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# Effect of a novel hydrogen sulfide donor on sperm physiology

**Diploma thesis**

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## Declaration

I declare that the Diploma Thesis "Effect of a novel hydrogen sulfide donor on sperm physiology" is my work and that I used only sources mentioned in the Bibliography section.

Prague, date: _____

**Acknowledgement**

I would like to express my gratitude to my supervisor, doc. Eliana Pintus, PhD., who dedicated considerable time to reviewing my diploma thesis and consistently provided guidance on how to complete each chapter successfully. Additionally, I am immensely thankful to my consultant, Maria Scaringi, who patiently assisted me with my diploma thesis's laboratory components. I would also like to extend my appreciation to my family and partner, who supported and encouraged me throughout the challenging journey of completing this diploma thesis.

# Effect of a novel hydrogen sulfide donor on sperm physiology

**Summary:**

Hydrogen sulfide ($H_2S$) donors are essential tools in exploring the physiological role of $H_2S$ in cell function. A wide range of $H_2S$ donors is available that mimics physiological $H_2S$ levels under in vitro conditions. Despite the significant advances in $H_2S$ research, the effects of these compounds on sperm function are not fully understood.

This diploma thesis aimed to investigate the effect of a novel *N*-thiocarboxyanhydride (NTA) derived from the amino acid valine (Val-NTA), which releases $H_2S$ in the presence of the ubiquitous enzyme carbonic anhydrase, on porcine sperm quality during semen storage. Specifically, sperm motility, kinetics, plasma membrane integrity, and acrosome integrity were evaluated in porcine sperm samples with or without NTA supplementation at 0 h (control group only), 17 h, and 45 h of semen storage at 17 °C.

The application of Val-NTA did not exhibit any positive influence on the sperm plasma membrane integrity, acrosomal integrity, motility, or kinetics. Additionally, the findings from this study demonstrated that Val-NTA exhibited dose-dependent toxicity towards spermatozoa, with specific concentrations resulting in a significant decrease in several sperm parameters. This was consistent with previous studies, which found that high concentrations of $H_2S$ donors could have negative effects on sperm motility and induce oxidative stress. Specifically, Val-NTA at the concentration of 100 μM was found to cause a drastic reduction in most of the sperm parameters evaluated.

The results also showed a decline in the sperm parameters during storage, which is consistent with previous research and might at least partly be linked to oxidative stress that can be provoked by the exposure of cells to low temperatures. The study suggests that different environmental conditions (the length of incubation, temperature, oxidative stress or variation of carbonic anhydrase levels in the porcine ejaculates) may have influenced the impact of Val-NTA on sperm plasma membrane integrity, acrosomal integrity, motility, and kinetics and that further research, specifically of the mitochondrial activity, is warranted to determine the potential of Val-NTA.

**Keywords:** boar spermatozoa, gasotransmitter, $H_2S$ donor, *N*-thiocarboxyanhydrides, sperm storage

# Table of contents

# 1 Introduction

Hydrogen sulphide ($H_2S$) is a gas involved in several cell's biological functions in cells. Studies have shown that $H_2S$ plays a vital role in several physiological processes such as modulation of ion channels, anti-inflammatory responses, regulation of oxidative stress, and cytoprotection (Kimura Y & Kimura H 2004; Lefer 2007; Hu et al. 2010; Kashfi & Olson 2013). $H_2S$ plays an important role in sperm metabolism and functionality. Several studies have suggested that $H_2S$ plays a role in promoting sperm motility, capacitation, and acrosome reaction. $H_2S$ can modulate the activity of enzymes involved in energy production in sperm, which is important for maintaining sperm motility and viability (Li et al. 2015). One way in which $H_2S$ promotes sperm motility is by increasing intracellular calcium levels, which are essential for sperm movement. Males with lower levels of $H_2S$ in their reproductive system often exhibit sub- or infertility. $H_2S$ has also been shown to activate mitogen-activated protein kinases, which are important for flagellar motility, hyperactivation, and the acrosome reaction (Wang et al. 2017). Regulation of calcium and potassium channels by $H_2S$ is also necessary for capacitation and acrosome reaction (Kulluru et al. 2017; Shefa et al. 2018) In particular, CatSper channels are critical for calcium influx and hyperactivation of sperm, while potassium channels are important for ATP generation by mitochondria and promoting sperm hyperactivity (Gupta et al. 2018; Kumar et al. 2019). Moreover, $H_2S$ can influence the smooth-muscle relaxation of the human corpus cavernosum (d'Emmanuele di Villa Bianca et al. 2009).

$H_2S$ donors are commonly used as tools to study the role of $H_2S$ in cellular function. These donors provide a means of mimicking physiological levels of hydrogen sulfide in vitro and thereby exploring its effects on cell function (Xu et al. 2019). $H_2S$ donors are compounds that release $H_2S$ in a controlled manner and have been used in various studies for their therapeutic potential (Wang et al. 2017, Xia et al. 2019; Pintus et al. 2020). However, the use of these donors has raised concerns about the uncontrollable release of $H_2S$ and the potential formation of toxic byproducts. There are two strategies for releasing $H_2S$, slow and fast (Rose et al. 2015). Sodium hydrosulfide (NaHS) releases $H_2S$ in a time-dependent manner, which allows for better control over the amount of $H_2S$ released. However, NaHS can also release other toxic sulfur-containing compounds, such as thiosulphide and sulphide, under certain conditions (Wu et al. 2016). Another $H_2S$ donor is GYY4137, which releases $H_2S$ at a slower rate than NaHS. This slower release can minimize the formation of toxic byproducts, but it also means that GYY4137 may not be as effective in delivering therapeutic levels of $H_2S$ (Li et al. 2008).

To address this problem, the present diploma thesis seeks to investigate the effect of *N*-thiocarboxyanhydride on the quality of porcine sperm during semen storage. NTAs are novel compounds that release $H_2S$ in the presence of carbonic anhydrase (CA), which is an enzyme that is widely present in cells (Haritos & Dojchinov 2005; Steiger et al. 2018; Lewinn et al. 2019). Precisely, the effects of Val-NTA on porcine sperm parameters and quality during semen storage will be evaluated. This research aims to expand our understanding of the role of $H_2S$ in sperm function.

# 2 Scientific hypothesis and objectives of the work

This diploma thesis aims to assess the effects of NTAs supplementation on porcine sperm samples by analyzing their motility, kinetics, plasma membrane, and acrosome integrity. This diploma thesis hypothesises that Val-NTA influences sperm motility and membrane integrity in a concentration-dependent manner with positive effects at the lowest, but negative effects at the highest concentrations. Because of Val-NTA $H_2S$ release half-life, which is estimated to be around 20 h, the samples will be collected at 0 h (control group only), 17 h, and 45 h of semen storage at 17°C. The results obtained from this study are expected to provide valuable insights into the impact of $H_2S$ on sperm quality and lifespan.

