chain reaction) and thus a lipid hydroperoxide forms (R-OOH). The complexes of transitional metals (particularly iron) can catalyze the breaking of the O-O bond from the hydroperoxide and the formation of an alkoxy radical; the process will lead to a breaking of the chain, releasing hydrocarbons or aldehydes of various sizes. The most stable and detectable final

products of lipid peroxidation are malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Malondialdehyde is soluble in the aqueous phase and can react with sub-layers such as DNA and proteins (Esterbauer et al., 1987).
4-Hydroxynonenal is a biologically active molecule and can cause the alteration of the activity of enzymes that contain thiol groups or can affect the GSH level (Uchida, 2003). In concentrations of picomolar level, it is chemotactic for the polymorphonuclear leukocytes, it inhibits cellular proliferation and it is mutagenic (Petersen and Doorn, 2004).
The involvement of free radicals in the reduction of sperm motility was first reported by Jones in 1979. He demonstrated that the peroxidation of the sperm membrane caused by ROS leads to the decrease of its flexibility and flagellum motility. The regeneration of the plasmatic membrane is not possible in the sperm cell, so any deterioration of it will affect the fertilization process. In mammals, the mature sperm cells have an increased content of polyunsaturated fatty acids bonded to phospholipids (Aitken et al., 1993; Lenzi et al., 2000). The phospholipids are distributed asymmetrically in the double lipid layer of the plasmatic membrane. The peroxidation of the membrane lipids, caused by the free radicals of oxygen, leads to the formation of hydroperoxides as first products. The hydroperoxides generate reactive forms which influence the activity of certain proteins and modify the physical properties of the membranes by shifting the orientation of the phospholipids and of their dynamics, and consequently, by the alteration of permeability and breaking of the membrane (Gadella et al., 1999). The ATPases located in the membrane are ionic pumps that regulate the intracellular concentration of nutrients and ions (sodium, calcium), their functioning being strongly connected to the membrane fluidity (Aitken and Clarkson, 1987). The loss of membrane fluidity leads to the alteration of the functioning of these pumps and the effect is the accumulation of these ions and finally the destruction of the cell (Aitken et al., 1993). The integrity of the acrosome and of the axoneme can be strongly affected by the lipid peroxidation which thus leads to the decrease in fertilization capacity, correlating negatively with the speed of *in vitro* fertilization (Zabludovsky et al., 1999).

2.3.1.2. Proteins

Recent studies have demonstrated that proteins are the initial targets of free radicals, before lipids or DNA (Dean et al., 1997; Du and Gebicki, 2004). The hydroxyl-type reactive oxygen species have the ability to bind covalently to proteins causing their aggregation. Subsequently, the modified proteins become targets of proteolysis, either in proteosomes or in lysosomes (Yu, 1994). This way, protein transporters or protein canals can be affected, and subsequently, transmembrane ionic gradients and thus the cell functions (Thomas et al., 1995).
Apart from the alteration of the membrane structure and fluidity, the peroxidation induced by ROS can also affect the thiol groups in the protein structure, changing

thus the sperm cell functions by increasing the susceptibility to be attacked by macrophages (Alvarez and Storey, 1989; Thomas, 1995). The oxidation of the tyrosine remains from the protein molecules induces the formation of crossed bonds between proteins, and thus modifications of the protein conformation which lead to the loss of the physiological functions of protein effectors (Beal, 2002). The oxidation of the lysine, proline, arginine and threonine remains leads to the formation of carbonyl-type protein groups. The cell can follow the path of proteolysis or of apoptosis, depending on the accumulation of carbonyl groups (Farout and Friguet, 2006).

2.3.1.3. Carbohydrates

The carbohydrates bonded either to proteins or to lipids are also sub-layers for peroxidation (Yu, 1994). The ions of transitional metals favor the self-oxidation of carbohydrates and generate carbonyl radicals and hydrogen peroxide (Dean et al., 1997).

2.3.1.4. The DNA

The research regarding male infertility caused by oxidative stress has shown a deterioration of the DNA integrity in the sperm nucleus by the modification of the nitrogenous bases or DNA fragmentation. The reactive species, such as hydroxyl radical, can cause severe alterations of the DNA by the irreversible modification of the nitrogenous bases (Evenson et al., 2002; Agarwal and Said, 2003).

