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The potential role of aminoguanidine against oxidative stress in boar spermatozoa

Diploma Thesis

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Declaration

I declare that the Diploma Thesis "The potential role of aminoguanidine against oxidative stress in boar spermatozoa" is my own work and that I used only sources mentioned in the Bibliography section.

Prague, 12. 4. 2018

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Bc. Martin Kadlec

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The potential role of aminoguanidine against oxidative stress in boar spermatozoa

Summary

Currently, modern pig reproduction is mainly performed through artificial insemination (AI), which is dependent on the production of high quality semen doses. Due to specific attributes of boar semen, the preparation of insemination doses represents an important challenge. One of such aspects is the unique composition of boar spermatozoa membrane, which contains high amount of polyunsaturated fatty acids (PUFA) making the spermatozoa highly sensitive to reactive oxygen species (ROS). This thesis is the first work dedicated to testing of the antioxidant capacity of aminoguanidine (AG) compound directly supplemented to boar semen samples under induced OS. In order to reduce male variability, the semen was mixed into pool for each of 5 replicates. Totally, semen of 15 boars was used. Oxidative stress was induced by incubating sperm samples with 0.05 mM $FeSO_4$ plus 0.5 mM sodium ascorbate (Fe^{2+}/ascorbate). The aminoguanidine was supplemented at following concentrations: 0.1 mM (Ag0.1-ox), 1 mM (Ag1-ox), and 10 mM. (Ag10-ox) All the samples were incubated at 38°C and evaluated at 2 and 3.5 hours except control sample, which was also evaluated at 0 hour. The assessment of motility included subjective evaluation and measurement of kinetic parameters by CASA. To establish the acrosomal status, normal apical ridge (NAR) test was performed. Moreover, the membrane status was analysed by PI/CFDA and HOST-eosin tests. Motility evaluation showed that AG was most effective at concentrations 1 mM and 10 m. Total sperm motility at 3.5 hours of sample incubation was almost 3 times higher in Ag10-ox than in control oxidised treatment (Ctr-ox). Moreover, several other kinetic parameters were improved in Ag1-ox and Ag10-ox: STR, VAP, VCL and VSL ($p>0.05$). Remarkably, the last three named parameters were more than two times higher compared to Ctr-ox. Additionally, all samples supplemented with AG showed higher percentage of acrosome and membrane intact sperm cells than Crt-ox ($p>0.05$). However, host-eosin test was only one that did not demonstrate significant difference between AG treatment and control group. Overall, AG showed substantial antioxidant capacity which resulted in increased motility and membrane integrity of boar spermatozoa under oxidative stress.

Keywords: aminoguanidine, antioxidant, boar spermatozoa, oxidative stress, reactive oxygen species

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1 Introduction

Since the 80's of twentieth century, the artificial insemination (AI) has become an important tool in pig industry (Rodríguez-Gil and Estrada, 2013). The main advantages of AI include the use of semen from boars of high genetic value to inseminate large number of sows, while high hygienic standards are maintained to prevent venereal disease transmission (Yeste, 2017). Although the first attempts to perform AI in swine were done at the beginning of the 20th century, its implementation for commercial use did not occur until several decades later. The development of more efficient semen extenders and the advances in AI techniques made AI in swine economically enough profitable for its application in commercial farms. Modern pig production is characterized by the utilisation of AI, which is practised around 90 % of exploitations in European countries (Rodríguez-Gil and Estrada, 2013).

Several basic issues had to be solved to efficiently handle collected semen, one of them is the generation of reactive oxygen species (ROS). It is well known that sperm cells are sensitive to free radicals like the ROS. The in vitro manipulation during assisted reproductive techniques increases the risk of exposing sperm cells to unnatural levels of ROS which afterwards may cause the oxidative stress in the cell. Although the sperm cell is endowed with biological systems to prevent the accumulation of ROS above the physiological levels necessary for several sperm functions, the ROS overproduction and consecutive oxidative stress may lead to cell damage. Oxidative stress is one of the most important factors contributing to low semen quality as it damages all cellular components including lipids, proteins, nucleic acids, and sugars (Bansal and Bilaspuri, 2011). Basically, whole sperm cell is altered and so are its functions. As the boar sperm cell is a very specifically constituted cell, even the smallest alteration may lead to its dysfunction.

Therefore, antioxidants are used to increase sperm protection against oxidative stress. They are compounds with ability to dispose, scavenge or suppress the formation of ROS. Some antioxidants are naturally present in the seminal plasma (SP) and in the cytoplasm of sperm cell. Yet these are not sufficient for the protection of stressed sperm cells during AI procedures, so more antioxidants are mixed into the semen doses. Natural antioxidants help the cell by neutralizing excessive ROS. They are composed of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR). Non-enzymatic antioxidants include dietary supplements such as vitamin C, zinc, taurine and others (Bansal and Bilaspuri, 2011).

The specific composition of sperm cell is crucial for performing its main task: the oocyte fertilization. Starting with the surface of boar sperm cell, there can be found specific differences to the somatic cells. The characteristic aspect of a sperm cell is the highly heterogenic surface. The lipid and protein composition of plasma membrane is specific for different regions of the sperm cell, so called membrane domains. This feature reflects the distinct surface qualities and the unique structure of this membrane. Moreover, the organization of different regions of sperm cell membrane set during the development in male genital tract changes within the female tract during the capacitation process through which the sperm cell acquires the fertilization capacity (Bonet et al., 2013).

As the plasma membrane is the frontier protecting the inner environment of the cell and allows the interaction between cell components and external environment, its alterations often affect the other essential organelles of sperm cell. To this purpose, different techniques are used in laboratories to evaluate the status of sperm cells and the semen of a given boar. These techniques are based on the knowledge of sperm cell structure and physiology.

2 Scientific Hypothesis and Objectives of the Work

The hypothesis of the present work was that aminoguanidine may protect boar spermatozoa against oxidative stress. The objective was to test the effects of aminoguanidine supplementation on sperm motility, kinetics, plasma membrane and acrosome integrity on boar semen samples under induced oxidative.

3 Literature Overview

3.1 AI and boar reproduction

The usage of AI in porcine industry is typical for developed western countries, yet its exploitation varies among different countries. The technique is widely extended across the Europe accounting for up to 80 % of reproductive gilts/sows in various countries. On the other hand, the use of AI in the US accounts for about half of the porcine reproduction.

Following the statistics at the start of second millennium, about 19 million of inseminations are performed across the world. Almost all inseminations are performed using the boar semen preserved at liquid state under 15 – 20 °C and 85 % of these inseminations are carried out the day after semen collection (Johnson et al., 2000).

The long-term storage of boar spermatozoa represents a difficult task to perform due to various boar semen characteristics such as the susceptibility to cold shock, which is the effect of rapid decline of temperature under 15°C. Due to specific membrane composition, boar spermatozoa are extremely sensitive to temperatures under 15°C which are irreversibly lowering its fertilization capacity. Therefore, the boar semen needs to be kept at moderately reduced temperatures. In consequence, the metabolism is only partially attenuated and the microbial conditions are more difficult to control (Gadea, 2003).

Moreover, specific metabolism changes are induced by diluents for preparation of insemination doses. In response to the dilution, spermatozoa increase their activity ensued by loss of motility and increase in membrane damage. This process is known as dilution effect (Johnson et al., 2000). The damage done to spermatozoa is even greater due to increased production of free radicals during in vitro storage (Hammerstedt. 1993).

Several dilutions used for liquid storage of boar semen are now widely used. Among these, Beltsville Thawing Solution (BTS) and Androhep diluents are the preferred ones. BTS diluent contains small amounts of potassium which helps to prevent intracellular potassium loss preserving motility during storage. It is classified as short-term diluent used for semen preservation for up to 3 days. On the other hand, Androhep is characterized as long-term diluent able to preserve semen doses for more than 4 days. This diluent contains Hepes, a substance used to pH regulation, and bovine serum albumin, a substance used to reduce effects of oxidative stress (Gadea, 2003).

Nevertheless, the research around the AI of pigs is focusing on finding more effective ways of long-term storage which could enable expedition of boar semen doses over long distances and make this technique more accessible. The investigation of novel protective substances may improve the protocols for semen storage and enhance the sperm fertility.

3.2 Boar semen

Boar ejaculate is creamy, slightly yellowish or greyish fluid which differs in various basic parameters compared to other livestock. The volume ranges averagely from 150 to 300 ml being greater than in any other domestic mammal. Naturally, the ejaculate is less concentrated, yet total sperm count reaches up 100×10^9 per ejaculate. The ejaculation of boar lasts up to 30 minutes which is much longer in comparison with other species of productive interest (Bonet et al., 2013[b]). The temperature of ejaculated semen is 37°C and pH varies from 6.85 to 7.9. The sperm fraction of boar ejaculate forms 10-30 % of total volume, the rest falls on the liquid fraction consisting of SP, whereas 90 % of SP originates from accessory sex glands. Three fractions may be distinguished in the boar ejaculate. The pre-spermatic fraction is produced by prostate, seminal vesicles and Cowper glands and does not contain spermatozoa. The volume of this fraction reaches approximately 10-15 ml. The second, spermatic fraction is the one that is collect during preparation of semen doses. The volume of this sperm-rich fraction is 150-200 ml and it contains prostate and seminal vesicle secretions. Post-spermatic fraction is of about same volume as the previous one, but it contains only substantially less spermatozoa. It is secreted by Cowper glands and the prostate; its role is to stimulate spermatozoa in female reproductive tract and therefore it is not collected (in order to maintain as low metabolism as possible) although it is the fraction with the greatest protective capacity due to high content of antioxidants (Sancho and Vilagran, 2013).

SP consists of carbohydrates, lipids, amino acids and proteins ensuring the nourishment, protection and transition of spermatozoa in female reproductive tract. The energy sources that help to maintain the motility of spermatozoa are fructose, glucose and sorbitol in boar SP (Peña et al., 2006; Sancho and Vilagran, 2013; Bonet et al., 2013). The protection of spermatozoa by SP is carried out through the action of antioxidants. Boar SP is characterized by high contents of SOD but also very low concentrations of catalase (Kowalowka et al., 2008).

3.2.1 Boar spermatozoon

The general organization of major structures of sperm cell is primarily given by the testicular development. This development results in typical sperm cell that can be divided into 3 main parts: a head, a midpiece, and a tail. This organization does not change after the spermatozoon leaves the testis. (Gadella, 2017) Yet, the testicular spermatozoa are not ready to fertilize an oocyte. First, the cytoplasmic droplet remains attached to the tail which is problematic since it restrains the movement of a sperm cell through the protective layers of an oocyte. But most importantly, spermatozoa leaving the testis are immotile (Dacheux et al., 2005) and do not possess surface factors and protein complexes that are requisite for the sperm-oocyte fusion (Burkin and Miller, 2000).

The formation of a boar sperm cell last about 34-36 days and it can be divided into two main phases: spermatocytogenesis, meiosis, and spermiogenesis. Spermatocytogenesis includes the transition of spermatogonia to early round spermatids through mitotic and meiotic divisions (Pinart et al., 1999, 2000). More specifically, diploid spermatogonia are divided up during the mitotic phase several times to give rise to primary spermatocytes (Garcia-Gil, 2004) and to further maintain a stem cell pool consisting of other spermatogonia (Ljiljak et al., 2012). Some of spermatogonia continue dividing and differentiate into primary spermatocytes (Garcia-Gil, 2002; Ljiljak et al., 2012). Primary spermatocytes enter the first meiotic division giving rise to haploid secondary spermatocytes. These undergo the second or equatorial meiotic division resulting in four round haploid spermatids (Pinart et al., 1999). Subsequently, the round spermatids differentiate into mature spermatozoa during the process of spermiogenesis (Fig. 1).