# 3 Literature overview

## 3.1 General aspects of the boar reproductive system

Boars go through puberty at around 8 to 9 months of age, although some breeds may mature earlier or later. During this time, boars experience physical changes that allow them to become sexually active and capable of breeding. One of the most notable changes that occur during boar puberty is an increase in testosterone production (Kuster & Althouse 2007). This hormone is responsible for the development of primary sexual characteristics, such as the growth of the testicles and penis, the development of the muscles, and the production of sperm (Bhasin & Jasuja 2018). Boars also begin to develop a musky odour as a result of the secretion of androstenone, a pheromone that plays a role in attracting sows for mating and is responsible for boar taint. Boars are non-seasonal breeders, which means that they can reproduce all year. Nevertheless, in the northern hemisphere, boars are typically more active from November to February, which coincides with the shorter days and colder temperatures of winter. During this time, the level of light exposure triggers hormonal changes in the boars that increase their libido and desire to mate. In contrast, during the summer months, when days are longer and temperatures are higher, boars tend to have lower reproductive activity. This occurs because the hormonal changes, that drive their sexual behaviour, are suppressed by increased exposure to light, moreover, their thermoregulation is less efficient and they can be more sensitive to heat stress (Berger et al. 1980; Mauget & Boissin 1987).

The structure of the male reproductive system in boars includes two testes, two epididymides, two deferent ducts, the urethra, the penis, and accessory sex glands like the seminal vesicles, prostate, and Cowper's glands (also known as bulbourethral glands; Constantinescu 2017). The testicle serves as the male gonad responsible for producing sperm and also as an endocrine gland that secretes hormones (Bonet et al 2013). The testicle, as shown in Figure 1, is enclosed by a tough, fibrous capsule called the tunica albuginea, which helps protect them from damage. Within the testes, there are coiled, tubular structures called seminiferous tubules, where sperm is produced through the process of spermatogenesis. The interstitium is the tissue that surrounds the seminiferous tubules in the testes (Kuster & Althouse 2007). It contains specialized cells called Leydig cells, which are responsible for androgens synthesis, namely testosterone and androstenedione. Sertoli cells are located in the seminiferous epithelium of the testes and are involved in sperm production and estrogen synthesis, among others. The testes and epididymides are located in the scrotum, which is situated in a sub-anal

position and is separated into two halves by a scrotal raphe. Likewise in most domestic animals, the testicular descent into the scrotal sac takes place just before birth (Bonet et al. 2013).

In the epididymis, which is located dorsally to the testes and divided into head, body and tail (as shown in Figure 1), the spermatozoa mature, acquire motility, and finally can fertilize. Testicular spermatozoa are not yet capable of fertilization, they need to mature in the tail of the epididymis. The mature spermatozoa are stored in the epididymal tail and await eventual ejaculation. During ejaculation, spermatozoa are transported through the ductus deferens towards the urethra, and along the way they acquire secretions from the prostate, seminal vesicles, and bulbourethral glands (Bonet et al. 2013). The seminal vesicles and the bulbourethral glands are very large whereas the prostate gland is relatively small. Boars possess a lengthy, fibroelastic penis featuring a sigmoid flexure and a spiralled apex that rests within the prepuce (Kuster & Althouse 2007). The semen or ejaculate is expelled by the penis in a process called ejaculation.

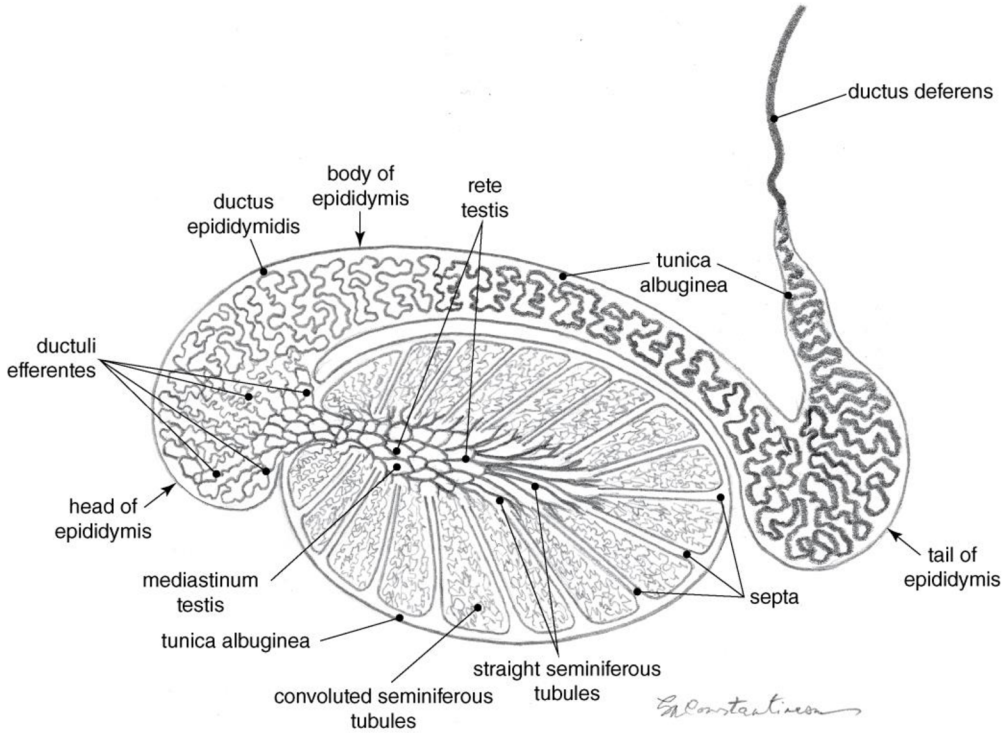


**Figure 1** Internal organization of testicles and epididymis (Kuster & Althouse 2007).

Semen is the biological fluid that is secreted by the male penis during ejaculation, is composed of two fractions: the spermatozoa or sperm cells, which form the cellular fraction, and the seminal plasma, which forms the liquid fraction (Chenoweth & Lorton 2014). The basic characteristics of boar ejaculate differ significantly from other livestock species. The volume of ejaculate is notably large, typically ranging from 150-300 ml, which is significantly higher

than other species. The texture of boar ejaculate is creamy and its colour is typically slightly greyish or yellowish. Due to the high volume, the concentration is quite low, the ejaculate contains approximately $100 \times 10^9$ sperm. Ejaculation itself takes a longer time, it can last up to 30 minutes. The temperature of the ejaculate is close to body temperature, i.e. 37 °C and the pH is characterized by a large range from 6.85-7.9 (Frunza et al. 2008). Boar sperm cells exhibit a linear osmotic response between the range of 185-900 mOsmol/kg (Gilmore et al. 1996). Precisely because of the low concentration, sperm only make up roughly 10-30% of the total volume of ejaculate, the remaining fraction is secreted by the accessory sex glands (Bonet et al. 2012). The daily sperm output of a boar can reach up to $20 \times 10^9$, and sperm concentration may reach as high as $200 \times 10^6$/mL. The total volume of the ejaculate can approach 500 mL (Frunza et al. 2008).

Duroc boars have relatively high-quality sperm compared to other breeds. Within healthy individuals, Duroc boars have higher sperm concentration, motility and normal morphology (Borg et al. 1993; Kondracki et al. 2012).