The DNA oxidation may lead to crossed bonds between DNA and proteins (e.g. between thymine and tyrosine). These bonds can interfere with processes such as replication, transcription of DNA repair. Because these crossed bonds occur in the presence of iron (Fe^{+2}), it was suggested to involve the hydroxyl radical in this process (the Fenton reaction) (Nakano et al., 2003). Other studies demonstrated that DNA oxidation can lead to the breaking of one or both catenae, which leads to the inactivation of certain genes (van Gent et al., 2001), of certain proteins (Moldovan and Moldovan, 2004) or to apoptosis (Ritch et al., 2000). The deterioration of DNA and the presence of apoptosis markers are connected to a process of deficient maturation either at nuclear level (protamination) or at cytosolic level (Sakkas et al., 2002). It was determined that there is a correlation between the apoptosis markers and the sperm parameters for inferior quality (Taylor et al., 2004). The final products of DNA peroxidation, 8-oxoguanine, 8-hydroxydeoxyguanosine and thymine glycol can be easily detected in urine (Bohr, 2002).

2.3.1.5. Mitochondrial DNA

The mitochondrial DNA does not form complexes with histones. The proteins of the mitochondrial complex I (NADH oxidoreductase), III (ubiquinona), cytochrome c oxidoreductase and IV (cytochrome oxidase) are encoded by the mitochondrial DNA. These complexes are located in the internal mitochondrial

membrane and represent the basis of the electron transporting chain. It is considered that the mitochondrion is the main formation place for the superoxide radical, the major source being complex III (Fleury et al., 2002; Chen, 2003). Thus, it can be explained why the mitochondrial DNA is the main target for the ROS attack (Cadenas and Davies, 2000). The mitochondrion contains a number of antioxidants that protect the sub-layers in this organelle against the destructions caused by ROS (examples of antioxidants are: GSH, NADH, thioredoxin, SOD-Mn, catalase, glutathione reductase and glutathione peroxidase) (Bai and Cederbaum, 2001; Fleury et al., 2002).

The ROS attack on the sperm mitochondria leads to the decrease of energy which is also reflected on the decrease of motility (by Lamirande et al., 1997). Other studies suggest that the reactive oxygen species are involved in the inhibition of the process of oxidative phosphorylation and of glycolysis, restricting thus the ATP production by the sperm cell. The enzymatic inhibition can be induced indirectly and by the products of lipid peroxidation, among which malondialdehyde (MDA), small concentrations of these substances being involved in the inhibition of a large number of cell enzymes, of anaerobe glycolysis, and of protein, DNA and RNA synthesis (Twigg et al., 1998; Whittington et al., 1999; Barroso et al., 2000; Kao et al., 2008).

2.4. Antioxidant defense systems at the level of the seminal material

Antioxidants are molecules capable of inhibiting directly the production of free radicals, of limiting their propagation or destroying the reactive oxygen species (Favier, 2003). They also act as protective agents against the cellular oxidative deteriorations induced by ROS or other free radicals (Halliwell and Gutteridge, 1995).

The antioxidants are classified into two categories: enzymatic and non-enzymatic (Silva, 2006).

2.4.1 The enzymatic antioxidant defense

Superoxide dismutase, catalase and glutathione peroxidase are among the enzymatic antioxidants present in the plasma (by Lamirande et al., 1997; Aitken, 1999). The coordinated activity of glutathione peroxidase, glutathione reductase and glutathione play a key role in the protection of sperm cells against the oxidative attack (Sikka, 2004).

The enzymes superoxide dismutase and catalase react with the $O_2^{-\bullet}$ and H_2O_2 radicals, respectively. Glutathione peroxidase, especially phospholipid glutathione peroxidase, eliminates the alkyl ($R^{\bullet}$), alkoxy ($RO^{\bullet}$) and peroxyl ($ROO^{\cdot}$) radicals which are generated in the oxidation systems of the membrane components.