The differentiation of spermatid into mature spermatozoon and its release to the lumen of seminiferous tubules takes 14 days in case of boar spermiogenesis. It is possible to distinguish up to nine types of spermatids by their morphology. These morphological types correlate with four differentiation phases: Golgi phase, cap phase, acrosome phase and maturation phase (Pinart, 1997; Pinart et al., 2000).

The formation of proacrosomal vesicles is the most characteristic aspect of Golgi phase. These vesicles are derived from Golgi apparatus and they fuse together to form acrosomal vesicle. The basal plate is formed at the end of this phase. The cap phase is characteristic by the extension of acrosomal vesicle over the nucleus forming a cap-like structure, the acrosome. This process is followed by the acrosome phase. During this phase the microtubules reorganize around the surface of nucleus forming a perinuclear manchette. This structure takes part in the nuclear elongation which is typical for this phase. During the last (maturation) phase,

spermatids possess well differentiated nucleus and acrosome. The nuclear manchette disappears and the annulus (Jensen's ring) is formed. Residual cytoplasm forms spherical mass between head and tail forming a typical structure for testicular sperm cell, so called proximal cytoplasmic droplet (Bonet et al. 2013). Along the differentiation of last stages of spermatogonia, the process of spermiation takes part. During spermiation, spermatids loose several organelles such as Golgi apparatus, endoplasmic reticulum or ribosomes which together form residual bodies which are subsequently phagocytosed by Sertoli cells (O'Donnell et al., 2011)

After leaving testes, mature spermatozoa are still unable to fertilize the oocyte. They travel through the rete testis into the epididimal ductus ending up in the epididymis where the process called epididymal maturation starts. The epididimal maturation lasts about 2 weeks in boars (Dacheux et al., 2005). During this process sperm surface is strongly modified, cytoplasmic droplet is disposed, and metabolism is changed, and the sperm cell becomes motile.

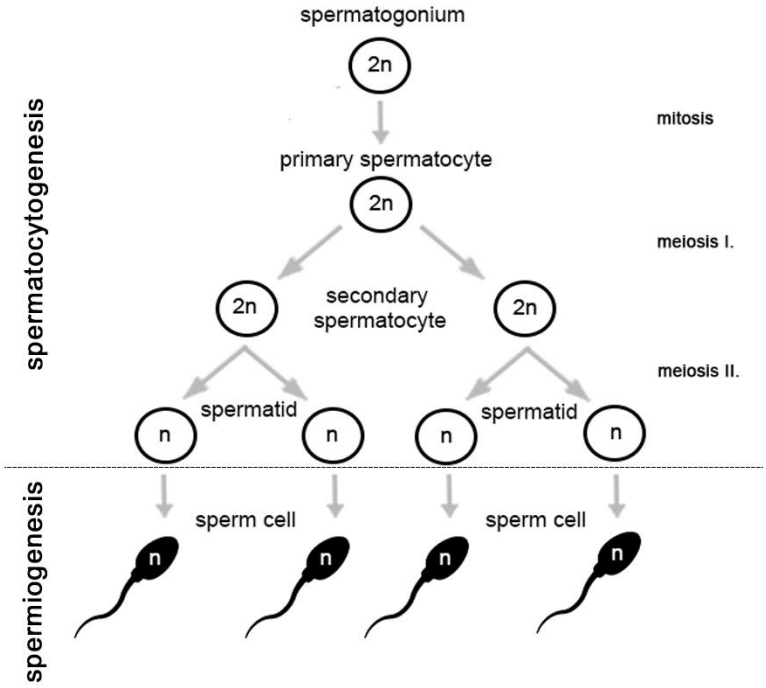

Figure 1. A simple scheme of spermatogenesis demonstrating the development of spermatogonium into a mature sperm cell through the processes of spermatocytogenesis and spermiogenesis. 2n: diploid cell; n: haploid cell. Kadlec, 2017.

Boar mature spermatozoa are relatively small elongated cells with length of about 44 µm (Briz 1994; Holt et al. 2010). Their morphology is designed according to specific aspect of their

function. Spermatozoa contain the male DNA and few more elements required to reach and fertilize oocyte. By the first look, it is possible to distinguish two main parts of a sperm cell: the head and the tail (Fig. 2). The head contains part of the male genetic information and acrosome, the structure necessary for the fusion with an oocyte. The tail gives the cell the ability to move, plus it can be considered as the cell reactor as it also contains mitochondria generating energy necessary for the sperm transportation. Both regions are connected by the neck and can be further subdivided into various cellular components, each with specific function. Such compartmentalization of sperm cell structure enables the cell to complete all the objectives it must undergo (Briz and Fàbrega, 2013).

3.2.1.1 Sperm head

Boar sperm head is a small and compact and there can be found a very limited number of components. Two main organelles may be distinguished in the head, the nucleus and the acrosome. Other structures located in the head are the postacrosomal dense lamina, the subacrosomal space, and the perinuclear fibrous material. The nuclear shape is given by the sperm genotype and it is specific for different species. Ejaculated healthy boar spermatozoon contains flattened nucleus with ovoidal shape. The length of the nucleus is about 6,6 μm and the nuclear thickness is different in the proximal and distal cephalic regions reaching from 220 nm to 320 nm respectively. Nucleus is a consistent structure containing extremely condensed chromatin fibres. The nucleus is accompanied by the sac-like acrosome located on the apical end of the head (Briz and Fàbrega, 2013).

Acrosome is a vesicle bounded by a membrane. It covers a large part of the nucleus forming a cap-like structure over the nucleus. There can be distinguished two parallel membranes in the acrosome. The inner membrane overlays the nuclear envelopment and continues to the posterior margins verging into outer membrane lying beneath the plasmalemma. The space between those two membranes is filled with amorphous material forming the acrosomal matrix. It is mainly composed by hydrolytic enzymes. Acrosome is divided into three segments: the apical segment, the principal segment and the equatorial segment. These segments vary in the expansion with apical segment being the most expanded one (Briz and Fàbrega, 2013).

The postacrosomal dense lamina (PDL) is a homogenous layer lying under the plasmalemma and coincides with the part of the nucleus that is not covered by the acrosome

membrane. PDL enclose the so called postacrosomal region of the head. In difference to the outer acrosomal membrane, PDL is firmly attached to plasma membrane. Together with inner acrosomal membrane is sets the boundary of the subacrosomal space lying between those two layers and the nucleus. This space is filled with perinuclear fibrous material coating the nucleus (Briz and Fàbrega, 2013).

3.2.1.2 Connecting piece

The connecting piece, also called the neck, is a short linking segment between the nucleus and the tail. The neck is firmly attached to the distal end of the nucleus. The neck can be divided into several major parts: the basal plate, the laminar bodies, the capitulum, the segmented columns, the basal body, and the axoneme.

The composition of basal plate is quite like the one of postacrosomal dense lamina, and it is adhered to the outer membrane of nuclear envelope. The laminar bodies are formed by the excrescent nuclear envelope. The envelope forms folds containing chromatin-free nuclear space (Briz and Fàbrega, 2013).

3.2.1.3 Tail

There can be plainly distinguished three segments in the spermatozoon tail: the midpiece, the principal piece, and the terminal piece. The midpiece is also called mitochondrial region and it starts at the distal end of connecting segment and ends at the annulus, also called Jensen's ring. Main parts of the midpiece are: the axoneme, the mitochondrial sheath, the outer dens fibres, and the peripheral granules. The axoneme is located at the central axis of the midpiece and it is stretched along the whole tail. The mitochondrial sheath is positioned right beneath the plasmalemma and contains elongated mitochondria which are deployed in a spiral pattern along the underlying axoneme. The outer dense fibres represent filamentous cytoskeletal structures composed of electrodense material located between the mitochondrial sheath and axoneme. They are extended along the midpiece and the principal piece. The proximal part of midpiece is the only location where can be found the peripheral granules consisting of electrodense material (Briz and Fàbrega, 2013).

The principal piece represents the longest section of the tail which is extended from the Jensen's ring to the proximal end of the terminal piece. There can be found several structures in principal piece: the fibrous sheath, the outer dense fibres, the axoneme, and the Jensen's ring. The fibrous sheath replaces the mitochondrial sheath of the principal piece. It is composed of

dorsal and ventral column united by series of ribs equally distributed around the principal piece. The dense fibres can be found only in the first third of the principal piece.

The terminal piece is the shortest part of the tail consisting only of the axoneme enclosed by the plasmalemma (Briz and Fàbrega, 2013)

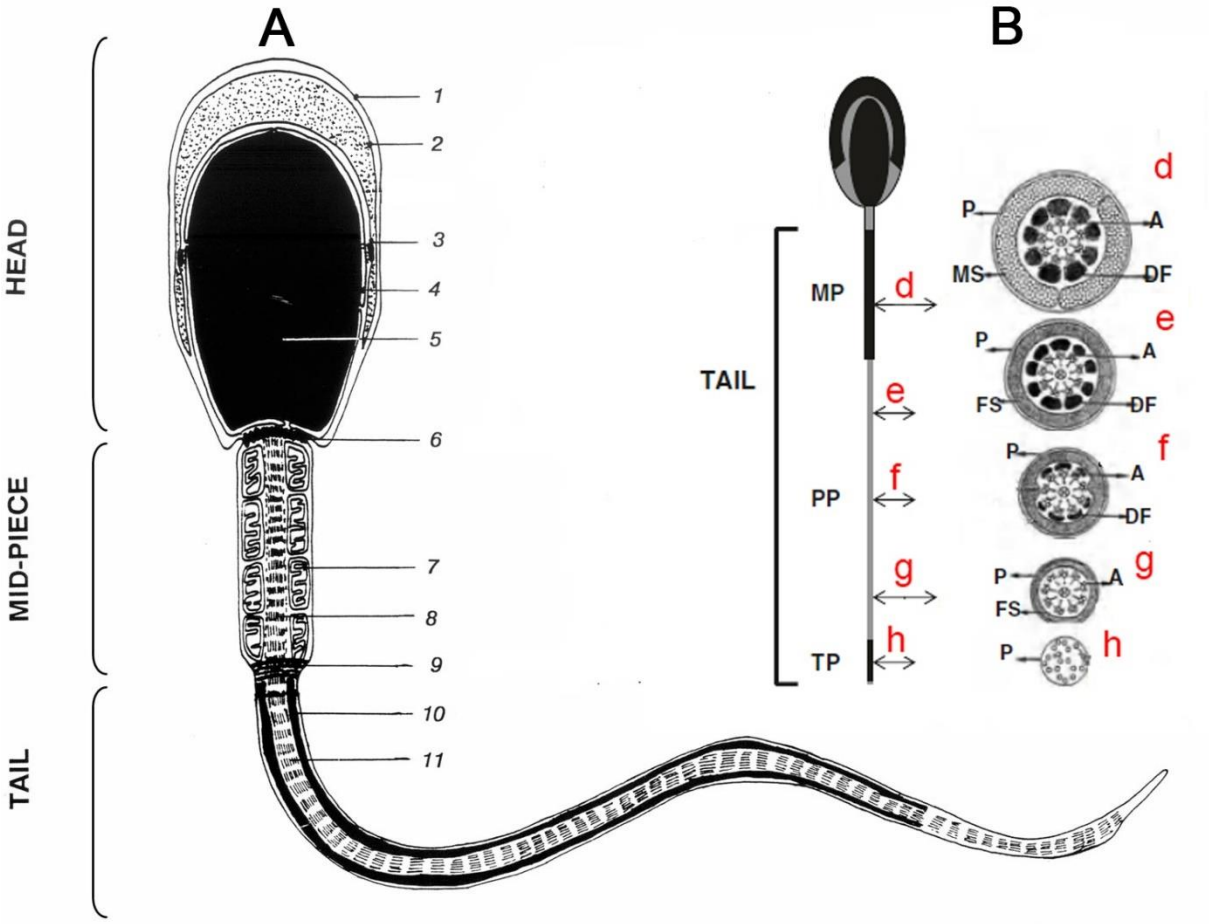

Figure 2. The diagram chart of boar spermatozoon. **(A)** A sectional view of the sperm cell. plasma membrane (1); outer acrosomal membrane (2); inner acrosomal membrane (3); nuclear envelope (4); nucleus (5); posterior ring and neck (6); mitochondria (7); proximal part of the flagellum (8); annular ring (9); fibrous sheath (10); axoneme and outer dens fibers (11). (Gadella et al., 2008). **(B)** Boar spermatozoon tail and its cross sections. d: midpiece (MP); e: proximal region of principal piece (PP); f: Intermediate region of principal piece; g: distal region of principal piece; h: terminal piece (TP); P: plasmalemma; A: axoneme; DF: dens fibres; MS: mitochondrial sheet; FS: fibrous sheet. (Bonet et al., 2013).