Boars have a unique aspect in their ejaculate excretion, which is divided into three fractions. The first fraction, known as the pre-spermatic phase (5-20 %), consists of secretions from the prostate, seminal vesicles, and Cowper's glands without any sperm. The second fraction, or sperm-rich fraction (≥60 %), contains a large amount of sperm along with secretions from the prostate and Cowper's glands. In the last fraction, called the post-spermatic phase, the amount of sperm decreases, and the secretions of accessory glands become predominant. (Sancho et al. 2004; Rodriguez-Martinez et al. 2009).

### 3.1.1 Boar spermatozoon

The primary role of spermatozoa is to fertilize the oocyte by reaching it. Sperm are small and mobile cells that carry the male's genetic information, i.e., DNA, to the next generation. They have a distinctive morphology and genome. Like other animals, ejaculated porcine spermatozoa are divided into two main parts. The first part is the head, which contains the haploid genome and the acrosome, required for the recognition and fusion with the oocyte. The second part is the tail, responsible for sperm motility and contains mitochondria, which generate energy (Hancock 1956; Briz & Fàbrega 2013). Mitochondria play a crucial role in the regulation of intracellular calcium levels, which are essential for various processes such as fertilization. Furthermore, mitochondria functions are involved in sperm maturation, DNA packaging and defects that may contribute to male infertility (Hirata et al. 2002).

The length of mature boar spermatozoa is approximately 43-45 μm. The head, which is flattened on both sides and oval in shape, is about 7 μm long, 0.4 μm thick and up to 3.7 μm in the widest area. The two surfaces of the head are not identical, while one is completely flat, the other is characterized by a crescent-shaped apical process 0.4 μm in width and reaching up to 1.2 μm in length. The tail is cylindrical in shape and is divided into three parts: midpiece, principal piece, and terminal piece. The central part is 9 μm long and 0.7 μm in diameter; the main part measures approximately 26 μm and is 0.2 μm in diameter; the terminal part shows a length of 2.2 μm and a diameter of 0.2 μm. Among other things, there is a connecting part between the head and the tail, which is 0.7 μm long and 0.5 μm thick, the shape resembles a trapezoid, which with its wider base touches the head and the narrower basic middle part. Each of these regions is characterized by different anatomy corresponding to their function (Fawcett 1958; Frunza et al. 2008; Briz & Fàbrega 2013).

The head of sperm (as shown in Figure 2) is relatively simple in structure, composed of only a small number of organelles. The two main organelles are the nucleus and the acrosome. There are also other structures present, such as the subacrosomal space and the perinuclear fibrous material, which are embedded in the postacrosomal dense laminae. The shape of the nucleus varies greatly among animal species and is highly influenced by their genome. Healthy boar sperm have a flattened head with an egg-shaped nucleus (Nicander & Bane 1962). The nucleus of the sperm contains condensed chromatin, known as heterochromatin. The acrosome, which is a membranous organelle surrounding a large portion of the nucleus, consists of both internal and external parallel membranes. The inner membrane is situated close to the nuclear membrane, and the outer membrane is present beneath the plasmalemma at the posterior edges. The space between these membranes is filled with an amorphous material consisting mainly of hydrolytic enzymes. The acrosome itself is further subdivided into three parts - the apical segment, the main segment, and the equatorial segment. The postacrosomal dense lamina is a uniform layer below the plasmalemma, enveloping the areas of the nucleus that are not beneath the inner membrane of the acrosome. In contrast to the acrosome, it is securely attached to the plasma membrane of the head. The boundary of the subacrosomal space, which is positioned between these layers and the nucleus, is designated by the postacrosomal dense lamina and inner acrosomal membrane. This space encompasses the perinuclear fibrous material that covers the nucleus (Fawcett 1958; Briz & Fàbrega 2013).

The connecting piece (as shown in Figure 2) is a brief segment that links the sperm head to the tail. It is attached to the distal end of the nucleus and can be divided into several parts including the basal plate, laminar bodies, capitulum, segmented columns, basal body, and

axoneme. The basal lamina, which shares a similar composition to the postacrosomal dense laminae, is firmly connected to the outer membrane of the nuclear envelope. Laminar bodies are formed by folds of the nuclear envelope that encase the nuclear space without chromatin. The capitulum is made up of segmented columns that terminate in the middle portion of the tail and have an electrodense structure. Segmented columns are made up of two types of bars with alternating periodicity, one eminent and the other less defined. The basal body is formed by microtubular triplets, which are arranged in the depressions of the capitulum. The basal body gives rise to the axoneme (Fawcett 1958; Nicander & Bane 1962; Briz & Fàbrega 2013).

The structure of the sperm tail (as shown in Figure 2) is composed of three segments, namely, the midpiece, principal piece, and terminal piece. The midpiece is located adjacent to the connecting piece and ends at the Jensen's ring or annulus. It contains several structures such as the axoneme, mitochondrial capsule, outer dense fibres, and peripheral granules. The axoneme is positioned at the centre and extends throughout the length of the tail. The mitochondrial capsule is located beneath the plasmalemma and houses mitochondria distributed along the axoneme. The outer dense fibres are cytoskeletal filaments composed of dense material and are found between the mitochondrial sheath and axoneme along the midpiece and the principal piece. A peripheral granule made up of electrodense material can be seen in the proximal part of the midpiece. The longest part of the tail is the principal segment, which extends from Jensen's ring to the proximal end of the terminal segment. The major structures in this segment are the fibrous sheath, outer dense fibres, axoneme, and Jensen's ring. The mitochondrial sheath is replaced by a fibrous sheath, which consists of 10 dorsal and ventral columns connected by ribs and spaced evenly around the principal segment. The dense fibres are found in the proximal region of this segment. The terminal segment is the shortest part of the tail, and only the axoneme enclosed in the plasma membrane is present in this region (Fawcett 1958; Nicander & Bane 1962; Briz & Fàbrega 2013).