2.4.1.1. Superoxide dismutase (SOD)

The SOD enzyme catalyzes the dismutation of the superoxide radical into hydrogen peroxide and oxygen. It eliminates both the intracellular superoxide

radicals and the extracellular ones and, in cooperation with the catalase and the glutathione peroxidase/reductase system, it prevents the action of H_2O_2 which initiates the formation of hydroxyl radicals (Alvarez et al., 1987; Jeulin et al., 1989).

$$2\ O_2^{-\cdot} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Superoxide dismutase has 3 isoenzymes (Zelko et al., 2002):
- cytosolic copper/zinc superoxide dismutase (Cu/ZnSOD)
- intramitochondrial manganese superoxide dismutase (MnSOD)
- extracellular copper/zinc superoxide dismutase

The enzyme is present both in the seminal plasma and in the sperm cell cytoplasm (Mennella and Jones, 1980; Zini et al., 1993). The cytosolic SOD has the major activity as it is responsible for the elimination of superoxide radicals from the cytosol (Yu, 1994). The Mn-SOD is responsible for the elimination of the $O_2^{-\bullet}$ radicals resulted from the oxidative metabolism in the mitochondria. The SOD enzymes (Mn-SOD and Cu/Zn-SOD) are present in larger quantity in the middle piece compared to the head and the tail of the sperm cells (Alvarez and Storey, 1983a). Its addition to the dilution media protects the sperm cells against the oxidative attack (Kobayashi et al., 1991). Also, SOD prevents the processes of premature hyperactivation and capacitation induced by the superoxide radicals before ejaculation (Peeker et al., 1997). The high levels of SOD induce peroxidative deteriorations by the generation of an increased quantity of hydrogen peroxide, being associated with the deficient functioning of sperm cells, while a reduced activity of SOD is associated to an optimal sperm activity (Aitken et al., 1996).

2.4.1.2. Catalase (CAT)

Catalase detoxifies the intra and extracellular hydrogen peroxide by catalyzing its transformation into water and oxygen, but also by inhibiting NADPH oxidase and thus the inhibition of producing the superoxide (Jeulin et al., 1989). The activity of the catalase was detected both in the seminal plasma and in the human sperm cell.

$$2\ H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

The studies demonstrated that there is a significant correlation between the deficiency of the activity of seminal catalase and human male fertility (Alkan et al., 1997; Miesel et al., 1997; Sanocka et al., 1997). The evaluation of the toxicity of hydrogen peroxide, appreciated by the decreased motility, on the bull, ram, mouse, rabbit and human sperm cells demonstrated the existence of significant

differences among species, the rabbit semen being the most enduring (Storey, 2008).

2.4.1.3. The glutathione peroxidase / reductase system (GPx/GR)

The importance of this enzymatic system is central in the antilipoperoxidative defense (Dandekar et al., 2002). Glutathione peroxidase (GPx) is an antioxidant enzyme that contains selenium in the catalytic centre. The presence of selenium in the structure of this enzyme can explain its importance in male infertility. Having glutathione reduced (GSH) as electron donor, the enzyme eliminates the peroxyl radicals from various peroxides, including the hydrogen peroxide.

$$2GSH + 2HO^{\cdot} \xrightarrow{GPx} GSSG + 2H_2O$$

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

The activity of the enzyme glutathione peroxidase (selenium dependent GPx) in the seminal plasma varies considerably among species:

-in bull, it has an important protective role against ROS attack (Kantola et al., 1988), as the seminal plasma contains high levels of selenium.

-the GPx activity is relatively low in the human and ram seminal plasma and absent in the boar and stallion seminal plasma, where the selenium level is substantially lower compared to the bull one (Saaranen et al., 1989).

-in birds, both the sperm cells and the seminal plasma contain SOD and GPx (Surai et al., 2000)

Recent studies have shown that individuals with abnormal enzymes from the phospholipid glutathione peroxidase family (PHGPx) display anomalies of the sperm mitochondria. The abnormal expression of this enzyme can lead, in humans, to oligoasthenozoospermia (Imai et al., 2001). The resumed PHGPx activity leads to the improvement of the structural integrity, of motility and viability of human sperm cells (Foresta et al., 2002).

