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Influence of gasotransmitters on the quality and storage of boar semen

doctoral thesis

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DECLARATION

I declare that doctoral thesis on topic „Influence of gasotransmitters on the quality and storage of boar semen“ I prepared independently, and used only the sources that I cited and listed in the literature.

In Prague day: 2020

signature of author ……..…….……

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I dedicate my dissertation to my son Nemanja Kralj.

List of abbreviations

AECOPD	acute exacerbation of chronic obstructive pulmonary disease
AG	aminoguanidine
AI	artificial insemination
ATP	adenosine triphosphate
ALH	amplitude of lateral head displacement
BCF	beat-cross frequency
CAT	catalase
CSE	cystathionine γ-lyase
CBS	cystathionine β-synthetase
cGMP	cyclic guanosine 3′,5′-monophosphate
COPD	chronic obstructive pulmonary disease
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
GTP	guanosine 5′-triphosphate
GYY4137	morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate
H_2S	hydrogen sulphide
HCL	hydrochloric acid
HOST	hypo-osmotic solution
K_{ATP}	ATP-sensitive potassium channel
LIN	linearity
L-NAME	Nω-Nitro-L-arginine methyl ester hydrochloride
LDL	low density lipoproteins
MDA	malondialdehyde
MMP	mitochondrial membrane potential
3MP	3-mercaptopyruvate
3-MST	3-mercaptopyruvate sulfurtransferase
NaHS	sodium hydrosulphide
Na_2S	sodium sulphide
NO	nitric oxide
NOS	nitric oxide synthase

nNOS	neural nitric oxide synthase
PUFA	polyunsaturated fatty acids
PLP	pyridoxal-5′-phosphate
PM	progressive motility
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
sGC	soluble guanylate cyclase
SNP	sodium nitroprusside
SMCs	smooth muscle cells
SOD	superoxide dismutase
STR	straightness
TM	total motility
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight-line velocity
WOB	wobble coefficient

The content

Abstract

The successful reproductive biotechnology and assisted reproduction methods also depends on the quality of semen. During collection and dilution of boar semen is important to do it carefully and properly to maintain its quality during storage. Boar sperm contains high concentrations of polyunsaturated fatty acids which are very susceptible to lipid peroxidation and serve as prefered substrates of reactive oxygen species. Oxidative stress is important factor which decreases the quality of semen. Using chemicals which have antioxidant properties is useful to decrease oxidative stress. It is known that gasotransmitters such as nitric oxide (NO) and hydrogen sulphide (H_2S) have a role in physiology. NO has dual effect, low concentration mostly has positive effect, high concentrations negative.

In this study we evaluate the effect of donor NO, sodium nitroprusside (SNP) and Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) or aminoguanidine (AG) as non-selective and selective nitric oxide synthase (NOS) inhibitors, respectively. The aim of this study was to evaluate the effect of NO on boar sperm motility, membrane integrity and acrosomal status during 72 h of semen storage at 17ºC. The effect on non-selective nitric oxide synthase (NOS) inhibitor L-NAME on boar semen stored at 17 °C in the concentration 0.625 mM did not show effect on quality, in contrast higher concentrations showed negative effect. This results suggests that low NO levels are necessary for boar sperm physiology. A significant increase of the amplitude of lateral sperm head displacement (ALH), and both curvilinear and straight-line velocity (VCL and VSL, respectively) was observed at 72 h of semen storage in samples treated with 0.625 mM selective AG, probably because of the antioxidant properties of this selective NOS inhibitor.

Concerning NO donor, we found that SNP concentrations from 18.75 till 150 μM had mostly no or only negative effects on boar sperm parameters during semen storage.

We also evaluated the *in vitro* effect of NOS inhibitor AG on boar sperm cells under induced oxidative stress. Our results demonstrate that AG mostly abolishes the deleterious effects of oxidative stress *in vitro* in boar spermatozoa. In virtue of its antioxidant capacities, AG preserves the boar sperm motility, reduces the lipid peroxidation, protects the plasma membrane and acrosome integrity under oxidative stress. Interestingly, AG 1 mM mostly

eliminates the negative effects of oxidative stress given that, except for the lipid peroxidation, all sperm parameters did not differ or even showed better performance than those of control samples without the reactive oxygen species (ROS) generating system.

It is known that H_2S has role in many diseases, but his role in sperm physiology still is not known. That´s way we evaluated effect of H_2S donors on boar semen under induced oxidative stress. The aimed of this study was to determine the total antioxidant capacities of sodium sulfide (Na_2S) and morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate (GYY4137), which are fast- and slow-releasing H_2S donors, respectively, and to test whether H_2S donors are able to protect spermatozoa against oxidative stress. We used slow releaser based on his rate of H_2S release, GYY4137, and fast releaser Na_2S. Within the range of concentration tested (3-300μM), GYY413 showed positive effect on boar sperm motility, whereas Na_2S was beneficial at the lowest concentration but detrimental at the highest. It is interesting that this two donors showed different effects on boar semen. Moreover both H_2S donors preserve sperm motility and reduce acrosome loss, although the effects were both dose and donor dependent.

1. Introduction

Reproductive biotechnology such as for example artifical insemination (AI) is wide spread in animal reproduction. The biggest problem is the quality of the semen used for (AI). On quality of semen also has influence stress *in vitro* conditions during collection, dilution and storage of boar semen.

AI of swine is widely practiced in countries with intensive pig production. Using AI is a very useful tool to introduce superior genes into sow herds, with a minimal risk of disease and better exploitation of boars.

There are three important aspects which should be considered before using semen for AI: only semen from healthy boars should be used; the quality of the semen; the semen processing procedure. The results of AI largely depend on the semen quality and the insemination procedure. The quality of boar ejaculate is necessary to be evaluated immediately after extraction, after dilution, and before use. Semen quality assessment aims to eliminate ejaculates with low quality and to improve the results of fertilization. Sperm analysis includes macroscopic and microscopic assessment.

The nitric oxide (NO) influences the sperm physiology in dose-dependent manner. At low concentrations, NO has positive effects in human spermatozoa: it improves sperm motility and viability, accelerates capacitation, hyperactivation, and acrosome reaction, and also promotes fusion with oocyte. However, high concentrations of NO have negative effects and disturb those processes. The aim of first part of this study was to evaluate the effect of NO donor sodium nitroprusside (SNP) and nitric oxide synthase (NOS) inhibitors Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) and aminoguanidine (AG) on boar sperm motility, membrane integrity and acrosomal status during 72 h of semen storage at 17ºC. According to results in first part of experiment in second part we evaluated effect of AG on total antioxidant capacity, lipid peroxidation, motility, plasma membrane integrity, and acrosomal status under induced oxidative stress.

The same like NO, hydrogen sulphide (H_2S) has dual effect, at low concentrations has positive effect on all those processes, but at high concentrations has negative effects and disturb those processes. Considering all these roles which have these two gasotransmitters we decided to investigate more their role on quality of boar semen. The aim of this part of study was to evaluate the *in vitro* effect of fast- and slow-releasing donors of H_2S sodium sulphide (Na_2S) and morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate (GYY4137),

respectively, on sperm (total antioxidant capacity, motility, mitochondrial status, plasma membrane integrity, lipid peroxidation, and acrosomal status) under induced oxidative stress.

2. Literature research

2.1. Spermatogenesis in the boar

Spermatogenesis is the sequence of cytological events resulting in the formation of mature spermatozoa from spermatogonia (Knobil and Neil, 2006).

Spermatogenesis is a process in which a series of divisions and the transformation of cells with a diploid number of chromosomes, create spermatozoa with haploid number chromosomes (Jotanović and Savić, 2017). The process of spermatogenesis in boars starts between 4-6 months of age in the majority of breeds, except Chinese fertile breeds where it occurs earlier (Haley et al., 1990). As described by Kyriazakis and Whittemore (2006), first ejaculations in boars occur between 5-6 months of age, and number of spermatozoa and volume of ejaculate continuously increase during the first 18 months of their life.

Nevertheless, the achievement of sexual maturity is related to the characteristics of the breed and the hybrid of the boars, the intensity of their daily growth, diet, accommodation and the annual season, as well as individual characteristics of boars (Jotanović and Savić, 2017).

The process of spermatogenesis consists of several phases (spermatogoniogenesis, meiotic division, spermiogenesis and spermiation), and it lasts about two months (Jotanović and Savić, 2017). There are four steps that together constitute spermatogenesis: stem cell renewal by the process of mitosis; stem cell amplification by mitosis and differentiation; reduction of chromosomal number by meiosis; and the transformation of the round spermatid into the spermatozoa by a series of changes involving no further cell division (spermiogenesis) (Kretser et al., 1998).

Spermatozoa are the “secretory product” of the spermatogenic proces. In order to enable a continuous production of “cells”, the process of spermatogenesis must involve a continuous replication of stem cells to produce cohorts of cells that can proceed through the subsequent changes (Knobil and Neil, 2006). The single stem cells which are randomly arranged along the basement membrane, and which undergo infrequent mitotic division are spermatogonial stem cells (de Rooij and Russell, 2000). Spermatogonial cells undergo several rounds of mitosis, beginning the pathway by which groups of spermatogonia then proceed to enter meiosis and first are termed primary spermatocytes and then secondary spermatocytes

(Zini and Agarwal, 2011). Meiosis consists of two cell divisions: in the first, the chromosomes of primary spermatocytes appear as pairs of chromatids, subsequent to which heterologous chromosomes pair by synapsis to form bivalents (Jotanović and Savić, 2017). Each member of the bivalent pair subsequently moves to the daughter cells, secondary spermatocytes, which contain haploid number of chromosomes (Knobil and Neil, 2006). After the first meiotic division, secondary spermatocytes undertake the second meiotic division (Zini and Agarwal, 2011). The relatively infrequent appearance of these cells in sections of the testis (Heller and Clermont, 1964) indicates a short life span before they complete meiosis to form spermatids. Secondary spermatocytes divide to form spermatids, haploid cells that are transformed during the spermiogenesis into spermatozoa (Knobil and Neil, 2006). The transformation of spermatids into spermatozoa involves a quite interesting but complex sequence of events that constitute the process of spermiogenesis (Garcia-Gil et al.,2002).

After a series of cellular divisions and the transformation of the resulting spermatids into spermatozoa, they are not immediately capable for fertilization. It is necessary to spend some time in the epididymis, in which they become mature and are kept until they are thrown into the outer environment through the ejaculation. According to Uremović and Uremović (1997) the process of spermatogenesis in boar lasts 40 to 45 days, and still spermatozoa spend in the epididymis another 10 days. Therefore, the complete formation of mature spermatozoa lasts for a total duration of 50 to 55 days. Stančić (1994) states that the process of spermatogenesis in testis lasts 40-50 days, and is followed by a period of the maturation of the resulting spermatozoa in epididymis that lasts about 15 days, which makes that the first mature spermatozoa can be founded in ejaculate after 60-65 days. Sexual inactivity results in the accumulation of sperm within the extragonadal ducts and subsequent losses via micturition (Chenoweth and Lorton, 2014).

2.2. The composition of boar semen and methods for sperm collection

The main function of the boar reproductive system is the production and the ejaculation of semen, composed of a cell fraction, the spermatozoa, and a noncellular fraction, the seminal plasma, which is constituted by a mix of secretions from the testes, the epididymis, and the accessory sex glands (Knobil and Neill, 2006).

The components of boar seminal plasma are organic and inorganic compounds, namely carbohydrates, lipids, amino acids and proteins of high and low molecular weight (Pursel et al., 1973, Mann and Lutwak-Mann, 1982). Boar seminal plasma contains fructose, glucose and sorbitol as its main sugar components. According to Chenoweth and Lorton (2014), in male reproduction the important factors are: the production of adequate numbers of viable sperm, and capacity to be used for semen collection and AI. Different methods of collecting ejaculates from boars are: with artificial vagina, electroejaculation and the gloved-hand method. The most common is the gloved-hand method (Bonet et al., 2013). The boar ejaculate is divided in three fractions: prespermatic, spermatic, and postspermatic (Briz, 1994, Sancho, 2002). Prespermatic fraction does not contain spermatozoa and it is between 10-15 ml. It is product of prostata, seminal vesicles Cowper or bulbourethral glands. Spermatic fraction is product of prostata and seminal vesicles, the volume is among 70-100 ml and the color is milky-white. It contains 0.5×10^9 and 1×10^9 spermatozoa per ml. Postspermatic fraction is product of prostata and Cowper glands and it is gelatinous consistency. This fraction has volume between 150-200 ml. It is of pale white color and contains a small number of spermatozoa, less than 10^6 spermatozoa per ml. The volume of boar ejaculate varies between 150 and 300 ml and in some cases can achieve 500 ml (Martín, 1982, Garner and Hafez, 1996, Rothschild, 1990, Pinart et al., 1999, Sancho, 2002). The concentration of spermatozoa in boar ejaculates varies between 10×10^9 and 100×10^9 spermatozoa (Crabo, 1997, Sutkeviciene et al., 2005). The pH values in boar ejaculate varies between 6.85 and 7.9.

According to Briz (1994) and Holt et al., (2010), the mature boar spermatozoa is an elongated cell of about 43–45 μm in length with two major distinguishable regions, the head and the tail, separated by a short linking segment called the connecting piece.

The oval shaped sperm head in the pig is comprised of three main functional components; the plasma membrane, acrosome, and nucleus (Spencer, 2010). The tail originates from the centrioles and consists of four main segments; the neck, middle (mid) piece, principal piece, and end piece (Spencer, 2010).

2.3. Evaluation of boar semen

2.3.1. The qualitive and quantitative parameters of fresh boar semen

Analyzing the quality of boar semen includes the following key measurements: semen volume, sperm concentration, total number of spermatozoa per ejaculate, sperm viability, sperm motility, and sperm morphology (Table 1). There are numerous types of spermatozoa movement, and progressive motility is of fundamental importance in the daily control quality of semen. The boar semen contains a higher number of spermatozoa which have circular movement, in comparision with other species such as bull, boar, deer, and horse (Johnson et al., 2000).

Sperm morphology is an important criterion for the assessment of sperm fertilizing ability. Consequently, a proper evalution of the morphological characteristics of spermatozoa represents a fundamental part of a routine seminal analysis of boar ejaculates (the percentage of mature, immature spermatozoa, sperm abnormalities or aberrant spermatozoa) (Bonet et al., 2000).

Table 1. Parameters of boar semen quality.

Paremeters		**Values**	**References**
Volume		150-300 ml	Pruneda *et al.,* 2005, Smital, 2009, Yeste *et al.,* 2010
Number of spermatozoa		$10 \times 10^9 – 100 \times 10^9$	Crabo, 1997, Sutkeviciene *et al.,* 2005
Viability		> 75%	Hugues and Varley, 1984, Sancho, 2002
Motility	total motility	> 80%	Hugues and Varley, 1984, Sancho, 2002
	progressive motility	> 60%	
Morphology	normal spermatozoa	> 80%	Bonet *et al.,* 2000
	mature spermatozoa	80 – 95%	
	immature spermatozoa	5 – 15%	
	abberant spermatozoa	5%	
pH		7.2 – 7.5	Johnson *et al.,* 2000
Osmolality		~300m $Osm \cdot Kg^{-1}$	Martín, 1982

2.4. Cryopreservation of boar semen

Cryopreserved boar sperm has been available for commercial use since 1975 (Johnson, 1985). Cryopreservation is the most efficient method for long-term preservation of mammalian sperm (Yeste, 2015). During cryopreservation boar semen is exposed to physical and chemical stress, less than 50% of the spermatozoa survive with fertilizing ability maintained (Waterhouse et al., 2006). Many researchers contributed the protocol how to cryopreserve boar semen including Pursel and Johnson (1971, 1975), Westendorf et al., (1975), Paquignon and Courot (1976), Larsson et al., (1977), Larsson (1978), Bwanga (1991), Johnson et al., (2000), and Eriksson and Rodrigues-Martinez (2000) and Athurupana et al., (2015). The steps of cryopreservation are a reduced (1:1) extension with buffer and a period of equilibration in the presence of homologous seminal plasma to promote the capacity of spermatozoa to withstand thermal shock events usually done while cooling the semen to 15ºC. After that a separation of the bulk of seminal plasma by centrifugation, thus reconcentrating the spermatozoa, inclusion of chilling

protectants (lactose and egg yolk) during cooling to 5ºC.

Then inclusion of penetrating cryoprotectants (the most commonly used glycerol at a 4% final concentration) immediately prior to freezing to enable rapid decreases of temperature, which are usually effected by programmable freezers. According to the boar sperm cryopreservation protocols, centrifugation is a necessary step before freezing. It facilitates the removal of seminal plasma and concentrates the spermatozoa so that they can be diluted later with freezing extenders (Zhang et al., 2012). Results indicated that the high g-force (2400×g) and short centrifugation time (3 min) achieved high sperm recovery and yield and that, there was a positive effect on the cryosurvival of boar sperm (Carvajal et al., 2004). The optimal level of, concentration of boar spermatozoa is considered to be about 1×10^9 spz/ml (Einarsson, 1971, Bolarín et al., 2006, Roca et al., 2003). There are more reasons for the negative effects of cooling, freezing and thawing, which are mainly caused by lipid phase transitions, ice crystallization and osmotic-induced water fluxes, and subsequent membrane reorganizations influence membrane integrity, structure and function (Hammerstedt et al., 1990). Successful freezing of boar semen depends on many factors and their interactions which influence the capacity of spermatozoa to survive freezing and thawing. These factors may be classified into two categories: 1. internal or fixed factors, such as the inherent characteristics of spermatozoa, and differences between individual boars and ejaculates, and 2. external factors, such as composition of diluents, type and concentration of cryoprotective agents, rates of dilution, cooling, equilibration, and method of semen freezing and thawing (Johnson et al., 2000). In the freezing protocol, it is possible to influence, manipulate or modify the external factors, but the internal factors cannot be influenced. Rapid cooling of spermatozoa from body temperature to temperatures close to freezing irreversibly reduces viability of spermatozoa (Fernández-Santos et al., 2006). Boar spermatozoa are very susceptible to cold shock. Damage to sperm, known as cold shock, is observed as an irreversible loss of motility upon rewarming (Parks, 1997), and a loss of selectivity in membrane permeability (Medeiros et al., 2002). Additionally, during cryopreservation, boar spermatozoa are sensitive to cellular injury and mechanical stress of ice formation all around of the cell (Yeste, 2015). This phenomenon occurs when freshly ejaculated boar spermatozoa are cooled quickly from body temperature to temperatures below 15°C. Another critical temperature zone which spermatozoa have to traverse are -15 to - 60°C during freezing and thawing, and both of these events are potentially harmful (Eriksson and Rodrigues-Martinez, 2000). The most critical variables which influence sperm cryosurvival and thus are a prerequisite for an optimal sperm cryopreservation protocol are the cooling and thawing rates

(Groβfeld et al., 2008).

The most appropriate freezing rate is defined as the fastest one that allows extracellular water freezing without intracellular ice formation. The optimal cooling rate for freezing boar spermatozoa is - 30°C/min (Devireddy et al., 2004). These cooling rates show acceptable post-thaw sperm survival rates (>50%) and are possible for a high proportion of boars (Groβfeld et al., 2008). In cryopreserved semen, the fertilizing ability is lower than at fresh or refrigerated semen (Rath et al., 2009). In their research Fraser et al. (2014) referred that boar sperm stored for a period of 4 years undergoes reduction of different sperm parameters, such as membrane integrity, motility, and mitochondrial function. In (Table 2) is writen recommended protocol for cryopreservation according to previous studies.

Table 2. Recommended protocol for cryopreservation and thawing of boar semen according to listed authors.

Processes	**Values**	**References**
Centrifugation	2400 × g/ 3min	Caravajal *et al.,* 2004
Concentration of semen	1×10^9 spz/ml	Einarsson, 1971, Bolarin *et al.,* 2006, Roca *et al.,* 2003
Extenders	egg yolk based glycerol free extender contains 100 mM trehalose	Athurupana *et al.,* 2015
Packaging	0.5 ml straws	Athurupana *et al.,* 2015 Didion et al., 2013
Optimal cooling rate	-30°C/min	Devireddy *et al.,* 2004
Critical temperature range	-15°C to -60°C	Yeste, 2015
Thawing rate	at 50°C for 20 seconds or at 70°C for 8 seconds	Thurston *et al.,* 2001, Tomás et al., 2014 Athurupana *et al.,* 2015

2.4.1. Differences in sperm freezability among breeds, boars, and within the same ejaculate

Differences exist between breeds, boars, in the same breed, and even between ejaculates from the same boar (Yeste, 2013). Ejaculates from Duroc boars exhibit better freezability than those of Landrace boars, which is confirmed by the higher percentage of spermatozoa with intact plasma membrane and intact acrosome in post-thawed samples from Duroc males (Waterhouse et al., 2006). According Roca et al. (2006) Landrace and Pietrain boars have higher post-thaw sperm motility, membrane integrity, mitochondrial membrane potential, and acrosomal integrity than Large White, Duroc, and Yorkshire boars. Farrowing rates is 20 % higher in post-thawed spermatozoa from Large White boars as compared to Landrace boars, whereas farrowing rates of fresh semen from the same boars were higher for Landrace than for Large

White (Johnson et al., 1981, 1982; Thurston et al. 2001).The differences among breeds, and even among individual boars in fertilizing ability, are related to differences in sperm head dimensions (Peña et al., 2006, Saravia et al., 2007), in the way that high-fertility boars producing spermatozoa with smaller and shorter heads than low-fertility boars (Hirai et al., 2001). In boars, not all ejaculates present the same ability to withstand freeze-thawing. Because of this reason, boars and their ejaculates are classified as 'good' or 'bad' freezers (Watson, 1995) and as 'good freezability ejaculates' (GFE) or 'poor freezability ejaculates' (PFE) (Casas et al., 2009). Variability among boars to sustain sperm cryopreservation could be related to differences in seminal plasma composition (Roca et al., 2006). The supplementation of freezing extender with seminal plasma from boars with good sperm freezability could eventually improve the ability of boar spermatozoa to sustain freezing-thawing processes (Hernandez et al., 2007), improve sperm motility (Rodriguez-Martinez, 1991), maintain acrosome integrity (Maxwell et al., 1996), delay capacitation-like changes (Pursel et al., 1973), and increase the resistance to cold shock (Watson, 2000) or oxidative stress (Roca et al., 2005).

2.4.2. Extenders and antioxidants

The extenders for boar semen may be divided into extenders without buffer, such as egg yolk-glucose, egg yolk-lactose and egg yolk-saccharose-ethylenediamine tetra-acetic acid (EDTA), Mg and Ca salts; and extenders with buffering capacity, such as glycine-phosphate and glucose-phosphate, egg yolk-glucose-citrate, egg yolk-glucose-citrate-EDTA-potassium-unitol-urea, Beltsville F3, Beltsville F5, Tes-tris-fructose-citrate-egg yolk, TEST, Tes-NaK-glucose-egg yolk, Tris-fructose-EDTA-egg yolk, and Tris-glucose-EDTA-egg yolk (Saravia, 2004). The freezing extenders for boar semen usually contain egg yolk plus other agents such as buffers, additives and cryoprotectants (Gutiérez-Pérez et al., 2009). Egg yolk is widely used as a cryoprotective agent in semen freezing extenders for domestic animals to protect the spermatozoa from cold shock during cryopreservation (Jian-Hong et al., 2006). Consequently, the low density lipoproteins (LDL) are commonly used in the extenders in concentration 9%. The most common cryoprotectant for boar sperm is glycerol in concentrations of 2 to 4% in the freezing medium (Corcuera et al., 2007). Amides, especially dimethylacetamide (DMA) and dimethylformamide (DMF), can successfully replace glycerol as penetrating cryoprotectants in freezing protocols for boar semen (Bianchi et al., 2008).

Among the most commonly used extender additives are sugars, proteins and lipoproteins, detergents, anti-oxidants and seminal plasma (Saravia, 2004). The combination of sugar with glycerol has been found indispensable in providing protection during freezing, because the osmotic properties of the sugar provide extracellular protection (Salamon, et al., 1973). It occurs that sugars do not penetrate plasma membranes and raising the percentage of unfrozen water at a given temperature or reducing the concentration of salts in the unfrozen extracellular water (Mazur, 1984). Athurupana et al. (2015), found that using egg yolk-based glycerol-free extender containg 100mM trehalose with appropriate protocol resulted in successful maintenance of all sperm parameters. Other cryoprotectants for boar semen are: sorbitol, mannitol, ribitol, erythritol, threitol, and ethylene glycol whose effects also occur along with a selection of sugars, including trehalose, sucrose, glucose, and fructose. Gómez-Fernández et al. (2012) tested effect of different monosaccharides (glucose, galactose, fructose) and disaccharides (lactose, sucrose, melabiose, theralose) on boar sperm quality after cryopreservation and found that freezing extenders supplemented with each of the monosaccharide presented smaller cryoprotective effect than the control extender supplemented with lactose. The addition of glutathione to the thawing extender resulted in a significant increase in sperm fertilizing ability (Gadea et al., 2004). Jeong et al. (2009) found in their research that a-tocopherol, supplemented at 200 μM concentration in boar semen extender during cryopreservation had a positive effect on postthawed sperm survivability. The addition of antioxidant vitamin E in boar semen before cryopreservation positively affected motility after thawing (Peña et al., 2003). Seminal plasma contains abundant antioxidants (Makker et al., 2009), and evidence suggests that as long as spermatozoa are suspended in SP, they are protected from oxidative damage (Saleh and Agarwal, 2002). L - Glutamine has the ability to cryoprotect boar sperm based on enhancement of post-thaw sperm motility, and it can be used as a partial glycerol substitute in the freezing extender (de Mercado et al., 2009).

2.4.3. Velocity of cryopreservation of boar semen

Boar spermatoza pass through physical and chemical stresses during cooling and freezing as a result of ice formation around of cells (Medrano et al., 2002). To protect spermatozoa from damage during cooling and freezing it is necessary to use apropriate cooling rate (Fiser and Fairful, 1990). Fiser and Fairful (1990), fouded that freezing boar semen in 3% of glycerol at

cooling rate of 30ºC/min provide optimal survival of spermatoza. The optimal cooling rate is between 3ºC min^{-1} and 5ºC min^{-1}, and during freezing between 20ºC min^{-1} and 50ºC min^{-1} (Bwanga, 1991). Deka et al., (2015) tested different freezing rates (30ºC/min, 50ºC/min, and 70ºC/min), and thawing methods (37 ºC for 30 seconds, 50 ºC for 12 seconds and 70 ºC for 10 seconds), the best results on sperm quality was at freezing rate 30ºC/min with the thawing method of 70ºC for 10 seconds.

2.4.4. Thawing of boar semen

During thawing the boar semen is once again exposed to all of the damaging effects of the cooling and freezing phases, however in reverse order (Holt, 2000). After rapid freezing cryoinjury, a high warming velocity is essential for the cryosurvival of spermatozoa (Mazur, 1985). The important factor which affects the survival of spermatozoa is the rate of thawing through the critical temperature range, in which spermatozoa are sensitive, and it ranges between - 15°C and - 60°C (Yeste, 2015). The effectivenes of thawing rate depends on the original rate of freezing (Mazur, 1985). The most effective thawing rate for boar semen is at 50º C for 20 seconds in water, thus limiting the cell's exposure to these harsh conditions and regrowth of ice crystals (Thurston et al., 2001, Tomás et al., 2014). Athurupana et al. (2015), found that rapid thawing of boar semen at 70°C for 8 seconds followed by stabilizing procedure at 39°C for 52 seconds maintained the all parameters of semen. Selles et al., (2003) founded that thawing of boar semen at temperature 50ºC for 12 seconds has better *in vitro* fertilisation (IVF) results, showed a higher number of sperm per penetrated oocyte and a near 10 points higher rate of pronuclear formation in comparison with thawing at 37ºC for 30 seconds. Moreover, a faster thawing rate is beneficial for parameters of the semen quality (Salamon et al., 1973, Pursel and Johnson, 1976). In their research Bamba and Cran (1985) found that fast warming of boar semen in the temperature range between 5°C and 37°C damaged acrosome membranes. The freeze-thawing of boar spermatozoa impaired sperm motility, membrane and acrosome integrity (Yeste et al., 2013, Okazaki et al., 2009), dramatically increased the frequency of spermatozoa with damaged plasma membranes (Ortman and Rodriguez-Martinez, 2010).