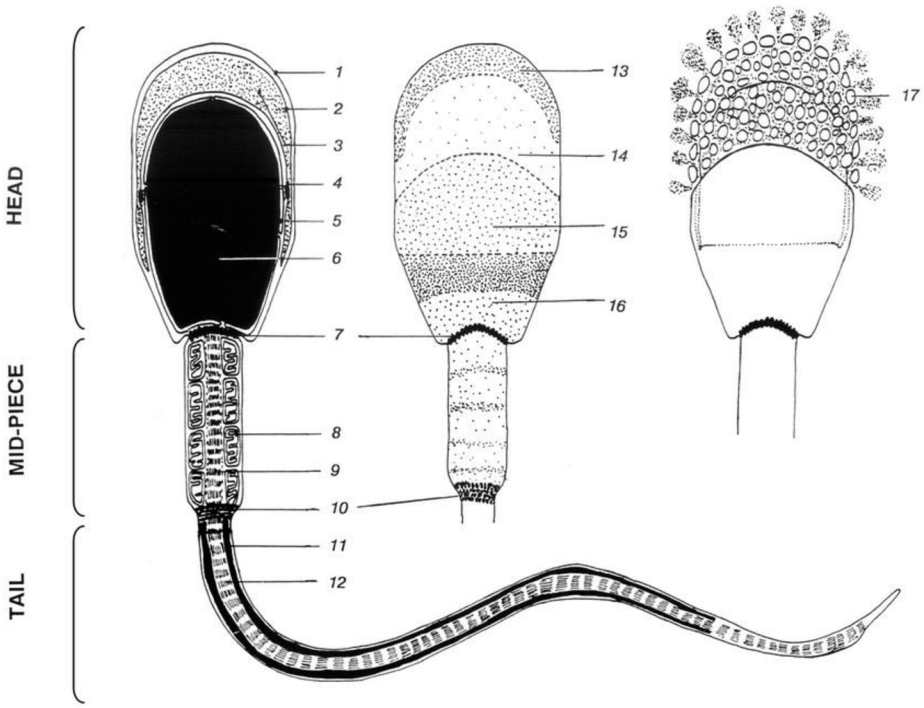


**Figure 2** Schematic representation of the boar sperm cell. 1. plasma membrane; 2. outer acrosomal membrane; 3. acrosomal enzyme matrix; 4. inner acrosomal membrane; 5. nuclear envelope; 6. nucleus; 7. posterior ring and neck; 8. mitochondria; 9. proximal part of the flagellum; 10. Jensen´s ring; 11. fibrous sheath; 12. axoneme + outer dense fibers; 13. apical ridge; 14. pre-equatorial; 15. equatorial; 16. post-equatorial; 17. the acrosome reaction and fusions between the plasma membrane and the outer acrosomal membrane (Briz & Fàbrega 2013).

Spermatogenesis is a key biological phenomenon important for male reproduction. This process includes several consecutive mitotic divisions, two meiotic divisions and the formation of haploid spermatids, which are transformed into immature spermatozoa during spermiogenesis (as shown in Figure 3). Chemical or physical agents that affect sperm quality can easily interrupt or damage spermatogenesis, which is essential for successful fertilization

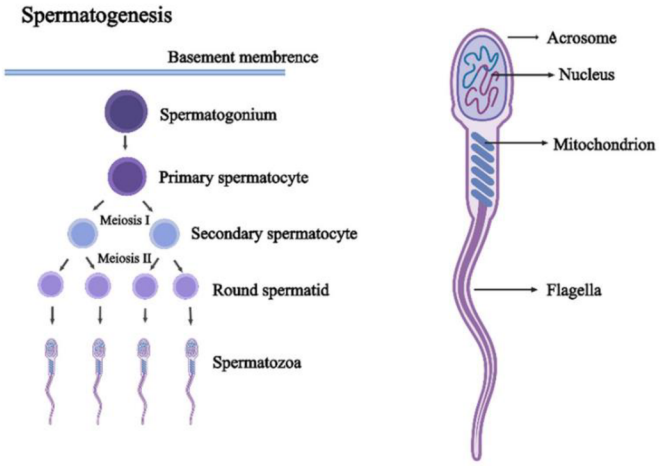


**Figure 3** Schematic representation of spermatogenesis and strucuture of spermatozoa (Yan et al 2022).

(Frankenhuis et al. 1982). Spermatogenesis takes place in the testicles, specifically in the seminiferous tubules, which contain three types of germ cells: spermatogonia, spermatocytes, and spermatids. The process of boar spermatogenesis can be divided into three phases: the proliferative or mitotic phase, the meiotic phase, and the haploid or post-meiotic phase. During the proliferative phase, spermatogonia differentiate into spermatocytes, which then enter meiosis, while a subpopulation of spermatogonial stem cells constantly renews itself to maintain a steady output of sperm (Frankenhuis et al. 1982). During the post-meiotic phase, haploid spermatids differentiate into spermatozoa. However, at this stage, the spermatozoa cannot move progressively or fertilize an egg. It is only after passing through the tail of the epididymis that the spermatozoa acquire these abilities. Spermatogonia are almost undifferentiated cells that enter a series of mitotic divisions. According to the degree of differentiation, spermatogonia are divided into A and B with other subgroups. Primary and secondary spermatocytes then undergo meiotic division, when the primary spermatocytes are still similar to germ cells, and DNA replication occurs in their nucleus (Chenoweth & Lorton 2014). This process can be divided into 12 distinct steps, including the remodelling of the spermatid nucleus and the development of sperm accessory structures such as the flagellum and acrosome. The length of the cycle of seminiferous epithelium is estimated to be 8.6 days, with eight stages each one lasting approximately 1 day (Swierstra 1968). The duration of spermatogenesis from an undifferentiated A-type spermatogonium to a spermatozoon is estimated to be around 40 days (França & Cardoso 1998). Boar spermatozoa undergo final maturation during epididymal transit, acquiring the potential for fertilization and motility. This process involves the modification of existing sperm proteins and the absorption of new ones from the epididymal fluid. The sperm cytoplasmic droplet slides down the boar sperm tail during transport from the head to the tail of the epididymis, with a greater proportion of boar spermatozoa retaining their distal cytoplasmatic droplet until after ejaculation. The epididymal passage takes approximately 9-10 days, and a single tail of epididymis can store 120-165 $\times 10^9$ spermatozoa. The epididymal reserve could be replenished within 3-5 days after depletion (Frankenhuis et al. 1982). Major secretory proteins in the boar epididymis include clusterin, glutathione peroxidase, and lactoferrin (Syntin et al. 1996). For successful fertilization to occur, there are important factors such as a sufficient amount of viable sperm in the ejaculate, the ability to perform a sexual act, and/or the possibility of removing the ejaculate artificially, thus giving the possibility of artificial insemination (AI; Sakkas et al. 2015).

### 3.1.2 Boar seminal plasma

Seminal plasma refers to the liquid part of the ejaculate that does not contain any sperm cells. It is a mixture of fluids from different parts of the male reproductive system, including the testes and epididymis, as well as secretions from the accessory sex glands, such as the seminal vesicles, prostate, and bulbourethral glands. These glands are classified as exocrine glands, as they release their secretions into the urethra. Androgen hormones play a crucial role in regulating the activity of these glands. In boar, it is interesting and exceptional that spermatozoa only represent 10-30 % of the ejaculate, the rest being seminal plasma. More precisely, 55-75 % of the volume of seminal plasma comes from the prostate and urethral gland, 10-25 % from the bulbourethral glands and 15-25 % from the seminal vesicles. The characteristic of seminal plasma is a pH between 7.3-7.9 and a water content of 94-98 %. Among other things, it contains organic and inorganic substances, mainly proteins (Bonet et al. 2013).

Seminal plasma supports boar spermatozoa after ejaculation and up until they pass through the uterotubal junction. It has multiple proposed functions, including protection against immune attack by uterus-infiltrating leucocytes, maintenance of a non-capacitated sperm state, and metabolic support for spermatozoa (Rodriguez-Martinez et al. 2009). Seminal plasma components, such as micro RNAs and cytokines, may stimulate gene expression in the uterus to prime the uterine lining for conception and implantation (Wang et al. 2011). In boars, seminal plasma may contain factors that help reject cytoplasmic droplets by ejaculated spermatozoa (Harayama et al. 1996). Boar seminal plasma is rich in inositol, fructose, bicarbonate, magnesium, and calcium, and has the highest zinc content among all studied body fluids (Rodriguez-Martinez et al. 2009). The major proteins in boars are spermadhesins, prostatic secretory protein (PSP), and sperm surface protein (DQH) glycoproteins (Jonakova et al. 2016). Spermadhesins are molecules that attach primarily to the surface of the acrosome during ejaculation and are believed to play a role in the binding of sperm to the oviductal lining, which forms the sperm reservoir. Certain spermadhesins are broken down by proteolytic enzymes and released from the sperm surface during capacitation, allowing the sperm to detach from the oviductal reservoir in response to ovulation (Dostálová et al. 1994). High concentrations of certain seminal plasma proteins are associated with high fertility in AI boars, while others may be more abundant in low-fertility ones (Flowers et al. 2016).