Glutathione reductase

Glutathione reductase is involved in the regeneration, at intracellular level, of the glutathione reduced by the reduction of the oxidized glutathione (GSSG) to GSH (Storey et al., 1998). The selective inhibition of this enzyme reduces the GSH amount needed to maintain the GPx activity by exposing the sperm cells to oxidative stress (Williams and Ford, 2004).

$$GSSG + NADPH + H^{+} \longrightarrow 2GSH + NADP^{+}$$

2.4.2. The non-enzymatic antioxidant defense

Seminal plasma contains a high number of non-enzymatic antioxidants, among which, vitamin C, vitamin E, glutathione reduced, urate, ubiquinone and bilirubin,

with a role in the capture and elimination of free radicals (chain-breaking) (Agarwal and Saleh, 2002). Other non-enzymatic antioxidants are present in the sperm cell, either in the cytosol (vitamin C, taurine, hypotaurine, vitamin A, pyruvate and GSH), or in the structure of the sperm cell membranes (vitamin E, coenzyme Q) and they have a preventive role (Agarwal et al., 2005).
Vitamins A, C and E have a role in the prevention of lipid peroxidation and in DNA protection against free radicals. In patients infected with HIV, the administration of these vitamins leads to the decrease in the number of modified bases in the DNA structure and in the level of malondialdehyde (MDA) (Jaruga et al., 2002). Vitamin E and coenzyme Q are lipophilic and therefore can neutralize the lipid radical derivatives in the membranes (Navarro et al., 1998). Coenzyme Q is located exclusively in the internal mitochondrial membrane. Both vitamin C and coenzyme Q have the property to regenerate oxidized vitamin E and can neutralize the hydrogen peroxide present in the hydrophile media (cytosol or extracellular fluids) (Beyer, 1994). Certain carotenoids (e.g. β-carotene) act as eliminators of the superoxide radicals, protecting the cells against lipid peroxidation (Paiva and Russell, 1999; Sies and Stahl,1995).

2.4.2.1. Vitamin E (α-tocopherol)

Vitamin E is a primary liposoluble antioxidant in biological systems and the main antioxidant in the membranes structure. Its concentration in the membranes is rather small (around 0.05-0.1 nmol/mg protein, less than 1/1000-2000 membrane phospholipids). On the other hand, the speed of generating lipid radicals in the membranes can, under certain conditions, reach values of 1-5 nmol/mg protein (Sikka, 2004). Under normal conditions, due to the non-enzymatic regeneration cycle that occurs in the membranes and to the enzymatic regeneration path which occurs in the mitochondrion and microsomes, the vitamin E deficiency is rather rare. However, under conditions of oxidative stress (centrifuging, "cold shock"), the addition of vitamin E to the dilution medium in various concentrations led to the sustaining or improvement of the sperm cell motility (Verma and Kanwar, 1999; Askari et al., 1994). The research developed within our lab on ram seminal material demonstrated that the addition of antioxidants (vitamin E 1.0mM) to the dilution-freezing medium increases the motility and the structural and functional integrity of the sperm cell, but also reduces the level of lipid peroxidation, confirming the fact that antioxidants are efficient in the prevention of the rapid drop in motility which normally occurs at cooling and that they maintain motility under conditions of oxidative stress. Also, it was demonstrated that there is a significant negative correlation between the antioxidant capacity and the level of lipid peroxidation (Anghel et al, 2010, 2011). Similar results were obtained on cryopreserved buck seminal material (Anghel et al, 2010). Vitamin E neutralizes efficiently all the three important types of ROS (the superoxide and hydroxyl radicals, and hydrogen peroxide) (Agarwal et al., 2004). It reacts directly with the

peroxyl radicals ($ROO^{\cdot}$) which it transforms into lipid hydroperoxydes which will be subsequently eliminated by the glutathione proxidases. Also, it interrupts the chain of lipid peroxidation by ending the propagation of the chain reaction (Wayner et al., 1987; Agarwal and Prabakaran, 2005).

2.4.2.2. Vitamin C (ascorbic acid)

Vitamin C is the main antioxidant in the seminal plasma of fertile males, providing over 65% of the total antioxidant activity. The concentration of ascorbate in the human seminal plasma is 10 times higher compared to blood plasma. However, in large doses, in the presence of certain cations such as iron or copper, vitamin C leads to an increase in the production of ROS (Agarwal et al., 2004). It is a hydrophile molecule and it acts as an antioxidant by reacting with $O_2^{\cdot -}$, $OH^{\cdot}$ and lipid hydroperoxides. Vitamin C acts synergically with vitamin E by reducing the radical α- tocopheryl to α-tocopherol (Jialal et al., 1990), the major role being to prevent the lipid peroxidation (Wang et al., 2002).