2.5. Oxidative stress

The spermatozoa are able to generate reactive oxygen species (ROS) (Aitken et al., 2012), and a major source of ROS are the mitochondria in the sperm midpiece (Storey, 2008). Boar spermatozoa are very susceptible to ROS (Awda et al., 2009). The phospholipids of the sperm plasma membrane have a high content of polyunsaturated fatty acids (PUFA) which are very susceptible to lipid peroxidation (Storey, 2008), and serve as preferred substrates for ROS and HO generation in membranes (Brouwers et al., 2005). Lipid peroxidation disrupts membrane structure and function by disordering membrane phospholipid structure and changing membrane fluidity (Sevanian et al., 1988). Free radicals that can oxidize PUFA are hydroxyl (OH*) , superoxide (O_2^-), hydroperoxyl (HO_2^*), alkoxyl (RO^*), and peroxyl (ROO*) (Štefan et al., 2007). The reaction of free radicals and PUFA forms a lipoperoxyl free radical which attack the neighboring molecule, PUFA, and starts a chain reaction with harmful effects in the membrane structure. This process is called lipid peroxidation. Vitamin E prevents or stops the chain reaction (lipid peroxidation) and converts free radicals into harmless forms. It works by donating a hydrogen atom to a fatty acid (or superoxide) radical and thus preventing further damage (Tappel, 1962; 1980). During the process, α-tocopherol is converted into an α-tocopheroxyl radical which is more stable than peroxyl radical and does not react with PUFA (Burton et al., 1989). The next step is a reaction of α-tocopheroxyl radical with another radical by the formation of a nonradical product or regenerate to α-tocopherol (Tappel, 1962; 1980). One molecule of vitamin E can protect 100 or even more molecules of PUFA from oxidative damage. Spermatozoa produces ROS by abnormal spermatoza, and increased ROS production by spermatozoa is associated with a decreased mitochondrial membrane potential (MMP) (Wang et al., 2003).

ROS has dual effects on sperm function, at low concentrations has positve effects, inducing sperm capacitation (Ford, 2004), hyperactivation (de Lamirande and Gagnon, 1993), acrosome integrity (Aitken et al., 1995), and sperm-oocyte fusion (Aitken et al., 1989), while, in contrast, high concentrations of ROS damage DNA (Bennetts et al., 2005), inhibit sperm-oocyte fusion (Aitken et al., 1989), and reduce sperm motility in humans (de Lamirande and Gagnon, 1992), horses (Baumber et al., 2000), and pigs (Guthrie and Welch, 2006).

2.6. Gasotransmitters – NO and H_2S

Gasotransmitters are small, gaseous molecule which has important roles in organism (Wareham et al., 2018). Gasotransmitters-principally nitric oxide (NO), carbon monoxide (CO), and hydrogen sulphide (H_2S), are endogenous signaling molecules that plays a significant role in the biomedical, clinical, and health sciences (Wang, 2004). NO is a colourless gas, and a biologically active free radical with a time decay 3-5 seconds (Herrero and Gagnon, 2001). It is sharp, sweet odor, boiling point is -151.7 °C, melting point is -163.6 °C. It is noncombustible but accelerates the burning of combustible material, very toxic by inhalation and skin absorption. NO is produced by the oxidation of L-arginine guanidine to form NO and L-citrulline catalyzed by the enzyme NOS (Palmer et al., 1996). The NO production occurs through the action of one of three NOS isoforms (O'Bryan et al., 2000).

Gasotransmitter H_2S is a colourless, flammable, watersoluble gas with the characteristic smell of rotten eggs or the obnoxious odor of a blocked sewer, has a molecular weight of 34.08 and a vapor density (d) of 1.19, boiling point is -60.3°C, melting point is -82.3°C, and freezing point is -86°C (Wang, 2012. Ning et al., 2018). Collectively, these endogenous molecules of gases or gaseous signaling molecules compose a family of „gasotransmitters", and the regulation of ion channels by gasotransmitters, either directly via chemical modification of ion channel proteins or indirectly via second messengers, exerts significant influence on cellular functions (Wang, 2004). H_2S it is not nearly toxic as CO and NO, NO produces ROS and induces oxidative stress (Wei et al., 2000), in contrast of NO H_2S does not produce oxidative stress, it protects the cells against oxidative stress (Whiteman et al., 2004).

2.6.1. *NOS isoforms*

There are three NOS isoforms: a constitutive neural (nNOS) and endothelial (eNOS); and inducible (iNOS) (reviewed by Herrero et al., 2003).

The isoform nNOS is Ca^{2+} and calmodulin-dependent and is expressed at a high rate in the brain (Mayer et al., 1990), in skeletal muscles, pancreatic β-cells, penis, the pituitary gland, adrenal medulla and macula densa and distal nephron of kidney (Wilcox et al., 1992).

The isoform eNOS is also constitutive and Ca^{2+} and calmodulin-dependent and is mostly expressed in vascular endothelial cells, however, it has been found to be also

expressed in pyramidal neurons of hippocamus, syncytiotrophoblasts, platelets, in testes and ejaculates (Mehta et al., 1994).

The isoform iNOS is induced in a wide range of cell types and tissues after they are exposed to cytokines and bacterial products (Stuehr et al., 1991). The iNOS is most actively involved in the generation of NO (Kim et al., 2007).

All three NOS isoforms were detected in many body tissues including the reproductive system in mammalian species (Staicu and Matas Parra, 2017) (Tab. 3).

Tab. 3. Summary of in vitro studies and the techiques used to identify NOS isoforms in different species (Staicu and Matas Parra, 2017).

Identified isoforms	Species	Techniques	References
nNOS	Human	Immunofluorescence	Herrero et al., 1996 b
eNOS	Human	Immunocytochemistry	O'Bryan et al., 1998
nNOS, iNOS, eNOS	Mouse	Kinetic assays measuring the conversion of L-arginine to L-citruline	Herrero et al., 1997
nNOS, eNOS	Bull	Modified griess reaction, Western blot, Immunofluorescence	Meiser and Schulz, 2003
nNOS, iNOS, eNOS	Boar	Western blot	Aquila et al., 2011
nNOS, eNOS	Stallion	Flow cytometry Western blot	Ortega Ferrsuola et al., 2009
nNOS, iNOS, eNOS	Cat	Histochemistry Immunohistochemistry Western blot	Liman and Alan, 2016

2.6.2. Biological functions of NO

The NOS synthesize NO by converting L-arginine to L-citrulline, are located in tissues of urinary and male reproductive system and act as key regulators for sexual function, female

and male reproduction etc. (Buzazdic et al., 2015, Hirst and Robson, 2010).

The target of NO in smooth muscle is, the enzyme soluble guanylate cyclase (sGC), which catalyzes the conversion of guanosine 5‘-triphosphate (GTP) into the second-messenger molecule cyclic guanosine 3‘,5‘- monophosphate (cGMP). sGC exists as a heterodimer composed of subunits α 1 and β 1 (Denninger and Marletta, 1999). The N - terminal of the β1 – subunit contains a histidine residue, which functions as an axial ligand for the enzyme heme group. Activation of the enzyme when NO interacts with the heme, dissociating it from the histidine residue and in effect exposing its catalyc site to GTP (Hobbs, 1997). NO enhances sperm motility by the activation of sGC, the subsequent synthesis of cGMP, and the activation of cGMP dependent protein kinase.

NO is a potent vasodilator and neurotransmitter and has been implicated in numerous physiological, pharmacological, and pathological processes (Moncada et al., 1991). It is also an essential mediator in the female (Yallampalli et al., 1993, Rosselli et al., 1994) and male reproductive tracts (Adams et al., 1992).

NO is involved in the induction and maintenance of erection through hemangiectasis and corpus cavernosum relaxation (Bivalacqua et al., 2004). The nNOS and iNOS were found in the central nervous system, especially the hypothalamic area that control sexual behavior and also regulate penile erection (Azadzoi et al., 2004, Bivalacqua et al., 2004), whereas eNOS were found in the penis and pelvic area of the urethra but less in the body part of the penis (Mizusawa et al., 2002), and enables cavernous body dilate and maintains the status of erection (Burnet et al., 1996). Massive amount of produced by hyperactive nNOS or highly expressed iNOS can contribute to process such as neurodegeneration, inflamation, and tissue damage (Hermann et al., 2012). NO is involved as mediator of penile erection, by neuronal isoform of NOS which is localized in the penile innervation and NOS inhibitors selectively block erections (Burnet et al., 1996). Burnett et al., (1996) confirmed this activity in their research by treatment with L-NAME completely prevents erections in mice. NOS/NO has roles in monitoring the levels of hormones and cytokines, indirectly controlling the processes of spermatogenesis (Lee and Cheng, 2008). Increasing evidences have been indicating that NOS and NO are associated with male infertility. Previous studies suggest that NO influences the sperm physiology in a way that seems strongly dose-dependent (Balercia et al., 2004). The effect of NO seems to be dual, low concentration of NO improves sperm motility, while high concentration contributes to adverse effect (Doshi et al., 2012). Low concentrations of NO increase spermatozoa hyperactivation, whereas high concentrations decrease the hyperactivated spermatozoa motility (Miraglia et al., 2011).

The concentration of NO influences the acrosome reaction, and increases amount of sperm was observed to undergo the acrosome reaction with the presence of NO donor compound (Donelly et al., 1997). Low concentration of NO also plays a role in the maintenance of human sperm viability after cryopreservation and post-thaw sperm (Balercia et al., 2004, Doshi et al., 2012).

Low concentrations of NO donor SNP enhanced sperm motility in hamsters as well as in teleosts (Creech et al., 1998, Yeoman et al., 1998). SNP was beneficial for maintenance of thaw-sperm motility in human semen by reducing lipid peroxidative damage to sperm membranes in concentrations 50 and 100 nM (Hellstrom et al., 1994).

High concentrations of SNP exert negative effects on this sperm parameter (Rosselli et al., 1995, Weinberg et al., 1995, Hassanpour et al., 2007, Rahman et al., 2014). Herrero et al. (1994) reported that a significant decrease on mouse sperm motility was observed in semen samples treated with SNP in a higher concentration of 300 μM, otherwise incubating semen in concentration of 150 μM, did not show any effect. Human semen treated with 0.25-2.5 mM SNP revealed significantly less sperm bound to the zona pellucida compared with the control group (Rosselli et al., 1995). Low level of NO donor induces human sperm capacitation (Zini et al., 1995), NO donor compounds significantly benefit the capacitation, whereas NO inhibitors decrease this process (Herrero et al., 1999).

NOS activity can be inhibited competitively by L-NAME. The inhibitory effect can be overcome by large doses (5 mM) of L-NAME (Sakuma et al., 1988).

NOS inhibitor L-NAME may improve sperm count and morphology that are associated with infertility in varicocelized rat (Bahmanzadeh et al., 2008). This researchers made experiment with varicocele (partial spermatic vein ligation) on rats. This rats were treated with 10mg/kg of L-NAME and founded that sperm count and normal morphology increased in treated rats. Therefore, it might be considered that NO is an important mediator in the pathogenesis of varicocele. Use of NOS inhibitor in infertile men with varicocele may be useful (Bahmanzadeh et al., 2008).

Adding L-arginine in a bicarbonate -CO_2 - buffered medium, mNCSU-37 induces NO synthesis and acrosome loss of boar spermatozoa (Funahashi, 2002).

L-NAME inhibits fertilization in a dose-dependent manner, and its effects is stereospecific (Herrero et al., 1996a). However, Sengoku et al. (1998) found that low concentrations of NO enhance sperm capacitation of human spermatozoa, whereas they show no effect on sperm motility, viability and acrosome reaction. According to Herrero et al. (2003), different

concentrations of NOS inhibitors added to the capacitating media reduce the fertilization rate in a dose dependent manner.

On the other hand, AG acts as a competitive specific inhibitor of the iNOS isoform (Misko et al., 1993) and has the capacity to decrease ROS formation, lipid peroxidation, and cell apoptosis (Giardino et al., 1998). In this way, recent studies show that AG improves sperm parameters in varicocelized rats that exhibit high NO and ROS levels (Abbasi et al., 2011a, 2011b, Alizadeh et al., 2010, 2016), but little is still known on the effect of this iNOS selective inhibitor on boar sperm parameters during semen storage. Effect of NO on boar sperm is shown in Fig 1. (Staicu and Matas Parra, 2017).

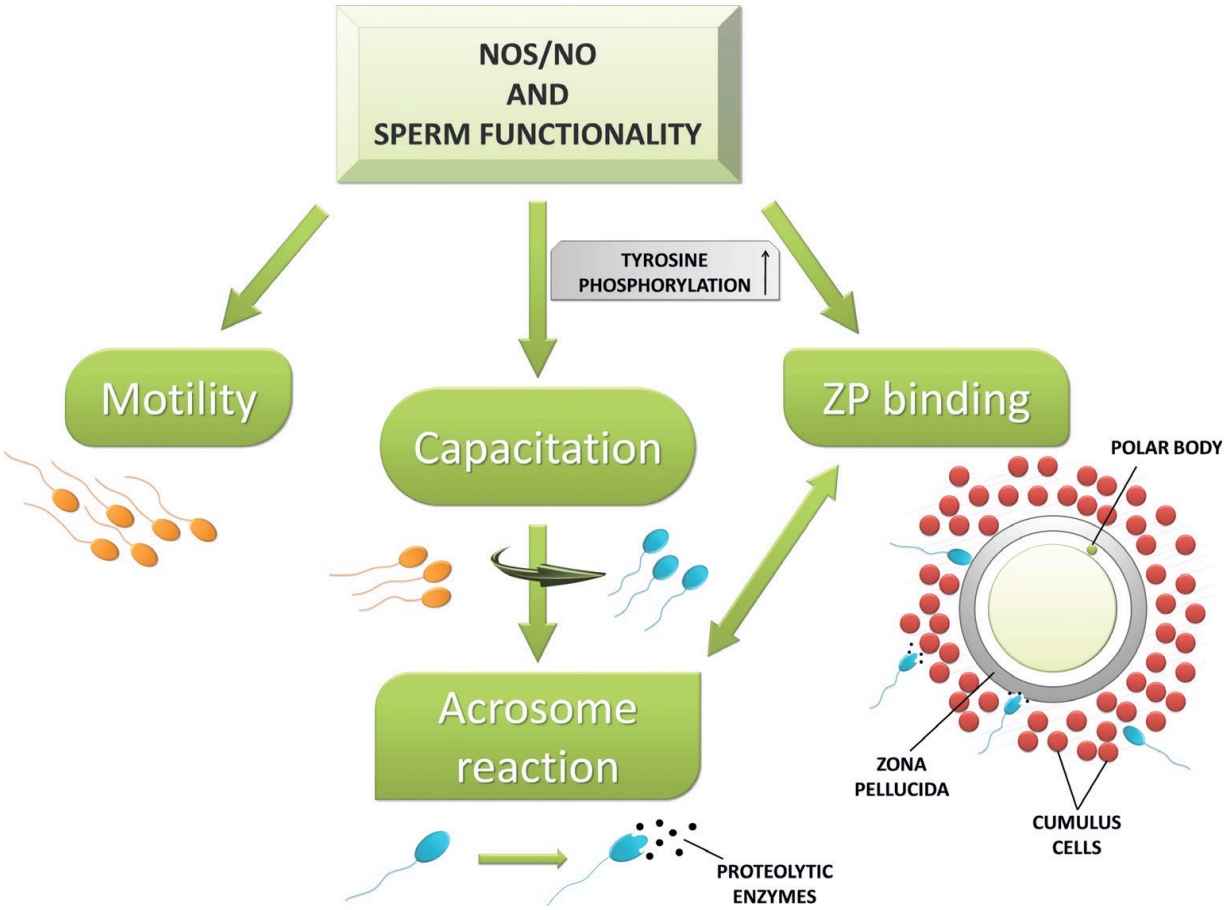

Fig. 1. Some aspects of the sperm physiology modulated by the NOS/NO system. At physiological levels, endogenous NO has a beneficial role in maintaining sperm motility, enhances tyrosine phosphorylation, which, in turn, promotes the capacitation process. NO also

increases the sperm-zona pellucida binding ability and leads to a rise in the percentage of reacted spermatozoa, especially in the presence of follicular fluid or protein-enriched extracts of follicular fluid (Staicu and Matas Parra, 2017).

2.6.3. H_2S synthesizing enzymes

Exists three enzymes which are able to produce hydrogen sulphide: cystathionine γ-lyase (CSE), cystathionine β-synthetase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST) (Kamoun, 2004). Both CSE and CBS are pyridoxal 5′-phosphate (PLP) dependent and utilize L-cysteine as substrate whilst 3-MST is non-PLP dependent and uses 3-mercaptopyruvate as substrate (Shibuya et al. 2009). H_2S is produced outright from CBS and CSE, but the sulfur from 3-mercaptopyruvate is transferred to cysteine on the 3-MST enzyme forming a persulfide (Ishigami et al., 2009). CSE produces H_2S through an α,β-elimination reaction with L-cysteine (Singh et al. 2009), CBS via a β-replacement reaction involving L-cysteine, and 3-MST by metabolizing 3-mercaptopyruvate (3-MP) (Kimura, 2015). The same like NO, H_2S is only present for very short periods before it is converted to more stable and less active compounds. The H_2S remains in the atmosphere for approximately 0.93 day in the summer and 41.9 days in winter becoming converted to sulfur dioxide and sulfuric acid (Bottenheim and Strausz, 1980). H_2S is synthesized by human cells, from head to foot (Farrugia and Szurszewski, 2014). Nearly two thirds of H_2S at physiologic pH exists as hydrosulfide anion (HS^-), a powerful nucleophile, rather than the acid (H_2S) (Bouillaud and Blachier 2011). Critical pH to release H_2S from acid-labile sulfur which is localized mainly in mitochondria is 5.4 (Ishigami et al., 2009). H_2S donors are divided into fast and slow releasers based on their rate of H_2S release, and the most used fast releasers are sodium sulphide (Na_2S) and sodium hydrosulphide (NaHS) (Drapala et al., 2017). Slow releasing H_2S-donor is morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate (GYY4137) (Drapala et al., 2017).

2.6.4. Biological function of H_2S

At beginning scientists considered H_2S as an agent which was of toxicological impact, after some time a gas of interest to pharmacologists to a molecule which now occupies a very

much more central position in gas physiology and with growing therapeutic potential (Huang and Moore, 2015). H_2S has a physiological role, it is founded in relatively high endogenous levels (50-160 μM in the brain of rats (Goodwin et al., 1989), humans (Warenycia et al., 1989) and cattle (Savage and Gould, 1990). The physiological role is induced by exogenous H_2S donor at physiologically relevant concentrations (Wang, 2012). Concentration of H_2S in the brain which is two time higher than endogenous concentration of H_2S in the rat is lethal (Warenycia et al., 1989), >320 PM inhibit synaptic transmission in the hippocampus (Abe and Kimura, 1996).

H_2S is a gasotransmitters like NO, and it is involved in many processes ranging from inflammatory response through to neural control (Wright et al., 2012). H_2S has many roles in body such us: regulates the activities of serotonergic neurons, release of corticotropin-releasing hormone (Kombian et al., 1993, Russo et al., 2000), relaxes smooth muscle (Zhao et al., 2001), protects neurons and cardiac muscle from oxidative stress (Kimura et al., 2006) and regulates insulin secretion (Yang et al., 2005). In their research Chen et al., (2005) founded that endogenous serum H_2S levels was altered in chronic obstructive pulmonary disease (COPD) in man in way that endogenous serum H_2S levels were higher in patients with COPD in comparision to patients with acute exacerbation of COPD (AECOPD). The higher levels of H_2S also was founded in sputum patients with asthma than healthy patients (Saito et al., 2013).

Such NO H_2S also has dual effect, in low concentration has positive effect in contrast in high concentrations has negative effects. H_2S at physiologically high levels affects the structures and functions of the human body at molecular, cellular, tissue, and system levels (Wang, 2003). H_2S acts on vascular smooth muscle and causes vasodilatation via a number of specific pathways including the potassium-activated ATP channels (K_{ATP}) (Szabó, 2007). In blood of different species H_2S exists approximately: 15 sec in fish, 51 sec in cows, 130 sec in rats, and 76 sec in pigs (Whitfield et al., 2008). H_2S has important regulatory roles in chronic respiratory diseases (Chen and Wang, 2012), cardiovascular (Yang et al., 2008), and metabolic disorders (Yang et al., 2011).). H_2S relaxes vascular tissues at physiologically relevant concentrations, an effect mediated by the activation of adenosine triphosphate (ATP) sensitive K^+ (K_{ATP}) channels in vascular smooth muscle cells (SMCs). In his research Wang (2003) founded that intravenous injection of H_2S provoked a transient, but significant, decrease in mean arterial blood pressure of anesthetized rats. In different species such as rats, mice, cows,

guinea pigs, sheep, and humans H_2S induces vasorelaxation in blood vessels (Wang, 2012). H_2S dilates blood vessels by opening of K_{ATP} channels in vascular smooth muscle (Zhao et al. 2001) as well as potentially via intracellular acidification by activating of the Cl^-/HCO_3^- exchanger (Lee et al., 2007). H_2S relaxes smooth muscle cells, and on that way has role in the penile physiology (d'Emmanuele di Villa Bianca et al., 2015), and this is disovered in corpus cavernosum function in animal models and human tissues (d'Emmanuele di Villa Bianca et al. 2011; Qiu et al. 2012). This role also was tested by Srilatha et al., (2006), who founded that the intra-cavernous injection of sodium hydrogen sulfide (NaHS) resulted in a significant increase in penile length and cavernous pressure in primates. In their research Wang et al., (2018) founded that H_2S may play an important role in male infertility, protects both the testes and the sperm in mice by its anti-inflammatory and anti-oxidative effects. In the male reproductive system, the expression of H_2S-generating enzymes has been reported in spermatozoa (Martínez-Heredia et al., 2008; Wang et al., 2018), which strongly suggests that this gasotransmitter is involved in sperm physiology to some extent.

In their research Sugiura et al. (2005) detected CBS and CSE enzymes: CBS was detected in Leydig, Sertoli, and germ cells, whereas CSE was detected in Sertoli and immature germ cells involving spermatogonia. Also other researchers detected H_2S generating enzymes,

Li et al., (2015) founded CBS, CSE, and 3MST in mouse testicular tissue. Exogenous application of H_2S may protect germ cells by preservation of mitochondrial function and stimulation of anti-oxidant activity (Li et al., 2015). Gao et al., (2019), detected CBS and CSE in rat epididymis, and founded that its plays an important role in the maintenance of high K^+ concentration in rat cauda epididymal intraluminal fluid what results in maintenance of the cauda epididymal sperm in quiescent dormant state before ejaculation.

NaHS and Na_2S are the simplest H_2S donors dissolves instantaneously in aqueous solution and produces only sulfide species (Lee and Deng, 2015). Using donors of H_2S Na_2S or NaHS at micromolar concentration in many studies it is demonstrated that has the cytoprotective (antinecrotic or antiapoptotic) effects (Whiteman et al., 2004; Rinaldi et al., 2006), which may be related to its ability to neutralize a variety of reactive species including oxyradicals (Geng et al., 2004) and peroxynitrite (Whiteman et al., 2004), hypochlorous acid (Whiteman et al., 2005).

H_2S produces decreases in arterial pressure, heart rate, and at higher doses cardiac output, for this reasons donors of H_2S may be useful in the treatment of cardiovascular diseases

(Yoo et al., 2015). The donors of H_2S, Na_2S and NaHS in concentrations 0.03 – 0.5 mg/kg produced doserelated decreases in systemic arterial pressure and in heart rate of the rat. Decreases in systemic arterial pressure in response to the H_2S donors are associated with dose-dependent decreases in heart rate and at higher doses significant decreases in cardiac output (Yoo et al., 2015).

In their research Zhao et al., (2016) has demonstrated that H_2S and/or NH_3 disrupted multiple signaling pathways to diminish boar spermatozoa motility *in vitro*. It occurs by decreasing production of ATP and AKT levels, activation of AMPK and PTEN, and an increase in ROS. Using it together Na_2S (50 mg/kg) and NH_4Cl (50 mg/kg) significantly decreased motility of mouse spermatozoa and increased the percentage of immotile spermatozoa *in vivo* (Zhao et al., 2016). As described Wang et al., (2018) GYY4137 in used concentration 2.5 μM improved sperm motility greatly in asthenospermic semen samples with a reduced H_2S concentration in the seminal plasma. In contrast, addition of GYY4137 had no effect on motility in asthenospermic samples with a normal seminal H_2S concentration.

In their research Drapala et al., (2017) founded that parenteral Na_2S, but not GYY4137 because of its high stability at plasma pH, lowers arterial blood pressure in rats. In contrast of this Li et al., (2008) found that intravenous administration of GYY4137 (26.6 to 133 μmol/kg body weight) decreases blood pressure in rats. The slow releasing donor of H_2S GYY4137 inhibits the development of atherosclerosis (Liu et al., 2013), myocardial ischemia (Lee et al., 2014) and cancer (Bucci et al., 2012). Grambow et al., (2014) founded that donor of H_2S GYY4137 delayes venular thrombus formation, increases tail bleeding time threefold and suggests a potential antithrombotic effect. Donor of H_2S GYY4137, in concentration (100 mM) protects human pulmonary arterial cells from hyperoxia, inhibited cellular and mitochondrial ROS production and reversed hyperoxia-induced alveolar cell growth arrest (Vadivel et al. 2014). Qazabard et al., (2014) founded that GYY4137 in concentrations (100-400 μM) significantly increased the life span of the worms, increasing both the growth rate and activity. GYY4137 attenuated oxidative stress and spermatogenic cell apoptosis in rats (Ning et al., 2018).

3. Hypotheses and aims

We set the hypothesis:

1. NO can improve motility, viability and acrosomal status of boar semen.
2. Inhibition of intracellular NO production affects motility, viability and acrosomal status of boar semen.
3. Selective inhibitor of NOS AG may palliate the deleterious effects of oxidative stress in sperm samples.
4. H_2S protects spermatozoa against the deleterious effects of oxidative stress, a condition that is common to several male fertility disorders.

To test the formulated hypothesis, we set the following aims:

1) To evaluate the effect of SNP as NO donor on motility, viability and acrosomal status of boar semen during long-term preservation.
2) To evaluate the effect of AG and L-NAME as NOS inhibitors on motility, viability and acrosomal status of boar semen.
3) To evaluate the *in vitro* effects of AG on sperm (total antioxidant capacity, lipid peroxidation, motility, plasma membrane integrity, and acrosomal status) under induced oxidative stress.
4) To evaluate the *in vitro* effect of fast- and slow-releasing donors of H_2S (Na_2S and GYY4137), respectively, on sperm (total antioxidant capacity, motility, mitochondrial status, plasma membrane integrity, lipid peroxidation, and acrosomal status) under induced oxidative stress.

4. Materials and Methods

4.1. Materials for realized experiments

4.1.1. Experiment 1.

Commercial sperm doses from boars (age: 2.7 ± 1.1 years old, mean ± SD, N = 11) of different breeds (e.g. Pietrain, Duroc, Czech Landrace, Přeštice Black-Pied) were purchased from a breeding company (Chovservis, Hradec Králové, Czech Republic). Boars were fed standard mixtures of cereals and proteins in the form of dry complete feed mixtures or liquid feeds. Sperm-rich fractions were collected every week (once per week) by gloved-hand method, diluted with Solusem® extender (pH ≈ 7, osmolality ≈ 300 mOsm/kg; AIM Worldwide, Vught, Netherlands), and transported to the laboratory at 17 °C. Only sperm samples with at least 75% motile spermatozoa and less than 25% sperm abnormalities were used for the experiments. To reduce the effect of male variability, equal volume of semen samples from different boars was 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, 15 aliquots were made. Equal volume of inhibitors of NOS L-NAME) and AG or donor of NO SNP stock solutions were added to the sperm samples at a tenfold dilution. Therefore, the final concentrations of NOS inhibitors (both L-NAME and AG) on sperm samples were: 0.625, 1.25, 2.5, 5, and 10 mM. The final concentrations of NO donor (SNP) on sperm samples were: 18.75, 37.5, 75, and 150 μM. For the control samples, equal volume of physiological solution was used. All sperm samples were stored at 17 °C.

Sperm motility, membrane integrity and acrosomal status were analysed at 0 (control only), 4, 24, 48, and 72 h of storage after incubating sperm samples at 38 °C for 15 minutes. The experiment was replicated 5 times.

4.1.2. Experiment 2.

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 for each replicate. 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.

After that, samples were randomly allocated into 5 groups: control (CTR), control under oxidative stress (CTR-ox), and 3 treatments of aminoguanidine under oxidative stress (10, 1, and 0.1 mM, respectively). AG was freshly prepared the day of the experiment (stock solution: 0.2 M) by dissolving AG in phosphate buffered saline (PBS) and diluted with sperm samples to give a final concentration of 10, 1, and 0.1 mM. For CTR-ox samples, equal volume of PBS solution was added. Oxidative stress was induced by 0.05 mM $FeSO_4$ and 0.5 mM sodium ascorbate (Fe^{2+}/ascorbate) (Brzezińska-Ślebodzińska et al., 1995).

The experiment was replicated 5 times using 5 different semen pools.

All sperm analyses were performed at 0h (control only), 2h, and 3.5h of incubation in a water bath at 38 °C.

4.1.3. Experiment 3 and 4.

Comercial sperm doses from 15 different boars were purchased 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 for each replicate. After that the semen was centrifugated at 167g/min during 3 min at 17 °C, to remove

nonmotile spermatozoa. 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.

Than 8 aliquots were made: control (CTR), control under oxidative stress (CTR-ox), 3 treatments of GYY4137 (donor of H_2S) under oxidative stress (300 µM, 30 µM, 3 µM, respectively), and 3 treatments of donor of H_2S Na_2S under oxidative stress (300 µM, 30 µM, 3 µM respectively). GYY4137 was prepared in PBS and stored in freezer at -20 °C till usage (stock solution: 20 mM). Na_2S was freshly prepared the day of the experiment (stock solution: 20 mM) in PBS. Both donors separately were diluted with sperm samples to give a final concentration of 300 µM, 30 µM, and 3 µM. For CTR-ox samples, equal volume of PBS solution was added. Oxidative stress was induced by 0.1 mM $FeSO_4$ and 0.5 mM $Fe^{2+/}$ascorbate.