## 3.2 Collection and storage of the porcine ejaculate

Semen for insemination can be obtained in two ways, using an artificial vagina filled with warm water and compressed air, or without an artificial vagina, by the so-called manual method "in hand" or "the glove" technique (King & Macpherson 1973). Nowadays, "the glove" technique is the most commonly used technique for boar sperm collection. The main physiological requirement for ejaculation in the boar is to ensure pressure on the helical end of the penis, which is best provided by the technician's hand. Another valued advantage of the manual method is the substantial reduction of microbial contamination of semen. Polyvinyl gloves are used, latex gloves could be toxic for the sperm. Also, rapid cooling could cause damage to sperm, therefore the collection container has to be prewarmed. The semen is collected in a single-use thin-walled collector from a non-spermicidal bag inserted and caught through an opening in a solid heat-insulating container with a handle. A protective cellulose gauze is attached to the collection bag with a rubber band, which is used to capture impurities from the surrounding environment and the post-sperm fraction. To obtain high-quality sperm during collection, it is essential to maintain a high level of hygiene before and during the collection process. This includes ensuring the cleanliness of the boars, washing or cleaning the abdominal area before collection, and creating optimal physiological conditions for ejaculation. It is also important to prevent the pre-spermatic fraction from entering the sperm collector to ensure the quality of the collected sperm (Maes et al. 2011; Rodriquez et al. 2017).

Freshly diluted semen is frequently used for AI on the day of or within a few days following collection. Two crucial factors play a significant role in the storage of liquid boar semen: the temperature at which the semen is collected and stored, and the composition of the storage medium (Watson 1995; Johnson et al. 2000). Empirical evidence has determined that the ideal storage temperature for liquid-preserved boar semen ranges between 15 °C and 20 °C. Although lower temperatures can effectively inhibit bacterial growth, they may prove problematic due to the high susceptibility of boar spermatozoa to chilling injury. Conversely, higher storage temperatures are known to cause energy deficiency as a result of insufficient suppression of metabolic cell activity (Henning et al. 2022). The membrane of boar spermatozoa has a different phospholipid composition compared to other species like, for instance, bull spermatozoa. Due to the low ratio of cholesterol to phospholipids and the asymmetrical distribution of cholesterol within the membrane, boar spermatozoa are highly sensitive to cold temperatures. This sensitivity can result in increased membrane permeability and loss of controlled membrane processes (De Leeuw et al. 1990). Similarly, sperm motility

can be influenced by sperm storage (Henning et al. 2012). To avoid cold shock, it is important to keep ejaculates at a temperature above 15 °C for several hours before use to increase their resistance to cold temperatures. Specialized containers are used during semen dilution to prevent contact with colder surfaces, and the temperature is lowered gradually to avoid sudden changes (Watson 1995; Johnson et al. 2000). Two common protocols for dilution are used: a one-step dilution with either preheated extender (~ 33 °C) or extender at room temperature, or a two-step dilution (1:1) with preheated extender (~ 33 °C) followed by a second dilution in either preheated or room temperature extender. Once the final dilution is made, commercial doses are filled and the semen is allowed to cool gradually to 17 °C before use (Maes et al. 2011).

Porcine spermatozoa are highly vulnerable to low temperatures and rapid cooling due to the specific composition of their cell membrane, which can lead to cold shock, as noted by De Leeuw et al. (1990). To address this issue, various techniques have been established, including incubating sperm at ambient temperature for several hours to induce cold resistance, utilizing seminal plasma to protect spermatozoa, and implementing controlled freezing protocols. (Watson 1995). Freezing boar semen is still challenging due to variations in the freezability of individual boar's semen. Different extenders are used for frozen semen than those used for liquid semen. Egg yolk is added to protect the sperm membrane during cooling because it contains low-density lipoproteins and cholesterol. Cryoprotectants, such as glycerol, are added in low concentrations to minimize membrane damage during freezing. Furthermore, sugars and synthetic detergents are added, with the latter improving the cell membrane's stability (Johnson et al. 2020). The thawing process of semen is a crucial factor to maintain sperm motility and acrosome integrity. However, both the freezing and thawing procedures can lead to changes in the plasma membrane, which is why different protocols are available. The use of frozen semen has improved fertility outcomes, with cervical insemination resulting in a farrowing rate of 75 % (Roca et al. 2003). However, the freezing and thawing steps are time-consuming, which limits their use in specific scenarios such as long transportation times and the preservation of valuable genetic material (Maes et al. 2011).

## 3.3 Impact of oxidative stress on sperm storage

In mammals, there is a balance between the presence of antioxidants and pro-oxidants in the body under normal conditions. Sperm also possesses an antioxidant defence mechanism to prevent oxidative damage. However, if the production of pro-oxidants, or reactive oxygen species (ROS), exceeds the capacity of antioxidants, it can lead to oxidative stress. Oxidative stress occurs when there is an imbalance between ROS and the organism's ability to detoxify these reactive intermediates or repair the damage caused by them (Halliwell & Gutridge 1989).

When the level of ROS is high, it can cause an imbalance between oxidants and antioxidants in the body. ROS are produced as a result of normal oxygen metabolism and include both oxygen radicals and non-radical oxidizing agents. Oxygen radicals, such as superoxide ($O_2^-$), hydroxyl ($-OH$), peroxyl ($HO_2$), and hydroperoxyl radicals, contain one or more unpaired electrons. Non-radical by-products of oxygen metabolism include ozone ($O_3$), perchloric acid ($HClO_4$), and hydrogen peroxide ($H_2O_2$), which can be converted into radicals when they encounter free radicals (Bayr 2005). One example can be the peroxidation of membrane lipids, with the simultaneous creation of a lipid radical and lipid hydroperoxide, the peroxyl radical can extract a hydrogen atom from an unsaturated fatty acid (Sanocka & Kurpisz 2004).

Both intrinsic and extrinsic factors have been identified as major sources of ROS for spermatozoa both in domestic and wild animals (Pintus & Ros-Santaella 2021). Among extrinsic factors, sperm handling (e.g., dilution or centrifugation) and storage (i.e., cooling and freezing–thawing procedures) have been shown to provoke oxidative stress in the sperm cells (Bucak et al 2008). Sperm cells usually depend on their internal antioxidant system to protect against ROS. However, as sperm cells develop and mature, most of the cytoplasmic content is lost, leaving them with a limited antioxidant capacity to deal with an excess of ROS (Bansal & Bilaspuri 2011). Furthermore, storage methods, as mentioned earlier, can expose the sperm cells to cold temperatures and atmospheric oxygen, which may lead to higher ROS production and greater susceptibility to lipid peroxidation (Bucak et al. 2008).