2.4.2.3. Glutathione is considered one of the most important components of the antioxidant defense in the living cell. Glutathione reduced (GSH) is a tripeptide with role in the elimination of the hydroxyl radicals and of the singlet oxygen. Glutathione reduced acts as hydrogen donor, being thus a substrate for certain antioxidant enzymes, including Se-dependent glutathione peroxidase (GPx) which decomposes the hydrogen peroxide and the glutathione S-transferases, GSTs, which catalyze the conjugation reactions between glutathione and the cellular components modified by the ROS attack. When the oxyradicals are present in large quantities, the GSSG concentration increases, and the glutathione reduced / glutathione oxidized (GSH/GSSG) ratio decreases. This ratio is often used as an indicator of the level of oxidative stress in the cells. The maintaining of glutathione as reduced, thus the maintaining of a reducing medium in the cell, is crucial for the organisms that periodically go through states of oxidative stress. By determining the GSH and GSSG concentration, we can evaluate both the level of the components of the antioxidant defense and the degree of oxidative stress in various metabolic systems.

Glutathione and selenium have an essential role in the formation of phospholipid hydroperoxide glutathione peroxidase, an enzyme present in the middle piece of the sperm cell. The glutathione deficiency may lead to the instability of the middle piece, resulting thus an alteration of motility (Lenzi et al., 2002).

2.5. The effect of conservation on the viability and motility of the sperm cell

The reduced fertility associated to artificial insemination with frozen semen is attributed to the processes that occur during freezing, when 10-50% of the sperm cells do not endure this process and die (Watson, 2000). Under the conditions of natural mating, the semen is exposed mostly to anaerobe conditions which limit

the generation of ROS. However, under preservation conditions, the semen is exposed to oxygen and the various stages during processing may also lead to the increase of ROS production and decrease of the antioxidant defense (dilution of the seminal plasma, deterioration of enzymes during freezing). These processes cause the cell deterioration due to temperature changes, formation of ice crystals, DNA lesions, oxidative stress and toxicity of the cryoprotectors (Ball and Vo, 2001).

In order to be considered viable and with fertile potential, the sperm cells must have normal morphology and metabolic activity, and intact membranes.

The reduction of temperature during cooling and freezing may affect motility, the integrity of the structural components and of the sperm cell respiratory capacity. The osmotic and oxidative stress set in during the freezing-thawing process causes deteriorations of the plasmatic and acrosomal membranes, but also induces precocious capacitation (Zamfirescu and Anghel, 2010).

Motility is an important and indispensible characteristic of the sperm cell because it must move through the female reproductive tract and reach the fertilization situs. The studies demonstrated that the level of ROS correlate negatively with the sperm cells motility. The ability of the sperm cell to maintain motility in the presence of ROS is tightly connected to the antioxidant capacity and the protective role of the epididymal fluids and seminal plasma. The *in vitro* experiments showed that motility can be affected temporarily or definitively (Cayli et al., 2004). Excessive ROS quantities lead to the rapid drop of the ATP level and the result is the reduction of the axonemal proteins phosphorylation speed, which causes a temporary alteration of motility. The peroxidative deteriorations of the sperm cell membrane and of the axonemal proteins are the cause for the permanent alteration of motility (Agarwal et al., 2005).

According to the studies of Aitken and Fisher (1994), the biosynthethis capacity of the sperm cell is limited, which makes impossible the replacement of the deteriorated molecules. Moreover, the antioxidant enzymes are located especially at the middle piece, most of the membrane that covers the head and the tail of the sperm cell remaining unprotected. This is why the seminal plasma will have the most important role in the protection of the sperm cell against the attack of ROS generated either by the sperm cell or by the semen phagocytes.

The limited degree of knowledge in terms of the action of oxidants and antioxidants in the semen of farm animals may be responsible for the inefficient results obtained in several species after the use of semen subjected to the procedures of cryopreservation and techniques of assisted reproduction such as artificial insemination or *in vitro* fertilization. This is why the studies involving oxidative stress caused by the manipulation procedures may provide solutions for the use of antioxidants in order to optimize the reproduction techniques. The antioxidant properties of the media normally used for the preservation of semen

are little known, and the effects of a supraproduction of ROS in these solvents are rather little characterized.

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