The experiment for the total antioxidant capacity of H_2S donors was replicated 4 times, and the ffect of H_2S donors on boar sperm parameters under induced oxidative stress was replicated 6 times. All sperm analyses were performed at 0h (control only), and 3.5h of incubation in a water bath at 38 °C.

4.2. Methods used in experiments

4.2.1. Assessment of sperm motility

A sperm aliquot (5 µl) was loaded into a pre-warmed (38 ºC) Makler chamber (Sefi-Medical instruments, Haifa, Israel; chamber depth: 10 µm) or into a pre-warmed 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).

The sperm kinetic parameters were obtained by analysing six random fields: total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz), linearity (LIN, %), straightness (STR %), and wobble coefficient (WOB, %).

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 (Pintus et al. 2018) A minimum of 200 motile sperm cells were analysed per sample.

4.2.2. Assessment of sperm head membrane integrity

The assessment was performed as previously described (Grieblová et al. 2017). Briefly, sperm samples were incubated with carboxyfluorescein diacetate (stock solution: 0.46 mg/ml in dimethyl sulfoxide), propidium iodide (stock solution: 0.5 mg/ml in PBS), and formaldehyde solution (0.3% in PBS) 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.

Assessment of sperm tail membrane integrity

The assessment was performed as previously described (Grieblová et al. 2017), using a hypo-osmotic solution (HOST) consisting of 7.35 g/l sodium citrate and 13.51 g/l fructose. Briefly, sperm samples were diluted into pre-warmed HOST solution and incubated for 30 minutes at 38 °C. At the end of the incubation, samples were fixed using a formaldehyde solution (3% in PBS). In each sample 200 spermatozoa were evaluated using phase-contrast microscopy (40× objective) and the sperm cells showing swollen tails were considered to have

an intact tail membrane.

4.2.3. Assessment of acrosomal status

The sperm samples were fixed in 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.

4.2.4. Assessment of acrosome reaction

Acrosome reaction was evaluated according to the protocol described by García-Vázquez et al., (2015). Briefly, sperm samples were smeared onto glass slides, air-dried, and fixed with methanol for 10 minutes at room temperature. Then, samples were washed two times for 3 min with PBS and incubated with peanut agglutinin-fluorescein isothiocyanate (PNA-FITC, stock solution: 200 µg/ml in PBS) for 10 minutes at 37 °C in the dark. Finally, the samples were washed for 5 minutes with PBS, covered with cover glass and evaluated under epi-fluorescence microscopy (40× objective). Two-hundred spermatozoa were evaluated and the spermatozoa showing no fluorescence over the acrosome were considered as acrosome-reacted spermatozoa.

4.2.5. Assesment of sperm mitochondrial status

Mitochondrial status was evaluated as previously described (Fraser et al., 2002), with minor modifications. Briefly, aliquots of sperm samples were incubated with rhodamine 123 (5 mg/ml, w/v, in dimethyl sulfoxide, DMSO) and propidium iodide (0.5 mg/ml, w/v, in PBS) for 15 minutes at 38 °C in the dark. Subsequently, samples were centrifuged at 500 g for 5 minutes, the supernatant was removed, and the sperm pellet was resuspended in PBS. Then, 200 spermatozoa were evaluated by using epifluorescence microscopy (40× objective; Nikon Eclipse E600, Nikon, Tokyo, Japan): the spermatozoa showing bright green fluorescence in the midpiece were considered to have active mitochondria.

4.2.6. Assessment of lipid peroxidation

Lipid peroxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay, as previously described (Brzezińska-Ślebodzińska et al., 1995; Domínguez-Rebolledo et al., 2010). At the end of each incubation time, sperm aliquots were collected and stored at -80 °C till analysis. After thawing samples were thoroughly mixed with stock solution containing 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid and 0.25 N HCl. This mixture was heated at 90 °C for 15 min, and then the reaction was stopped by placing the tubes in ice-cold water for 5 min. After that the tubes were centrifuged at 20000 × *g* during 15 min to pellet the precipitate, and the clear supernatant was collected and transferred to other clean tubes. The liquid (supernatant) is transferred in glass cuvettes. The absorbance of the sample was measured by spectrophotometry at 532 nm. Standard curve was established using known concentrations of 1,1,3,3-tetramethoxypropane (malondialdehyde, MDA). The levels of lipid peroxidation are shown as µmol of MDA per 10^8 spermatozoa.

This assay was run in duplicate per each sample.

4.2.7. Assessment of total antioxidant capacity

At the end of each incubation time, 300 µl of sample were centrifuged at 2,000 × g during 10 minutes. Then 150 µl of supernatant was collected and stored at -80 °C till analysis. The reagent 1 (R1) and reagent 2 (R2) were prepared according to Erel, (2004). R1 is put in plastic cuvettes, in R1 was added sperm sample and immediately readed first absorbance as sample blank, after that is added R2, incubated 5 min, mixed and readed once more. The total antioxidant capacity was determined by spectrophotometry (Libra S22, Biochrom, Harvard Bioscience Company, Cambridge, United Kingdom) at 660 nm using the method described by Erel, (2004). Standard curve was established using known concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The total antioxidant capacity present in the sample cause a reduction in absorption proportional to their concentration and was expressed as mmol/l Trolox equivalents.

This assay was run in duplicate per each sample.

4.3. Statistical analysis

All statistical analyses were performed using the SPSS 20.0 statistical software package (IBM Inc, Chicago, IL, USA). First two experiments was replicated 5 times, the experiment for the total antioxidant capacity of H_2S donors was replicated 4 times, and the ffect of H_2S donors on boar sperm parameters under induced oxidative stress was replicated 6 times. The Shapiro-Wilk test was applied to check for the normal distribution of data. The repeated measures ANOVA or Friedman tests were used to check for differences in sperm parameters in the control group during the different times of incubation. The generalized
linear model (GZLM) was performed to analyze the effects of the treatments and storage times on sperm variables.

In third experiment Shapiro-Wilk's and Levene's tests were used to analyse the normal distribution and the variance homogeneity of the data, respectively. The Mann–Whitney U-test was applied to check for differences between the total antioxidant capacities of Na2S and GYY4137 at the same concentration, whereas the repeated-measures Friedman test was used to compare the total antioxidant capacities of the H_2S donors across the incubation times. GZLM was performed to analyse the effects of the type and concentration of H_2S donor on the sperm variables. In all experiments data are shown as mean ± SD, or as mean ± SE. Statistical significance was set at $p<0.05$.

5. Published articles

5.1. Effect of nitric oxide on boar sperm motility, membrane integrity, and acrosomal status during semen storage

There is a lot of evidence that NO/NOS pathways are involved in the regulation of sperm motility, viability, capacitation, hyperactivation, acrosome reaction, and fertilizing ability (e.g. Herrero and Gagnon 2001, Ješeta et al. 2017). Although intracellular NO is essential for proper sperm function, NO has dose-dependent dual effect on sperm motility. While low concentrations of SNP, a NO donor, increase sperm motility (Hellstrom et al. 1994, Zhang and Zheng 1996), high concentrations of this compound exert negative effects on this sperm parameter (Rosselli et al. 1995, Weinberg et al. 1995, Hassanpour et al. 2007, Rahman et al. 2014). On the other hand, AG acts as a competitive specific inhibitor of the iNOS isoform (Misko et al. 1993) and has the capacity to decrease ROS formation, lipid peroxidation, and cell apoptosis (Giardino et al. 1998).

In this article we tested selective (iNOS), non-selective NOS inhibitor, and NO donor on boar sperm parameters. The aim of this study was to evaluate the effects of NO on boar sperm motility, membrane integrity, and acrosomal status during 72 h of semen storage at 17 °C. For this purpose, we used SNP as a NO donor, and L-NAME or AG as non-selective and selective NOS inhibitors, respectively. The sperm analyses were performed at 0 (control only), 4, 24, 48, and 72 h of semen storage. Concernig to the effect of L-NAME, a non-selective NOS inhibitor, our results are in agreement with previous study, it has negative effect on sperm motility by decreasing the percentage of sperm cells showing rapid progressive motiliy as well as by increasing immotile spermatozoa (human: Rosselli et al. 1995, ram: Hassanpur et al. 2007). In contrast of this inhibitor we founded that AG after 48h and 72h of semen storage in all experimental groups ($p<0.05$), except those treated with the lowest (0.625 mM) and the highest (10 mM) concentrations significantly increased sperm motility index (SMI). Also the lowest concentration 0.625 mM of AG increased sperm kinetic parameters ALH, VCL, and VSL after 72h of storage, which are related to boar fertility (Broekhujise et al. 2012). The positive effects of AG on boar sperm motility might be due to its antioxidant properties and

scavenger activity against free radicals like ROS and RNS (Yildiz et al. 1998). In this way, Abbasi et al. (2011a, 2011b) and Alizadeh et al. (2010, 2016) have shown that AG improves sperm parameters (i.e. concentration, motility, viability, normal morphology, mitochondrial membrane potential, and DNA integrity) in varicocelized rats, where the upregulated iNOS expression leads to high oxidative stress on sperm cells. We therefore hypothesize that the antioxidant properties of AG may protect boar sperm cells against ROS during semen storage.

In this study we found that SNP at concentrations ranging from 18.75 to 150 μM showed mostly no or negative effects on boar sperm motility and kinetics.

Similarly to our findings, previous studies have shown that concentrations of the NO donor SNP ranging from 0.1 to 2,500 μM have no or only negative effects on sperm motility (Rosselli et al. 1995, Weinberg et al. 1995, Rodriguez et al. 2005, Hassanpour et al. 2007, Rahman et al. 2014). In this way, Balercia et al. (2004) have found that astenoozoospermic men exhibit higher levels of NO than those of normozoospermic men. In the same study, authors also found that NO levels were negatively related to the sperm motility, VCL, and VSL, providing further support to our findings. On the other hand, positive effects of SNP on sperm motility and viability were observed at much lower concentrations (i.e. 25-100 nM) (Hellstrom et al. 1994, Zhang and Zheng 1996). The negative effects of SNP might be related to the caspase activation that promotes cell apoptosis (Moran et al. 2008). In this way, Zhang and Zheng (1996) found that concentrations of SNP higher than 100 nM show detrimental effects on sperm viability both in fertile and asthenozoospermic infertile men. In addition to this mechanism, more recently Rahman et al. (2014) found that SNP decreases sperm kinetic parameters by increasing intracellular Fe^{2+} and ROS levels and by decreasing Ca^{2+} and adenosine triphosphate (ATP) levels. Although several studies show that SNP induces the acrosome reaction in capacitated spermatozoa (human: Revelli et al. 2001, bull: Rodriguez et al. 2005, boar: Hou et al. 2008, mouse: Rahman et al. 2014), as expected, we found no effect on the acrosome integrity given that in our experimental design the semen was evaluated under no capacitating conditions.

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5.2. Aminoguanidine protects boar spermatozoa against the deleterious effects of oxidative stress

AG is a selective inhibitor of the iNOS isoform (Misko et al., 1993), which releases large amount of NO• and is found in spermatozoa and activated leukocytes, among others (Dixit and Parvizi, 2001: Aquila et al., 2011). Our findings indicate that AG can be employed for palliating the effects of oxidative stress in sperm cells, in agreement with what previously found in other cells and tissues lung: (Eroglu et al., 2008); bladder: (Abraham et al., 2009); kidney: (Abo-Salem, 2012); testis: (Oguz et al., 2013). Similarly, Abbasi et al. 2011a and 2011b and Alizadeh et al. 2010 and 2016 found that the *in vivo* administration of AG improves the sperm concentration, motility, viability, normal morphology, mitochondrial membrane potential, and DNA integrity in varicocelized rats, where the upregulation of the iNOS isoform may lead to high levels of ROS in the semen. Our results confirm that Fe^{2+}/ascorbate induces a state of oxidative stress characterized by increased levels of lipid peroxidation and reduced sperm motility as previously described (Brzezińska-Ślebodzińska et al.,1995; Guthrie and Welch, 2007). In this study we also found that this ROS generator decreases the sperm head plasma membrane and acrosome integrity and increases the incidence of acrosome-reacted spermatozoa. In contrast to our findings, Guthrie and Welch (2007) found that Fe^{2+}/ascorbate did not affect the sperm membrane integrity (i.e. viability), perhaps because in their study a smaller Fe^{2+}/ascorbate concentration was employed (i.e. 1 µM and 30 µM *vs.* 50 µM and 500 µM, respectively). Our findings also confirm that AG has powerful antioxidant abilities against the oxidative stress induced by Fe^{2+}/ascorbate, as previously described by Yildiz et al. (1998). Irrespectively of the incubation time, AG 10 and 1 mM showed stronger antioxidant capacity than that of control samples with or without induced oxidative stress. The total antioxidant capacity of AG 1 mM was on average 0.5 mmol/l Trolox equivalents, which is within the range described in the boar seminal plasma (Zakošek et al., 2014; Barranco et al., 2015). A greater total antioxidant capacity of the seminal plasma contributes to the ability of boar sperm cells to better sustain the preservation process (liquid-storage and cryopreservation) and is also positively related to the fertility outcomes and litter size (Barranco et al., 2015). In this way, our results indicate the aminoguanidine 1 mM shows a total antioxidant capacity similar to that of boar seminal plasma, which provides further support to the beneficial effects of this compound on boar sperm parameters under induced oxidative stress.

The results of this study show that under induced oxidative stress, AG better preserves sperm motility, plasma membrane and acrosome integrity, being these parameters correlated with male fertility in humans (Khatun et al., 2018) and other species bulls: (Kastelic and Thundathil, 2008); boars: (Jung et al., 2015); stallions: (Love, 2016). Interestingly, at 3.5h of incubation, AG 10 and 1 mM showed percentages of motile sperm cells that were more than twofold greater than those of control samples under oxidative stress. Nevertheless, it is important to highlight that sperm cells treated with AG 10 mM showed rapid curvilinear trajectories with remarkable low values of progressive and linear motility. A plausible explanation of this phenomenon might be due to the antioxidant capacity of AG 10 mM (2 mmol/l Trolox equivalents), which is quite above the physiological range reported in the boar seminal plasma (Zakošek et al., 2014; Barranco et al., 2015). Because certain levels of ROS are required for a normal sperm function (Agarwal et al., 2014; Aitken, 2017), AG 10 mM may reduce the amount of ROS in such a way that it impairs some sperm kinetic parameters, but it does not affect the sperm plasma membrane and acrosome integrity. In this sense, the protective effects on the sperm plasma membrane and acrosome integrity in samples treated with AG 10 were also confirmed by the smaller levels of lipid peroxidation. On the other hand, AG 0.1 mM was able to only partially prevent the damages caused by Fe^{2+}/ascorbate on sperm parameters. In contrast, although the sperm tail integrity tended to be greater in sperm samples supplemented with AG, there were no differences among the latter and control group treated with Fe^{2+}/ascorbate, likely because of the variability among replicates. Another important finding of this study is that AG protects the acrosome integrity and reduces the incidence of acrosome reaction in non-capacitating conditions. It is well known that acrosome integrity is a requisite to the acrosome reaction, which must timely occur in order to allow the penetration of the spermatozoon through the protective barriers of the oocyte (Yeste, 2013). In the porcine species, a partially induced acrosome reaction in the preincubation or fertilization media has been invoked as an important cause of polyspermy, which is one major challenge in the assisted reproductive technologies of this species (Funahashi, 2003). The exposure of boar spermatozoa to a ROS generating system triggers the acrosome reaction (Awda et al., 2009), which may lead to a reduced fertilizing potential. Based on our findings, we can therefore speculate that AG may increase the fertilization potential of porcine spermatozoa by preventing a precocious acrosome reaction under oxidative stress.

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5.3. Divergent effect of fast-and slow-releasing H_2S donors on boar spermatozoa under oxidative stress

H_2S is a gas which plays important roles in health and disease, together with CO and NO (Kimura and Kimura, 2004; Wang, 2012) .The production of H_2S occurs via two pathways – enzymatic and non-enzymatic. Enzymatic occurs through three enzymes that are CSE, CBS and 3-MST. On the other hand, non-enzymatic production of H_2S occurs through glucose, glutathione, inorganic and organic polysulphides (present in garlic) and elemental sulfur (Kolluru et al. 2013). On the basis of their release mechanism, H_2S donors can be classified in two categories: slow- and fast-releasing agents. Among the fast-releasing H_2S donors, the inorganic salts Na_2S and NaHS. Slow releasing donor of H_2S is GYY4137 (Rose et al., 2015). The aim of this study was to evaluate the total antioxidant capacity and stability of the H_2S donors Na_2S and GYY4137 under standard conditions (38 °C, pH≈7) and at different times (i.e. 20, 120, and 210 minutes) during the incubation (experiment I). Based on the results from experiment I and the physiological total antioxidant capacity of boar seminal plasma (Barranco et al., 2015), we then established a suitable range of concentrations of Na_2S and GYY4137 to be tested in boar sperm samples under a ROS-generating system (experiment II). In presented results it is clearly that Na_2S and GYY4137 show different total antioxidant capacities and stabilities under standard conditions (38 °C, physiological pH) and after different periods (20, 120, and 210 minutes) of incubation. Our results also reveal that the total antioxidant capacity of Na_2S is less stable than that of GYY4137, although the latter's total antioxidant capacity tends to increase over time. In this study GYY4137 shows higher total antioxidant capacity than that of Na_2S after any incubation time, with the total antioxidant capacity of Na_2S at 150 µM even being undetectable by spectrophotometry after 20 minutes of incubation.

Our results show that both H_2S donors partly palliate the damages provoked by oxidative stress in sperm cells, supporting the results found previously in other cells (neurons (Kimura and Kimura, 2004; Kimura et al 2010), gastric cells (Yonezawa et al.,2007), testicular germ cells (Li et al., 2015)) and organs (lungs (Han et al., 2011; Benetti et al., 2013, testes (Ning et al., 2018)). In this study we found that the slow-releasing H_2S donor GYY4137 did not show any cytotoxic effect. Moreover, 3 and 30 μM GYY4137 preserved the boar sperm motility against the detrimental effects of oxidative stress,at both concentrations, the percentage of motile sperm cells was almost 50% higher than that of the CTR-ox samples and no kinetic parameters differed from those of the CTR group without oxidative stress. Moreover, 30 μM GYY4137 samples showed a higher percentage of progressive motility than those in the CTR-ox group. In our study we did not find any effect of GYY4137 on the levels of lipid peroxidation, which may be due to the different cell type and donor concentration used in our study. Our findings also show that, in comparison with GYY4137, the effects of Na_2S were markedly dose dependent. At a concentration of 3 μM, this fast-releasing H_2S donor preserves boar sperm motility (40.5% higher than that in the CTR-ox samples), whereas it shows clear cytotoxic effects at 300 μM. This pattern may reflect the well-known biphasic biological dose–response curve of H_2S: it acts as an antioxidant at low concentrations, but a pro-oxidant at high concentrations (Szabo et al., 2014). Interestingly, we also found that 30 μM Na_2S was not cytotoxic (i.e. no effect was observed on the mitochondrial activity, plasma membrane integrity, acrosome integrity, or lipid peroxidation), but it strongly inhibited sperm motility. As in our findings, Zhao et al. (2016) found that 25 μM Na_2S inhibits boar sperm motility although it does not affect the viability and mitochondrial membrane potential. In our study, the inhibitory effects of Na_2S on sperm motility were analyzed using by by Computer-Assisted Sperm Analysis (CASA).

Overall, 30 μM Na_2S remarkably decreased the sperm motility, VAP, and VSL, but it did not affect the remaining motion parameters. In spite of some variability among replicates, a small percentage of sperm cells showed very slow but progressive movement. With regard to the mechanism of action, Zhao et al. (2016) found that the inhibitory effects of Na_2S on sperm motility are related to the adenosine 5′-monophosphate-activated protein kinase (AMPK) and protein kinase B (AKT) pathways whereas a more recent study revealed that H_2S promotes the secretion of K^+ in the epididymis; this suppresses sperm motility and may contribute to keeping the sperm cells in a quiescent state before ejaculation (Gao et al., 2019). In contrast to the effects observed at low concentrations, high concentrations of Na_2S showed clear detrimental effects: no motility or active mitochondria were observed in any sample treated with 300 μM Na_2S. The toxic effects were acute and led to immediate failure of sperm motility and mitochondrial activity (personal observations). This phenomenon can be explained by the high levels of ROS induced by Na_2S (Zhao et al, 2016), an explanation corroborated by the increased levels of lipid peroxidation and the low percentage of sperm with intact plasma membrane and acrosome (NAR) found in our study. Our results also show that Na_2S and GYY4137 partly reduce the damage induced by high ROS levels on acrosomal status by decreasing the percentage of acrosome-lost (PNA-FITC) spermatozoa relative to that in the CTR-ox group. However, neither of the two donors showed any positive effect on the acrosome integrity evaluated by the NAR test. During handling and storage semen decrease sperm quality and fertilising ability, probably because of oxidative stress (Kumaresan, et al., 2009; Lopez Rodriguez et al., 2017) so H_2S donors may be useful for the optimisation of boar semen extenders.

Further studies are nevertheless required to investigate the mechanisms of action of H_2S donors in sperm cells under oxidative stress.

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5.4. Cryopreservation of boar semen

The sperm cryopreservation is the most efficient method for longterm storage of the semen in domestic animals (Yeste 2015). However, only 1% of the boar semen that is used for AI throughout the world is cryopreserved (Techakumphu et al. 2013). The remaining 99% is preserved in liquid form at 16-20ºC (Saravia et al. 2005). In comparison with other mammalian species, boar spermatozoa have low survivability rates during the freezing-thawing process and the short lifespan of the surviving spermatozoa (Mazur et al. 2008; Techakumphu et al. 2013) due to their high cold shock sensitivity. Approximately 40 to 50% of boar spermatozoa do not survive cryopreservation. Intracellular ice crystal formation is a major cause of the damage sustained by spermatozoa during the freezing process (Johnson et al., 2000; Watson, 2000). Boar spermatozoa are very susceptible to peroxidative damage because of high content of polyunsaturated fatty acids (Brouwers et al. 2005; Esmaeili et al., 2015), and low level of cholesterol in plasma membrane. Among the reasons for this restricted use of frozen boar semen are great variability of semen quality and freezability between breeds as well as individual boars and even the season. The review is focused on using various additives to freezing and thawing extenders and freezing protocols for efficient cryopreservation The most critical variables that influence sperm cryosurvival and thus serve as prerequisites for an optimal sperm cryopreservation protocol are the cooling (Said et al., 2010) and thawing rates. The optimal cooling rate for freezing boar spermatozoa is - 30°C/min, while the most suitable rate for thawing is 1200°C/min. The most effective thawing rate for boar semen is 1200 to 1800 ºC/min (20 sec in 50 ºC water), which limits the exposure of the cell to such harsh conditions as well as regrowth of ice crystals (Fiser et al., 1993; Thurston et al., 2001). Extender additives are usefull to maintain sperm quality during freeze/thawing of boar sperm. The most used additives are: egg yolk (Bathgate et al., 2008); low density lipoproteins (LDL) Jian-Hong et al., 2006;

Jing et al., 2007); glycerol (Corcuera et al., 2007); L-Glutamine (De Mercado et al., 2009); sodium dodecyl sulphate (SDS) (Axner et al., 2004; Pursel et al., 1978); sugars (trehalose, sucrose, lactose, glucose, fructose) (Hu et al., 2009; Malo et al., 2010; Athurupana et al., 2015; Gomez-Fernandez et al., 2012); antioxidants (glutathione (Gadea et al., 2004), α-tocopherol (Jeong et al., 2009), seminal plasma (Barranco et al., 2015), vitamin E (Peña et al., 2003), ascorbic acid (Breininger and Beconi, 2014; Varo-Ghiuru et al., 2015), lutein (Varo-Ghiuru et al., 2015), superoxide dismutase (SOD) (Roca et al., 2005), catalase (CAT) (Roca et al., 2005)).

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6. Conclusions

The aim of this thesis was to prove that gasotransmitters NO and H_2S has role on boar sperm physiology. Also one aim of the thesis was to describe the literature available about the cryopreservation of boar semen. In our experiments we proved that NO and H_2S has role on boar sperm physiology. Our results show that low concentration of iNOS inhibitor AG increases sperm kinetics and may indicate the potential use of this selective iNOS inhibitor to palliate the effects of oxidative stress during semen storage, what we confirmed in *experiment 2*. Our findings show that AG mostly abolishes the deleterious effects of oxidative stress *in vitro* in boar spermatozoa. AG preserves the boar sperm motility, reduces the lipid peroxidation, protects the plasma membrane and acrosome integrity, and decreases the incidence of acrosome reaction under oxidative stress. High concentrations of both selective and non-selective NOS inhibitors negatively affect sperm kinetics and acrosome integrity, which suggests that low NO levels are necessary for boar sperm physiology. Concerning NO donor, we found that SNP concentrations from 18.75 till 150 μM had mostly no or only negative effects on boar sperm parameters during semen storage. Our findings clearly show that Na_2S has a shorter and less stable total antioxidant capacity than that of GYY4137. However, it is important to stress out that the antioxidant capacity of GYY4137 tends to increase over time. We also found that both H_2S donors preserve sperm motility and protect the acrosomal status against the detrimental consequences of oxidative stress, although the effects were clearly both dose and donor dependent. Taken together, our results suggest that Na_2S and GYY4137 may be used as in vitro therapeutic agents against oxidative stress in sperm cells, although the optimal therapeutic range varies between H_2S donors. This study provides evidence about the antioxidant properties of two H_2S donors, Na_2S and GYY4137. The results from this study increase the understanding of the role of NO and H_2S on boar sperm physiology.

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8. Attachments

1) Jovičić, M., Pintus, E., Fenclová, T., Šimonik, O., Chmelíková, E., Ros-Santaella, J. L., Sedmíková, M. 2018. Effect of nitric oxide on boar sperm motility, membrane integrity, and acrosomal status during semen storage. Polish Journal of Veterinary Sciences. 21 (1). 73 - 82.
2) Pintus, E., Kadlec, M., Jovičić, M., Sedmíková, M., Ross-Santaella, J. L. 2018. Aminoguanidine Protects Boar Spermatozoa against the Deleterious Effects of Oxidative stress. 2018. Pharmaceutics.10 (4). 212.
3) Pintus, E., Jovičić, M., Kadlec, M., Ros-Santaella, J. L. 2020. Divergent effect of fast- and slow- releasing H_2S donors on boar spermatozoa under oxidative stress. SCIENTIFIC REPORTS. 10. 6508.
4) Jovičić, M., Chmelíková, E., Sedmíková, M. 2020. CRYOPRESERVATION OF BOAR SEMEN. Czech Journal of Animal Science. 65 (04). 115 - 123.

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Original article

Effect of nitric oxide on boar sperm motility, membrane integrity, and acrosomal status during semen storage

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Abstract

Nitric oxide (NO) is a major gasotransmitter involved in several physiological processes of male reproduction. There is, nevertheless, little information concerning the role of NO during semen storage. The aim of this study was to evaluate the effect of NO on boar semen stored at 17°C for 72 h. For this purpose, sperm samples were treated with 0.625, 1.25, 2.5, 5, and 10 mM aminoguanidine (AG) or Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), a selective and non-selective NO synthase (NOS) inhibitor, respectively. Moreover, sodium nitroprusside (SNP), a NO donor, was used at the dose of 18.75, 37.5, 75, and 150 µM. Sperm motility, membrane integrity, and acrosomal status were evaluated at 0, 4, 24, 48, and 72 h of semen storage. A significant increase of the amplitude of lateral sperm head displacement (ALH), and both curvilinear and straight-line velocity (VCL and VSL, respectively) was observed at 72 h of semen storage in samples treated with 0.625 mM AG, probably because of the antioxidant properties of this NOS inhibitor. Contrarily, 0.625 mM L-NAME showed no effect on boar sperm parameters during the entire period of semen storage. Moreover, AG and L-NAME at 10 mM negatively affected sperm kinetics and acrosome integrity, which may provide further support to the notion that low NO levels are necessary for a normal sperm function. The concentrations of SNP used in this study had mostly no or negative effects on boar sperm parameters during semen storage. In conclusion, the results from this study increase the understanding of the role of NO on boar sperm physiology.

Key words: boar spermatozoa, nitric oxide, nitric oxide synthases, NO donor, NOS inhibitor

Introduction

Nitric oxide (NO) is one of the signalling molecules responsible for the regulation of sperm function (Herrero and Gagnon 2001). Intracellular NO is formed from guanidine by nitric oxide synthase-catalysed reaction. There are three nitric oxide synthase (NOS) isoforms: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). The nNOS and eNOS isoforms are constitutive and produce small amounts of NO, whereas the iNOS isoform produces large amounts of NO (Dixit and Parvizi 2001, Herrero and Gagnon 2001). The NOS isoforms have been described in human (Herrero et al. 1996), mouse

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(Herrero et al. 1996), and boar (Aquila et al. 2011) spermatozoa. There is a lot of evidence that NO/NOS pathways are involved in the regulation of sperm motility, viability, capacitation, hyperactivation, acrosome reaction, and fertilizing ability (e.g. Herrero and Gagnon 2001, Jeseta et al. 2017).