3.2.2 Sperm cell membrane

Although there is reduced number of cellular organelles and amount cytosol in a sperm cell, its plasma membrane structure is more complex, than the majority of other somatic cell types. Due to the relatively high number of different membrane proteins and lipids, the sperm

cell membrane may be defined by high level of molecular mosaicism (Briz and Fàbrega, 2013). Additionally, the sperm cell membrane must undergo a lot of changes during the maturation and capacitation in the female reproductive tract (Brewis and Gadella, 2009). This membrane reorganization is characterized by the change in the protein and lipid composition and mutual proportion of both (Flesch and Gadella, 2000).

Yet, the composition of the membrane is not the same along the sperm cell. Five membranes domains are set in the sperm cell, each closely associated with underlying cell compartment and involved in different aspects of cell function. Three of them can be distinguished on the head. One domain is covering the apical ridge, another one is covering the principal segment, and the last one is found in the equatorial section of the sperm head. Two additional membrane domains can be distinguished on the tail, one over the midpiece and the other one over the principal piece of the tail (Briz and Fàbrega, 2013). The membrane specialization allows the independent interaction of underlying structures (Curry and Watson, 1995). Specifically, the acrosome apical section is responsible for recognition and binding to the oocyte, the principal section of the acrosome is responsible for the acrosome reaction (AR), and the acrosome equatorial section is necessary for the fusion with the oocytes plasma membrane. The reason of the presence of two different plasmatic membrane domains on the tail is yet not understood. Nevertheless, it is quite possible, that the different membrane domains of the tail ensure the optimal sperm motion (Brewis and Gadella, 2009)

3.2.2.1 The lipid composition of boar sperm membrane

As Singer and Nicolson described in 1972, the cell membrane as a lipid bilayer with phospholipids and cholesterol being the most important lipids. The lipids influence greatly the functional properties of a cellular membrane. In particular, proportion of lipids present in membrane have great impact on the membrane fluidity, fusion behaviour and interaction with proteins.

Boar sperm membrane is characterized by high content of ether-linked phospholipids and lipids with polyunsaturated aliphatic chains (Evans et al. 1980). On the other hand, relatively less cholesterol is found in the boar sperm membrane with ratio to phospholipids of about 0.12. Seventy percent of lipids are phospholipids, whereas the choline is the most abundant one followed by steroids (Nikolopoulou et al. 1985). Other 25 % of sperm membrane lipids are represented by neutral lipids. The glycolipids represent the 5 % of remaining lipids present in sperm membrane (Mann and Lutwak-Mann 2012). There is higher-percentage of n-6 than of n-

3 polyunsaturated fatty acids (PUFA), whereas the docosapentaenoic acid is the most abundant one. Due to the high content of PUFA in phospholipid, the boar sperm cell is more sensitive to the lipid peroxidation caused by ROS (Am-in et al., 2011).

3.3 Oxidative stress

Oxidative stress may be defined as the state when the ROS cause increased cellular damage. This may happen when the biological antioxidant systems are failing to reduce ROS production to physiological levels. Under physiological conditions, ROS are involved in crucial reproductive processes such as sperm capacitation, hyperactivation, sperm-oocyte interactions and others. On the other hand, the increased amount of ROS may be detrimental for spermatozoa and lead to infertility (Fig. 3). All cellular components are potential targets of oxidative stress (Bansal and Bilaspuri, 2011). To mention several examples of detrimental effects of ROS, it should be mentioned that ROS increase DNA fragmentation, modify the cytoskeleton and disrupt the axoneme development (Cerolini et al., 2000). Following the explanation of Awda et al. (2009), ROS damage the DNA through the effect of hydroxylation causing detrimental changes that may be mutagenic or lethal for the cell. Various ways of motility impairment by ROS are hypothesized. ROS may interfere with oxidative phosphorylation and glycolysis leading to ATP depletion. ROS may also negatively affect the phosphorylation of axonemal proteins, which is necessary for sperm motility. ROS also make the cell insensitive to Ca^{2+} signals which ultimately inhibit the sperm fusion with the oocyte. The insensitivity of the cell to Ca^{2+} signal may be related to lipid peroxidation.

Regarding the effects of ROS on spermatozoa, there is a great focus on previously mentioned lipid peroxidation of sperm membrane. The boar sperm membrane contains high concentration of PUFA. Being the preferred substrate for ROS, PUFA are susceptive to peroxidation and consequently the boar sperm membrane is more sensitive to the effect of ROS. As the effect of lipid peroxidation, the phospholipid structure changes altering the fluidity and functionality of the membrane (Cerolini et al., 2000; Awda et al., 2009). Ultimately, lipid peroxidation of the membrane leads to the loss of motility and reduced fertility potential of spermatozoa. The process is frequent in infertile individuals (Cerolini et al., 2000).

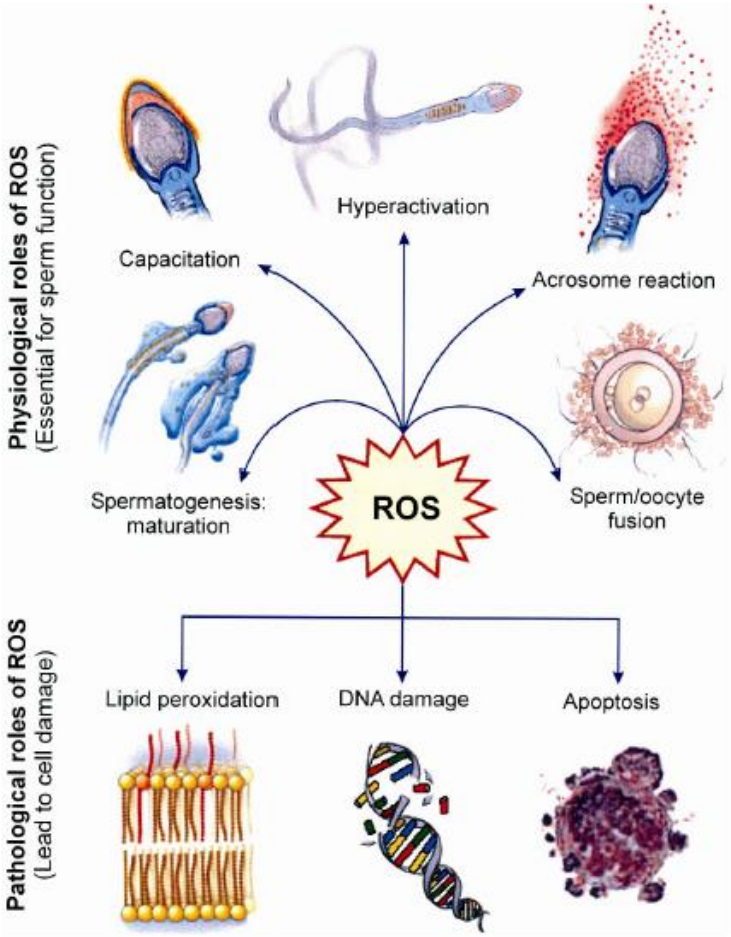

Figure 3. The physiological roles of ROS versus pathological effects of excessive ROS levels. (Kothari et al., 2010).

As De Lamirande and Gagnon (1995) state, the intracellular origin of ROS may be linked to oxygen metabolism which result in production of superoxide anion, hydrogen peroxide and hydroxyl radical. These ROS are also produced extracellularly by leukocytes present in SP. Under physiological conditions, antioxidant components of plasma protect spermatozoa from ROS produced by leukocytes. The participation of leukocytes in creating the oxidative stress becomes apparent when the SP is removed. This factor is especially considered in case of *in vitro* fertilization, since it has great impact on its effectiveness. In addition to leukocytes, excessive levels of ROS may be generated by defective or immature spermatozoa. It is thought to be due to the defects of sperm midpiece which is associated with accumulation of residual cytoplasm. In consequence to this, the spermatozoa exhibit increased quantities of cytoplasmic enzymes such as lactic acid dehydrogenase, creatine phosphokinase and glucose-6-phosphate dehydrogenase. The latter is thought to take part in oxidative stress generation as it is indirectly

involved in dihydronicotinamide adenine dinucleotide phosphate (NADPH) production. NADPH is one of the sources of free electrons that produce superoxide radical (Aitken, 1995).

Following the review of Bansal and Bilaspuri (2011), ROS include wide group of compounds and may be further divided. Radicals such as hydroxyl ion, superoxide, nitric oxide (NO) and others represent only a part of known ROS acting within the cellular ambient. Other class of ROS is represented by nonradicals such as single oxygen, lipid peroxides, hydrogen peroxide. ROS may be also further dived by distinguishing the subclass of reactive nitrogen species represented by free nitrogen radicals.

3.3.1 Nitric oxide production and function

NO represents a free radical gaseous molecule with very short biological half-life. It takes part in regulation of reproductive function. Specifically, it takes part in induction of sperm capacitation and acrosomal reaction and it also modulate sperm motility. NO is synthesized from L-arginine by three different isoforms of NO synthase (NOS): neuronal (nNOS), endothelial (eNOS), and inducible NOS (iNOS) (Türkyilmaz et al., 2004; Lampiao et al., 2006). Following the review of Herrero and Gagnon (2001), the nNOS and eNOS represent two forms of constitutive NOS (cNOS) isolated from different tissues.

Both types of cNOS generate subtle amount of NO that performs various physiological functions. It has been proven that the activity of cNOS may be stimulated by sex hormones, (e.g. estradiol) which induce the production of mRNA encoding both types of cNOS (Herrero and Gagnon, 2001).

Nevertheless, this type of transcriptional induction was first proven for the other isoform of NOS, the iNOS. This enzyme was first observed in macrophages, which use it to increase NO production to cytotoxic levels protecting the body from uncontrolled cellular growth and foreign microorganism. In difference to cNOS, this isoform requires substantially less calcium for activation and forms stronger bound with calmodulin signal molecule. The iNOS is mainly connected with pathological conditions and inflammatory processes, but it was also proven that it takes part in physiological actions such as ovulation, pregnancy and labor. Actually, iNOS has been located in almost every tissue under investigation including reproductive systems (Herrero and Gagnon, 2001).

It has been proven, at least in human and mouse, that spermatozoa possess NOS and consequently are able to increase the NO production themselves. As mentioned above, second

important source of NOS and NO production in relation to semen, are the leucocytes present in SP (Herrero and Gagnon, 2001).