Excessive ROS can also be induced by various factors such as exposure to ionizing radiation (Sadani & Nadkarni 1997), metabolic activation of xenobiotics (Akiyama 1999), inflammatory processes (Villegas et al. 2003), heightened cellular metabolism (Hollan 1996), or reduced antioxidant capacity (Hsu et al. 1998).

## 3.4 Role of hydrogen sulfide ($H_2S$) in male reproduction

Hydrogen sulfide is a gas molecule, also known as a gasotransmitter, that plays a crucial part in cellular functions (Kimura Y & Kimura H 2004). Gasotransmitters, which include $H_2S$, carbon monoxide, and nitric oxide, were discovered in the late 1980s and have emerged as essential cellular messengers, much like neurotransmitters and humoral factors (Brune & Ullrich 1987; Palmer et al. 1987; Goodwin et al. 1989). $H_2S$ interacts directly with the main signalling pathways of various diseases, such as cardiovascular, neurological, and other chronic disorders. In diseases, the $H_2S$ levels may be disturbed, and the administration of exogenous $H_2S$ can alleviate the symptoms of these diseases (Lefer 2007; Hu et al. 2010; Kashfi & Olson 2013).

$H_2S$ is an endogenous gas that is naturally located in various body organs like, for instance, the brain, the heart, and the organs of the digestive system. It is formed mainly by the enzymes - cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-mercapto pyruvate sulfur transferase (3-MST). The 3-MST is a zinc-dependent enzyme, meanwhile, CBS and CSE are pyridoxal-5-phosphate-dependent enzymes (Kulluru et al. 2017). These enzymes are mainly found in specific parts of the body, such as the brain and nervous system, mitochondria, and certain cells in the cytosol. Research has found that enzymes responsible for producing $H_2S$ can also be found in the testis (Sugiura et al. 2005), epididymis (Gao et al. 2019), penile corpus cavernosum (D'Emmanuele di Villa Bianca et al. 2009), and spermatozoa (Martínez-Heredia et al. 2008). This suggests that $H_2S$ is involved in the normal metabolism of sperm. In the testes, two enzymes produce $H_2S$. One of these enzymes, CBS, can be found in Leydig cells, Sertoli cells, and germ cells. The other enzyme, CSE, is only found in Sertoli cells and developing germ cells, such as spermatogonia (Sugiura et al. 2005).

$H_2S$ is described in the human body as a relaxant of blood vessels and smooth muscle cells (Yang et al 2008). For instance, $H_2S$ mediates human corpus cavernosum smooth-muscle relaxation by enzymes such as L-cysteine (D'Emmanuele di Villa Bianca et al. 2009). Research has shown that the seminal plasma of infertile males contains lower levels of $H_2S$ compared to fertile males, indicating a possible link between $H_2S$ and male fertility (Wang et al. 2017).

$H_2S$ is a product of L-cysteine and L-homocysteine, which can be obtained directly from food or via the methionine transsulfuration pathway (Olson & Straub 2016). One way $H_2S$ can be produced is through alpha-ketoglutarate metabolism, where 3-MST is generated via cysteine aminotransferase and alpha-ketoglutarate or by D-amino acid oxidase and D-cysteine (Olson 2018). Another possible production route is the reduction of thiols by catalase (Olson 2019).

Mitochondrial complex I, which contains a large concentration of cysteine, is another potential source. Additionally, $H_2S$ can be generated by a non-enzymatic pathway using persulfides, polysulfides, or sulfur bound to the cellular reservoir (Kulluru et al. 2017). The bound sulfur provides $H_2S$ in neural cells in the presence of glutathione and cysteine (Shefa et al 2018). $H_2S$ can be oxidized by enzymes such as sulfide quinone oxidoreductase (SQR), thiosulfate transferase, and sulphide oxidase in mitochondria. Cysteine dioxygenase and ethylmalonic encephalopathy 1 are also possible enzymes for $H_2S$ catabolism. Non-enzymatic catabolism of $H_2S$ can occur through its interaction with $O_2$, $H_2O_2$, $O_2^-$, or peroxynitrite ($NO_3^-$; Rose et al. 2017).

$H_2S$ is also recognized to impact proteins via modifications that occur after the proteins are synthesized, known as post-translational modifications (Yang 2017). $H_2S$ interacts with cysteine and converts cysteine −SH groups to −SHH groups through persulfidation, leading to protein enrichment. This modification allows $H_2S$ to impact cell signalling by reducing the sulfur atoms of cysteine and $H_2S$ to the –2 oxidation state. Before this event, oxidation to S- via persulfidation is required (Mustafa et al. 2009). Persulfide enzymes like 3-MST, CSE, CBS, or SQR transfer persulfides to another protein directly or via a secondary carrier, resulting in target specificity. The oxidized products of $H_2S$, polysulfides and persulfides, may have higher cellular activity due to their higher reactivity and autoxidation rate compared to $H_2S$. Therefore, persulfide transfer is likely the primary mechanism of $H_2S$ signalling (Mishanina et al. 2015).

### 3.4.1 $H_2S$ in sperm cells

Studies have suggested that physiological levels of $H_2S$ are important for sperm motility, capacitation, and the acrosome reaction. $H_2S$ has been shown to increase sperm motility by increasing intracellular calcium levels, which are essential for sperm movement. Wang et al. (2017) measured $H_2S$ concentration in seminal plasma, and low levels were found in sub-fertile or infertile patients. The experiment showed that $H_2S$ plays role in the activation of mitogen-activated protein kinases (MAPK). These kinases are important for flagellar motility, hyperactivation and the acrosome reaction. In men with asthenozoospermia, lower levels of $H_2S$ in seminal plasma were observed, but the application of an $H_2S$ donor (NaHS) improved sperm motility. However, high concentrations of $H_2S$ were found to have a negative effect on sperm motility and hypermotility. Similarly, high concentrations of $H_2S$ induced by $H_2S$ donors in boar sperm cells were also found to cause negative effects, including oxidative stress (Zhao et al. 2016). The toxicity of high concentrations of $H_2S$ is attributed to its inhibition of cytochrome *c* oxidase, a key enzyme involved in cellular respiration and ATP production

(Szabo et al. 2014). Xia et al. (2019) studied PI3K/Akt pathway and proved that $H_2S$ is involved in its activation. $H_2S$ can regulate the $Ca^{2+}$ and $K^+$ channels, especially Catsper, which are necessary for capacitation and acrosome reaction (Kulluru et al. 2017; Shefa et al. 2018). Moreover, the regulation of $K^+$ channels affects ATP generation by mitochondria and thus promotes sperm hyperactivity, which is important for fertilization (Gupta et al. 2018; Kumar et al. 2019). A recent study showed that boar spermatozoa rely specifically on oxidative phosphorylation for ATP generation which is important for flagellar movement (Nesci et al. 2020). Researchers led by Lorian et al. (2019) conducted a study to investigate the potential protective effects of NaHS on rat sperm parameters that had been damaged by induced varicocele. The findings of the study suggested that administering NaHS resulted in improved sperm motility and viability, as well as preservation of normal morphology when compared to the varicocele group. Pintus et al. (2020) experimented to investigate whether GYY4137 possesses antioxidant properties that can protect boar sperm from induced oxidative stress. The researchers treated the sperm samples with three different concentrations of GYY4137 and assessed various sperm parameters, including motility, mitochondrial status, acrosomal status, plasma membrane integrity, and lipid peroxidation. According to the results, GYY4137 was effective in preserving sperm motility and protecting the acrosomal status under oxidative stress conditions, although it did not exhibit significant effects on mitochondrial status, plasma membrane integrity, or lipid peroxidation.