Although intracellular NO is essential for proper sperm function, NO has dose-dependent dual effect on sperm motility. While low concentrations of sodium nitroprusside (SNP), a NO donor, increase sperm motility (Hellstrom et al. 1994, Zhang and Zheng 1996), high concentrations of this compound exert negative effects on this sperm parameter (Rosselli et al. 1995, Weinberg et al. 1995, Hassanpour et al. 2007, Rahman et al. 2014). The high levels of reactive oxygen and nitrogen species (ROS and RNS, respectively) are considered to be main components of oxidative stress and one major cause of poor sperm quality in humans (Balercia et al. 2004, Uribe et al. 2015). The stress produced by high ROS and RNS levels decrease total and progressive sperm motility, kinetics, and mitochondrial membrane potential (Uribe et al. 2015, Jeseta et al. 2017). Likewise, the inhibition of NOS by Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) inhibits sperm motility (Hassanpour et al. 2007) and acrosome reaction (Hou et al. 2008). On the other hand, aminoguanidine (AG) acts as a competitive specific inhibitor of the iNOS isoform (Misko et al. 1993) and has the capacity to decrease ROS formation, lipid peroxidation, and cell apoptosis (Giardino et al. 1998). In this way, recent studies show that AG improves sperm parameters in varicocelized rats that exhibit high NO and ROS levels (Abbasi et al. 2011a, 2011b, Alizadeh et al. 2010, 2016), but little is still known on the effect of this iNOS selective inhibitor on boar sperm parameters during semen storage.

The aim of this study was to evaluate the effects of NO on boar sperm motility, membrane integrity, and acrosomal status during 72 h of semen storage at 17°C. For this purpose, we used SNP as a NO donor, and L-NAME or AG as non-selective and selective NOS inhibitors, respectively. The sperm analyses were performed at 0 (control only), 4, 24, 48, and 72 h of semen storage.

Materials and Methods

Preparation of NOS inhibitors and NO donor

Stock solutions of L-NAME and AG were prepared by dilution of chemicals in physiological solution (NaCl 0.9%, w/v) at concentrations of 6.25, 12.5, 25, 50, and 100 mM. Stock solutions of SNP were prepared by dilution of compound in physiological solution (NaCl 0.9%, w/v) at concentrations of 187.5, 375, 750, and 1,500 µM. Stock solutions were stored at -20°C till usage.

Sperm samples collection and processing

Commercial sperm doses from boars (age: 2.7 ± 1.1 years old, mean ± SD, $N = 11$) of different breeds (e.g. Pietrain, Duroc, Czech Landrace, Přeštice Black-Pied) were purchased from a breeding company (Chovservis, Hradec Králové, Czech Republic). Boars were fed standard mixtures of cereals and proteins in the form of dry complete feed mixtures or liquid feeds. Sperm-rich fractions were collected every week (once per week) by gloved-hand method, diluted with Solusem® extender (pH ≈ 7, osmolality ≈ 300 mOsm/kg; AIM Worldwide, Vught, Netherlands), and transported to the laboratory at 17°C. Only sperm samples with at least 75% motile spermatozoa and less than 25% sperm abnormalities were used for the experiments. To reduce the effect of male variability, equal volume of semen samples from different boars was mixed. Then, sperm concentration was ckecked using a Bürker chamber and samples were further diluted with Solusem® to get a final concentration of 20 × 10^6 spermatozoa/ml. Then, 15 aliquots were made. Equal volume of L-NAME, AG or SNP stock solutions were added to the sperm samples at a tenfold dilution. Therefore, the final concentrations of NOS inhibitors (both L-NAME and AG) on sperm samples were: 0.625, 1.25, 2.5, 5, and 10 mM. The final concentrations of NO donor (SNP) on sperm samples were: 18.75, 37.5, 75, and 150 µM. For the control samples, equal volume of physiological solution was used. All sperm samples were stored at 17°C. Sperm motility, membrane integrity and acrosomal status were analysed at 0 (control only), 4, 24, 48, and 72 h of storage after incubating sperm samples at 38°C for 15 minutes. The experiment was replicated 5 times.

Assessment of sperm motility

A sperm aliquot (5 µl) was loaded into a pre-warmed (38°C) Makler chamber (Sefi-Medical instruments, Haifa, Israel; chamber depth: 10 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

Table 1. Effect of selective iNOS inhibitor on boar sperm motility during semen storage at 17°C.

Parameters	Treatments	Time				
		0h	4h	24h	48h	72h
SMI (%)	CTR	63.00 ± 4.47	62.00 ± 4.47	58.00 ± 4.47	53.50 ± 2.24	49.00 ± 2.24
	AG 0.625 mM		61.50 ± 4.87	61.00 ± 5.76	59.50 ± 5.42	54.00 ± 8.77
	AG 1.25 mM		63.50 ± 6.02	65.00 ± 10.16	**63.50 ± 6.98***	**59.50 ± 14.62***
	AG 2.5 mM		67.00 ± 8.37	65.00 ± 5.00	**66.50 ± 2.24****	**67.00 ± 7.79*****
	AG 5 mM		62.00 ± 4.47	65.00 ± 5.00	**62.00 ± 6.47***	**60.50 ± 9.91****
	AG 10 mM		62.00 ± 4.47	59.50 ± 1.12	57.50 ± 3.95	55.00 ± 9.52
ALH (µm)	CTR	2.91 ± 0.19	3.15 ± 0.34	3.12 ± 0.43	2.88 ± 0.14	2.64 ± 0.22
	AG 0.625 mM		3.24 ± 0.27	3.15 ± 0.19	3.17 ± 0.43	**3.36 ± 0.62*****
	AG 1.25 mM		3.03 ± 0.31	3.25 ± 0.16	3.02 ± 0.44	**3.12 ± 0.45***
	AG 2.5 mM		3.03 ± 0.17	2.95 ± 0.32	2.89 ± 0.31	2.76 ± 0.60
	AG 5 mM		3.04 ± 0.24	**2.70 ± 0.40***	**2.46 ± 0.53***	2.47 ± 0.50
	AG 10 mM		2.76 ± 0.37	**2.16 ± 0.23*****	**2.33 ± 0.38****	**2.15 ± 0.57***
VAP (µm/s)	CTR	35.17 ± 3.62	37.33 ± 6.04	37.03 ± 6.99	32.90 ± 3.47	30.31 ± 3.70
	AG 0.625 mM		33.30 ± 4.42	32.21 ± 2.52	32.51 ± 4.38	33.20 ± 4.78
	AG 1.25 mM		34.35 ± 5.21	32.06 ± 5.88	32.78 ± 4.21	30.28 ± 5.14
	AG 2.5 mM		33.20 ± 5.79	**30.90 ± 1.55***	31.68 ± 1.82	28.39 ± 3.34
	AG 5 mM		34.21 ± 2.57	**26.75 ± 2.49*****	**26.62 ± 3.16***	26.53 ± 3.73
	AG 10 mM		**28.23 ± 1.31*****	**22.98 ± 2.46*****	**23.36 ± 1.67*****	**20.61 ± 0.76*****
VCL (µm/s)	CTR	83.00 ± 2.32	89.38 ± 10.44	94.55 ± 12.71	82.37 ± 10.57	75.44 ± 8.73
	AG 0.625 mM		85.06 ± 13.05	85.29 ± 9.35	84.03 ± 13.51	**86.33 ± 11.71***
	AG 1.25 mM		88.52 ± 2.77	87.69 ± 3.54	78.61 ± 11.01	71.87 ± 14.07
	AG 2.5 mM		87.95 ± 5.85	**79.96 ± 5.65****	73.45 ± 8.84	69.93 ± 19.85
	AG 5 mM		92.83 ± 4.71	**66.77 ± 5.96*****	**64.86 ± 11.20*****	**63.04 ± 10.74***
	AG 10 mM		80.18 ± 9.48	**58.27 ± 7.40*****	**61.43 ± 9.44*****	**53.70 ± 11.82*****
VSL (µm/s)	CTR	25.64 ± 3.84	23.32 ± 1.83	23.43 ± 3.73	21.83 ± 3.07	20.40 ± 1.38
	AG 0.625 mM		24.76 ± 3.42	23.49 ± 4.08	23.17 ± 3.76	**25.06 ± 5.93***
	AG 1.25 mM		23.29 ± 4.59	22.24 ± 5.26	24.54 ± 4.81	23.88 ± 4.42
	AG 2.5 mM		21.57 ± 3.17	20.71 ± 1.36	22.96 ± 2.26	20.79 ± 3.32
	AG 5 mM		**18.29 ± 2.47***	**17.23 ± 2.03****	18.32 ± 1.97	18.94 ± 3.33
	AG 10 mM		**14.45 ± 1.05*****	**14.93 ± 1.52*****	**14.38 ± 1.66*****	**14.47 ± 9.21****

Asterisks indicate significant differences between the treatment and the control within each given time (*p<0.05; **p≤0.01; ***p≤0.001). SMI: sperm motility index; ALH: amplitude of lateral head displacement; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; CTR: control; AG: aminoguanidine. Data are shown as mean ± SD.

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°C (Tokai Hit, Shizuoka, Japan) and a DMK 23UM021 digital camera (The Imaging Source, Bremen, Germany). A total of four descriptors of sperm kinetics were recorded analyzing 6 randomly selected fields and a minimum of 200 sperm cells per sample: average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), amplitude of lateral head displacement (ALH, µm). The standard parameter settings were as follows: frames per second: 60; minimum of frames acquired: 21; VAP ≥ 10 µm/s to classify a spermatozoon as motile.

Assessment of sperm head membrane integrity

The assessment was performed as previously described (Grieblová et al. 2017). Briefly, sperm samples were incubated with carboxyfluorescein diacetate (stock solution: 0.46 mg/ml in dimethyl sulfoxide), propidium iodide (stock solution: 0.5 mg/ml in PBS), and 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.

Assessment of sperm tail membrane integrity

The assessment was performed as previously described (Grieblová et al. 2017), using a hypo-osmotic solution consisting of 7.35 g/l sodium citrate and

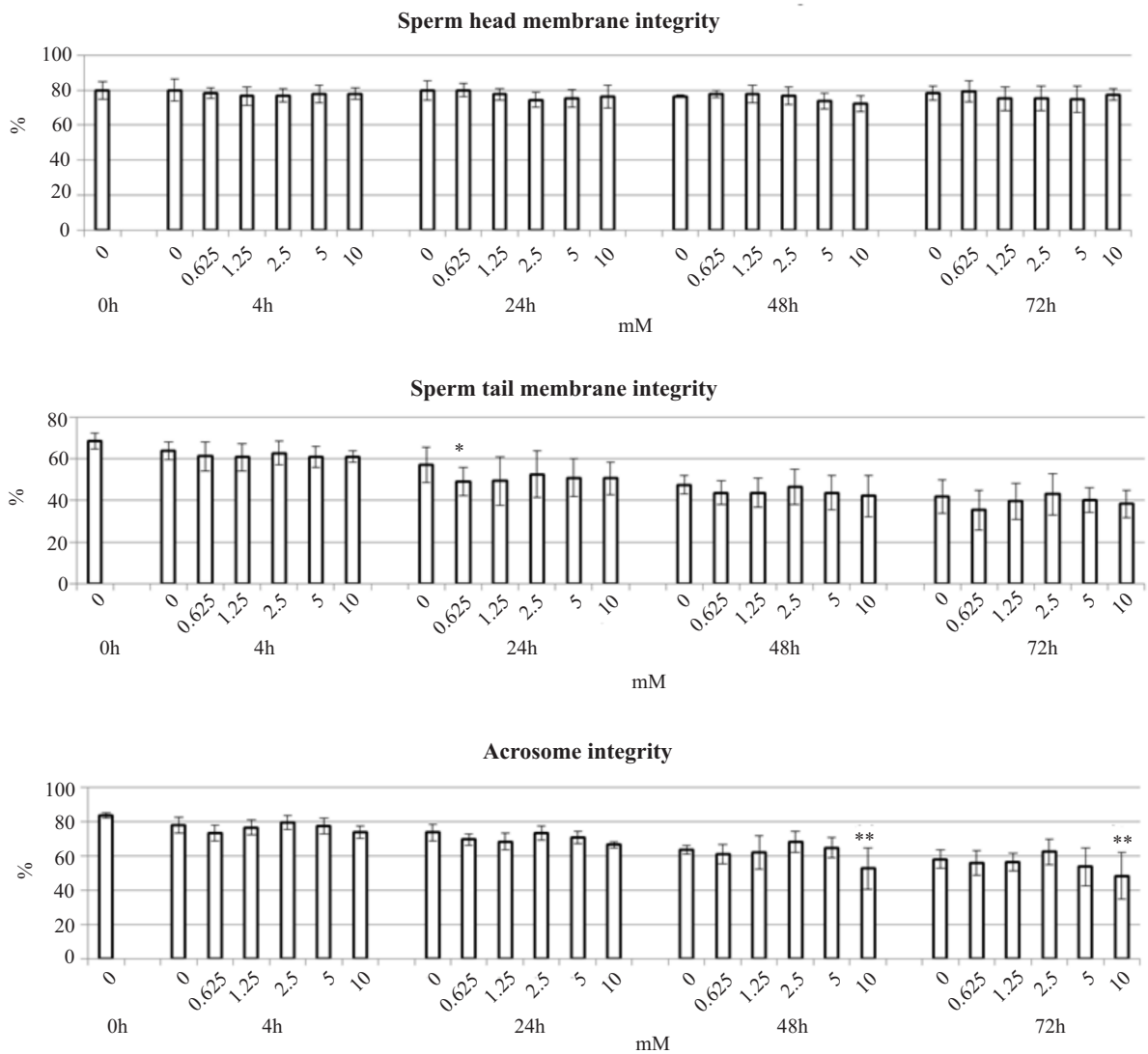

Fig. 1. Effect of selective iNOS inhibitor (aminoguanidine) on boar sperm membrane and acrosome integrity during semen storage at 17°C.
Asterisks indicate significant differences between the treatment and the control within each given time (*p<0.05; **p≤0.01). Data are shown as mean ± SD.

13.51 g/l fructose. Briefly, sperm samples were diluted into pre-warmed HOST solution and incubated for 30 minutes at 38°C. At the end of the incubation, samples were fixed using a formaldehyde solution (3%). In each sample 200 spermatozoa were evaluated using phase-contrast microscopy (40× objective) and the sperm cells showing swollen tails were considered to have an intact tail membrane.

Assessment of acrosomal status

The sperm samples were fixed in 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.

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 (GZLM) to analyze the effects of the treatments and storage times on sperm variables. Data are shown as mean ± SD. Statistical significance was set at $p<0.05$.

Results

Effect of selective iNOS inhibitor on boar sperm parameters

Our results show that AG significantly improved the SMI at 48 h and 72 h of semen storage in all experimental groups ($p<0.05$), except those treated with the lowest (0.625 mM) and the highest (10 mM) concentrations ($p>0.05$; Table 1). Concerning sperm kinetic parameters, at 72 h of semen storage we found that 0.625 mM AG increased ALH, VCL, and VSL ($p<0.001$, $p=0.042$, and $p=0.036$, respectively; Table 1). In contrast, at 72 h of storage, the highest concentration of AG (10 mM) significantly decreased all kinetic parameters ($p<0.05$). The AG has no effect on the sperm head membrane integrity at any concentration and storage time in comparison with the control group ($p>0.05$; Fig. 1). Conversely, the lowest concentration of AG (0.625 mM) significantly decreased the percentage of sperm with intact tail membrane at 24 h of storage ($p=0.046$; Fig. 1), whereas the highest concentration of AG (10 mM) gave a significantly lower percentage of sperm with intact acrosome at 48 h and 72 h of storage ($p=0.003$ and $p=0.008$, respectively; Fig. 1). No effect was found on the sperm tail membrane and acrosome integrity in samples treated with the remaining AG concentrations ($p>0.05$).

Effect of non-selective NOS inhibitor on boar sperm parameters

Overall, we found that L-NAME had no effect on boar sperm motility and kinetics at the lowest concentration (0.625 mM; $p>0.05$), whereas negative effects on ALH and VCL were observed in samples treated with 5 and 10 mM concentrations during whole period of semen storage ($p<0.05$; Table 2). However, because less than 5% motile sperm were observed at 72 h of semen storage at 5 and 10 mM L-NAME concentrations, sperm kinetics could not be evaluated. In the same way, sperm membrane and acrosome integrity were not affected by L-NAME at the lowest concentration (0.625 mM) at any time of storage ($p>0.05$), whereas the highest concentration of this NOS inhibitor (10 mM) negatively affected all parameters starting from 24 h of semen storage ($p<0.001$, Fig. 2).

Effect of NO donor on boar sperm parameters

As shown in Table 3, we found that SNP at concentrations ranging from 18.75 to 150 µM showed mostly no or negative effects on boar sperm motility and kinetics. Concerning the SMI, for example, SNP did not have any effect at the lowest concentration (18.75 µM) during the entire period of storage ($p>0.05$), whereas the highest concentration (150 µM) of this NO donor significantly decreased the SMI at 48 h and 72 h of storage ($p=0.001$ and $p<0.001$, respectively). A significant increase of the SMI was observed only at 24 h of semen storage in samples treated with 37.5 µM SNP ($p=0.021$). Concerning sperm kinetics, ALH was significantly decreased at 4 h of semen storage at SNP concentrations of 18.75, 75, and 150 µM ($p=0.026$, $p=0.043$, and $p=0.026$, respectively), but at 24 h this parameter was significantly decreased at concentrations of 37.5, 75, and 150 µM ($p=0.006$, $p=0.001$, and $p=0.002$, respectively). Moreover, SNP significantly decreased VCL at any concentration and storage time ($p<0.01$), although differences were not significant at 72 h in samples treated with 18.75 µM SNP ($p>0.05$). Moreover, while the lowest SNP concentration (18.75 µM) did not show any effect on the sperm membrane and acrosome integrity during whole period of semen storage ($p>0.05$; Fig. 3), we found that 37.5 µM SNP increased the percentage of sperm with intact sperm tail membrane at 48 h and 72 h of semen storage ($p=0.007$ and $p=0.001$, respectively; Fig. 3). Conversely, the highest concentration of this NO donor (150 µM) significantly decreased the percentage of sperm with intact head membrane at 72 h of semen storage ($p<0.001$).

Discussion

The results of this study indicate that the inhibition of NO by selective or non-selective NOS inhibitors shows different effects on boar sperm motility, membrane integrity and acrosomal stutus during sperm storage. At 72 h of sperm storage, for instance, the selective iNOS inhibitor AG at concentration of 0.625 mM increased sperm kinetic parameters like ALH, VCL, and VSL, which are related to boar fertility (Broekhujise et al. 2012). The positive effects of AG on boar sperm motility might be due to its antioxidant properties and scavenger activity against free radicals like ROS and RNS (Yildiz et al. 1998). In this way, Abbasi et al. (2011a, 2011b) and Alizadeh et al. (2010, 2016) have shown that AG improves sperm parameters (i.e. concentration, motility, viability, normal morphology, mitochondrial membrane potential, and DNA integrity) in varicocelized rats, where the upregulated iNOS expression leads to high oxidative stress on sperm cells. We therefore hypothesize that the antioxidant properties of AG may protect boar sperm cells against ROS during semen storage. On the contrary, the negative effects of high AG concen-

Table 2. Effect of non-selective NOS inhibitor on boar sperm motility during semen storage at 17°C.

Parameters	Treatments	Time				
		0h	4h	24h	48h	72h
SMI (%)	CTR	63.00 ± 4.47	62.00 ± 4.47	58.00 ± 4.47	53.50 ± 2.24	49.00 ± 2.24
	L-NAME 0.625 mM		63.00 ± 4.47	59.00 ± 2.24	53.00 ± 5.70	52.00 ± 4.47
	L-NAME 1.25 mM		62.00 ± 4.47	56.00 ± 2.24	47.50 ± 3.95	43.00 ± 5.70
	L-NAME 2.5 mM		62.00 ± 4.47	52.00 ± 3.26	**44.50 ± 5.12***	**36.00 ± 4.18****
	L-NAME 5 mM		59.50 ± 3.71	**47.00 ± 2.09****	**33.00 ± 6.71*****	**27.00 ± 6.94*****
	L-NAME 10 mM		**52.50 ± 3.54***	**39.00 ± 6.75*****	**23.00 ± 5.70*****	**19.00 ± 4.18*****
ALH (µm)	CTR	2.91 ± 0.19	3.15 ± 0.34	3.12 ± 0.43	2.88 ± 0.14	2.64 ± 0.22
	L-NAME 0.625 mM		3.15 ± 0.21	3.32 ± 0.26	3.26 ± 0.39	2.87 ± 0.24
	L-NAME 1.25 m		M3.01 ± 0.28	3.01 ± 0.34	2.99 ± 0.48	2.38 ± 0.46
	L-NAME 2.5 mM		2.93 ± 0.22	2.73 ± 0.44	**2.36 ± 0.19****	1.73 ± 0.24
	L-NAME 5 mM		**2.58 ± 0.36****	**2.30 ± 0.31*****	**1.86 ± 0.18*****	N/A
	L-NAME 10 mM		**2.36 ± 0.35*****	**2.19 ± 0.34*****	**1.20 ± 0.53*****	N/A
VAP (µm/s)	CTR	35.17 ± 3.62	37.33 ± 6.04	37.03 ± 6.99	32.90 ± 3.47	30.31 ± 3.70
	L-NAME 0.625 mM		38.91 ± 5.82	35.53 ± 5.38	30.50 ± 6.42	30.19 ± 3.71
	L-NAME 1.25 mM		37.71 ± 6.32	34.07 ± 6.02	31.69 ± 9.19	**23.84 ± 1.81***
	L-NAME 2.5 mM		36.89 ± 3.76	**30.69 ± 4.46***	**26.27 ± 6.33***	**18.33 ± 4.24*****
	L-NAME 5 mM		32.87 ± 5.32	**25.69 ± 1.91*****	**16.79 ± 1.40*****	N/A
	L-NAME 10 mM		**28.61 ± 5.37*****	**19.39 ± 2.20*****	**14.85 ± 1.41*****	N/A
VCL (µm/s)	CTR	83.00 ± 2.32	89.38 ± 10.44	94.55 ± 12.71	82.37 ± 10.57	75.44 ± 8.73
	L-NAME 0.625 mM		94.96 ± 9.64	92.79 ± 10.56	86.09 ± 11.11	82.93 ± 6.51
	L-NAME 1.25 mM		86.68 ± 6.21	87.47 ± 13.47	83.00 ± 15.71	67.23 ± 6.89
	L-NAME 2.5 mM		85.63 ± 3.69	**81.21 ± 13.00***	**69.08 ± 10.88***	**52.79 ± 4.01*****
	L-NAME 5 mM		**77.59 ± 7.75***	**66.08 ± 10.12*****	**53.27 ± 3.99*****	N/A
	L-NAME 10 mM		**65.56 ± 10.96*****	**59.14 ± 14.9*****	**38.31 ± 21.23*****	N/A
VSL (µm/s)	CTR	25.64 ± 3.84	23.32 ± 1.83	23.43 ± 3.73	21.83 ± 3.07	20.40 ± 1.38
	L-NAME 0.625 mM		25.04 ± 2.37	25.47 ± 3.49	21.63 ± 6.32	22.94 ± 4.62
	L-NAME 1.25 mM		25.92 ± 3.75	25.79 ± 4.12	24.42 ± 8.44	19.62 ± 2.44
	L-NAME 2.5 m		M26.09 ± 2.24	24.07 ± 3.47	21.90 ± 6.18	15.94 ± 4.33
	L-NAME 5 mM		22.84 ± 3.06	21.01 ± 1.25	**12.91 ± 3.49*****	N/A
	L-NAME 10 mM		21.16 ± 2.41	**15.09 ± 3.62*****	**13.60 ± 0.03****	N/A

Asterisks indicate significant differences between the treatment and the control within each given time (*p<0.05; **p≤0.01; ***p≤0.001). SMI: sperm motility index; ALH: amplitude of lateral head displacement; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; CTR: control; L-NAME: Nω-Nitro-L-arginine methyl ester hydrochloride; N/A: not available. Data are shown as mean ± SD.

trations (10 mM) might be explained by the inhibition of catalase activity leading to a reduced hydrogen peroxide (H_2O_2) removal (Ou and Wolff 1993). Given that the catalase content in boar semen is low (Foote 1962), the consequently increase of H_2O_2 levels may promote cell membrane damage by lipid peroxidation, which in turn may decrease sperm motility and acrosome integrity. Nevertheless, further studies have to be performed in order to evaluate the effects of AG treatment on boar sperm parameters under induced oxidative stress.

Our findings concerning the effect of L-NAME, a non-selective NOS inhibitor, are overall in agreement with previous studies showing that L-NAME negatively affects sperm motility by decreasing the percentage of sperm cells showing rapid progressive motiliy as well as by increasing immotile spermatozoa (human: Rosselli et al. 1995, ram: Hassanpur et al. 2007). In human spermatozoa, Lewis et al. (1996) found that L-NAME decreases VAP, VCL, and VSL, which were also negatively affected in our study. Because of its non-selective inhibitory activity against NOS isoforms, the negative effects of L-NAME might be the consequence of extremely low NO levels. Under no capacitating conditions, as in our study, boar spermatozoa produce low and constant levels of NO

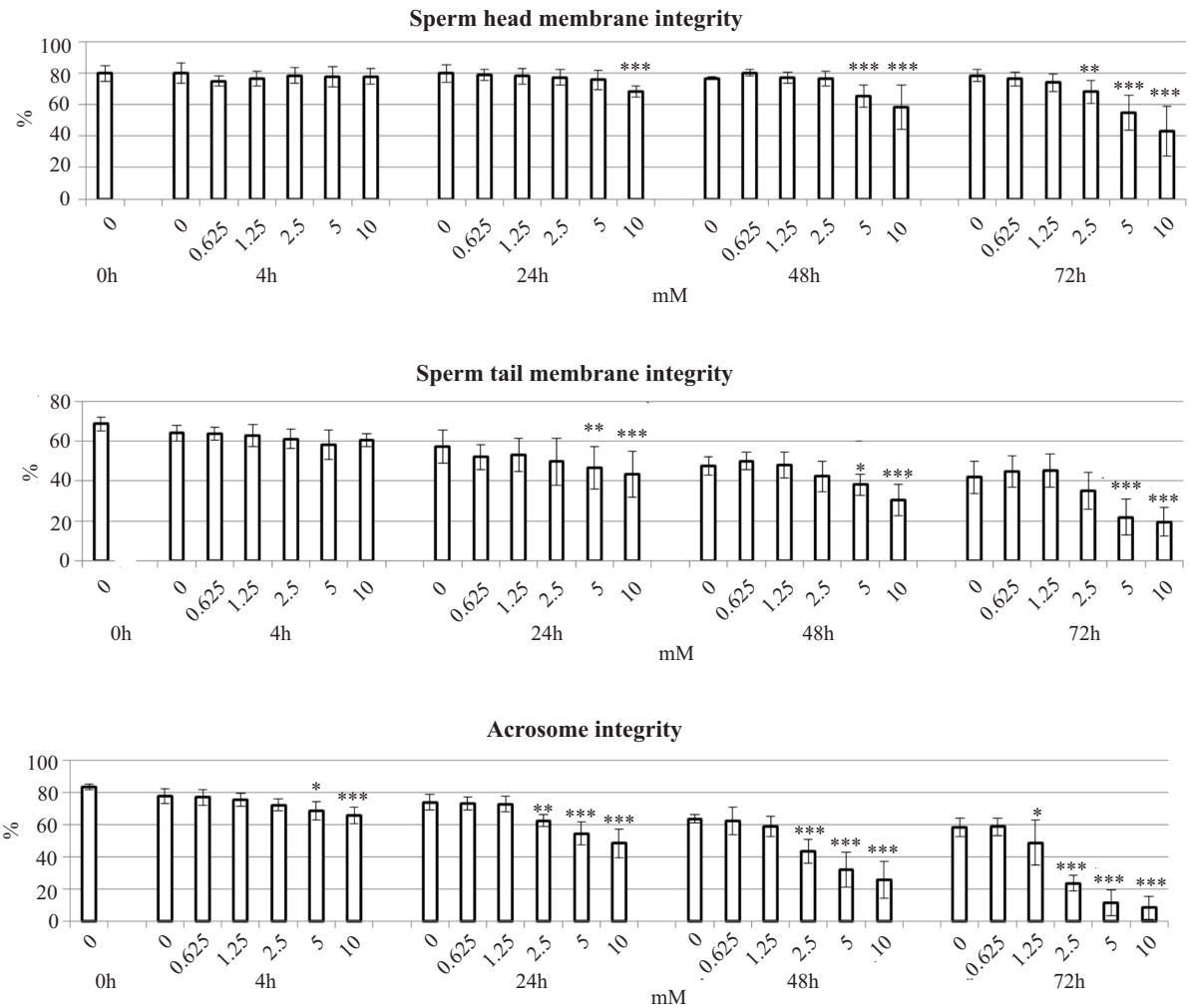

Fig. 2. Effect of non-selective NOS inhibitor (L-NAME) on boar sperm membrane and acrosome integrity during semen storage at 17°C.
Asterisks indicate significant differences between the treatment and the control within each given time (*p≤0.05; **p≤0.01; ***p≤0.001). Data are shown as mean ± SD.