Various studies have investigated the influence of NO-releasing compounds on sperm parameters including motility. It was demonstrated, that while in low concentrations NO boosted up the sperm motility, in high concentrations it had opposite effect due to inhibition of sperm respiration (Rosselli et al., 1995; Lewis et al., 1996). Moreover, it has been observed that increased NO production may lead to premature sperm hyperactivation which complicates the sperm movement through lower female reproductive tract. It has been also observed, that NO affects sperm capacitation as the NO-releasing compound has accelerated capacitation process. It seems that NO regulates the tyrosine phosphorylation which is one of the basic processes of intracellular signalization which takes part also in sperm capacitation (Herrero and Gagnon, 2001).

NO seems to play role also in AR, zona pellucida binding and fusion events. Excessive NO causes rearrangements of membrane phospholipids that prohibit the AR. On the other hand, too low levels of NO prohibit the AR by blocking the progesterone-induced acrosomal exocytosis induced by follicular fluid (Herrero and Gagnon, 2001).

Therefore, it is clear that NO takes part in crucial processes preceding the fertilization, which is in accordance to the approach, that ROS play important part in acquisition of fertilization capacity of spermatozoa (Herrero and Gagnon, 2001).

3.4 Antioxidants

To control the amount of ROS, scavenging systems are set within spermatozoon itself and seminal plasma too. These systems include enzymes such as superoxide dismutase (SOD) or catalase, which are further supported by substances with similar effects such as albumin, glutathione, pyruvate, taurine, vitamin E and vitamin C (De Lamirande and Gagnon, 1995).

Enzymatic antioxidants are also referred to as natural antioxidants. They can break the oxidative chain and doing so they reduce the oxidative stress. Particularly, the SOD scavenges the superoxide anion to less toxic hydrogen peroxide, which is further converted by catalase to O_2 and H_2O as shown in Fig.1. The hydrogen peroxide is naturally reduced to water and alcohol by glutathione peroxidase (GPx). GPx together with glutathione reductase (GR) help to ensure the protection from lipid peroxidation by maintaining physiological levels of glutathione and its oxidised form within the cell (Funahashi and Sano,2005; Maroušková et al., 2014).

Other part of natural antioxidant body complex is represented by synthetic antioxidants. This system is dependent on the dietary intake of antioxidant substances like vitamins, minerals and peptides. Glutathione is one of such substances and it serves as a cofactor to selenium-dependent GPx which takes part in H_2O_2 reduction. It is one of the most effective and protective agents against oxidative damage in bull and boar spermatozoa (Funahashi and Sano,2005).

The amino acid cysteine is a precursor that enhances the biosynthesis of glutathione. Therefore, it can augment indirectly the protection of membrane lipids and proteins against free radicals. Cysteine also demonstrated cryoprotective effect on the functional integrity of axosome and mitochondria leading to viability, membrane integrity and motility improvement. This effect has been observed also in case of liquid stored boar spermatozoa (Bansal and Bilaspuri, 2011). Funahashi and Sano (2005) report positive effect on sperm viability and oocyte-penetration capacity by adding cysteine to the preservation media.

Among many other antioxidants, inositol may be mentioned as it improves the abundance of morphologically normal spermatozoa and enhances the scavenging activity of glutathione. Taurine also acts as non-enzymatic free radical scavenger when spermatozoa are exposed to aerobic condition and during cryopreservation by increasing catalase concentration. Bovine serum albumin represents an effective non-enzymatic antioxidant protecting sperm membrane integrity (Bansal and Bilaspuri, 2011).

3.4.1 Aminoguanidine

Various guanidine compounds have been tested for antioxidant capacity, aminoguanidine (AG) among them. It is a chemical compound soluble in water formed by L-arginine linked to hydrazine (Misko et al., 1993). The potential role in regulating biological processes as ROS scavenger and iNOS inhibitor has been quite widely investigated. Various studies investigated the potential effects of aminoguanidine as diabetes medication, other studies focused on its use during cancer treatment and stroke (Corbett et al. 1992; Courderot-Masuyer et al., 1999; Sun et al., 2010; Janakiram and Rao, 2012). Several other studies focused on aminoguanidine's beneficial roles in solving reproduction-related issues (Abbasi et al., 2011; Oguz et al., 2013; Alizadeh et al., 2016). Courderot-Masuyer et al. (1999) demonstrated scavenging activities of aminoguanidine against hydroxyl and peroxyl radicals. Yildiz et al. (1998) verified its scavenging activities against peroxynitrite and oxygen free radicals and demonstrated for the first time the scavenging ability of guanidine compounds against hypochlorous acid. Corbett et al. (1992) proved that aminoguanidine specifically inhibits iNOS.

As the cNOS ensures the physiological production of NO signalling molecule, its inhibition is not desirable, which comes even more evident regarding the reported toxic effects of non-selective inhibitors (Wolf and Lubeskie, 1995). In difference, the iNOS is involved in various pathologies such as septic shock, inflammatory conditions of joints, intestine, and CNS (Boer et al., 2000).

The mechanism of iNOS inhibition by AG was studied by Wolf and Lubeskie (1995) who demonstrated some crucial facts about its interactions with NOS. Essentially, AG represents a substrate for iNOS competitive to natural one, the l-arginine which is always present in the cell. One of the important discoveries was that AG has more than 50 times higher affinity to iNOS than cNOS. These data were further investigated by Boer et al. (2000) who came to conclusion that the selective inhibition and potency of NOS inhibitors is only given by their affinity toward different isoforms. Wolf and Lubeskie (1995) also noted that the l-arginine and AG affinity to iNOS is comparable, being slightly higher in AG. On the other hand, cNOS prefers arginine as a substrate as its affinity to cNOS is more than 1000 times higher than the one of AG.

Once the production of iNOS is induced, the enzyme is active for longer time periods and able to form NO. This is in contrary to cNOS which is naturally present in cells and active only under specific stimuli. For its inactivation specific stimuli are required (such as Ca^{2+} upregulation, CaM and NADPH presence). Moreover, to inactivate any NOS isoform, the metabolically active enzyme must be present (Wolf and Lubeskie, 1995).

Bryk and Wolf (1998) state in their work, that AG requires NADPH and oxygen as cosubstrates. Regarding the potency of aminoguanidine as iNOS inhibitor, Boer et al. (2000) designated AG as a weak iNOS inhibitor which requires mM concentration to demonstrate inhibitory activity under *in vitro* conditions. On the other hand, Corbett et al. (1996) evaluated AG as an effective and low-cost selective iNOS inhibitor under both *in vitro* and *in vivo* conditions and they have recommended the administration of injections of AG 2-3 times a day containing 200-400 mg per kg of body weight. Philis-Tsimikas et al. (1995) state that dosis of 50-1000 µmol/l of AG prevented lipid peroxidation which was later demonstrated by Giardino et al. (1998) under *in vivo* conditions using rat as model organism. Both previous studies agree in that the AG antioxidant effect is dependent on the AG concentration and that too low concentrations of AG may even promote ROS and lipid peroxidation.

4 Material and Methods

4.1 Reagents

Reagents were purchased from Sigma-Aldrich (Prague, Czech Republic), unless otherwise stated.

4.2 Sperm samples collection and processing

Commercial sperm doses from 15 boars of different breeds (i.e. Czech Landrace, Czech Large White, Pietrain, Duroc, and Přeštice Black-Pied) and hybrid genetic lines were purchased from a breeding company (Chovservis, Hradec Králové, Czech Republic). Ejaculates were collected by gloved-hand method, diluted with Solusem® extender (AIM Worldwide, Vught, Netherlands) and transported to the laboratory at 17 °C. Only sperm samples with at least 75% motile spermatozoa were used for these experiments. To reduce the effect of male variability, equal volumes of sperm doses from three boars were mixed. Then, sperm concentration was checked using a Bürker chamber and samples were further diluted with Solusem® to get a final concentration of 20×10^6 spermatozoa/ml. Then, samples were randomly allocated into 5 groups: control (CTR), control under oxidative stress (CTR-ox), aminoguanidine 10 mM under oxidative stress (Ag10-ox), aminoguanidine 1 mM under oxidative stress (Ag1-ox), and aminoguanidine 0.1 mM under oxidative stress (Ag0.1-ox). Aminoguanidine was freshly prepared the day of the experiment by dilution in phosphate buffered saline (PBS) solution and diluted with sperm samples to give a final concentration of 10, 1, and 0.1 mM. For both CTR and CTR-ox samples, equal volume of PBS solution was added. Oxidative stress was induced by incubating sperm samples with 0.05 mM $FeSO_4$ and 0.5 mM sodium ascorbate (thereafter Fe^{2+}/ascorbate**;** Brzezińska-Ślebodzińska et al. 1995, Dominguez-Rebolledo et al. 2010). Equal volume of Solusem® was added to the CTR. The experiment was replicated 5 times using 5 different pools.

4.3 Assessment of sperm motility

A sperm aliquot (5 µl) was loaded into a pre-warmed (38 ºC) Spermtrack (PROiSER R+D S.L., Paterna, Spain; chamber depth: 20 µm). Sperm motility was evaluated subjectively by estimating the percentage of motile spermatozoa to the nearest 5% and the quality of movement (QM) using a scale from 0 (lowest: no motility) to 5 (highest: progressive and vigorous movements). The sperm motility index (SMI) was calculated according to the formula: [%

individual motility + (QM × 20)]/2 (Comizzoli et al. 2001). Sperm kinetics was assessed by a Computer Assisted Sperm Analysis (CASA) (NIS-Elements, Nikon, Tokyo, Japan and Laboratory Imaging, Prague, Czech Republic), which consists of an Eclipse E600 tri-ocular phase contrast microscope (Nikon, Tokyo, Japan), equipped with a 10× negative phase-contrast objective (Nikon, Tokyo, Japan), a warming stage set at 38 ºC (Tokai Hit, Shizuoka, Japan), and a DMK 23UM021 digital camera (The Imaging Source, Bremen, Germany). A total of nine descriptors of sperm kinetics were recorded analyzing 6 randomly selected fields and a minimum of 200 sperm cells per sample: total motility (%), progressive motility (%), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight linear velocity (VSL, μm/s), amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz), linearity (LIN, %), and straightness (STR, %). The standard parameter settings were as follows: frames per second: 60; minimum of frames acquired: 31; VAP ≥ 10 μm/s to classify a spermatozoon as motile, STR ≥ 80 % to classify a spermatozoon as progressive.

4.4 Assessment of sperm head membrane integrity

The assessment was performed as previously described (Harrison and Vickers, 1990; Grieblová et al. 2017). Briefly, 30 μl of sperm samples were incubated with pre-warmed freshly prepared staining solution composed of 160 μl PBS, 4 μl carboxyfluorescein diacetate (CFDA, stock solution: 0.46 mg/ml in dimethyl sulfoxide), 4 μl propidium iodide (PI, stock solution: 0.5 mg/ml in PBS), and 2 μl formaldehyde solution (0.3%) for 10 minutes at 37 °C in the dark. Then, 200 spermatozoa were evaluated in each sample using epi-fluorescence microscopy (40× objective) and the sperm cells showing complete green fluorescence of the head were considered to have an intact head membrane (Fig. 4).