### 3.4.2 Antioxidative properties of $H_2S$

The antioxidant properties of hydrogen sulfide are due to its ability to scavenge ROS such as superoxide anions, hydroxyl radicals, and hydrogen peroxide. This process reduces the levels of ROS and protects cells from damage caused by oxidative stress. This antioxidative effect of $H_2S$ has been observed in various cell types, including neurons (Kimura Y & Kimura H 2004), gastric tissue (Yonezawa et al. 2007), endothelial cells (Han et al. 2011) or sperm cells (Pintus et al. 2020). Apart from its ROS scavenging ability, $H_2S$ can also enhance the body's natural antioxidant defence mechanisms. Studies have revealed that $H_2S$ can elevate the expression of various antioxidant enzymes like superoxide dismutase (SOD), and glutathione peroxidase (GPx). By upregulating these enzymes, $H_2S$ can effectively clear ROS and protect the body against oxidative stress (Li et al. 2015; Shefa et al. 2018). Moreover, $H_2S$ can regulate redox-sensitive signalling pathways and modulate the expression of genes involved in oxidative stress responses. By modulating these pathways, $H_2S$ can improve cellular redox status and mitigate the damaging effects of oxidative stress (Ning et al. 2018).

## 3.5 $H_2S$ donors

$H_2S$ donors can be classified into two categories based on how they release $H_2S$: fast-releasing and slow-releasing agents. NaHS and sodium sulfide ($Na_2S$) are two inorganic salts that belong to the fast-releasing group and have been studied extensively. However, the amount of $H_2S$ released by these fast-releasing donors may not accurately represent the levels found naturally in cells and tissues. On the other hand, slow-releasing $H_2S$ donors release $H_2S$ in smaller amounts, but at a steady, slow rate. This method of release is more similar to the natural conditions in the body (Rose et al. 2015). The administration of exogenous $H_2S$ is crucial for studying and comprehending its physiology in the body. This is achieved through the use of $H_2S$ donors that aim to mimic the production of this endogenous gasotransmitter (Xu et al. 2019). $H_2S$ is a gaseous molecule, therefore with a short half-life in a biological environment (DeLeon et al. 2012). GYY4137 is a phosphino-dithioate derived from Lawson´s reagent. It undergoes slow hydrolysis in water to release $H_2S$, although the efficiency of sulfide release remains low. The rate of hydrolysis is pH dependent and slow at physiological pH, contrasting the rapid release of $H_2S$ by inorganic salts (Li et al. 2008). This slow-releasing $H_2S$ donor was reported to have the ability to act as a vasorelaxant (Li et al. 2008), lowering blood pressure, and even had an antithrombotic effect (Grambow et al. 2014) or antioxidant properties in boar sperm cells (Pintus et al. 2020). One of the different ways to effectively obtain exogenous $H_2S$ is through carbonyl sulfide (COS), which is converted to $H_2S$ in cells by the enzyme CA (Haritos & Dojchinov 2005; Steiger et al. 2018; Lewinn et al. 2019 A). One option is to use thiocarbonate to produce COS, however, the by-products can be toxic, making $H_2S$ research difficult (Zhao et al. 2017). Further studies have indicated that toxicity is most likely related to the rate of cleavage of the ester group (Lewinn et al. 2019 B; Powell et al. 2019). One class of COS-mediated $H_2S$ donors with benign by-products are the *N*-thiocarboxyanhydrides.

### 3.5.1 *N*-thiocarboxyanhydrides (NTAs)

*N*-thiocarboxyanhydrides (NTAs) are a newly discovered class of $H_2S$ donors using COS with benign by-products (Powell et al. 2016). Hirschmann et al. (1971) first reported on the use of NTAs as an alternative to *N*-carboxyanhydrides for synthesizing oligopeptides in solution. Nowadays, NTAs have been synthesized from *N*-alkyl amino acids and polymerized to form polypeptoids (Tao et al. 2014). Ring-opening polymerization of NTAs results in the loss of COS, thus it have been considered as potential COS donors following ring-opening by a

biological nucleophile, such as an amine. After COS release, conversion into $H_2S$ is expected to occur through rapid enzymatic conversion by CA or slower hydrolysis (Powell et al. 2016).

CA is a metalloenzyme that plays a crucial role in maintaining the acid-base balance in various tissues and biological fluids (Carter 1972). There are 11 active types of CA isozymes and 3 inactive types (Hewett-Emmett & Tashian 1996; Fujikawa-Adachi et al. 1999). CA-VI is a salivary or secreted carbonic anhydrase that is produced and secreted into saliva by the serous acinar cells of the parotid and submandibular glands (Murakami & Sly 1987). Nishita et al. (2011) investigated the concentration of Ca-VI in seminal plasma and established that it is a constituent of seminal plasma. Similarly, Pintus et al. (2023) quantified the levels of Ca-VI in both boar seminal plasma and spermatozoa and discovered that the seminal plasma contains a greater abundance of Ca-VI. CA is an enzyme that is naturally present in organisms, particularly in seminal plasma and spermatozoa. Its essential role is to catalyze the conversion of NTAs into $H_2S$. Recent research conducted by Powel et al. (2016) demonstrated that the exogenous addition of CA enzyme is not required for NTAs treatment, as the concentration of endogenous CA widely distributed in mammalian cells is adequate to facilitate the swift conversion of COS into $H_2S$.

One of the first NTAs was derived from sarcosine (as shown in Figure 4), where the *N*-alkyl amino acid releases COS with the by-product sarcosine. Another option was NTAs with bio-orthogonal handles for polymeric and peptide materials that release COS/$H_2S$. However, the speed of release was problematic, so several new NTAs containing natural amino acids were proposed. These NTAs should undergo hydrolysis, and produce COS and only the amino acids as a by-product. The substituent on the α-carbon should affect the rate of COS release via hydrolytic ring opening (Powell et al. 2016).

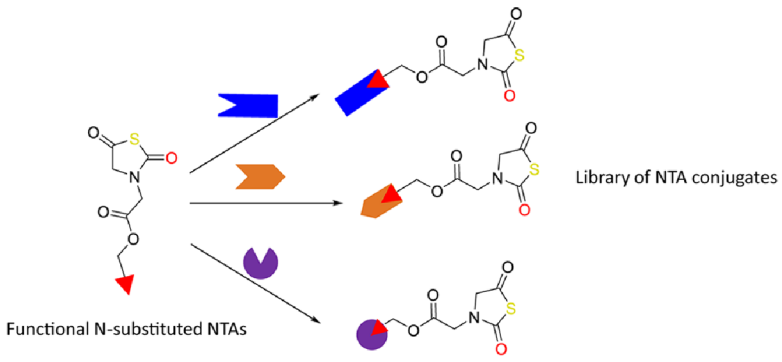


**Figure 4** First delivered *N*-thiocarboxyanhydrides (NTA) from sarcosine, and possible NTA conjugates (Powell et al 2019).