(Hou et al. 2008), which are important for sperm function (Awda et al. 2009). In human, for instance, NO stimulates sperm motility via activation of the soluble guanylate cyclase/cyclic guanosine monophosphate (sGC/GMP) pathway (Miraglia et al. 2011). The inhibition of the sGC/GMP pathway by NOS inhibitors may represent another plausible mechanism responsible for the decreased boar sperm motility in presence of high concentrations of L-NAME or AG. However, in pathological conditions like varicocele, L-NAME exerts positive effects on sperm concentration and morphology, but not on motility (Bahmanzadeh et al. 2008).

Similarly to our findings, previous studies have shown that concentrations of the NO donor SNP ranging from 0.1 to 2,500 µM have no or only negative effects on sperm motility (Rosselli et al. 1995, Weinberg et al. 1995, Rodriguez et al. 2005, Hassanpour et al. 2007, Rahman et al. 2014). In this way, Balercia et al. (2004) have found that astenoozoospermic men exhibit higher levels of NO than those of normozoospermic men. In the same study, authors also found that NO levels were negatively related to the sperm motility, VCL, and VSL, providing further support to our findings. On the other hand, positive effects of SNP on sperm motility and viability were observed at much lower concentrations (i.e. 25-100 nM) (Hellstrom et al. 1994, Zhang and Zheng 1996). The negative effects of SNP might be related to the caspase activation that promotes cell apoptosis (Moran et al. 2008). In this way, Zhang and Zheng (1996) found that concentrations of SNP higher than 100 nM show detrimental effects on sperm viability both in fertile and asthenozoospermic infertile men. In addition to this mechanism, more recently Rahman et al. (2014) found that SNP decreases sperm kinetic parameters

Table 3. Effect of NO donor on boar sperm motility during semen storage at 17°C.

Parameters	Treatments	Time				
		0h	4h	24h	48h	72h
SMI (%)	CTR	63.00 ± 4.47	62.00 ± 4.47	58.00 ± 4.47	53.50 ± 2.24	49.00 ± 2.24
	SNP 18.75 µM		60.50 ± 4.47	58.50 ± 10.55	55.50 ± 6.22	43.50 ± 14.96
	SNP 37.5 µM		62.50 ± 4.33	**68.00 ± 3.71***	54.50 ± 10.95	45.50 ± 16.24
	SNP 75 µM		60.50 ± 4.47	59.00 ± 7.83	48.00 ± 11.65	**40.00** ± 19.96*
	SNP 150 µM		60.00 ± 6.37	50.50 ± 10.52	**39.00 ± 8.94*****	**31.00 ± 17.82*****
ALH (µm)	CTR	2.91± 0.19	3.15 ± 0.34	3.12 ± 0.43	2.88 ± 0.14	2.64 ± 0.22
	SNP 18.75 µM		**2.72 ± 0.12***	2.95 ± 0.52	2.92 ± 0.47	2.74 ± 0.27
	SNP 37.5 µM		2.78 ± 0.21	**2.54 ± 0.21****	2.60 ± 0.53	2.58 ± 0.22
	SNP 75 µM		**2.76 ± 0.20***	**2.46 ± 0.15*****	2.57 ± 0.16	2.68 ± 0.21
	SNP 150 µM		**2.72 ± 0.14***	**2.48 ± 0.71****	2.87 ± 0.53	2.39 ± 0.00
VAP (µm/s)	CTR	35.17 ± 3.62	37.33 ± 6.04	37.03 ± 6.99	32.90 ± 3.47	30.31 ± 3.70
	SNP 18.75 µM		32.54 ± 3.47	**27.97 ± 6.41*****	28.38 ± 4.73	26.13 ± 4.05
	SNP 37.5 µM		**30.98 ± 4.53***	**28.32 ± 3.98*****	**25.09 ± 4.17****	29.68 ± 2.86
	SNP 75 µM		32.57 ± 3.26	**27.31 ± 5.08*****	**26.50 ± 2.87***	**23.20 ± 5.04****
	SNP 150 µM		**29.90 ± 7.70****	**25.87 ± 4.47*****	**24.86 ± 4.10****	30.17 ± 1.48
VCL (µm/s)	CTR	83.00 ± 2.32	89.38 ± 10.44	94.55 ± 12.71	82.37 ± 10.57	75.44 ± 8.73
	SNP 18.75 µM		**74.45 ± 1.54****	**66.67 ± 5.14*****	**66.17 ± 12.84****	64.46 ± 9.23
	SNP 37.5 µM		**68.79 ± 7.39*****	**62.32 ± 5.40*****	**55.80 ± 8.93*****	**59.23 ± 1.96****
	SNP 75 µM		**69.14 ± 5.66*****	**59.47 ± 5.95*****	**58.53 ± 3.02*****	**51.38 ± 2.42*****
	SNP 150 µM		**68.55 ± 9.57*****	**57.72 ± 1.93*****	**54.59 ± 7.96*****	**57.07 ± 3.41****
VSL (µm/s)	CTR	25.64 ± 3.84	23.32 ± 1.83	23.43 ± 3.73	21.83 ± 3.07	20.40 ± 1.38
	SNP 18.75 µM		26.29 ± 3.38	22.04 ± 7.08	22.68 ± 6.48	21.12 ± 5.00
	SNP 37.5 µM		25.06 ± 3.74	23.62 ± 4.57	21.30 ± 4.21	26.59 ± 3.16
	SNP 75 µM		26.71 ± 3.99	22.43 ± 5.73	22.51 ± 3.21	**18.71 ± 7.03***
	SNP 150 µM		23.61 ± 6.23	21.03 ± 5.08	19.61 ± 5.23	26.55 ± 0.80

Asterisks indicate significant differences between the treatment and the control within each given time (*p<0.05; **p≤0.01; ***p≤0.001). SMI: sperm motility index; ALH: amplitude of lateral head displacement; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; CTR: control; SNP: sodium nitroprusside. Data are shown as mean ± SD.

by increasing intracellular Fe^{2+} and ROS levels and by decreasing Ca^{2+} and adenosine triphosphate (ATP) levels. Although several studies show that SNP induces the acrosome reaction in capacitated spermatozoa (human: Revelli et al. 2001, bull: Rodriguez et al. 2005, boar: Hou et al. 2008, mouse: Rahman et al. 2014), as expected, we found no effect on the acrosome integrity given that in our experimental design the semen was evaluated under no capacitating conditions.

Conclusion

Our results show that low concentration of AG increases sperm kinetics and may indicate the potential use of this selective iNOS inhibitor to palliate the effects oxidative stress during semen storage. Moreover, high concentrations of both selective and non-selective NOS inhibitors negatively affect sperm kinetics and acrosome integrity, which suggests that low NO levels are necessary for boar sperm physiology. Concerning NO donor, we found that SNP concentrations from 18.75 till 150 µM had mostly no or only negative effects on boar sperm parameters during semen storage. In conclusion, the results from this study increase the understanding of the role of NO on boar sperm physiology.

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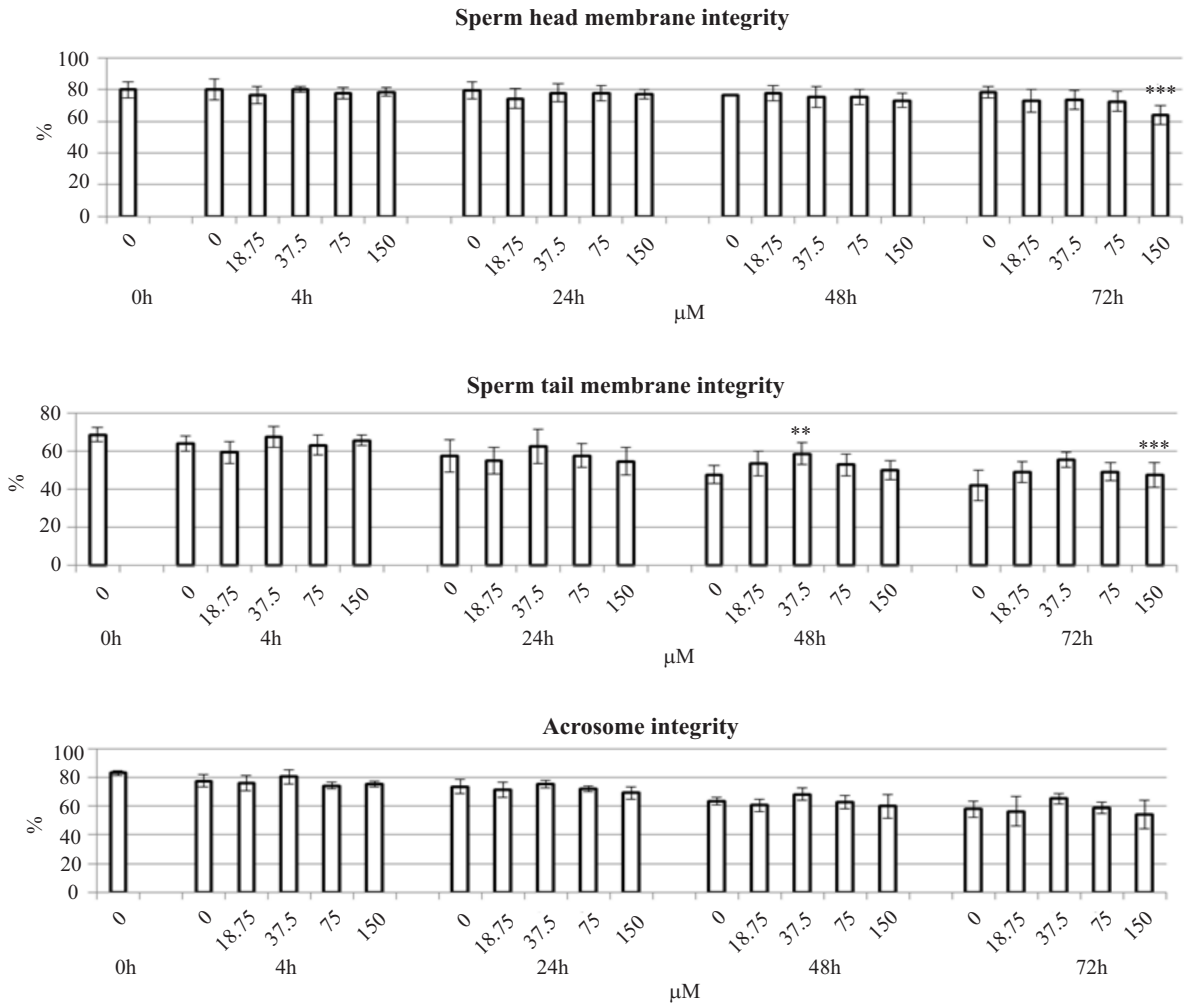

Fig. 3. Effect of NO donor (SNP) on on boar sperm membrane and acrosome integrity during semen storage at 17°C. Asterisks indicate significant differences between the treatment and the control within each given time (**p≤0.01; ***p≤0.001). Data are shown as mean ± SD.

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Aminoguanidine Protects Boar Spermatozoa against the Deleterious Effects of Oxidative Stress

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Abstract: Aminoguanidine is a selective inhibitor of the inducible nitric oxide synthase (iNOS) and a scavenger of reactive oxygen species (ROS). Numerous studies have shown the antioxidant properties of aminoguanidine in several cell lines, but the in vitro effects of this compound on spermatozoa under oxidative stress are unknown. In this study, we tested the hypothesis that aminoguanidine may protect against the detrimental effects of oxidative stress in boar spermatozoa. For this purpose, sperm samples were incubated with a ROS generating system (Fe^{2+}/ascorbate) with or without aminoguanidine supplementation (10, 1, and 0.1 mM). Our results show that aminoguanidine has powerful antioxidant capacity and protects boar spermatozoa against the deleterious effects of oxidative stress. After 2 h and 3.5 h of sperm incubation, the samples treated with aminoguanidine showed a significant increase in sperm velocity, plasma membrane and acrosome integrity together with a reduced lipid peroxidation in comparison with control samples ($p < 0.001$). Interestingly, except for the levels of malondialdehyde, the samples treated with 1 mM aminoguanidine did not differ or showed better performance than control samples without Fe^{2+}/ascorbate. The results from this study provide new insights into the application of aminoguanidine as an in vitro therapeutic agent against the detrimental effects of oxidative stress in semen samples.

Keywords: antioxidant capacity; lipid peroxidation; nitric oxide; oxidative stress; sperm velocity

1. Introduction

Oxidative stress arises when the production of the reactive oxygen species (ROS) overwhelms the intrinsic antioxidant defense of a biological system, leading to cell damage and death [1]. As a result of their metabolic activity, cells normally produce ROS, which are also required at certain levels for processes, such as cell signaling, mitochondrial function, and immune response [2–4]. Several factors (e.g., age, cigarette smoke, and ionizing radiation) and pathological conditions (e.g., cancer, diabetes, and infections) can also increase the amount of ROS to be above physiological levels, leading to oxidative stress. Across cells, spermatozoa are particularly susceptible to the damage caused by oxidative stress due to the high content of polyunsaturated fatty acids in their membranes and their limited antioxidant defence [5–7]. Despite certain levels of ROS being required for normal sperm function, their overproduction (due to pathological conditions, semen handling and storage) is detrimental for male fertility both in humans [8,9] and domestic animals [6,10].

Nitric oxide ($NO^{\bullet}$) is a short-living gas and a free radical that participates in many physiological (e.g., immune response, regulation of vascular tone and permeability) and pathological (e.g., cancer and neurological diseases) processes [11–14]. In the male reproductive system, $NO^{\bullet}$ contributes to penile erection, sperm motility, capacitation, hyperactivation, and acrosome reaction [15,16].

In biological systems, $NO^{\bullet}$ can be generated through non-enzymatic pathways by either direct disproportionation or reduction of nitrite under acidic and highly reduced conditions [17]. However, $NO^{\bullet}$ is mainly synthesized from L-arginine by three NO synthase (NOS) isoforms: Neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). All isoforms play a major role in the control of reproductive processes [18] and are expressed in human, mouse, and boar spermatozoa [19–21] among others. Unlike the other isoforms, iNOS is calcium independent and generates a large amount of $NO^{\bullet}$ over prolonged periods (from seconds to days) [18]. Moreover, the iNOS isoform is expressed during inflammation or infection in activated leukocytes [13], which are the main source of ROS in the semen together with abnormal spermatozoa [22,23]. Therefore, the inhibition of the iNOS isoform may contribute by protecting against the detrimental effects of the oxidative stress in the semen.

Aminoguanidine is a selective inhibitor of the iNOS isoform [24] and a scavenger of hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl ($^{\bullet}OH$) and peroxynitrite ($ONOO^{\bullet}$) radicals [25]. Moreover, aminoguanidine was the first inhibitor of the advanced glycation pathway [26] with similar effects to those of the polyamines, spermine, and spermidine, which are abundant in sperm samples [27]. In a recent study conducted by our research group [28], we found that aminoguanidine improves some sperm kinetic parameters during boar semen storage at 17 °C. Moreover, aminoguanidine protects against the negative effects of oxidative stress induced by environmental pollutants [29] and pathological conditions, such as varicocele [30–33]. Nonetheless, the in vitro effects of aminoguanidine on sperm cells under induced oxidative stress are still unknown.

The aim of this study was to evaluate the in vitro effects of aminoguanidine on sperm cells under induced oxidative stress. Due to its powerful antioxidant activity, we hypothesized that aminoguanidine may protect against the deleterious effects of oxidative stress in sperm samples. To test our hypothesis, sperm samples were treated with Fe^{2+}/ascorbate, which induces lipid peroxidation by catalyzing the production of $^{\bullet}OH$, the most potent free radical known [34]. The total antioxidant capacity, lipid peroxidation, sperm kinetics, plasma membrane integrity, and acrosomal status were evaluated in samples treated with aminoguanidine (10, 1, and 0.1 mM) and compared to those of control samples with or without oxidative stress. The results from this study indicate that aminoguanidine could be used as an efficient in vitro therapeutic agent for the treatment of sperm disorders associated with oxidative stress.

2. Materials and Methods

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

2.1. Collection and Processing of Sperm Samples

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). Sperm-rich fractions were collected by the 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 for each replicate. After this, 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. The samples were then randomly allocated into five groups: Control (CTR), control under oxidative stress (CTR-ox), and three treatments of aminoguanidine under oxidative stress (10, 1, and 0.1 mM, respectively). Aminoguanidine was freshly prepared on the day of the experiment (stock solution: 0.2 M) by dissolving aminoguanidine hydrochloride in phosphate buffered saline (PBS) and diluted with sperm samples to give a final concentration of 10, 1, and 0.1 mM. For CTR-ox samples, an equal volume of PBS solution was added. Oxidative stress was induced by 0.05 mM $FeSO_4$ and 0.5 mM sodium ascorbate (Fe^{2+}/ascorbate), a ROS generating system that is specific for inducing lipid peroxidation [35]. The experiment was replicated five times using five different semen pools.

All sperm analyses were performed at 0 h (after 20 min of incubation, control only), 2 h, and 3.5 h of incubation in a water bath at 38 °C (Supplementary Dataset).

2.2. Assessment of Total Antioxidant Capacity

At the end of each incubation time, 300 µL of each sample was centrifuged at 2000× *g* for 10 min. After this, 150 µL of supernatant was collected and stored at −80 °C until analysis. The total antioxidant capacity was determined by spectrophotometry (Libra S22, Biochrom, Harvard Bioscience Company, Cambridge, UK) at 660 nm using the method described by Erel [36]. A standard curve was established using the known concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The total antioxidant capacity was expressed as mM Trolox equivalents. This assay was run in duplicate for each sample.

2.3. Assessment of Sperm Motility

A sperm aliquot (5 µL) was loaded into a pre-warmed (38 °C) Spermtrack chamber (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 following formula: [% individual motility + (QM × 20)]/2. Sperm kinetics were assessed by 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 °C (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 after analyzing six random fields: Total motility (TM, %), progressive motility (PM, %), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight-line 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; and STR ≥ 80% to classify a spermatozoon as progressive. A minimum of 200 motile sperm cells were analyzed per sample.

2.4. Assessment of Lipid Peroxidation

Lipid peroxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay as previously described [35,37]. At the end of each incubation time, sperm aliquots were collected and stored at −80 °C until analysis. The absorbance of the sample was measured by spectrophotometry at 532 nm. A standard curve was established using the known concentrations of 1,1,3,3-tetramethoxypropane (malondialdehyde, MDA). The levels of lipid peroxidation are shown as µmol of MDA per 10^8 spermatozoa. This assay was run in duplicate for each sample.

2.5. Assessment of Sperm Plasma Membrane Integrity

The assessment of head membrane integrity was performed, as previously described [38,39]. Briefly, sperm samples were incubated with carboxyfluorescein diacetate (stock solution: 0.46 mg/mL in dimethyl sulfoxide), propidium iodide (stock solution: 0.5 mg/mL in PBS), and formaldehyde solution (0.3%) for 10 min at 37 °C in the dark. After this, 200 spermatozoa were evaluated in each sample using epi-fluorescence microscopy (40× objective) and the sperm cells showing complete green fluorescence over the head were considered to have an intact head membrane. The tail membrane integrity was determined using the hypoosmotic swelling test as previously described [39,40]. Briefly, sperm samples were diluted into a pre-warmed hypoosmotic solution (7.35 g/L sodium citrate and 13.51 g/L fructose) and incubated for 30 min at 38 °C. At the end of the incubation, 200 spermatozoa

were evaluated using phase-contrast microscopy (40× objective) and the sperm cells showing swollen tails were considered to have an intact tail membrane.

2.6. Assessment of Acrosomal Status

In order to determine the percentage of sperm cells with a normal apical ridge (NAR) [41], the samples were fixed in 2% glutaraldehyde solution and examined under phase contrast microscopy (40× objective). Two-hundred spermatozoa were evaluated for each sample. The percentage of damaged acrosomes was determined according to the protocol described by García-Vázquez et al. [42]. Briefly, sperm samples were smeared onto glass slides, air-dried, and fixed with methanol for 10 min at room temperature. After this, samples were washed twice with PBS and incubated with peanut agglutinin-fluorescein isothiocyanate (PNA-FITC, stock solution: 0.2 mg/mL in PBS) for 10 min at 37 °C in the dark. Finally, the samples were washed for 5 min with PBS and evaluated under epi-fluorescence microscopy (40× objective). Two-hundred spermatozoa were evaluated and the spermatozoa that showed no fluorescence over the acrosome were considered to be damaged spermatozoa.

2.7. Statistical Analysis

The statistical analyses were performed using the SPSS 20.0 statistical software package (IBM Inc, Chicago, IL, USA). The Shapiro-Wilk test was applied to check for a normal distribution of the data. The repeated measures ANOVA or Friedman tests were used to check for differences in sperm parameters in the control group during the different times of incubation. The generalized linear model (GZLM) was performed to analyze the effects of the treatments and storage times on sperm variables. Data are shown as mean ± standard error (SE). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Total Antioxidant Capacity

As shown in Table 1, the total antioxidant capacity of the CTR samples did not change during the whole incubation ($p > 0.05$). At each incubation time, there were also no differences between CTR and CTR-ox groups ($p > 0.05$). Irrespective of the incubation time, 10 mM aminoguanidine showed greater total antioxidant capacity than CTR-ox group ($p < 0.001$ at both incubation times), while 1 mM aminoguanidine showed greater total antioxidant capacity at 3.5 h of incubation only ($p = 0.031$).

Table 1. Total antioxidant capacity of boar sperm samples submitted to oxidative stress (CTR except) and supplemented with aminoguanidine.

Treatment	Time (h)	Total Antioxidant Capacity (mM)
CTR	0	0.4 ± 0.1 ^{a}
CTR	2	0.1 ± 0.0 ^{a,A}
CTR-ox	2	0.4 ± 0.2 ^{A}
Ag10-ox	2	2.1 ± 0.1 ***
Ag1-ox	2	0.5 ± 0.1
Ag0.1-ox	2	0.4 ± 0.3
CTR	3.5	0.2 ± 0.0 ^{a,A}
CTR-ox	3.5	0.2 ± 0.0 ^{A}
Ag10-ox	3.5	2.2 ± 0.1 ***
Ag1-ox	3.5	0.5 ± 0.1 *
Ag0.1-ox	3.5	0.2 ± 0.1

Total antioxidant capacity is expressed as Trolox equivalents. Different superscript lower-case letters indicate significant differences ($p < 0.05$) among times for the control samples without induced oxidative stress. Different superscript upper-case letters indicate significant differences ($p < 0.05$) within each given time between the control samples with and without induced oxidative stress. The asterisks indicate significant differences (* $p < 0.05$; *** $p \leq 0.001$) within each given time between the treatments and the control samples submitted to induced oxidative stress. CTR = control; ox = samples submitted to induced oxidative stress; Ag10 = 10 mM aminoguanidine; Ag1 = 1 mM aminoguanidine; and Ag0.1 = 0.1 mM aminoguanidine. Data are shown as mean ± standard error of 5 replicates.

Furthermore, after 2 h and 3.5 h of incubation, 10 and 1 mM aminoguanidine showed greater total antioxidant capacity than CTR samples ($p < 0.05$).

3.2. Sperm Motility and Kinetics

At 2 h of incubation, there were no significant differences in any sperm kinetic parameter between CTR and CTR-ox groups ($p > 0.05$, Figure 1 and Table 2). Conversely, at 3.5 h of incubation, sperm kinetic parameters (except for the BCF, LIN, and STR) were negatively affected by this ROS generator ($p < 0.05$, Figure 1 and Table 2).

Overall, our results show that aminoguanidine preserved sperm motility under oxidative stress conditions (Figure 1 and Table 2). At both times of incubation, the TM of samples that were treated with 10 and 1 mM aminoguanidine were significantly greater than those of CTR-ox group ($p < 0.05$) with a two-fold increase at 3.5 h of sperm incubation. Nevertheless, it is important to highlight that despite the great percentage of motile spermatozoa observed during the whole incubation, samples treated with 10 mM aminoguanidine tended to display a relatively non-progressive and circular movement. In this way, at 2 h of incubation, samples treated with 10 mM aminoguanidine showed a greater percentage of motile sperm cells, SMI, TM and VCL, but smaller percentage of PM, BCF, LIN, and STR compared to those of CTR-ox samples ($p < 0.05$). There was also a significant increase in the percentage of motile sperm cells, SMI, and TM at 3.5 h of incubation in samples treated with 0.1 mM aminoguanidine compared to those of CTR-ox samples ($p < 0.05$). While there were no differences in the other kinetic parameters at this aminoguanidine concentration, they tended to be greater than those of CTR-ox group ($p > 0.05$).

Interestingly, irrespective of the incubation time, there was no difference in any sperm kinetic parameter between 1 mM aminoguanidine and CTR samples ($p > 0.05$). Conversely, 10 mM aminoguanidine showed greater TM, VCL, and ALH, but smaller PM, BCF, LIN, and STR than CTR group at 2 h of incubation ($p < 0.05$). Sperm kinetic parameters in samples treated with 0.1 mM aminoguanidine did not differ or were significantly smaller than the CTR group ($p < 0.05$).

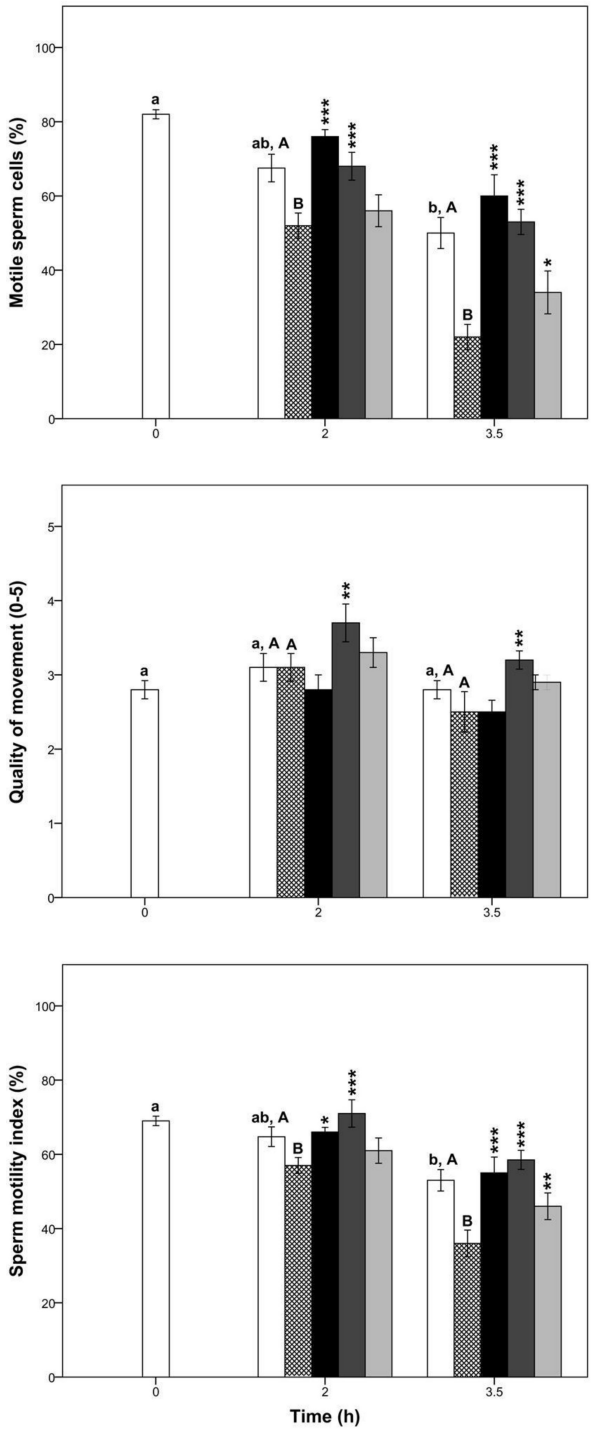

Figure 1. Percentage of motile sperm cells, quality of movement, and sperm motility index in boar samples submitted to oxidative stress (CTR except) and supplemented with aminoguanidine. Different superscript lower-case letters indicate significant differences ($p < 0.05$) among times for the control group without induced oxidative stress. Different superscript upper-case letters indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress within each given time. The asterisks indicate significant differences between the treatment and the control submitted to induced oxidative stress within each given time (* $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). White bars = control samples; crossed bars = control samples under induced oxidative stress; black bars = 10 mM aminoguanidine; dark grey bars = 1 mM aminoguanidine; and light grey bars = 0.1 mM aminoguanidine. Data are shown as mean ± standard error of 5 replicates.

Table 2. Boar sperm kinetics in samples submitted to oxidative stress (CTR except) and supplemented with aminoguanidine.