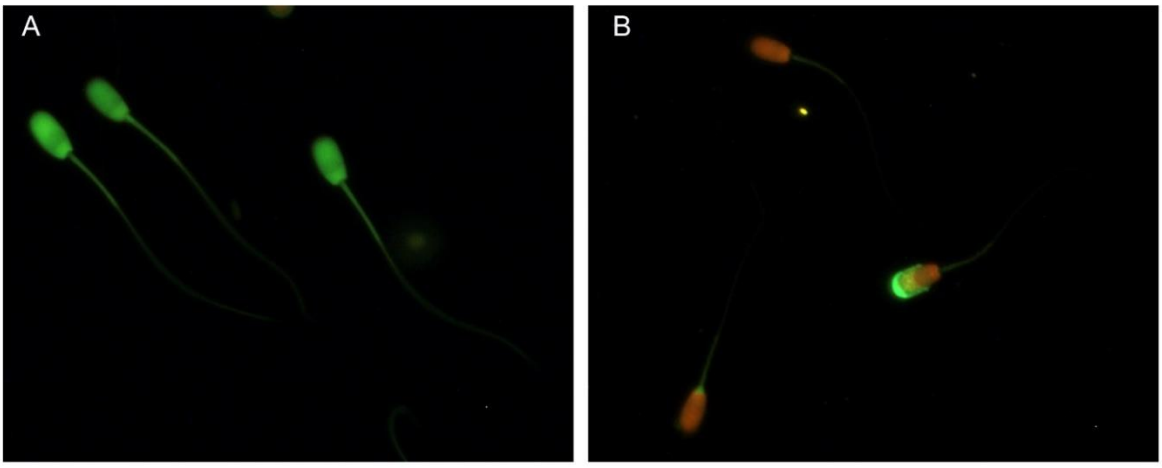

Figure 4. Sperm classification by PI/CFDA. A) PI^-/CFDA green-head sperm: intact head membrane; B) PI^+/CFDA red-head: damaged head integrity; red and green-head: transitional stage of damaged head integrity. CFDA: carboxyfluorescein diacetate; PI: propidium iodide. Ros-Santaella, 2017.

4.5 Assessment of sperm tail and head membrane integrity

The assessment was performed using a hypo-osmotic solution consisting of 7.35 g/L sodium citrate and 13.51 g/L fructose (Jeyendran et al., 1984). Samples (50 µl) were diluted in 150 µl of pre-warmed HOST solution and incubated for 30 min at 38 °C. After 28 min of incubation, 40 µl of 1.5% eosin in hypo-osmotic solution was added to 200 µl of sperm sample and incubated for another 2 min. At the end of the incubation, sperm cells were evaluated using a phase-contrast microscopy (40× objective) and classified into four subpopulations based on the staining of the head and the swelling of the tail (Fig. 5):

- Type I: head-pink and tail-non-swollen;
- Type II: head-white and tail-non-swollen;
- Type III: head-pink and tail-swollen;
- Type IV: head-white and tail-swollen.

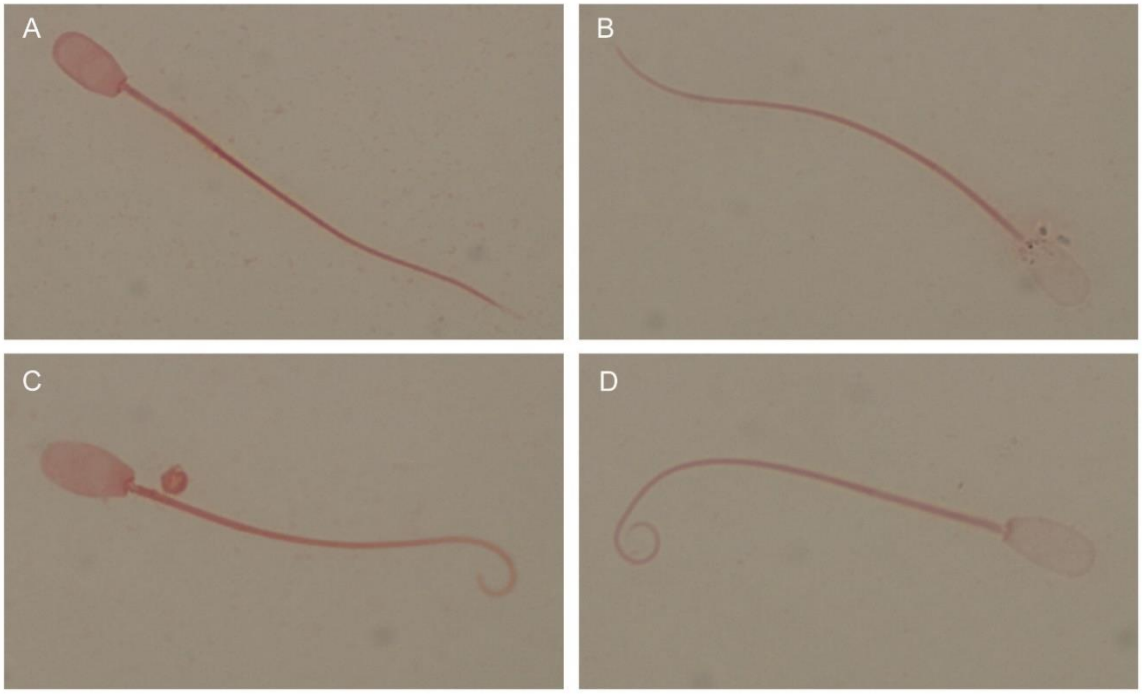

Figure 5. Sperm classification by HOST/E. A) Type I: red head and straight tail, damaged head and tail membranes; B) Type II: white head and straight tail, intact head and damaged tail membranes; C) Type III: red head and swollen tail, damaged head and intact tail membranes; D) Type IV: white head and swollen tail, intact head and tail membranes. HOST/E: HOST combined with eosin staining. Ros-Santaella, 2017.

4.6 Assessment of acrosomal status

For acrosomal status evaluation, 20 µl of sperm samples were diluted in 80 µl of PBS. The sperm samples were fixed in 10 µl of 2% glutaraldehyde solution and examined under phase contrast microscopy (40× objective). In each sample 200 sperm cells were evaluated and the percentage of sperm cells with a normal apical ridge (NAR; Pursel et al. 1972) was determined.

4.7 Statistical analysis

All statistical analyses were performed using the SPSS 20.0 statistical software package (IBM Inc, Chicago, IL, USA). We used a generalized linear model to analyse the effects of the treatments and storage times on sperm variables. Data are shown as mean ± SD. Statistical significance was set at $p < 0.05$.

5 Results

5.1 Subjective evaluation of motility

As shown in Fig. 6, the sperm motility percentage was higher in all aminoguanidine treatments than in the control-ox group ($p<0.05$) except the Ag0.1-ox at 2 h of sperm incubation ($p>0.05$). Also, the Ag0.1-ox treatment has shown lower sperm motility percentage than the other two aminoguanidine treatments ($p<0.05$) in any time of incubation. It is worth to highlight that the Ag10-ox treatment presented higher percentage of motile sperm cells than the control group at 3.5 h of sperm incubation ($p<0.05$).

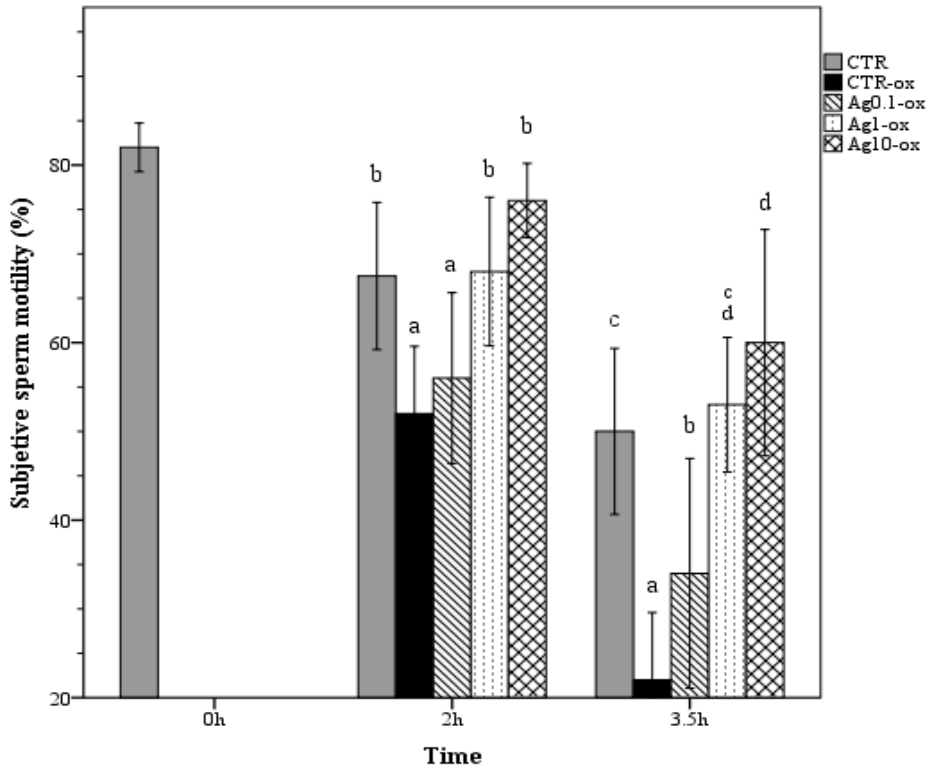

Figure 6. Subjective sperm motility in boar sperm samples under induced-oxidative stress (ox) with or without aminoguanidine supplementation. Different letters indicate significant differences ($p<0.05$) within each given time. Ag10 = aminoguanidine 10 mM; Ag1 = aminoguanidine 1 mM; Ag0.1 = aminoguanidine 0.1 mM. Data are shown as mean ± standard deviation.

At two hours of incubation, there was noted better quality of sperm movement in Ag1-ox treatment than in any other ($p<0.05$). Moreover, the Ag1-ox also presented better results than CTR-ox group at 3.5 h of incubation ($p<0.05$). Surprisingly, the Ag10-ox group did not significantly differ from CTR-ox treatment at any time of evaluation (Fig. 7).

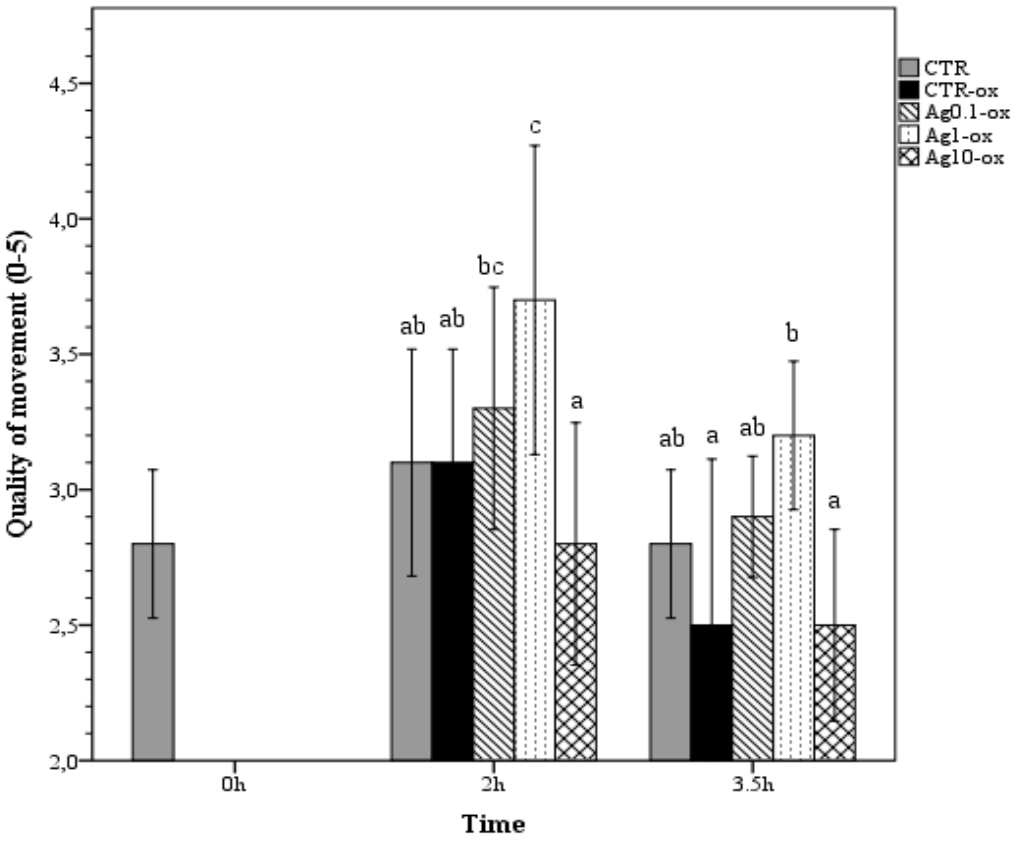

Figure 7. Quality of movement of boar sperm samples under induced-oxidative stress (ox) with or without aminoguanidine supplementation. Different letters indicate significant differences (p<0.05) within each given time. Ag10 = aminoguanidine 10 mM; Ag1 = aminoguanidine 1 mM; Ag0.1 = aminoguanidine 0.1 mM. Data are shown as mean ± standard deviation.