Treatment	Time (h)	TM (%)	PM (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)	ALH (μm)	BCF (Hz)	LIN (%)	STR (%)
CTR	0	77.2 ± 3.5 ^{a}	44.9 ± 0.9 ^{a}	40.6 ± 1.3 ^{a}	80.8 ± 1.9 ^{a}	30.6 ± 1.0 ^{a}	3.0 ± 0.1 ^{a}	13.1 ± 0.2 ^{a}	38.0 ± 0.9 ^{a}	75.5 ± 0.8 ^{a}
CTR	2	63.1 ± 6.1 ^{a,A}	62.4 ± 5.6 ^{b,A}	39.2 ± 2.8 ^{a,A}	67.5 ± 3.2 ^{b,A}	36.0 ± 2.5 ^{ab,A}	2.7 ± 0.1 ^{b,A}	14.4 ± 0.4 ^{b,A}	51.7 ± 1.4 ^{b,A}	89.7 ± 0.5 ^{b,A}
CTR-ox	2	54.2 ± 4.7 ^{A}	61.2 ± 5.4 ^{A}	41.4 ± 3.0 ^{A}	68.1 ± 5.3 ^{A}	38.9 ± 2.8 ^{A}	2.8 ± 0.2 ^{A}	14.7 ± 0.2 ^{A}	55.9 ± 1.9 ^{A}	92.3 ± 0.9 ^{A}
Ag10-ox	2	83.0 ± 1.6 ***	42.0 ± 2.8 ***	42.2 ± 3.3	92.9 ± 8.6 ***	30.2 ± 1.7 **	3.3 ± 0.3	12.7 ± 0.4 ***	34.2 ± 1.8 ***	70.8 ± 2.3 ***
Ag1-ox	2	65.6 ± 1.4 *	63.2 ± 2.6	45.0 ± 4.9	79.6 ± 9.4	40.7 ± 4.1	3.1 ± 0.4	14.0 ± 0.4	50.8 ± 2.3 *	88.6 ± 1.4 *
Ag0.1-ox	2	56.7 ± 4.7	58.7 ± 3.1	39.4 ± 4.0	68.4 ± 9.0	35.8 ± 3.1	2.7 ± 0.3	14.4 ± 0.4	52.5 ± 3	89.5 ± 2.2
CTR	3.5	60.0 ± 6.3 ^{a,A}	62.8 ± 6.0 ^{b,A}	41.5 ± 3.3 ^{a,A}	68.9 ± 4.2 ^{b,A}	38.6 ± 3.3 ^{b,A}	2.7 ± 0.2 ^{ab,A}	14.8 ± 0.4 ^{b,A}	54.6 ± 1.9 ^{b,A}	90.9 ± 1.0 ^{b,A}
CTR-ox	3.5	24.9 ± 4.4 ^{B}	43.7 ± 8.0 ^{B}	29.4 ± 1.4 ^{B}	45.5 ± 2.9 ^{B}	27.9 ± 1.4 ^{B}	1.9 ± 0.1 ^{B}	15.1 ± 0.4 ^{A}	62.3 ± 2.4 ^{B}	93.6 ± 1.4 ^{A}
Ag10-ox	3.5	69.0 ± 5.6 ***	52.6 ± 4.0	36.0 ± 3.2	78.1 ± 6.6 ***	29.3 ± 2.5	2.8 ± 0.2 ***	12.9 ± 0.4 ***	39.4 ± 1.6 ***	80.5 ± 1.7 ***
Ag1-ox	3.5	59.0 ± 3.8 ***	63.8 ± 3.3 ***	40.4 ± 2.5 **	69.2 ± 3.1 **	37.7 ± 2.5 **	2.8 ± 0.1 **	14.2 ± 0.4	54.1 ± 1.2 ***	91.6 ± 0.6
Ag0.1-ox	3.5	39.6 ± 4.8 *	53.1 ± 5.5	32.7 ± 1.9	53.0 ± 5.0	30.8 ± 1.5	2.3 ± 0.2	15.0 ± 0.4	59.1 ± 2.5	93.0 ± 0.8

Different superscript lower-case letters in the same column indicate significant differences ($p < 0.05$) among times for the control group without induced oxidative stress. Different superscript upper-case letters in the same column indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress within each given time. The asterisks indicate significant differences between the treatment and the control submitted to induced oxidative stress within each given time (* $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). TM = total motility; PM = progressive motility; VAP = average path velocity; VCL = curvilinear velocity; VSL = straight-line velocity; ALH = amplitude of lateral head displacement; BCF = beat-cross frequency; LIN = linearity; STR = straightness; CTR = control; ox = samples submitted to induced oxidative stress; Ag10 = 10 mM aminoguanidine; Ag1 = 1 mM aminoguanidine; and Ag0.1 = 0.1 mM aminoguanidine. Data are shown as mean ± standard error of 5 replicates.

3.3. Lipid Peroxidation

The oxidative stress induced by Fe^{2+}/ascorbate provoked a significant increase in sperm lipid peroxidation at 2 h and 3.5 h of semen incubation ($p < 0.05$; Figure 2) compared to the CTR group. On the other hand, CTR samples did not change their levels of MDA during the entire period of semen incubation ($p > 0.05$). Interestingly, at 2 h of incubation, all aminoguanidine treatments showed lower levels of MDA than those of CTR-ox group ($p < 0.05$). Conversely, at 3.5 h of incubation, only 10 and 1 mM aminoguanidine showed lower levels of lipid peroxidation than CTR-ox samples (both $p < 0.001$).

As expected, all aminoguanidine treatments showed greater levels of lipid peroxidation than CTR samples ($p < 0.001$).

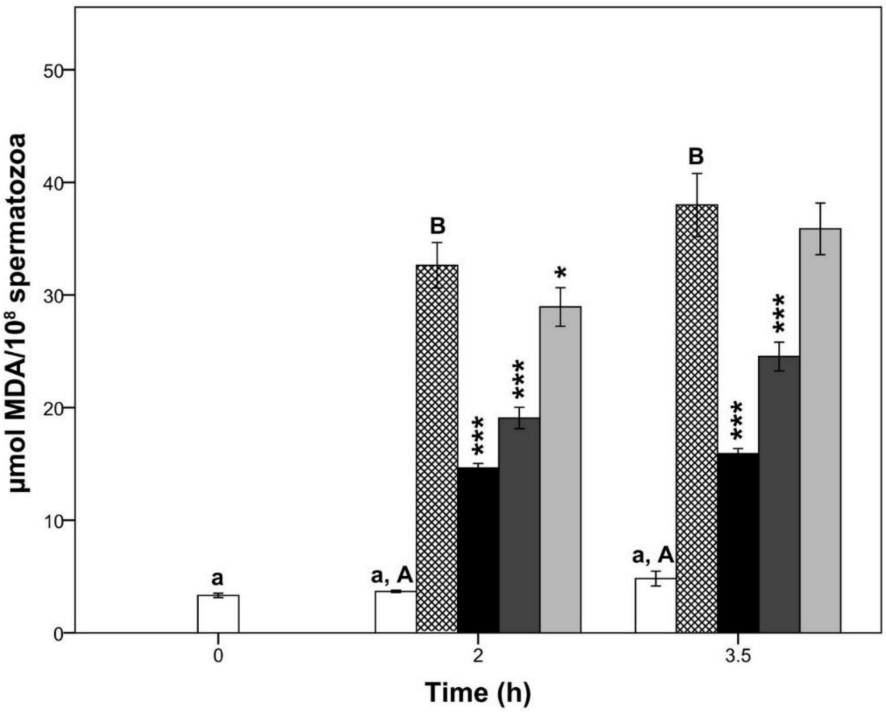

Figure 2. Lipid peroxidation in boar sperm samples submitted to oxidative stress (CTR except) and supplemented with aminoguanidine. Different superscript lower-case letters indicate significant differences ($p < 0.05$) among times for the control group without induced oxidative stress. Different superscript upper-case letters in the same column indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress within each given time. The asterisks indicate significant differences between the treatment and the control submitted to induced oxidative stress within each given time (* $p < 0.05$; *** $p \leq 0.001$). MDA = malondialdehyde; white bars = control samples; crossed bars = control samples under induced oxidative stress; black bars = 10 mM aminoguanidine; dark grey bars = 1 mM aminoguanidine; and light grey bars = 0.1 mM aminoguanidine. Data are shown as mean ± standard error of 5 replicates.

3.4. Sperm Plasma Membrane Integrity and Acrosomal Status

In general, the oxidative stress induced by Fe^{2+}/ascorbate impaired the sperm plasma membrane and acrosome integrity of CTR-ox samples (Table 3). Moreover, we found that aminoguanidine protects the sperm head plasma membrane and acrosome integrity against oxidative stress (Table 3). While there were no significant differences in the sperm tail plasma membrane integrity between the values of CTR-ox samples and those of aminoguanidine treatments ($p > 0.05$), there was a trend for the latter to be greater at any concentration used.

Table 3. Boar sperm plasma membrane integrity and acrosomal status in samples submitted to oxidative stress (CTR except) and supplemented with aminoguanidine.

Treatment	Time (h)	Intact Head Plasma Membrane (%)	Intact Tail Plasma Membrane (%)	Normal Apical Ridge (%)	Damaged Acrosome (%)
CTR	0	83.2 ± 0.6 ^{a}	27.9 ± 2.5 ^{a}	94.5 ± 0.3 ^{a}	2.1 ± 0.2 ^{a}
CTR	2	76.4 ± 0.5 ^{b,A}	23.0 ± 4.6 ^{ab,A}	92.1 ± 0.5 ^{b,A}	3.2 ± 0.4 ^{b,A}
CTR-ox	2	71.9 ± 0.7 ^{B}	16.3 ± 4.5 ^{A}	87.6 ± 1.3 ^{B}	3.9 ± 0.4 ^{A}
Ag10-ox	2	78.8 ± 0.6 ***	24.3 ± 4.3	93.1 ± 0.5 ***	2.7 ± 0.3 **
Ag1-ox	2	81.6 ± 1.0 ***	20.2 ± 3.8	91.4 ± 1.1 ***	2.2 ± 0.2 ***
Ag0.1-ox	2	74.3 ± 0.3 *	21.0 ± 4.7	91.1 ± 0.5 ***	3.3 ± 0.4
CTR	3.5	69.3 ± 1.2 ^{c,A}	20.4 ± 4.0 ^{b,A}	88.7 ± 0.8 ^{c,A}	4.7 ± 0.3 ^{c,A}
CTR-ox	3.5	62.8 ± 0.8 ^{B}	14.1 ± 3.7 ^{A}	84.9 ± 1.0 ^{B}	6.2 ± 0.3 ^{B}
Ag10-ox	3.5	73.5 ± 0.8 ***	22.0 ± 3.9	90.3 ± 1.0 ***	4.2 ± 0.4 ***
Ag1-ox	3.5	78.3 ± 1.1 ***	18.1 ± 4.5	90.6 ± 1.1 ***	3.1 ± 0.2 ***
Ag0.1-ox	3.5	67.7 ± 1.2 ***	16.0 ± 4.61	90.5 ± 0.6 ***	5.3 ± 0.3 *

Different superscript lower-case letters in the same column indicate significant differences ($p < 0.05$) among times for the control group without induced oxidative stress. Different superscript upper-case letters in the same column indicate significant differences ($p < 0.05$) between the control group with and without induced oxidative stress within each given time. The asterisks indicate significant differences between the treatment and the control submitted to induced oxidative stress within each given time (* $p < 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). CTR = control; ox = samples submitted to induced oxidative stress; Ag10 = 10 mM aminoguanidine; Ag1 = 1 mM aminoguanidine; and Ag0.1 = 0.1 mM aminoguanidine. Data are shown as mean ± standard error of 5 replicates.

We also found that 10 and 1 mM aminoguanidine showed a greater percentage of intact sperm head plasma membrane at 2 h ($p = 0.025$ and $p < 0.001$, respectively) and 3.5 h of sperm incubation (both $p < 0.001$) than CTR samples. Moreover, at 2 h and 3.5 h of incubation, 1 mM aminoguanidine showed a lower percentage of damaged acrosome than that of the CTR group ($p = 0.011$ and $p < 0.001$, respectively).

4. Discussion

The present study provides the first piece of evidence that aminoguanidine notably reduces the detrimental effects of oxidative stress in boar sperm cells in vitro. Our results clearly show that aminoguanidine has powerful antioxidant capacity, preserves the sperm motility, reduces the lipid peroxidation, and protects the plasma membrane and acrosome integrity under induced oxidative stress. Interestingly, except for the levels of MDA, sperm parameters of samples treated with 1 mM aminoguanidine did not differ or even showed better performance than those of control samples without the ROS-generating system, which demonstrates that the deleterious effects of oxidative stress were mostly abolished. As there was no cytotoxic effect shown in any sperm parameter, our results suggest that aminoguanidine could potentially be a treatment for impaired semen quality associated with high ROS levels.

Aminoguanidine is a selective inhibitor of the iNOS isoform [24], which releases large amounts of $NO^{\bullet}$ and is found in spermatozoa and activated leukocytes [18,21]. In human semen, the presence of abnormal spermatozoa and activated leukocytes increases the amount of ROS over physiological levels, which causes sperm DNA damage, lipid peroxidation, and poor motility [5]. In this sense, Balercia et al. [43] found that asthenozospermic men show greater levels of $NO^{\bullet}$ than normozoospermic men and that the concentration of this gasotransmitter were negatively correlated with the sperm motility. In this way, our findings indicate that aminoguanidine can be employed for protecting against the effects of oxidative stress in sperm cells, which is consistent with the findings in other cells and tissues (lung: [44]; bladder: [45]; kidney: [46]; testis: [29]). Similarly, Abbasi et al. [30,31] and Alizadeh et al. [32,33] found that the in vivo administration of aminoguanidine improves the sperm concentration, motility, viability, normal morphology, mitochondrial membrane potential,

and DNA integrity in varicocelized rats where the upregulation of the iNOS isoform may lead to high levels of ROS in the semen.

As previously described in boar semen [35,47], our results confirm that Fe^{2+}/ascorbate induces a state of oxidative stress characterized by increased levels of lipid peroxidation and reduced sperm motility. In addition, we also found that this ROS generator negatively affects the sperm head plasma membrane and acrosome integrity. In contrast to our findings, Guthrie and Welch [47] found that Fe^{2+}/ascorbate did not affect the sperm membrane integrity (i.e., viability). This is possibly because a smaller Fe^{2+}/ascorbate concentration was employed (i.e., 1 μM/30 μM) in their study. Our findings also confirm that aminoguanidine has powerful antioxidant abilities against the oxidative stress induced by Fe^{2+}/ascorbate, as previously described by Yildiz et al. [25]. Irrespective of the incubation time, 10 and 1 mM aminoguanidine showed stronger antioxidant capacity than that of control samples with or without induced oxidative stress. The total antioxidant capacity of 1 mM aminoguanidine was 0.5 mM Trolox equivalents on average, which is within the range described in the boar seminal plasma [48,49]. A greater total antioxidant capacity of the seminal plasma contributes to the ability of boar sperm cells to better sustain the preservation process (liquid-storage and cryopreservation), which is also positively related to the fertility outcomes and litter size [49]. In this way, our results indicate that 1 mM aminoguanidine shows a total antioxidant capacity similar to that of boar seminal plasma, which provides further support for the beneficial effects of this compound on boar sperm parameters under induced oxidative stress.

The results of this study show that under induced oxidative stress, aminoguanidine better preserves sperm motility, plasma membrane and acrosome integrity. These three parameters are correlated with male fertility in humans [50] and other species (bulls: [51]; boars: [52]; stallions: [53]). Interestingly, at 3.5 h of incubation, 10 and 1 mM aminoguanidine showed more than twice the percentage of motile sperm cells compared to control samples under oxidative stress. Nevertheless, it is important to highlight that sperm cells treated with 10 mM aminoguanidine showed rapid curvilinear trajectories with remarkably low values of progressive and linear motility. A plausible explanation of this phenomenon might be due to the antioxidant capacity of 10 mM aminoguanidine (2 mM Trolox equivalents), which is quite above the physiological range reported in the boar seminal plasma [48,49]. As certain levels of ROS are required for a normal sperm function [8,9], 10 mM aminoguanidine may reduce the amount of ROS in such a way that it impairs some sperm kinetic parameters, but it does not affect the sperm plasma membrane and acrosome integrity. In this sense, the protective effects on the sperm plasma membrane and acrosome integrity in samples treated with 10 mM aminoguanidine were also confirmed by the lower levels of lipid peroxidation. On the other hand, 0.1 mM aminoguanidine was able to only partially prevent the damage caused by Fe^{2+}/ascorbate in terms of sperm parameters. In contrast, although the sperm tail integrity tended to be greater in sperm samples supplemented with aminoguanidine, there were no differences among the latter and control group treated with Fe^{2+}/ascorbate, which is likely due to the variability among replicates. The boar sperm plasma membrane shows low tolerance to the hypoosmotic conditions, which varies across breeds and between boars within the same breed [54]. In this way, despite the fact that we used pooled semen in order to reduce the male variability, factors, such as the boar and breed, may have influenced our results by increasing the variability among replicates.

Another important finding of this study is that aminoguanidine protects the acrosome integrity as shown by the two techniques employed. It is well known that acrosome integrity is a requisite for the acrosome reaction, which must occur in a timely manner in order to allow the penetration of the spermatozoon through the protective barriers of the oocyte [55]. In the porcine species, a partially induced acrosome reaction in the preincubation or fertilization media has been found to be an important cause of polyspermy, which is one major challenge in the assisted reproductive technologies of this species [56]. The exposure of boar spermatozoa to a ROS generating system triggers the acrosome reaction [57], which may lead to reduced fertilizing potential. Based on our findings, we can therefore speculate that aminoguanidine may increase the fertilization potential of porcine spermatozoa by

preventing a precocious acrosome reaction under oxidative stress. Further studies, such as the in vitro and in vivo fertilizations, are needed to test our hypothesis.

In conclusion, the findings from this study demonstrate that aminoguanidine mostly abolishes the deleterious effects of oxidative stress in boar spermatozoa under in vitro conditions. Due to its antioxidant capacities, aminoguanidine preserves the boar sperm motility, reduces the lipid peroxidation, and protects the plasma membrane and acrosome integrity under oxidative stress. Interestingly, 1 mM aminoguanidine mostly eliminates the negative effects of oxidative stress as, except for the lipid peroxidation, all sperm parameters did not differ or even showed better performance than those of control samples without the ROS-generating system. As no cytotoxic effects were observed in any sperm parameters, our results suggest that aminoguanidine could be used as an effective in vitro therapeutic agent for the treatment of sperm disorders associated with oxidative stress.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4923/10/4/212/s1, Supplementary Dataset 1. Dataset of boar sperm parameters under induced oxidative stress (CTR except) and supplemented with aminoguanidine.

Availability of Materials and Data: All data generated or analyzed during this study are included in this article and Supplementary Information file.

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Divergent effect of fast- and slow-releasing H_2S donors on boar spermatozoa under oxidative stress

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Hydrogen sulphide (H_2S) is involved in the physiology and pathophysiology of different cell types, but little is known about its role in sperm cells. Because of its reducing properties, we hypothesise that H_2S protects spermatozoa against the deleterious effects of oxidative stress, a condition that is common to several male fertility disorders. This study aimed i) to determine the total antioxidant capacities of Na_2S and GYY4137, which are fast- and slow-releasing H_2S donors, respectively, and ii) to test whether H_2S donors are able to protect spermatozoa against oxidative stress. We found that Na_2S and GYY4137 show different antioxidant properties, with the total antioxidant capacity of Na_2S being mostly unstable and even undetectable at 150 µM. Moreover, both H_2S donors preserve sperm motility and reduce acrosome loss, although the effects were both dose and donor dependent. Within the range of concentrations tested (3–300 µM), GYY4137 showed positive effects on sperm motility, whereas Na_2S was beneficial at the lowest concentration but detrimental at the highest. Our findings show that Na_2S and GYY4137 have different antioxidant properties and suggest that both H_2S donors might be used as *in vitro* therapeutic agents against oxidative stress in sperm cells, although the optimal therapeutic range differs between the compounds.

Hydrogen sulphide (H_2S) is the most recently discovered gaseous molecule that participates in a variety of biological functions, as do nitric oxide (NO) and carbon monoxide (CO). In mammals, H_2S can be synthesised by enzymatic or non-enzymatic pathways[1]. Overall, it seems likely that most of the H_2S produced within an organism is generated by the H_2S-synthesising enzymes: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulphurtransferase (3-MST), with the latter coupled with cysteine aminotransferase (CAT)[2].

In the male reproductive system, the expression of H_2S-generating enzymes has been reported in the testis[3,4], epididymis[5], penile corpus cavernosum[6], and spermatozoa[7,8], which strongly suggests that this gasotransmitter is involved in sperm physiology to some extent. In a recent study, Wang *et al.* found that asthenospermic men show reduced levels of H_2S in their seminal plasma and that exogenous H_2S supplementation improves their sperm motility[8]. In contrast, in boar spermatozoa, H_2S exerts no or negative effects on sperm motility, viability, and mitochondrial membrane potential[9]. With both positive and negative effects documented, there is still controversy concerning the role of H_2S in sperm cells. This apparent discrepancy might, at least partly, be a result of H_2S dose- and donor-dependent effects[10].

According to their chemical structure and source, H_2S donors include inorganic salts and derivatives of phosphorodithioate, garlic extracts, thioaminoacids, and anti-inflammatory drugs[11]. On the basis of their release mechanism, H_2S donors can be classified in two categories: slow- and fast-releasing agents. Among the fast-releasing H_2S donors, the inorganic salts sodium sulphide (Na_2S) and sodium hydrosulphide (NaHS) are probably most frequently employed in biological studies. Both salts can be dissolved in aqueous solution, leading to an instantaneous release of H_2S that mimics a bolus administration. Despite the common use of these donors in experimental studies, it is becoming increasingly clear that their gas release might not be representative of the physiological H_2S levels in tissues and cells[12]. On the other hand, slow-releasing H_2S donors, like the phosphorodithioate derivative GYY4137, produce a slow and continuous release of gas, which is more similar to the physiological conditions found within organisms[12]. For this reason, the use of different H_2S donors in studies is useful to elucidate the biological activity and possible therapeutic effects[12].

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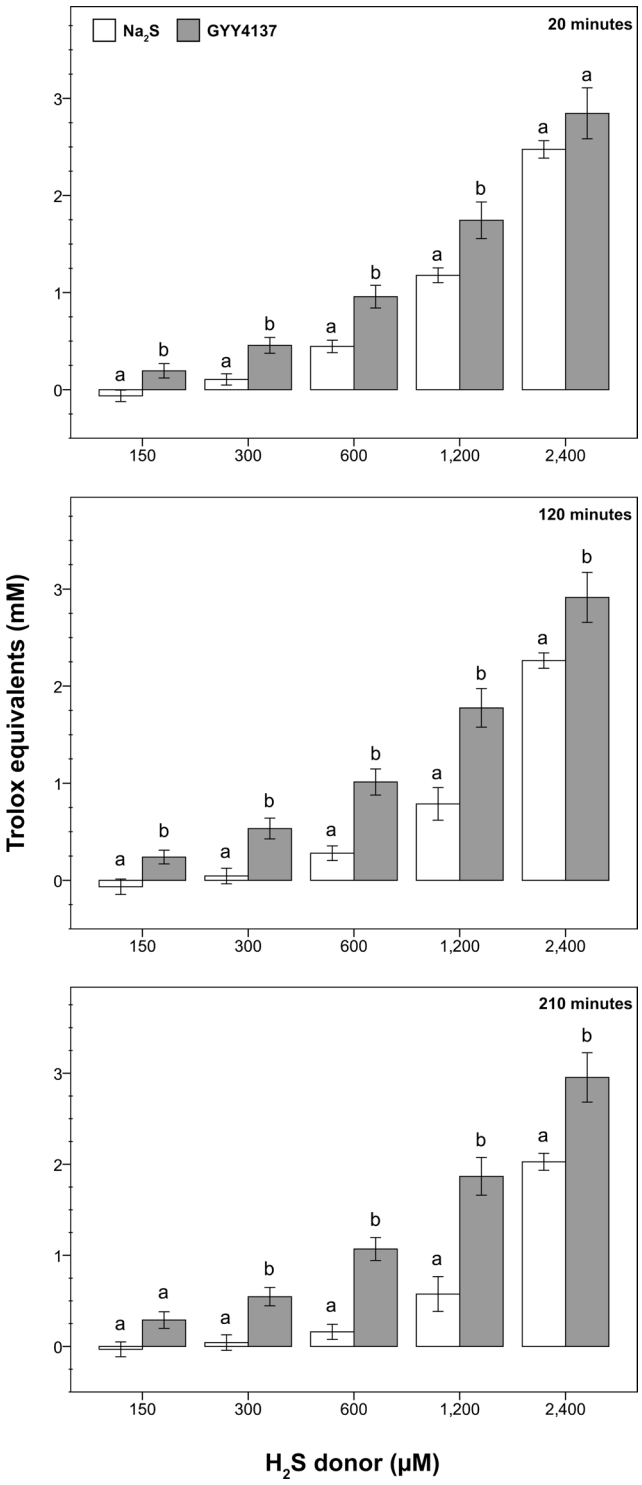

Figure 1. Total antioxidant capacity and stability of the H_2S donors Na_2S and GYY4137. The H_2S donors were incubated in phosphate-buffered saline solution at 38 °C in a water bath. Total antioxidant capacity is expressed as Trolox equivalents (mM). White histograms: Na_2S; grey histograms: GYY4137. Upper panel: 20 minutes of incubation; middle panel: 120 minutes of incubation; lower panel: 210 minutes of incubation. Different letters indicate significant differences ($p < 0.05$) between H_2S donors at the same concentration and incubation time. Data are shown as the mean±standard error of four replicates.

By virtue of its activity as a reducing agent, H_2S attenuates the damage induced by oxidative stress in different cells and tissues (e.g. neurons[13], gastric cells[14], lung cells[15]). Oxidative stress is an underlying condition common to several male reproductive disorders, in which high levels of reactive oxygen species (ROS) cause sperm dysfunction (e.g. decreased sperm motility, impaired membrane and DNA integrity, increased lipid peroxidation)

and infertility[16,17]. Previous studies have shown that H_2S is able to alleviate the effects of oxidative stress on testicular functions[4,8,18], but the *in vitro* effects of this gasotransmitter on sperm cells under a ROS-generating system still need to be elucidated.

The aim of this study was to evaluate the total antioxidant capacity and stability of the H_2S donors Na_2S and GYY4137 under standard conditions (38 °C, pH≈7) and at different times (i.e. 20, 120, and 210 minutes) during the incubation (experiment I). Because sperm motility under a ROS-generating system may drop in a few hours[19,20] and based on the opposite modalities of H_2S release by Na_2S and GYY4137 (i.e. fast and slow release, respectively), these incubation times were chosen to determine the dynamics of the antioxidant activity of each donor during the early, mid, and late stages of incubation. Based on the results from experiment I and the physiological total antioxidant capacity of boar seminal plasma[21], we then established a suitable range of concentrations of Na_2S and GYY4137 to be tested in boar sperm samples under a ROS-generating system (experiment II). Although some Na_2S and GYY4137 concentrations used in experiment II show a total antioxidant capacity that is below the range of detection by spectrophotometry, they were included in our experimental design because increasing evidence suggests that *in vivo* H_2S levels range from low μM to high nM[22]. Next, we evaluated the effects of both donors on sperm motility, mitochondrial activity, plasma membrane integrity, acrosomal status, and lipid peroxidation. The results from this study elucidate the role of H_2S donors in sperm samples under oxidative stress and the possible therapeutic implications of these compounds for alleviating the negative effects of ROS on sperm function.

Results

Experiment I. Total antioxidant capacity and stability of H_2S donors. As can be seen from Table 1, Na_2S and GYY4137 showed different total antioxidant capacities and stabilities during the incubation. Overall, the total antioxidant capacity of Na_2S significantly decreased between 20 and 210 minutes of incubation, whereas that of GYY4137 tended to increase during this period and was significantly higher after 210 minutes than after 20 minutes of incubation at 2,400 and 1,200 μM ($p < 0.05$). Moreover, GYY4137 showed detectable levels of total antioxidant capacity at all concentrations tested, whereas Na_2S was unstable within the range of 300 to 1,200 μM and was undetectable at 150 μM. Irrespective of the concentration considered, GYY4137 showed greater total antioxidant capacity than Na_2S (Fig. 1).

Experiment II. Effect of H_2S donors on boar sperm parameters under induced oxidative stress. *Sperm motility.* Overall, the effects of H_2S on boar sperm motility under induced oxidative stress were dose- and donor-dependent (Table 2, Fig. 2). Thus, 3 and 30 μM GYY4137 and 3 μM Na_2S preserved the sperm motility and kinetics under the ROS-generating system. Interestingly, all of the sperm kinetic parameters in these treatments did not differ from those of the control group without oxidative stress (CTR; $p > 0.05$). The results with both 3 and 30 μM GYY4137 showed higher percentages of total motility (TM) relative to that of the control group under oxidative stress (CTR-ox; $p < 0.01$). Moreover, a dose of 30 μM GYY4137 significantly increased the percentage of progressive motility (PM) over that in the CTR-ox group ($p = 0.040$). Although differences were not statistically significant, higher average path velocity (VAP) and straight-line velocity (VSL) were observed in samples treated with 3 μM GYY4137 than in those in the CTR-ox group ($p = 0.071$ and $p = 0.064$, respectively). On the other hand, the effects of Na_2S were markedly dose dependent. At 300 μM, this fast-releasing H_2S donor showed clear negative effects on sperm motility. No motile spermatozoa were observed in any replicate; therefore, no kinetics data could be provided. By contrast, at the lowest concentration, Na_2S significantly increased the percentage of motile sperm cells relative to that in the CTR-ox group ($p = 0.018$). At a concentration of 30 μM, Na_2S greatly decreased the TM, PM, VAP, and VSL ($p < 0.05$), although it did not affect the curvilinear velocity (VCL) and the remaining motion parameters in comparison with those of the CTR-ox group ($p > 0.05$). However, at this Na_2S concentration, we observed some variability among the replicates with the percentage of motile spermatozoa ranging from 0 to almost 30%. There were no differences between the CTR-ox and H_2S donor treatments in the amplitude of lateral head displacement (ALH), beat-cross frequency (BCF), linearity (LIN), straightness (STR), and wobble (WOB; $p > 0.05$).