Following the data presented in Fig. 8, significantly higher SMI was noted in Ag1-ox and Ag10-ox treatments compared to CTR-ox group at 2 hours of evaluation. The values of SMI at 3.5 h of sperm incubation were proven to be higher in all Ag treatments compared to CTR-ox group ($p<0.05$).

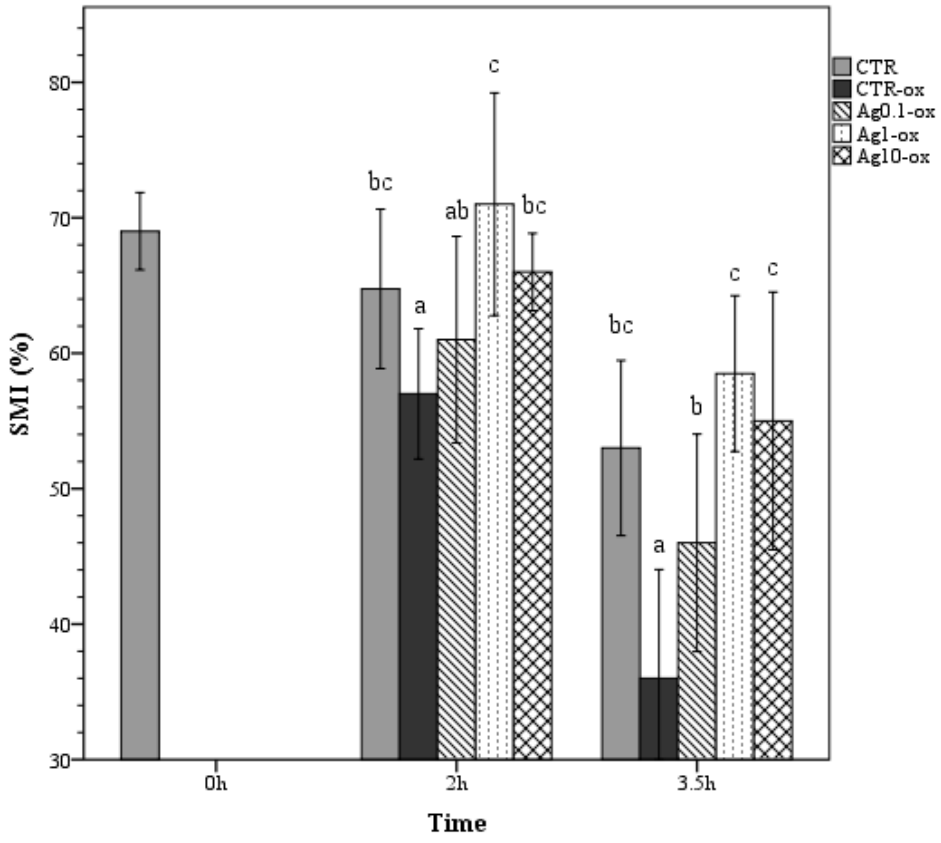

Figure 8. SMI values of boar sperm samples under induced-oxidative stress (ox) with or without aminoguanidine supplementation. Different letters indicate significant differences (p<0.05) within each given time. Ag10 = aminoguanidine 10 mM; Ag1 = aminoguanidine 1 mM; Ag0.1 = aminoguanidine 0.1 mM. Data are shown as mean ± standard deviation.

5.2 Evaluation of kinetic parameters at 2 hours by CASA

There was a significant movement improvement in Ag1-ox and Ag10-ox treatments compared to the CTR-ox (Tab. 1). Outstanding results were obtained with Ag10-ox treatment, since the percentage of motile spermatozoa was even higher than the one of control group (*p<0.05).*

The spermatozoa in CTR-ox treatment demonstrated significantly increased BCF and better LIN. Higher percentage of straight moving spermatozoa were found in Ag1-ox which was comparable to control group and both of them were superior to CTR-ox (*p<0.05).*

Table 1

Boar sperm kinetics under induced-oxidative stress (ox) with or without aminoguanidine supplementation at 2 hours of incubation.

Evaluation of sperm motility after 2 hours

	CTR	CTR-ox	Ag0.1-ox	Ag1-ox	Ag10-ox
Sperm motility (%)	63.12 ± 13.54^{ab}	54.20 ± 10.58^{a}	56.75 ± 10.55^{ab}	65.64 ± 3.10^{b}	83.05 ± 3.52^{c}
Sperm progressive motility (%)	62.42 ± 12.42^{b}	51.40 ± 29.77^{ab}	58.74 ± 6.90^{b}	63.15 ± 5.79^{b}	41.99 ± 6.23^{a}
ALH (µm)	2.03 ± 0.40^{a}	3.47 ± 3.59^{b}	1.89 ± 0.52^{a}	2.34 ± 0.56^{ab}	2.86 ± 0.61^{ab}
BCF (Hz)	11.49 ± 1.93^{a}	15.58 ± 9.69^{b}	11.10 ± 1.11^{a}	11.51 ± 0.93^{a}	11.68 ± 0.61^{a}
LIN (%)	37.88 ± 8.39^{a}	49.68 ± 24.57^{b}	36.41 ± 4.32^{a}	38.38 ± 4.66^{a}	30.43 ± 2.86^{a}
STR (%)	76.18 ± 7.08^{b}	64.87 ± 25.79^{a}	73.80 ± 3.60^{ab}	76.18 ± 3.35^{b}	67.13 ± 4.48^{ab}
VAP (µm/s)	27.37 ± 8.38^{a}	29.52 ± 10.38^{ab}	25.02 ± 8.03^{a}	31.47 ± 8.13^{ab}	36.13 ± 7.16^{b}
VCL (µm/s)	54.72 ± 7.68^{ab}	42.84 ± 16.35^{a}	49.97 ± 15.33^{ab}	61.74 ± 14.07^{b}	82.00 ± 16.83^{c}
VSL (µm/s)	24.47 ± 7.93^{a}	27.03 ± 9.54^{a}	22.05 ± 7.03^{a}	27.91 ± 7.05^{a}	25.67 ± 3.89^{a}

Different letters indicate significant differences (p<0.05) within each given time. Ag10 = aminoguanidine 10 mM; Ag1 = aminoguanidine 1 mM; Ag0.1 = aminoguanidine 0.1 mM. ALH: amplitude of lateral head displacement; BCF: beat cross frequency; LIN: linearity; STR: straightness; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight linear velocity.

5.3 Evaluation of kinetic parameters at 3.5 hours by CASA

As shown in Tab. 2, all samples supplemented with aminoguanidine demonstrated improved sperm motility in comparison with CTR-ox. Within the Ag treatments, there were less motile spermatozoa in Ag0.1-ox treatment than in the other two *($p<0.05$)*. Moreover, the Ag1-ox and Ag10-ox contained more than two times higher percentage of motile spermatozoa than the CTR-ox treatment (*$p<0.05$*). Comparing CTR-ox with the other treatments, the only one with significantly improved progressive motility was the Ag1-ox which was also equal to control group.

The spermatozoa in Ag1-ox and Ag10-ox treatments were significantly superior to Ctr-ox in several other parameters: STR, VAP, VCL and VSL. In case of VSL, the sperm velocity in Ag-1 and Ag-10 was more than two times higher. Moreover, these two Ag treatments were comparable to the control group (*$p>0.05$*). Remarkable velocity improvement was noted in Ag-10 treatment which showed almost 3 times higher average speed than the CTR-ox.

Table 2

Boar sperm kinetics under induced-oxidative stress (ox) with or without aminoguanidine supplementation at 3.5 hours of incubation.

Evaluation of sperm motility after 3.5 hours

	CTR	CTR-ox	Ag0.1-ox	Ag1-ox	Ag10-ox
Sperm motility (%)	60.04 ± 14.01^{c}	24.95 ± 9.85^{a}	39.55 ± 10.76^{b}	59.01 ± 8.53^{c}	69.14 ± 12.66^{c}
Sperm progressive motility (%)	62.79 ± 13.50^{b}	43.68 ± 17.84^{a}	53.09 ± 12.23^{ab}	63.80 ± 7.41^{b}	52.75 ± 9.16^{ab}
ALH (µm)	1.98 ± 0.55^{ab}	0.83 ± 0.18^{a}	1.28 ± 0.30^{ab}	1.95 ± 0.32^{ab}	2.22 ± 0.58^{b}
BCF (Hz)	11.59 ± 2.07^{a}	9.06 ± 1.91^{a}	10.00 ± 1.69^{a}	11.23 ± 1.08^{a}	10.87 ± 1.04^{a}
LIN (%)	39.09 ± 9.06^{a}	28.65 ± 10.06^{a}	33.80 ± 8.44^{a}	38.52 ± 5.64^{a}	31.46 ± 4.90^{a}
STR (%)	75.52 ± 8.17^{b}	60.52 ± 12.04^{a}	67.83 ± 8.69^{ab}	75.76 ± 4.88^{b}	71.89 ± 4.43^{b}
VAP (µm/s)	27.54 ± 10.09^{b}	9.77 ± 3.43^{a}	15.34 ± 4.05^{a}	26.02 ± 6.25^{b}	26.88 ± 8.15^{b}
VCL (µm/s)	52.05 ± 10.80^{b}	21.06 ± 4.49^{a}	31.03 ± 9.05^{a}	50.74 ± 7.76^{b}	62.61 ± 15.41^{b}
VSL (µm/s)	25.03 ± 10.00^{b}	8.54 ± 3.56^{a}	13.73 ± 3.84^{a}	23.65 ± 6.11^{b}	21.54 ± 6.62^{b}

Different letters indicate significant differences (p<0.05) within each given time. Ag10 = aminoguanidine 10 mM; Ag1 = aminoguanidine 1 mM; Ag0.1 = aminoguanidine 0.1 mM. ALH: amplitude of lateral head displacement; BCF: beat cross frequency; LIN: linearity; STR: straightness; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight linear velocity.

5.4 Evaluation of the effect of aminoguanidine on plasma membrane and acrosome integrity

The evaluation of plasma membrane provided some significant results in case of PI/CFDA test and normal apical ridge (NAR) test. The HOST – eosin test did not reveal any difference among treatments ($p > 0.05$).

5.4.1 Evaluation of head membrane by PI/CFDA

Independently of the time of incubation, the CTR-ox treatment showed higher percentage of spermatozoa with damaged head than all other treatments (Fig. 9). On the other hand, Ag1-ox and Ag10-ox had significantly higher percentage of PI-/CFDA+ (membrane intact) sperm cells than all other treatments at both times of evaluation ($p<0.05$). Noteworthy is the fact that Ag0.1-ox had same percentage of PI-/CFDA+ sperm cells as the control treatment ($p>0.05$).