Sperm mitochondrial status. At a concentration of 300 μM, Na_2S showed clear negative effects on boar sperm mitochondrial status. In every replicate, there were no spermatozoa with active mitochondria (Table 3, Fig. 3). There were no differences between the CTR-ox group and the remaining treatment groups ($p > 0.05$).

Sperm plasma membrane integrity and lipid peroxidation. There was no significant effect of GYY4137 on sperm plasma membrane integrity at any of the concentrations used ($p > 0.05$, Table 3). On the other hand, 300 μM Na_2S markedly impaired the plasma membrane integrity relative to the results obtained for the CTR-ox group ($p < 0.001$, Table 3, Fig. 3). No effects were observed at the remaining Na_2S concentrations ($p > 0.05$). A similar pattern was observed for the sperm lipid peroxidation: higher values of malondialdehyde (MDA) per 10^8 spermatozoa were observed in samples treated with 300 μM Na_2S than those in the CTR-ox group and the other treatment groups ($p < 0.01$, Fig. 4). No differences in the MDA levels were found between the CTR-ox group and the remaining treatment groups ($p > 0.05$).

Acrosomal status. We found that 300 μM Na_2S impaired the acrosome integrity (normal apical ridge or NAR test) relative to that of the CTR group, both with and without oxidative stress ($p < 0.001$, Table 3, Fig. 3). No differences were observed in the NAR test results between the CTR-ox group and the other treatment groups ($p > 0.05$). On the other hand, all treatments showed lower percentages of acrosome-lost spermatozoa (evaluated with peanut agglutinin–fluorescein isothiocyanate, PNA-FITC) than that of the CTR-ox group ($p \leq 0.001$, Table 3).

Treatment	Concentration (μM)	Time (min)		
		20	120	210
Na_2S	2,400	$2,474.6 \pm 89.8^{a}$	$2,262.3 \pm 79.4^{ab}$	$2,027.1 \pm 92.8^{b}$
	1,200	$1,178.1 \pm 75.7^{a}$	787.4 ± 168.1^{ab}	575.2 ± 191.3^{b}
	600	445.4 ± 64.6^{a}	278.6 ± 75.1^{ab}	160.2 ± 83.3^{b}
	300	105.5 ± 58.0^{a}	44.5 ± 80.1^{a}	42.6 ± 85.2^{a}
	150	n.d.	n.d.	n.d.
GYY4137	2,400	$2,845.9 \pm 262.7^{a}$	$2,913.8 \pm 257.3^{ab}$	$2,954.2 \pm 270.7^{b}$
	1,200	$1,745.0 \pm 188.8^{a}$	$1,775.9 \pm 199.1^{ab}$	$1,867.2 \pm 207.3^{b}$
	600	958.3 ± 117.2^{a}	$1,012.7 \pm 135.0^{a}$	$1,069.6 \pm 125.3^{a}$
	300	456.0 ± 81.6^{a}	532.8 ± 107.2^{a}	546.0 ± 101.1^{a}
	150	194.1 ± 73.7^{a}	239.5 ± 70.5^{a}	289.4 ± 91.3^{a}
PBS		n.d.	n.d.	n.d.

Table 1. Total antioxidant capacity and stability of the H_2S donors Na_2S and GYY4137. The H_2S donors were incubated in phosphate-buffered saline solution at 38 °C in a water bath. Total antioxidant capacity is expressed as Trolox equivalents (μM). Different superscripts indicate significant differences ($p < 0.05$) among times within each donor concentration. PBS: phosphate-buffered saline solution; n.d.: not detectable. Data are shown as the mean±standard error of four replicates.

Treatment	Conc. (μM)	Time (min)	TM (%)	PM (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)	ALH (μm)	BCF (Hz)	LIN (%)	STR (%)	WOB (%)
CTR		20	75.6 ± 2.9	52.8 ± 5.8	43.1 ± 1.7	83.8 ± 3.7	34.8 ± 1.6	3.1 ± 0.1	13.5 ± 0.4	41.7 ± 2.8	80.7 ± 2.8	50.1 ± 1.9
CTR		210	73.1 ± 3.4^{a}	70.8 ± 5.2^{ac}	42.0 ± 2.6^{a}	69.0 ± 5.1^{a}	37.8 ± 1.8^{a}	3.1 ± 0.6^{a}	15.6 ± 0.6^{a}	55.8 ± 3.0^{a}	89.9 ± 2.3^{a}	61.0 ± 2.3^{a}
CTR-ox		210	46.7 ± 8.7^{c}	65.8 ± 2.3^{a}	33.1 ± 5.4^{b}	52.7 ± 9.6^{ab}	30.8 ± 4.7^{a}	2.1 ± 0.4^{bc}	16.3 ± 0.5^{a}	62.9 ± 2.9^{a}	93.5 ± 1.2^{a}	66.4 ± 2.5^{a}
Na_2S-ox	300	210	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	30	210	7.9 ± 5.5^{d}	44.8 ± 5.5^{b}	21.7 ± 2.0^{c}	34.7 ± 2.7^{b}	20.3 ± 2.1^{b}	1.5 ± 0.1^{c}	15.1 ± 0.5^{a}	62.3 ± 1.6^{a}	94.3 ± 0.7^{a}	65.6 ± 1.3^{a}
	3	210	65.6 ± 6.2^{ab}	69.3 ± 5.1^{ac}	39.2 ± 2.6^{ab}	64.7 ± 8.0^{a}	35.3 ± 1.6^{a}	2.6 ± 0.2^{ab}	15.7 ± 0.5^{a}	58.1 ± 4.4^{a}	90.6 ± 2.8^{a}	63.0 ± 3.3^{a}
GYY4137-ox	300	210	57.2 ± 6.9^{bc}	70.5 ± 3.4^{ac}	35.5 ± 4.4^{ab}	56.0 ± 8.2^{a}	33.0 ± 3.8^{a}	2.3 ± 0.3^{abc}	16.0 ± 0.3^{a}	61.8 ± 3.0^{a}	93.0 ± 1.6^{a}	65.6 ± 2.4^{a}
	30	210	69.0 ± 6.2^{ab}	77.2 ± 2.5^{c}	38.1 ± 3.7^{ab}	59.0 ± 6.7^{a}	35.6 ± 3.3^{a}	2.5 ± 0.3^{ab}	16.2 ± 0.4^{a}	62.2 ± 2.8^{a}	93.0 ± 1.3^{a}	66.2 ± 2.2^{a}
	3	210	69.8 ± 7.7^{ab}	75.3 ± 3.7^{ac}	41.3 ± 4.0^{ab}	65.9 ± 7.5^{a}	38.0 ± 3.5^{a}	2.7 ± 0.3^{ab}	16.1 ± 0.5^{a}	59.3 ± 2.8^{a}	91.8 ± 1.5^{a}	63.8 ± 2.2^{a}

Table 2. Boar sperm motility and kinetics in samples submitted to oxidative stress and supplemented with the H_2S donors Na_2S and GYY4137. Different superscripts within the same column indicate significant differences ($p < 0.05$) among treatments within the same incubation time. Conc.: concentration; TM: total motility; PM: progressive motility; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; ALH: amplitude of lateral head displacement; BCF: beat-cross frequency; LIN: linearity (VSL/VCL); STR: straightness (VSL/VAP); WOB: wobble (VAP/VCL); CTR: control; ox: samples submitted to induced oxidative stress; n.a.: not available. Data are shown as the mean±standard error of six replicates.

Discussion

In this study, we provide the first evidence, to the best of our knowledge, that Na_2S and GYY4137 show different total antioxidant capacities and stabilities under standard conditions (38 °C, physiological pH) and after different periods (20, 120, and 210 minutes) of incubation. Our results also reveal that the total antioxidant capacity of Na_2S is less stable than that of GYY4137, although the latter's total antioxidant capacity tends to increase over time. This phenomenon should be taken into account in studies entailing cell incubation at 38 °C and at physiological pH, because the release of H_2S by GYY4137 is both pH and temperature dependent[12]. Moreover, GYY4137 shows higher total antioxidant capacity than that of Na_2S after any incubation time, with the total antioxidant capacity of Na_2S at 150 μM even being undetectable by spectrophotometry after 20 minutes of incubation. The patterns observed in the antioxidant capacities of these H_2S donors may not reflect their H_2S release, given that the inorganic salts Na_2S and NaHS lead to a larger but shorter gas release than GYY4137[23,24].

Our results show that both H_2S donors partly palliate the damages provoked by oxidative stress in sperm cells, supporting the results found previously in other cells (neurons[13,25], gastric cells[14], testicular germ cells[4]) and organs (lungs[15,26], testes[18]). In these studies, oxidative stress was induced by *in vivo* or *in vitro* ROS-generating systems, such as glutamate, hydrogen peroxide (H_2O_2), heat, tobacco smoke, ovalbumin sensitisation, and ischaemia-reperfusion injury. In our study, we used Fe^{2+}/ascorbate, which induces lipid peroxidation and catalyses the production of hydroxyl radicals ($^{\bullet}OH$), the most powerful free radical known, by the Fenton reaction[27]. Moreover, several previous studies[4,13–15,18,25,26] employed a single H_2S donor, whereas two H_2S-releasing agents were tested in our study. To date, few studies[8,23,28] have investigated the biological effects of both fast- and slow-releasing H_2S donors in cells under oxidative stress conditions. In agreement with these studies, our findings

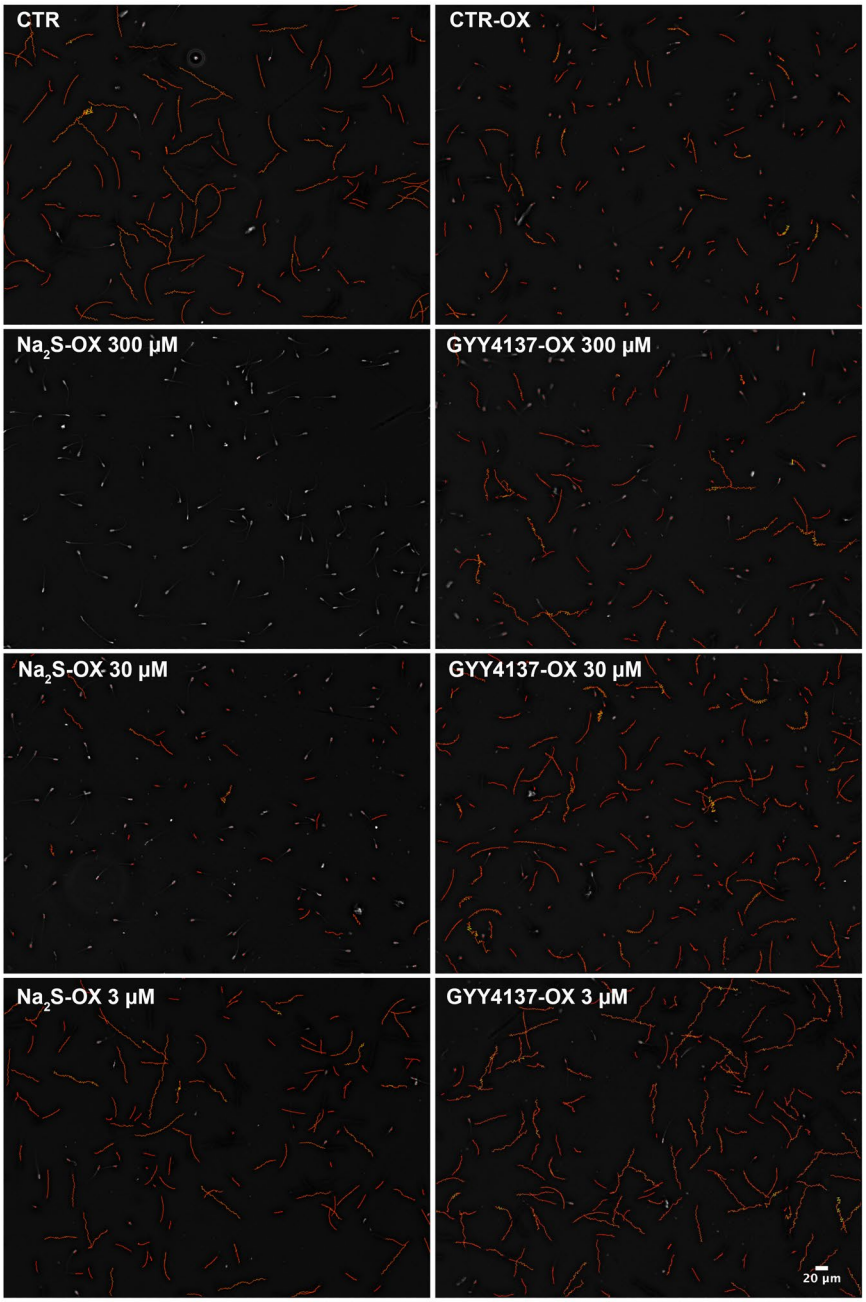

Figure 2. Boar sperm motility in samples submitted to oxidative stress and supplemented with the H_2S donors Na_2S and GYY4137. Representative images of sperm trajectories assessed by Computer-Assisted Sperm Analysis (CASA). Red trajectories show motile spermatozoa (cells are not shown because of overlapping with the trajectories), whereas immotile sperm cells are fully shown.

confirm that the effects of H_2S donors are not only dose but also donor dependent. Moreover, for the first time to the best of our knowledge, the *in vitro* effects of fast- and slow-releasing H_2S donors were evaluated in sperm cells in the presence of a ROS-generating system. Overall, we found that the slow-releasing H_2S donor GYY4137 did not show any cytotoxic effect. Moreover, 3 and 30 µM GYY4137 preserved the boar sperm motility against the detrimental effects of oxidative stress. Interestingly, at both concentrations, the percentage of motile sperm cells was almost 50% higher than that of the CTR-ox samples and no kinetic parameters differed from those of the CTR group without oxidative stress. Moreover, 30 µM GYY4137 samples showed a higher percentage of progressive motility than those in the CTR-ox group. However, in contrast to other studies[8,18], we did not find any effect of GYY4137 on the levels of lipid peroxidation, which may be due to the different cell type and donor concentration used in our study. Our findings also show that, in comparison with GYY4137, the effects of Na_2S were markedly dose dependent. At a concentration of 3 µM, this fast-releasing H_2S donor preserves boar sperm motility (40.5% higher than that in the CTR-ox samples), whereas it shows clear cytotoxic effects at 300 µM. This

Treatment	Conc. (μM)	Time (min)	Active mitochondria (%)	Intact plasma membrane (%)	Intact acrosome (NAR, %)	Acrosome loss (PNA, %)
CTR		20	56.0 ± 3.2	82.0 ± 2.2	95.1 ± 0.9	1.4 ± 0.3
CTR		210	62.8 ± 3.3^{a}	76.6 ± 2.3^{a}	94.8 ± 1.0^{a}	2.4 ± 0.2^{a}
CTR-ox		210	62.4 ± 1.2^{a}	67.8 ± 3.9^{abc}	92.9 ± 0.8^{a}	3.9 ± 0.7^{b}
Na_2S-ox	300	210	0^{b}	21.2 ± 6.0^{d}	31.1 ± 5.6^{b}	2.0 ± 0.3^{a}
	30	210	60.8 ± 5.1^{a}	66.1 ± 4.7^{bc}	94.6 ± 0.7^{a}	2.2 ± 0.4^{a}
	3	210	62.1 ± 3.3^{a}	74.8 ± 3.3^{abc}	92.8 ± 0.6^{a}	1.7 ± 0.4^{a}
GYY4137-ox	300	210	67.7 ± 2.8^{a}	70.8 ± 3.8^{abc}	93.0 ± 1.4^{a}	1.5 ± 0.4^{a}
	30	210	62.9 ± 2.9^{a}	75.8 ± 3.2^{a}	94.1 ± 0.9^{a}	2.3 ± 0.4^{a}
	3	210	63.1 ± 1.8^{a}	76.5 ± 3.6^{a}	94.2 ± 0.6^{a}	1.8 ± 0.4^{a}

Table 3. Boar sperm mitochondrial status, plasma membrane integrity, and acrosomal status in samples submitted to oxidative stress and supplemented with the H_2S donors Na_2S and GYY4137. Different superscripts within the same column indicate significant differences ($p < 0.05$) among treatments within the same incubation time. Conc.: concentration; NAR: normal apical ridge; PNA: peanut agglutinin-fluorescein isothiocyanate; CTR: control; ox: samples submitted to induced oxidative stress. Data are shown as the mean±standard error of six replicates.

pattern may reflect the well-known biphasic biological dose–response curve of H_2S: it acts as an antioxidant at low concentrations, but a pro-oxidant at high concentrations[29]. Interestingly, we also found that 30 μM Na_2S was not cytotoxic (i.e. no effect was observed on the mitochondrial activity, plasma membrane integrity, acrosome integrity, or lipid peroxidation), but it strongly inhibited sperm motility. As in our findings, Zhao *et al*. found that 25 μM Na_2S inhibits boar sperm motility although it does not affect the viability and mitochondrial membrane potential[9]. Likewise, NaHS has been shown to inhibit the motility of mouse and human spermatozoa[8,30]. In our study, the inhibitory effects of Na_2S on sperm motility were described by using a comprehensive set of kinetic parameters provided by Computer-Assisted Sperm Analysis (CASA). Overall, 30 μM Na_2S remarkably decreased the sperm motility, VAP, and VSL, but it did not affect the remaining motion parameters. In spite of some variability among replicates, a small percentage of sperm cells showed very slow but progressive movement. With regard to the mechanism of action, Zhao *et al*. found that the inhibitory effects of Na_2S on sperm motility are related to the adenosine 5′-monophosphate-activated protein kinase (AMPK) and protein kinase B (AKT) pathways[9], whereas a more recent study revealed that H_2S promotes the secretion of K^+ in the epididymis; this suppresses sperm motility and may contribute to keeping the sperm cells in a quiescent state before ejaculation[5]. Another plausible explanation for the reduced sperm motility elicited by Na_2S can be provided by the inhibitory effect of H_2S on cytochrome *c* oxidase (complex IV), the final component of the electron transport chain that plays a key role in aerobic respiration and adenosine triphosphate (ATP) generation[29]. Because mammalian spermatozoa devote most of the energy generated as intracellular ATP to motility[31], a reduction in ATP levels would lead to an immediate decrease in sperm motility. In this way, for instance, cardiac cells treated with 25 μM Na_2S showed over 80% decreased O_2 consumption rate relative to the baseline[32]. In contrast to the effects observed at low concentrations, high concentrations of Na_2S showed clear detrimental effects: no motility or active mitochondria were observed in any sample treated with 300 μM Na_2S. The toxic effects were acute and led to immediate failure of sperm motility and mitochondrial activity (personal observations). This phenomenon can be explained by the high levels of ROS induced by Na_2S[9], an explanation corroborated by the increased levels of lipid peroxidation and the low percentage of sperm with intact plasma membrane and acrosome (NAR) found in our study. In contrast to our findings, in testicular germ cells, Li *et al*. found that treatment with NaHS in the concentration range of 1–200 μM for 30 minutes does not affect cell viability, although cell injuries are induced at 1 mM[4]. This result might be because, unlike other male germ cells, spermatozoa have limited antioxidant defences. Spermatozoa lack the necessary cytoplasmic-enzyme repair systems, and their membranes are particularly rich in polyunsaturated fatty acids, which make these cells particularly susceptible to the damage caused by oxidative stress[16]. Another reason might be related to the use of open systems (e.g. four-well plates and Petri dishes) that lead to quick volatilisation of the gas during cell incubation[33]. It is known that Na_2S volatilises very quickly[33], and the use of closed systems, such as the one used in our study, delays this process and leads to more consistent results regarding the effects of H_2S donors on cell biology. Our results also show that Na_2S and GYY4137 partly reduce the damage induced by high ROS levels on acrosomal status by decreasing the percentage of acrosome-lost (PNA-FITC) spermatozoa relative to that in the CTR-ox group. However, neither of the two donors showed any positive effect on the acrosome integrity evaluated by the NAR test. This divergence could be explained by the different acrosomal attributes considered by these two methodologies: NAR evaluated in unstained samples by phase-contrast microscopy versus outer acrosomal membrane integrity assessed by PNA-FITC staining with epifluorescence microscopy[34,35]. Because acrosome integrity is a requisite for fertilisation and the ROS levels affect sperm function[36,37], it remains to be tested whether Na_2S and GYY4137 may preserve the fertilising potential of sperm cells under oxidative stress. Semen handling and storage decrease boar sperm quality and fertilising ability, probably because of oxidative stress[38,39], so H_2S donors may be useful for the optimisation of semen extenders used in artificial insemination programmes. In the porcine industry, higher efficiency of artificial insemination outcomes may have major economic implications because this assisted reproductive technology is the main tool for pig gene dissemination worldwide[40].

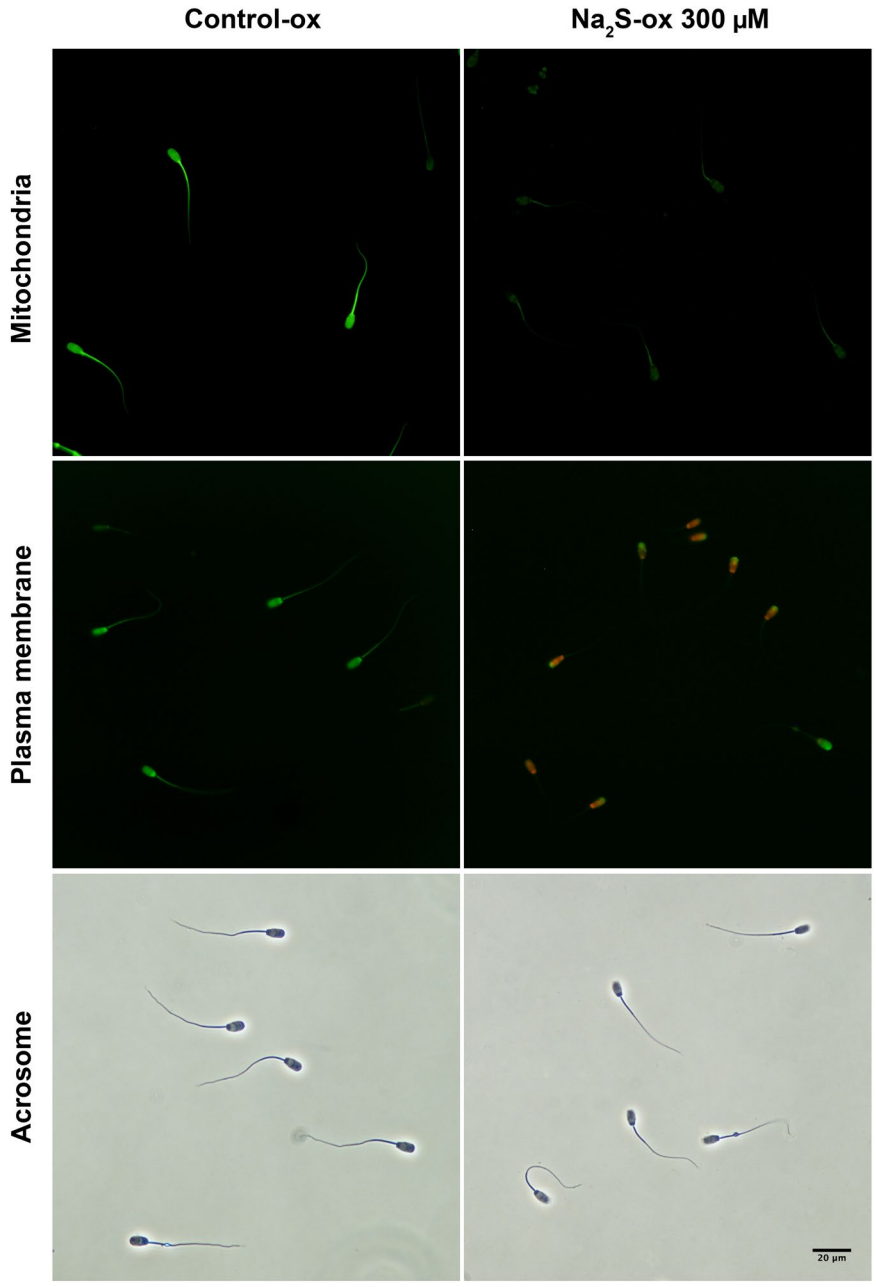

Figure 3. Detrimental effects of a high concentration of Na_2S, a fast-releasing H_2S donor, on the boar sperm mitochondrial status and the plasma membrane and acrosome integrities (normal apical ridge) under oxidative stress. Representative images of sperm cells assessed by epifluorescence microscopy (mitochondrial status was assessed by using rhodamine 123 and propidium iodide; plasma membrane integrity was assessed by using carboxyfluorescein diacetate and propidium iodide) or phase-contrast microscopy (the normal apical ridge was assessed after fixation with glutaraldehyde).

Several mechanisms are involved in the ROS scavenging properties of H_2S. One of these mechanisms involves H_2S itself by virtue of its reducing properties[13]. In this way, the total antioxidant capacities of Na_2S and GYY4137 were tested in this study based on the compounds' abilities to reduce 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) previously oxidised with H_2O_2[41]. Another mechanism that may explain the capacity of H_2S donors to palliate the damages provoked by oxidative stress concerns the enhancement of the cellular antioxidant defences. Previous studies found that H_2S increases glutathione synthesis, decreases ROS production, and stimulates the activities of superoxide dismutase, glutathione peroxidase, and glutathione reductase[4,9,13,25,26]. Moreover, H_2S decreases the apoptosis rate, increases the protein expression ratio of Bax/Bcl-2, and stimulates Cyp19 gene expression, among other effects[4,18,42]. Because spermatozoa are transcriptionally inactive[43], it is likely that H_2S donors protect sperm cells against ROS damage through their proper reducing activity, as well as by increasing the ratio of reduced to oxidised glutathione and stimulating superoxide

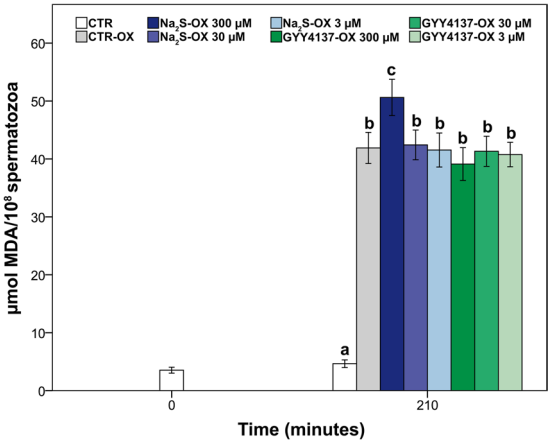

Figure 4. Lipid peroxidation in boar sperm samples submitted to oxidative stress and supplemented with the H_2S donors Na_2S and GYY4137. CTR: control; ox: samples submitted to induced oxidative stress; MDA: malondialdehyde. Different letters indicate significant differences ($p < 0.05$) among treatments. Data are shown as the mean±standard error of six replicates.

dismutase and glutathione peroxidase activities, which represent the major antioxidant defence system of sperm cells[44]. Further studies are nevertheless required to investigate the mechanisms of action of H_2S donors in sperm cells under oxidative stress.

In conclusion, our study provides evidence about the antioxidant properties of two H_2S donors, Na_2S and GYY4137; this evidence will be useful for future studies aiming to test the antioxidant effect of this gasotransmitter. Our findings clearly show that Na_2S has a shorter and less stable total antioxidant capacity than that of GYY4137; it is even undetectable by spectrophotometry at 150 µM. However, it is important to stress out that the antioxidant capacity of GYY4137 tends to increase over time. We also found that both H_2S donors preserve sperm motility and protect the acrosomal status against the detrimental consequences of oxidative stress, although the effects were clearly both dose and donor dependent. Within the range of concentrations tested (3–300 µM), GYY4137 showed positive effects on sperm motility, whereas Na_2S was detrimental at the highest concentration but beneficial at the lowest. Taken together, our results suggest that Na_2S and GYY4137 may be used as *in vitro* therapeutic agents against oxidative stress in sperm cells, although the optimal therapeutic range varies between H_2S donors.

Methods

Reagents were purchased from Sigma–Aldrich (Prague, Czech Republic), unless otherwise indicated.

Ethics statement. This study did not involve animal handling because the sperm samples were purchased as artificial insemination doses from a pig breeding company (Chovservis, Hradec Králové, Czech Republic).

Experiment I. This experiment was designed to evaluate the total antioxidant capacity and stability of Na_2S and GYY4137 at 20, 120, and 210 minutes during incubation at 38 °C in a water bath. The solutions were prepared shortly before the experiment and kept in microcentrifuge tubes tightly sealed with the attached cap (certified free of DNA, DNase, RNase, and endotoxins (pyrogens); material: virgin polypropylene; volume: 600 µl; Neptune Scientific, San Diego, CA, USA) during the whole incubation. For each concentration of H_2S donor, analyses were performed on the same tube throughout the incubation period. Moreover, each microcentrifuge tube contained the same volume (i.e. 200 µl) of H_2S donor or phosphate-buffered saline (PBS; blank) solution. The experiment was replicated four times.

H_2S donor preparation. Na_2S ($Na_2S \times 9\ H_2O$) and GYY4137 ($C_{11}H_{16}NO_2PS_2{\cdot}C_4H_9NO \times CH_2Cl_2$) were prepared in PBS (pH≈7) solution at final concentrations of 2,400, 1,200, 600, 300, and 150 µM.

Total antioxidant capacity of H_2S donors. The total antioxidant capacity was determined by spectrophotometry (Libra S22, Biochrom, Harvard Bioscience Company, Cambourne, United Kingdom) at 660 nm by using the method described previously[41]. The principle of this assay is based on the antioxidant's capacity to reduce ABTS previously oxidised with H_2O_2. A standard curve was established by using known concentrations of 6-hydroxy-2 ,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). The total antioxidant capacity was expressed as Trolox equivalents (µM or mM). The assay was run in duplicate for each sample.

Experiment II. This experiment was designed to test whether H_2S donors protect sperm cells against the deleterious effects of oxidative stress.

Sample collection and experimental design. Artificial insemination doses from 18 boars of different breeds were purchased from a pig breeding company. Sperm-rich fractions were collected by the gloved-hand method, diluted with Solusem® extender (AIM Worldwide, Vught, Netherlands; pH≈7), and transported to the laboratory at 17 °C.

The sperm motility and morphology were then checked, and only samples with at least 75% of motile and morphologically normal sperm were used for the experiments. Sperm samples from three boars were pooled to reduce the effect of male variability and were centrifuged at 167 *g* for 3 minutes at 17 °C to remove debris and dead sperm cells. The sperm concentration was then checked by using a Bürker chamber, adjusted to 30–40 × 10^6 spermatozoa/ml with Solusem®, and finally diluted 1:1 (v/v) with Solusem® supplemented with 0.2% (w/v) of bovine serum albumin (BSA; ethanol-fractionated lyophilised powder). Thus, the final sperm and BSA concentrations were 15–20 × 10^6 spermatozoa/ml and 0.1%, respectively. Sperm samples were then randomly split into eight microcentrifuge tubes tightly sealed with the attached cap (certified free of DNA, DNase, RNase, and endotoxins (pyrogens); material: virgin polypropylene; volume: 2 ml; Neptune Scientific, San Diego, CA, USA): CTR, CTR-ox, and Na_2S or GYY4137 at 300, 30, and 3 µM under oxidative stress. Oxidative stress was induced by adding a solution composed of 0.1 mM $FeSO_4$ and 0.5 mM sodium ascorbate (Fe^{2+}/ascorbate) to the sperm samples. Because the effects of this ROS-generating system are clearly evident after 210 minutes of sperm incubation[19], sperm analyses were performed at 0 hour (after 20 minutes of incubation for the CTR group only) and after 210 minutes of incubation at 38 °C in a water bath. The experiment was replicated six times with six independent semen pools.

Sperm motility. Sperm motility was evaluated by using CASA (NIS-Elements; Nikon, Tokyo, Japan, and Laboratory Imaging, Prague, Czech Republic), after loading 5 µl of sperm sample into a pre-warmed (38 °C) Spermtrack chamber (PROiSER R + D S.L., Paterna, Spain; chamber depth: 20 µm). A total of ten sperm kinetic parameters were obtained by analysing six random fields: TM (%), PM (%),VAP (µm/s), VCL (µm/s), VSL (µm/s), ALH (µm), BCF (Hz), LIN (VSL/VCL, %), STR (VSL/VAP, %), and WOB (VAP/VCL, %). The settings parameters were as follows: frames per second, 60; minimum frames acquired, 31; VAP ≥ 10 µm/s to classify a spermatozoon as motile, STR ≥ 80% to classify a spermatozoon as progressive[19]. A minimum of 200 sperm cells were analysed for each sample.

Sperm mitochondrial status. Mitochondrial status was evaluated as previously described[45], with minor modifications. Briefly, aliquots of sperm samples were incubated with rhodamine 123 (5 mg/ml, w/v, in dimethyl sulfoxide, DMSO) and propidium iodide (0.5 mg/ml, w/v, in PBS) for 15 minutes at 38 °C in the dark. Subsequently, samples were centrifuged at 500 *g* for 5 minutes, the supernatant was removed, and the sperm pellet was resuspended in PBS. Then, 200 spermatozoa were evaluated by using epifluorescence microscopy (40× objective; Nikon Eclipse E600, Nikon, Tokyo, Japan): the spermatozoa showing bright green fluorescence in the midpiece were considered to have active mitochondria.

Sperm plasma membrane integrity. The sperm plasma membrane integrity was evaluated as previously described[46,47]. Aliquots of sperm samples were incubated with carboxyfluorescein diacetate (0.46 mg/ml, w/v, in DMSO), propidium iodide (0.5 mg/ml, w/v, in PBS), and formaldehyde solution (0.3%, v/v) for 10 minutes at 38 °C in the dark. Then, 200 spermatozoa were evaluated by using epifluorescence microscopy (40× objective). The spermatozoa showing green fluorescence over the entire head area were considered to have intact plasma membrane.

Lipid peroxidation. Lipid peroxidation was assessed with the thiobarbituric acid reactive substances (TBARS) assay, as previously described[19,48]. At the end of each incubation period, sperm aliquots were collected and stored at −80 °C until analysis. The absorbance of each sample was then measured by spectrophotometry at 532 nm. A standard curve was established by using known concentrations of 1,1,3,3-tetramethoxypropane (MDA). The levels of lipid peroxidation are shown as µmol of MDA per 10^8 spermatozoa. The assay was run in duplicate for each sample.

Acrosomal status. Acrosome integrity was assessed after sample fixation in 2% (v/v) glutaraldehyde solution and by examination with phase-contrast microscopy (40× objective)[34]. For each sample, 200 spermatozoa were evaluated, and the percentage of sperm cells with NAR was determined. Acrosome loss was evaluated according to the protocol previously described[49]. Briefly, after methanol fixation and double washing with PBS, the samples were incubated with PNA-FITC (100 µg/ml, w/v, in PBS) for 10 minutes at 38 °C in the dark. Epifluorescence microscopy (40× objective) was used to evaluate 200 spermatozoa, and the cells showing no fluorescence over the acrosome were considered as acrosome-lost spermatozoa.

Statistical analysis. Data were analysed with the statistical program SPSS, version 20 (IBM Inc., Chicago, IL, USA). Shapiro-Wilk's and Levene's tests were used to analyse the normal distribution and the variance homogeneity of the data, respectively. The Mann–Whitney U-test was applied to check for differences between the total antioxidant capacities of Na_2S and GYY4137 at the same concentration, whereas the repeated-measures Friedman test was used to compare the total antioxidant capacities of the H_2S donors across the incubation times. The generalized linear model (GZLM) was performed to analyse the effects of the type and concentration of H_2S donor on the sperm variables. The statistical significance was determined at $p < 0.05$. Data are shown as the mean±standard error.

Data availability

All data generated or analysed during this study are included in this article and its supplementary information file.

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E.P. and J.L.R.-S. conceived and designed the experiments; E.P., M.J., M.K. and J.L.R.-S. performed the experiments; E.P. and J.L.R.-S. analysed the data; E.P. wrote the paper; E.P., M.J., M.K. and J.L.R.-S. revised and edited the paper.

Competing interests

The authors declare no competing interests.

Additional information

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Cryopreservation of boar semen

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Abstract: Sperm cryopreservation is the best technology for long-term storage of the semen. However, the damage of boar spermatozoa by cryopreservation is more severe than in other animal species and a standardized freezing protocol for efficient cryopreservation has not been established yet. Semen quality and freezability vary greatly between breeds as well as between individual boars and even the season. Boar spermatozoa are sensitive to low temperatures; they sustain damage and a high rate of mortality and freezing/thawing the boar semen may strongly impair the sperm function and decrease the semen quality. The freezability of boar semen can be influenced by a cryopreservation procedure, and also by using various additives to freezing and thawing extenders such as antioxidants. In order to obtain acceptable results after thawing the boar semen, it is necessary to combine an optimal amount of additives (glycerol, egg yolk, sugars, antioxidants), cooling and warming velocities.

Keywords: additives; antioxidants; extenders; freezability; sperm

Introduction

Sperm cryopreservation is the most efficient method for the long-term storage of semen in domestic animals (Yeste 2015). However, only 1% of the boar semen that is used for artificial insemination (AI) throughout the world is cryopreserved (Techakumphu et al. 2013). The remaining 99% is preserved in liquid form at 16–20 °C (Saravia et al. 2005).

Among the reasons for this restricted use of frozen boar semen are great variability of semen quality and freezability between breeds as well as individual boars and even the season. Frangez et al. (2005) found that it is the best to collect sperm from mature boars (20–24 months) two or three times per week. In their research Ratchamak et al. (2019) revealed no differences in sperm motility collected as sperm-rich fraction and bulk samples, and no differences in any sperm quality parameters after cryopreservation.

Moreover, in comparison with other mammalian species, boar spermatozoa have low survivability rates during the freezing/thawing process and the short lifespan of the surviving spermatozoa (Mazur et al. 2008; Techakumphu et al. 2013) due to their high cold shock sensitivity. Approximately 40% to 50% of boar spermatozoa do not survive cryopreservation. In comparison with insemination with fresh semen, insemination with frozen/thawed semen causes lower fertility rates, a decrease in farrowing rates by 20% to 30%, and smaller litter sizes (Saravia et al. 2005). Due to sublethal damage, fertility rates are still lower with frozen semen than they are with fresh semen, even when using similar numbers of motile sperm (Watson 2000).

The review is focused on using various additives to freezing and thawing extenders and freezing protocols for efficient cryopreservation.

Parameters of boar semen

Where analysing the quality and sperm fertilizing ability of boar semen, the following parameters are evaluated: semen volume, sperm concentration,

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Table 1. Parameters of boar semen quality

Paremeters		Values
Volume		150–300 ml
Concentration		$10 \times 10^9 – 100 \times 10^9$
Viability		> 75%
Motility	total motility	> 80%
	progressive motility	> 60%
Morphology	normal spermatozoa	> 80%
	mature spermatozoa	80–95%
	immature spermatozoa	5–15%
	abberant spermatozoa	5%
pH		7.2–7.5
Osmolality		~300 m Osm/kg

In Table 1 are written the values of parameters which good quality fresh boar semen should fulfil. It is written according to previous studies about the quality of boar semen

total number of spermatozoa per ejaculate, sperm viability, sperm motility and sperm morphology, percentage of mature, immature spermatozoa, sperm abnormalities or aberrant spermatozoa (Table 1).

However, for objective ejaculate evaluation several other markers such as measurement of sperm-produced reactive oxygen species, DNA fragmentation analysis and biomarkers (ubiquitin, aggresome) can be used (Sutovsky 2015).

Differences in sperm freezability

Differences exist between breeds, individual boars, within the same breed, and between ejaculates from the same boar (Techakumphu et al. 2013). Ejaculates from Duroc boars exhibit better freezability than those of Landrace boars; this is confirmed by the higher percentage of spermatozoa with intact plasma membranes and acrosomes in post-thawed samples taken from Duroc males (Waterhouse et al. 2006). According Roca et al. (2006), Landrace and Pietrain boars have higher post-thaw sperm motility, membrane integrity, mitochondrial membrane potential, and acrosomal integrity than do Large White, Duroc, and Yorkshire boars.

In their research Waterhouse et al. (2006) found differences within breeds male-to-male in post-thaw percentages of live sperm, which may be related with the amount of long-chain and polyunsaturated fatty acids in the sperm plasma membranes. There are consistent and genetically determined variations in frozen-thawed sperm quality and fertility rates between boars. The farrowing rate is 20% higher in post-thawed spermatozoa from Large White boars than it is in that of Landrace boars; however, the farrowing rate of fresh semen from the same boars is higher for Landrace boars than for Large White boars (Johnson et al. 1981; Johnson et al. 1982; Thurston et al. 2001).

Differences in fertilizing ability between breeds, and even between individual boars, are related to differences in sperm head dimensions (Pena et al. 2006; Saravia et al. 2007). High-fertility boars produce spermatozoa with smaller and shorter heads than low-fertility boars (Hirai et al. 2001). Genetically determined characteristics of spermatozoa membrane structure are also important for a predisposition to survive under cryopreservation stress (Watson 2000).

Not all boar ejaculates possess the same ability to withstand freeze/thawing. For this reason, boars and their ejaculates are classified as 'good freezers' or 'bad freezers' (Hernandez et al. 2007a) and as 'good freezability ejaculates' (GFE) or 'poor freezability ejaculates' (PFE) (Yeste et al. 2013). Variability of semen to sustain sperm cryopreservation could be related to differences in the seminal plasma composition (Roca et al. 2006). Supplementing freezing extenders with seminal plasma from boars with good sperm freezability could eventually improve the ability of boar spermatozoa to sustain the freezing-thawing process, improve sperm motility, maintain acrosome integrity, delay capacitation-like changes, and increase resistance to cold shock or oxidative stress (Roca et al. 2005; Hernandez et al. 2007b). Sperm freezability in boars can be modulated through diet modifications, as well as through changes in feeding regimens and trial lengths (Yeste et al. 2010; Yeste 2015).

Overconsumption of high-energy diets has negative effects (Rato et al. 2014); on the other hand, consumption of some dietary fats (Safarinejad et al. 2010), microminerals and vitamins will maintain and improve sperm quality in humans (Vujkovic et al. 2009; Prasad 2013). In fresh boar sperm sample, sperm motility is appreciably slower in comparison with other mammalian species such as horses, dogs, bulls, or mice (Quintero-Moreno et al. 2003; Mogas et al. 2011), also boar sperm cryosurvival

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after freeze/thawing is consistently lower in comparison with other species (Rodrigues-Martines and Wallgren 2011).

Cryopreservation of boar semen

During cryopreservation, boar semen is exposed to physical and chemical stress, and less than 50% of the spermatozoa survive with their fertilizing ability intact (Waterhouse et al. 2006). Many researchers created protocols for the cryopreservation of boar semen, e.g. Pursel and Johnson (1975), Larsson et al. (1977), Bwanga (1991), Johnson et al. (2000), and Athurupana et al. (2015). The steps of cryopreservation are: 1) reducing (1 : 1) extension with buffer and a period of equilibration in the presence of homologous seminal plasma. The step promotes the capacity of spermatozoa to withstand thermal shock events usually done while cooling the semen to 15 °C. 2) Separation of the bulk of seminal plasma by centrifugation, thus re-concentrating the spermatozoa. 3) The inclusion of chilling protectants (lactose and egg yolk) during cooling to 5 °C. 4) The inclusion of penetrating cryoprotectants (the most commonly used glycerol at a 4% final concentration) immediately prior to freezing to enable rapid decreases of temperature, which are usually effected by programmable freezers.

When following the cryopreservation protocols, centrifugation is a necessary step taken prior to freezing because it facilitates the removal of seminal plasma and concentrates the spermatozoa that are rediluted with freezing extenders (Zhang et al. 2012). The optimal concentration of boar spermatozoa is about 1×10^9 spz/ml (Bolarin et al. 2006). Carvajal et al. (2004) demonstrated that high *g*-force (2 400 × *g*) and short centrifugation time (3 min) achieved high sperm recovery and yield, as well as a positive effect on the cryosurvival of boar sperm. However, other researchers recommend other values for centrifugation rates, i.e. 300 × *g* for 10 min (Pursel and Johnson 1975), 800 × *g* for 10 min (Westendorf et al. 1975), 1 400 × *g* for 5 min (Larsson et al. 1977), and 700 × *g* for 3 min (Athurupana et al. 2015).

Cryopreservation produces physical and chemical stress on sperm structures and reduces post-thaw sperm viability and fertilizing ability. There are many reasons for the negative effects of cooling, freezing and thawing. Boar spermatozoa are very susceptible to peroxidative damage because of the high content of polyunsaturated fatty acids (Esmaeili et al. 2015) and low level of cholesterol in the plasma membrane (Cerolini et al. 2000). The main causes are lipid phase transitions, ice crystallization and osmotic-induced water fluxes. It results in membrane reorganizations that can disrupt membrane integrity, structure and function. The successful freezing of boar semen is dependent on many factors that affect the capacity of spermatozoa to survive freezing and thawing. These factors may be classified into two categories: 1) internal or fixed factors such as the inherent characteristics of spermatozoa or differences between boars and ejaculates and 2) external factors such as composition of diluents, types and concentrations of cryoprotective agents, dilution and cooling rates, equilibration, and semen freezing and thawing methods (Johnson et al. 2000; Watson 2000).

While internal factors cannot be improved by modification of the freezing protocol, external factors have the ability to improve semen freezability. Sperm selection through density gradient washing has also been reported to increase ejaculate freezability prior to cryopreservation (Macias Garcia et al. 2009).

The freeze/thaw processes of boar semen are important, but some authors reported that the length of the holding time (total length of storage time at temperature of 15–17 °C from semen collection to the beginning of cryopreservation procedures) affects also the ability of the sperm to withstand freeze/thaw procedures (Juarez et al. 2011; Alkmin et al. 2014).

However, regardless of the protocol used, boar spermatozoa are very susceptible to cold shock, and rapid cooling from body temperature to temperatures below 15 °C irreversibly reduces their viability (Johnson et al. 2000). During slow freezing, boar spermatozoa are also sensitive to cellular injury and mechanical stress caused by ice formation around of the cells (Rodriguez-Martinez and Wallgren 2011; Di Santo et al. 2012).

The most critical variables that influence sperm cryosurvival and thus serve as prerequisites for an optimal sperm cryopreservation protocol are the cooling (Said et al. 2010) and thawing rates. The most appropriate freezing rate is the fastest one that allows extracellular water freezing without intracellular ice formation. The optimal cooling rate

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for freezing boar spermatozoa is –30 °C/min, while the most suitable rate for thawing is 1 200 °C/min. These cooling and thawing rates lead to acceptable post-thaw sperm survival rates (> 50%) and are thus appropriate to the majority of boars (Devireddy et al. 2004).

In their research Horvath et al. (2018) found that it is possible to improve post-frozen-thawed semen quality by an appropriate application of hydrostatic pressure to boar semen during cryopreservation. Li et al. (2018) concluded that the cryostorage time affected sperm kinetics while the sperm cryopreserved longer than 2 years can significantly decrease post-thaw motility.

In cryopreserved semen, fertilizing ability is lower than that of fresh or refrigerated semen (Rath et al. 2009). When using frozen-thawed boar semen instead of fresh semen for artificial insemination (AI), the farrowing rates are reduced by 10% to 25% and the litter sizes are reduced by 1 to 3 piglets (Johnson et al. 1981; Waterhouse et al. 2006). The boar semen has to be frozen in much larger quantities in comparison with the bull semen. The pellets of approximately 200 ml volume or 10–15 ml tubes are used provided that they contain sufficient spermatozoa for each insemination (Holt 2000).

During thawing, boar semen is exposed to all of the same damaging effects of the cooling and freezing phases, albeit in reverse order (Holt 2000). A high warming velocity is essential for the cryosurvival of spermatozoa (Mazur 1984), particularly within the critical temperature range (–15 °C and –60 °C) in which spermatozoa are most sensitive (Yeste 2015). Mazur (1984) found that the thawing rate effectiveness is dependent on the used freezing rate. The most effective thawing rate for boar semen is 1 200 °C/min to 1 800 °C/min (20 s in 50 °C water), which limits the exposure of the cell to such harsh conditions as well as regrowth of ice crystals (Fiser et al. 1993; Thurston et al. 2001). Athurupana et al. (2015) found that rapidly thawing boar semen at 70 °C for 8 s followed by a stabilizing procedure at 39 °C for 52 s also maintained all semen parameters. Thawing similar to freezing impaired boar spermatozoa motility, membrane, and acrosome integrity (Okazaki et al. 2009; Yeste et al. 2013) and the number of spermatozoa with damaged plasma membranes dramatically increased (Ortman and Rodriguez-Martinez 2010). Bamba and Cran (1985) reported that rapidly warming boar semen between 5 °C and 37 °C damaged acrosome membranes. However, only a small proportion of spermatozoa will survive proper thawing if semen is frozen at suboptimum rates (Johnson et al. 2000). Freezing at the optimum rate and fastest thawing rates is beneficial for all parameters of the semen quality, including the motility and acrosome integrity of spermatozoa (Salamon et al. 1973; Pursel and Johnson 1975). A fast thawing rate could not be achieved effectively when the ejaculate is packed in maxi-straws of large diameter (Salamon et al. 1973; Weitze et al. 1987). Fiser et al. (1993) suggested to use of 0.5 ml plastic straws cooled by a flow of liquid nitrogen vapour (using a programmable freezing system) to properly control the freezing rate, on the other hand in the research of Eriksson and Rodriguez-Martinez (2000) the ejaculate was cryopreserved in 5-ml plastic bags.

The protocol for the crucial steps of cryopreservation and thawing of boar semen according to listed authors is summarized in Table 2.

Table 2. Protocol for cryopreservation and thawing of boar semen according to listed authors

Processes	Values	References
Centrifugation	2 400 × g/3 min	Caravajal et al. (2004)
Concentration of semen	1×10^9 spz/ml	Bolarin et al. (2006)
Extenders	egg yolk based glycerol free extender contuining 100 mM trehalose	Athurupana et al. (2015)
Packaging	0.5 ml straws	Athurupana et al. (2015)
Optimal cooling rate	–30 °C/min	Devireddy et al. (2004)
Critical temperature range	–15 °C to –60 °C	Yeste (2015)
Thawing rate	1 200 °C – 1 800 °C/min or 20 s in 50 °C water or at 70 °C for 8 s	Fiser et al. (1993); Thurston et al. (2001); Athurupana et al. (2015)

In Table 2 a protocol is written – the main steps for cryopreservation and thawing of boar semen according to previous studies

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Extenders and antioxidants

Defensive strategies are useful for the cryopreservation of boar semen which comprise the addition of different cryoprotectants, antioxidants, animal serum, antifreeze proteins, fatty acids to freezing media to protect sperm cells against damage (Hezavehei et al. 2018). Extenders may be divided into the following categories: (1) extenders without buffers, such as egg yolk-glucose, egg yolk-lactose and egg yolk-saccharose, ethylenediamine-tetraacetic acid (EDTA), and Mg and Ca salts; (2) extenders with a buffering capacity, such as glycine-phosphate and glucose-phosphate, egg yolk-glucose-citrate, egg yolk-glucose-citrate-EDTA-potassium-unitol-urea, Beltsville F3, Beltsville F5, Tes-tris-fructose-citrate-egg yolk, TEST, Tes-NaK-glucose-egg yolk, Tris-fructose-EDTA-egg yolk, and Tris-glucose-EDTA-egg yolk (Jian-Hong et al. 2006). Many researchers have used different extenders for the cryopreservation of boar semen.

Freezing extenders for boar semen usually contain egg yolk in addition to other agents, such as buffers, additives and cryoprotectants (Gutierez-Perez et al. 2009). Egg yolk is widely used as a cryoprotective agent in semen freezing extenders to protect the spermatozoa from cold shock during cryopreservation (Bathgate et al. 2006) Consequently, low density lipoproteins (LDL) are commonly used in the extenders at concentrations of 9% (Jian-Hong et al. 2006). The most common cryoprotectant for boar sperm is glycerol at concentrations of 2% to 4% (Corcuera et al. 2007). Higher concentrations of glycerol decrease survivability post-thaw and compromise acrosomes, resulting in reduced fertility (Holt 2000). Amides, especially DMA (dimethylformamide) and DMF (dimethylacetamide), can successfully replace glycerol as penetrating cryoprotectants used in freezing the boar semen. Although glycerol and amides can protect sperm cells during cryopreservation, their cryoprotectant properties are achieved through different mechanisms (Bianchi et al. 2008).

The most commonly used extender additives include sugars, proteins and lipoproteins, detergents, antioxidants. The combination of sugar and glycerol has been found to be indispensable in providing protection during freezing. This is so because the osmotic properties of sugar provide extracellular protection (Salamon et al. 1973). Sugars do not penetrate into plasma membranes; they increase the percentage of unfrozen water at a given temperature or reduce the concentration of salts in the unfrozen extracellular water (Mazur 1984). The use of cryoprotectants like trehalose, sucrose, lactose, glucose, and fructose that are added to the extenders results in enhancing spermatozoa viability and fertilization parameters after thawing the boar spermatozoa (Malo et al. 2010; Athurupana et al. 2015). Gomez-Fernandez et al. (2012) tested the effects of different monosaccharides (glucose, galactose, fructose) and disaccharides (lactose, sucrose, melabiose, trehalose) on boar sperm quality after cryopreservation and found that the freezing extenders supplemented with monosaccharides provided a lower cryoprotective effect than the extenders supplemented with lactose.

Gadea et al. (2004) found that adding glutathione to the freezing extender did not result in any improvement in either standard semen parameters or sperm fertilizing ability. In contrast, the addition of glutathione to the thawing extender resulted in a significant increase in sperm fertilizing ability. Jeong et al. (2009) found that the freezing extender supplemented with α-tocopherol (200 M) prior to cryopreservation had a positive effect on post-thaw boar spermatozoa survival. The addition of the antioxidant vitamin E to boar semen prior to cryopreservation positively affected motility after thawing (Pena et al. 2003). The seminal plasma (SP) contains abundant antioxidants (Makker et al. 2009), and evidence suggests that as long as spermatozoa are suspended in SP, they are protected from oxidative damage (Saleh and Agarwal 2002). In their research Barranco et al. (2019) tested the effect of seminal plasma cytokines (Barranco et al. 2015) on the cryopreservation of boar semen and they proved that seminal plasma cytokines are capable to sustain the preservation of sperm in frozen state. Varo-Ghiuru et al. (2015) found that adding lutein to the semen extender before freezing improved motility, acrosome integrity, plasma membrane function and DNA integrity of boar semen after thawing.

L-glutamine has the ability to cryoprotect the boar sperm by enhancing post-thaw sperm motility, and it can be used as a partial glycerol substitute in the freezing extenders (de Mercado et al. 2009). The addition of sodium dodecyl sulphate (SDS) to semen extenders increases the survival of spermatozoa (Axner et al. 2004). The addition of superoxide dismutase (SOD) and catalase (CAT) to a semen extender before cryopreservation re-

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Table 3. Extender additives used for the cryopreservation of boar semen

Additives		References
Egg yolk		Bathgate et al. (2008)
Low density lipoproteins (LDL)		Jian-Hong et al. (2006)
Glycerol		Corcuera et al. (2000)
L-glutamine		de Mercado et al. (2009)
Sodium dodecyl sulphate (SDS)		Axner et al. (2004)
Sugars	trehalose, sucrose, lactose, glucose, fructose	Malo et al. (2010); Fernandez et al. (2012); Athurupana et al. (2015)
Antioxidants	glutathione	Gadea et al. (2004)
	α-tocopherol	Jeong et al. (2009)
	seminal plasma	Barranco et al. (2015)
	vitamin E	Pena et al. (2003)
	ascorbic acid	Breininger and Beconi (2014); Varo-Ghiuru et al. (2015)
	lutein	Varo-Ghiuru et al. (2015)
	superoxide dismutase (SOD)	Roca et al. (2005)
	catalase (CAT)	Roca et al. (2005)

In Table 3 are listed all extender-additives used for the cryopreservation of boar semen in previous studies

sulted in better post-thaw sperm survival (Roca et al. 2005). Adding ascorbic acid to the thawing extender decreased lipid peroxidation in a dose-dependent manner and increased post-thaw motility in the boar sperm (Breininger and Beconi 2014; Varo-Ghiuru et al. 2015).

Extender additives used for the cryopreservation of boar sperm are summarized in Table 3.

Conclusion

Although the boar semen is sensitive to low temperatures and only the good quality semen is suitable for cryopreservation, development of new cryopreservation protocols is important, because the semen in liquid form can be stored for 7 to 8 days, but the cryopreserved semen can be stored for a much longer period of time and used when necessary. An optimal protocol for freezing and thawing of individual boar sperm as well as supplementation of extenders with buffers, additives and cryoprotectants is crucial for viability and fertility of spermatozoa. One solution is to add the antioxidant to the boar semen before cryopreservation to avoid oxidative damage of sperm cells. The other solution is to add the seminal plasma semen before cryopreservation and to store the cryopreserved sperm in liquid nitrogen no longer than 2 years. This paper will encourage researchers to continue work on the cryopreservation of boar semen for better understanding of the composition of sperm cells, and to create a protocol which will contribute to improving results in practice. This will result in better utilization of boar semen and greater economic profits.

Conflict of interest

The authors declare no conflict of interest.

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