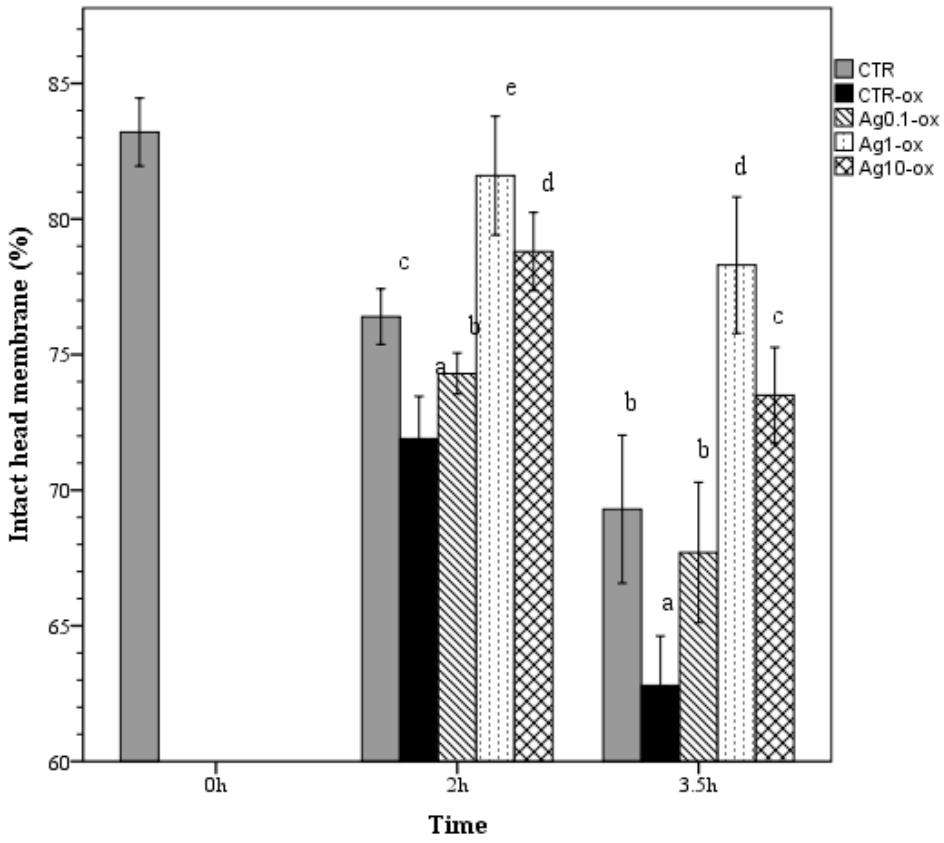

Figure 9. Sperm head membrane integrity assessment through the PI/CFDA test. Different letters indicate significant differences ($p<0.05$) within each given time. Data are shown as mean ± standard deviation.

5.4.2 Evaluation of membrane integrity by HOST - eosin test

The HOST-eosin test did not show any significant difference between treatments supplemented with aminoguanidine and control group ($p>0.05$). Nevertheless, irrespectively of the incubation time, all aminoguanidine treatments showed lower percentage of type I spermatozoa (damaged head and tail membrane) than the one of CTR-ox. This tendency corresponded with finding higher percentage of type IV (intact head and tail membrane) spermatozoa in same samples again compared to CTR-ox.

The evaluation revealed high variability of the data characterized by high standard deviation (SD). In case of type II sperm cells, average SD is 1.4 % while the average percentage of type II sperm cell across all measurements and treatments is 1.6 %. The relatively high standard deviation for the remaining types of spermatozoa evaluated by host-eosin test may be also seen in Tab. 3.

Table 3.

HOST-eosin test results after 2 and 3.5 hours of treatment incubation.

HOST-eosin test

	0 hours				
	CTR	**CTR-ox**	**Ag0.1-ox**	**Ag1-ox**	**Ag10-ox**
Type I (%)	69.9 ± 5.8	-	-	-	-
Type II (%)	2.2 ± 1.0	-	-	-	-
Type III (%)	11 ± 5,1	-	-	-	-
Type IV (%)	16.9 ± 6.3	-	-	-	-
	2 hours				
	CTR	**CTR-ox**	**Ag0.1-ox**	**Ag1-ox**	**Ag10-ox**
Type I (%)	76.1 ± 10.2	82.3 ± 9.9	78.5 ± 10.5	78.6 ± 8.7	74.2 ± 9.9
Type II (%)	0.9 ± 0.4	1.4 ± 1.2	0.5 ± 0.4	1.2 ± 1.0	1.5 ± 1.0
Type III (%)	13.2 ± 8.1	11.3 ± 7.8	14.2 ± 9.8	11.1 ± 4.2	15.8 ± 9.5
Type IV (%)	9.8 ± 3.3	5 ± 2.5	6.8 ± 2.1	9.1 ± 6.1	8.5 ± 1.3
	3.5 hours				
	CTR	**CTR-ox**	**Ag0.1-ox**	**Ag1-ox**	**Ag10-ox**
Type I (%)	78.5 ± 9.4	84.2 ± 8.5	81.75 ± 9.7	81.5 ± 9.5	74.6 ± 8.0
Type II (%)	1.1 ± 0.7	1.7 ± 0.7	2.4 ± 2.5	1.4 ± 1.0	3.4 ± 2.2
Type III (%)	12.9 ± 5.6	8.4 ± 6.1	11.1 ± 9.5	13.5 ± 9.1	13.3 ± 6.6
Type IV (%)	7.5 ± 5.1	5.7 ± 2.5	4.9 ± 1.6	4.6 ± 2.0	8.7 ± 2.9

Type I: stained head and non-swollen tail sperm; Type II: white head and non-swollen tail sperm; Type III: stained head and swollen tail sperm; Type IV: white head and swollen tail sperm.

5.4.3 Evaluation of the acrosome integrity by NAR test

All treatments supplemented with aminoguanidine showed higher percentage of sperm cells with undamaged acrosome than the CTR-ox treatment independently on the time of incubation ($p<0.05$). No differences were observed among treatments with different aminoguanidine concentration. An interesting founding was the fact, that the percentage of sperm cells with intact acrosome of Ag10-ox treatment at 2 hours of incubation did not differ

from that of the CTR sample at 0 hours of incubation ($p>0.05$). Complete results are demonstrated in figure 10.

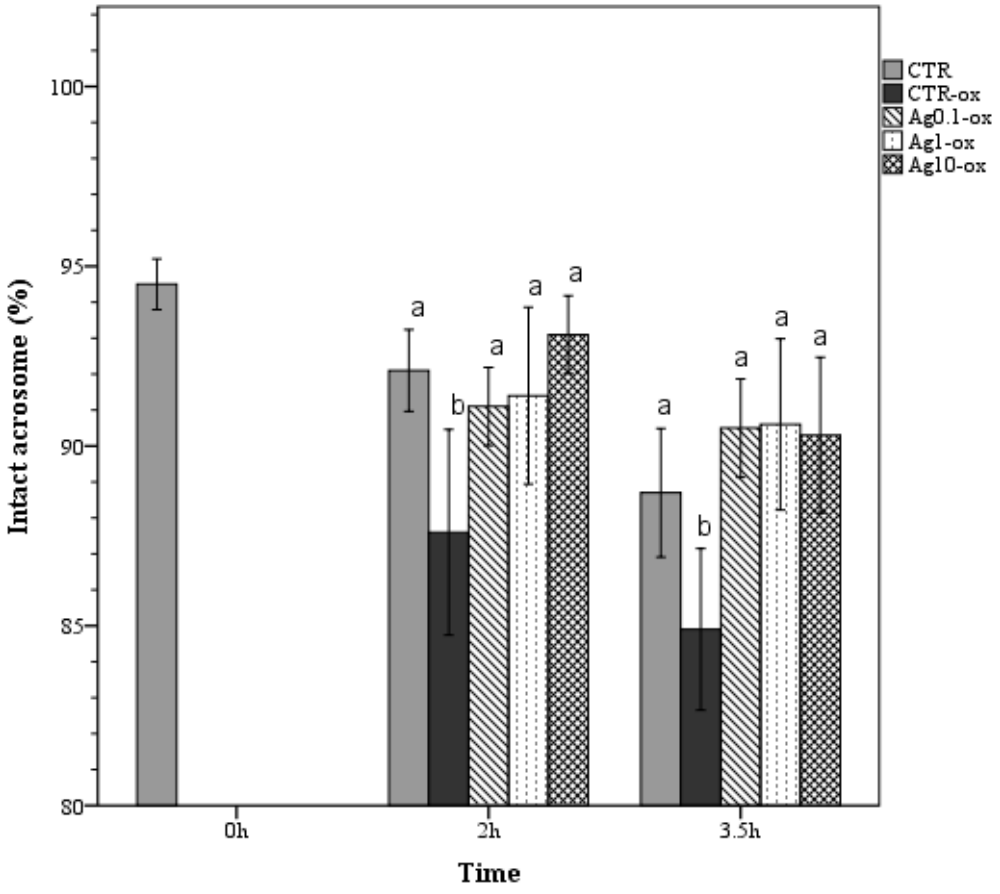

Figure 10. Acrosome integrity assessment through the normal apical ridge (NAR) test. Bars labelled by different letters differ significantly ($p<0.05$). Data are shown as mean ± standard deviation.

6 Discussion

Currently, the porcine reproduction in western countries is mainly performed using AI. Most of the semen doses used in AI are stored in liquid state (Johnson et al., 2000; Cerolini et al., 2000; Yeste 2017) as the boar spermatozoa are difficult to freeze while maintaining the fertilization capacity (Yeste, 2015, 2016). This is due to the lipid composition of the boar sperm membrane which is rich in PUFA being the preferred substrate for ROS. The consequent lipid peroxidation of membrane phospholipids changes its structure and functionality (Cerolini et al., 2000, 2001; Peňa et al., 2004; Am-in et al., 2011). Moreover, the sperm cells possess limited antioxidant capacity and mostly depend on the antioxidants present in seminal plasma. As the doses for AI are diluted by semen extenders, the antioxidant capacity of seminal plasma is consequentially reduced, and it has to be compensated by adding suitable substances with similar effect into the extender in order to maintain the fertility potential, which is closely related to sperm motility (Holt et al, 1997; Tardiff et al, 1999; Vyt et al., 2008). The intactness of membrane is related to an appropriate metabolism and motility of spermatozoa (Johnson et al., 2000; Zhang et al., 2015). In this thesis the antioxidant capacity of aminoguanidine was tested using Fe^{2+}/ascorbate which was successfully used to induce lipid peroxidation of sperm membrane as described in previous studies (Comaschi et al., 1989; Brzezińska-Ślebodzińska et al., 1995; Guthrie et al., 2008).

The results of this work demonstrate that aminoguanidine preserves the plasma membrane, acrosome integrity and motility of boar spermatozoa submitted to oxidative stress. The effective concentrations of aminoguanidine seemed to be 1 mM and 10 mM. Nor the subjective evaluation or CASA analysis showed significant differences between these two concentrations at 3.5 hours of incubation. To date, this is the first study that investigate the *in vitro* effect of AG on the quality of boar semen samples submitted to oxidative stress.

Subjective and CASA evaluation of sperm motility indicated that aminoguanidine at concentration 0.1 mM is not sufficient to prevent the oxidative damage induced by Fe^{2+}/ascorbate. Thus, the non-significant effect of aminoguanidine at the lowest concentration was in contrast to Ag1-ox and Ag10-ox. Both treatments at 3.5 hours of semen incubation clearly improved several sperm motility parameters: total sperm motility, STR, VAP, VCL, and VSL. Several studies report connections between previously listed sperm parameters and semen quality, fertility and the litter size (Hirai et al., 1997; Holt et al., 1997; Vyt et al., 2008).

In the present study, sperm total motility reached the highest percentage in Ag 10 treatment being 1.5 times higher than in Ctr-ox at 2 hours of semen incubation. Same situation occurred in case of VCL, which exceeded almost twice the VCL of Ctr-ox. Noteworthy is the fact that BCF at 2 hours of incubation was highest in the Ctr-ox sample and so was the LIN and ALH compared to control sample. The high value of BCF in the Ctr-ox group at 2 h of sperm incubation is interesting compared to the study performed by Holt et al. (1997) who observed minimal changes of BCF during 6 hours of semen incubation without any induced-oxidative stress. This may indicate the positive effect of AG on BCF parameter even at 2 hours of semen incubation, as all the Ag treatments demonstrated the same BCF as control sample. Regarding the other kinetic parameters, it should be mentioned the work of Schmidt and Kamp (2004) who investigated hyperactivation of boar spermatozoa under capacitating conditions using a CASA system. They noted that hyperactivated boar are characterized by increased VCL, ALH and reduced LIN, STR, and BCF. To define hyperactivity in boar spermatozoa they established the values for the following parameters: VCL > 97 μms/s LIN ≤ 31.7 % and ALH > 3.5 μm. Suarez (1992) also noted reduced VSL in boar spermatozoa hyperactivated *in vitro*. In the present study the AG 10 treatment at 2 hours of incubation showed similar values of kinetic parameters described by Schmidt and Kamp (2004) and Suarez (1992), although in this study sperm samples were evaluated under no-capacitating conditions. Evaluating Ctr-ox treatment at the same time of incubation, some evidence of hyperactivation was also found regarding the VSL, ALH, STR and BCF parameters. Another significant observation was noted comparing Ag 1 and control sample without Fe^{2+}/ascorbate as both treatments had similar motility parameters indicating that AG at concentration 1 mM successfully protected spermatozoa from the oxidative stress**.** These data may be also compared to work done by Vyt et al. (2008) who have investigated sperm motility parameters by CASA in correlation to fertility potential. Among other findings, they observed that sperm cells need certain time of incubation to acclimatize to the changes of temperature in order to gain accurate measurements by CASA. The acclimatization time was set to 45 minutes of incubation of semen which was incubated without any induced-oxidative stress. Vyt el al. (2008) comment in their work, that irrespectively of the time of incubation, all samples demonstrated the hyperactivation-like motility based upon the determining parameters set by Schmidt and Kamp (2004), but simultaneously the samples differed in several other parameters. Similar situation occurred in present work, when various treatments showed resemblance to hyperactivation-like motility except control group without oxidation**.** Therefore, it may be hypothesized that the time necessary for acclimatization could be affected by increased oxidative stress. Such theory seems

even more tempting taking in account the clear motility patterns after 3.5 hours of incubation. This contradictory result due to acclimatization period would be also backed up by observation in other studies implying the gradual increase of ROS in time (Johnson et al., 2000; Gadea, 2003; Kumaresem et al., 2009; Vongpralub et al., 2016) which could also explain the different patterns observed at different time of incubation.

Nevertheless, the positive effect of AG on sperm motility observed in this work is in agreement with other studies (Abbasi et al., 2011[a], 2011[b]; Oguz et al., 2012; Alizadeh et al., 2010, 2016). In all previously mentioned studies, rodents were used as model animals which were injected with AG. Oguz et al. (2012) tested the effect of intraperitoneal injections of AG on rats treated by 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), which is known to impair testicular function and induce oxidative stress with consequential lipid peroxidation of sperm. In this case, the administration of AG increased concentration and percentage of motile sperm. Similarly, sperm motility was improved, when mice with induced varicocele were treated by AG. Varicocele is related with increased NO production by iNOS. The positive effect of AG was attributed to its scavenging activities and reduction of iNOS activity, although it was not measured directly (Alizadeh et al., 2010, 2016). Very similar study was performed by Abbasi et al. (2011[a], 2011[b]) who tested epididymal sperm parameters and came to same conclusion that intraperitoneal injections of AG positively affects motility of spermatozoa under oxidative stress.

Bahmanzadeh et al. (2008) investigated the influence of non-selective guanidine L-NAME on the regulation of NO and its effect on semen parameters in mice with induced varicocele. L-NAME is another guanidine investigated for its ability to scavenge free radicals and reduce NOS activity. The work demonstrated positive influence of L-NAME on sperm motility through reducing NO radical amount. Nevertheless, other study performed by Francavilla et al. (2000) showed the negative effect of non-selective inhibition of NOS on spermatozoon binding to zona pellucida. This process is dependent on the expression of cNOS present in the spermatozoon. Moreover, Lewis et al. (1996) further demonstrated that L-NAME may negatively influence motility parameters (VSL, VCL, VAP, and percentage of progressive motile spermatozoa). These studies may support the advantages of AG as an antioxidant and selective NOS inhibitor compared to its non-selective analogue L-NAME in relation to motility.

The membrane integrity is one of the best indicators of sperm viability, closely related to motility and fertilization potential (Althouse and Hopkins, 1995; Juonala et al., 1999). Among the various techniques of staining, the ones using double fluorochrome labeling allow the

differentiation between intact and damaged membrane and have become the most used ones in twenty-first century (Sancho and Vilagran, 2013). In reaction to lipid peroxidation, the membrane structure and function is altered, and spermatozoa lose their function (Aitken 1995). The membrane integrity evaluation by PI/CFDA staining in this thesis clearly demonstrated positive effect of aminoguanidine irrespectively of the concentration used. This finding indicates the ability of aminoguanidine in preserving the sperm plasma membrane integrity under oxidative stress and thus increasing the number of live spermatozoa. These results are in agreement with the results reported by Alizadeh et al. (2016) who also used PI to differentiate viable spermatozoa and found positive effect of aminoguanidine. Regarding the use of light microscopy, Abbasi et al. (2011[a]) and Alizadeh et al. (2010) have stained semen samples with eosin to identify viable spermatozoa. They verified the positive effect of aminoguanidine injections in protecting the spermatozoa from oxidative stress in treated animals.

The host-eosin evaluation performed in this thesis did not reveal any significant difference between samples treated by aminoguanidine and control group, although samples treated with aminoguanidine tended to show smaller percentage of spermatozoa having both damaged sperm head and tail membranes At this purpose, it is important to bear in mind that the hypoosmotic test evaluates the functional integrity of the sperm tail, while the tests using fluorescent dyes and vital stains are focused on structural integrity of the head membrane (Brito et al., 2003; Yeste et al., 2010; Zubair et al., 2015) which comes as a first-hand explication comparing results of different membrane tests performed in this work. Moreover, Druart et al. (2009) observed a major negative influence of oxidative treatment on the osmotic resistance of boar spermatozoa. The negative influence is even more promoted by the relatively low tolerance of boar spermatozoa to the hypoosmotic conditions (Gilmore et al., 1996), which also vary across breeds and between boars within the same breed (Druart et al. 2009). These might be therefore some of the factors that may have affected the results of host-eosin test performed in this work.

The results of host-e test contrasted with clear pattern of the acrosome integrity test performed in this thesis. Regardless of the time of incubation, all AG treatments contained higher number of spermatozoa with intact acrosome than control sample with Fe^{2+}/ascorbate. Regarding acrosome status and membrane integrity, Zou and Yang (2000) found significant relation between those two parameters and suggested the relation to viability. This observation corresponds to observations made in this thesis. The positive effect of AG on acrosome integrity of boar spermatozoa under oxidative stress is in agreement with the results obtained by

PI/CFDA test, yet according to the NAR test any of the AG treatments proved to be superior to the other. This is overall in agreement with the observations of Althouse and Hopkins (1995) who showed that more accurate evaluation of membrane integrity is gained by fluorescent dyes. Nevertheless, the sustainment of acrosome integrity is crucial for successful acrosome reaction, which must be precisely timed in order to allow the penetration of a spermatozoon through the oocyte protective barriers (Kommisrud et al., 2002; Bonet et al., 2013). The generation of excessive ROS may trigger premature AR (Lamirande and O'Flaherty, 2007; Awda et al., 2009). Based upon the positive results during NAR test performed in this work, it is possible to speculate that AG could increase the fertilization potential of spermatozoa through reducing oxidative stress and protecting the acrosome integrity.

It seems suitable to mention few other aspects which highlight the protecting abilities of AG against oxidative stress observed in this thesis. The predominant external source of excessive ROS in semen is represented by leukocytes, which increase ROS production through iNOS (Aitken 1995; Kothari et al., 2010; Janakiram and Rao, 2012). This phenomenon is even more obvious under in vitro conditions when the antioxidant capacity of seminal plasma is reduced (Aitken, 1995) and ROS generation is further increased by spermatozoa themselves, due to the dilution effect and the consequential membrane damage (Johnson et al., 2000). As reviewed by Yeste and Castillo-Martín (2013) and Schubert et al. (2008), insemination is followed by influx of leucocytes into the female genital tract. The immunological response of female also depends on the sperm concentration and semen extenders and moreover the boar AI is characterized by usage of highly concentrated semen doses (Bonet et al., 2013). Therefore, the positive effect of AG on sperm motility and membrane integrity observed in this thesis may be further enhanced by the potential beneficial effect of iNOS inhibition within the female genital tract. Upon these remarks and results of this thesis, it would seem interesting to perform more experiments regarding AG scavenging activity against free radicals and its ability of iNOS inhibition.

7 Conclusion

Aminoguanidine has successfully protected boar spermatozoa against oxidative stress preserving motility and membrane integrity. Moreover, various kinetic parameters and membrane integrity (PI/CFDA) of samples supplemented with aminoguanidine at concentration 1 and 10 mM were comparable or superior to control group without induced oxidative stress. Even though host-eosin evaluation did not prove significant differences between control group and aminoguanidine treatments, the results of the other tests were explicit. The results from this thesis indicate that aminoguanidine is a promising antioxidant for reducing the deleterious effects of oxidative stress in boar spermatozoa.

8 Bibliography

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9 List of Abbreviations

A	Axoneme
Ag10	Sample with aminoguanidine at concentration 10 mM
Ag1	Sample with aminoguanidine at concentration 1 mM
Ag0.1	Sample with aminoguanidine at concentration 0.1 mM
AI	Artificial insemination
ALH	Amplitude of lateral head displacement
AR	Acrosome reaction
BCF	Beat-cross frequency
BTS	Beltsville Thawing Solution
CASA	Computer assisted sperm analysis
CFDA	Carboxyfluorescein diacetate
cNOS	Constitutive nitric oxide synthase
CTR	Control sample
DF	Dens fiber
eNOS	Endothelial nitric oxide synthase
Fe^{2+}/ascorbate	$FeSO_4$ plus sodium ascorbate
FS	Fibrous sheath
GPx	Glutathione peroxidase
GR	Glutathione reductase
HOST	Hypo-osmotic swelling test
HOST/E	Hypo-osmotic swelling test combined with eosin staining
iNOS	Inducible nitric oxide synthase
LIN	Linearity
MP	Midpiece
MS	Mitochondrial sheath
NADPH	Dihydronicotinamide adenine dinucleotide phosphate
NAR	Normal apical ridge
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
ox	induced-oxidative stress
P	Plasmalemma

PBS	Phosphate buffered saline
PDL	Postacrosomal dense lamina
PI	Propidium iodide
PP	Principal piece
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SMI	Sperm motility index
SOD	Superoxide dismutase
SP	Seminal plasma
STR	Straightness
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight linear velocity
TP	Terminal piece