Kaur et al. (2021) synthesized nine different NTAs (as shown in Figure 5) from natural L-amino acids. Seven of them were derived from canonical amino acids – Gly-NTA, Ala-NTA, Val-NTA, Leu-NTA, Ile-NTA, Phe-NTA and Pro-NTA and the other two were Aib-NTA, with two methyl groups on C-4, derived from 2-aminoisobutyric acid and β-Ala-NTA derived from β-alanine with a 6-membered NTA ring.

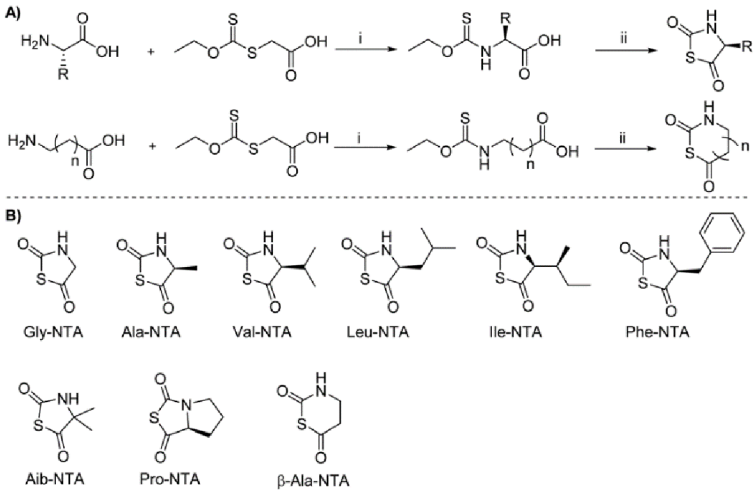


**Figure 5** (A) General scheme for synthesis of N-thiocarboxyanhydrides (NTA) used in Kaur et al (2021) experiment; (i) NaOH, methanol/$H_2O$ (ii) alcohol to bromide - $PBr_3$ (B) Chemical structures of nine NTAs

Val-NTA and other NTAs with hydrolytic ring-opening (such as Gly-NTA and Ile-NTA) were dissolved in 5 % DMSO-PBS (10 mM) in the presence of 300 nM CA and measured to determine the amount of $H_2S$ released from them using an electrochemical probe designed to detect $H_2S$. The NTAs with the larger substituents at C-4 exhibit more gradual release: Val-NTA – 96 min; Ile-NTA – 104 min, meanwhile Gly-NTA showed a sharp profile with a peaking time of 51 minutes. Half-life values were determined by the methylene blue method, where Val-NTA had the longest one: 20 h, compared to the shortest half-life of b-Ala-NTA, which had only 1.1 h. In this study, the authors also investigated the longer-term inhibitory effects of certain NTAs, like Val-NTA. To compare their effects with a commonly used slow-releasing $H_2S$ donor named GYY4137, they evaluated cell growth inhibition in MCF-7 cells (cell line of breast cancer obtained in the 1970s) and human colon adenocarcinoma HT-29 cells (cell line of colon cancer obtained in 1960s). Each cell line was incubated with various concentrations of the four NTAs for 72 hours, allowing to determine the $IC_{50}$ (half-maximal inhibitory concentration) value for each NTA. It was found that in MCF-7 cells, Gly-NTA and Ala-NTA had higher $IC_{50}$ values compared to GYY4137 (592 mM), while Val-NTA ($IC_{50}$ value of 289 nM) and Leu-NTA were more cytotoxic than GYY4137. Similar trends were observed in the

HT-29 cell line, with Val-NTA and Leu-NTA showing nearly 10-fold greater cytotoxicity compared to Gly-NTA and 5-fold greater toxicity than Ala-NTA and GYY4137. These results suggest that slow-releasing NTAs are significantly more potent inhibitors of cancer cell growth over 72 hours than fast-releasing NTAs, as observed in both cell lines (Kaur et al 2021).

NTAs can be used for enhancing sperm parameters. Pintus et al (2023) found that Gly-NTA and Leu-NTA had a positive influence on sperm mitochondrial activity in the presence or absence of ROS-generating systems without any negative effects on cellular function. Moreover, the tested NTAs tend to preserve redox balance under oxidative stress conditions. Pintus et al (2023) suggested that NTAs could be employed to investigate the role of $H_2S$ in sperm mitochondria-dependent events and further roles in fertilization and therapeutic protocols against oxidative stress in assisted reproductive technology (ART).

# 4 Materials and methods

All the counting procedures were performed by the mobile app Hemepath Counter by Alexander Thurman et al. (2015).

## 4.1 Reagents

All reagents were purchased from Sigma-Aldrich (Prague, Czech Republic) unless otherwise specified. Val-NTA was synthesised in the laboratory, as it is described by Kaur et al. (2021).

## 4.2 Sample collection and preparation

The porcine sperm-rich ejaculate fractions were collected from healthy and fertile Duroc boars using the gloved hand method at an animal breeding centre in Lipra Pork, Czech Republic. The samples were transported to the laboratory at 17 °C and diluted 1:1 with Beltsville Thawing Solution (BTS; Pursel & Johnson 1973), supplemented with gentamycin sulphate (250 mg/L).

### 4.2.1 Evaluation of morphology

The morphology was assessed by fixing 2 µL of the sample with 498 µL of 0.3% formaldehyde in phosphate-buffered saline (PBS). Samples were then evaluated under phase-contrast microscopy (40× objective). In each sample, 200 sperm cells were evaluated and classified as normal or abnormal. Sperm abnormalities were marked as abnormal head, abnormal flagellum, proximal or distal cytoplasmic droplet or other defects. The samples that showed at least 75 % of normal spermatozoa were chosen and pooled to analyse the effect of individual variability. Overall, boar ejaculates used in this thesis showed 82.75±7.64 % normal spermatozoa, 1.00±0.58 % abnormal sperm heads, 4.62±3.30 % abnormal sperm flagella, 4.75±2.06 % proximal cytoplasmic droplets, 5.25±5.10 % distal cytoplasmic droplets, and 1.62±1.11 % other defects (mean±SD).

### 4.2.2 Evaluation of sperm concentration

For the evaluation of sperm concentration, 5 µL of the pool was diluted in 995 µL of 0.3 % formaldehyde. The samples were carefully mixed and 10 µL were placed into the Bürker chamber. Then the spermatozoa were counted under phase-contrast microscopy (10× objective). Overall, the sperm concentration of pooled sperm samples used in this thesis was 342±107×$10^6$ spermatozoa/mL (mean±SD). Then, pooled sperm samples were diluted to 20×$10^6$ spermatozoa/mL in BTS.

### 4.2.3 Preparation of Valine *N*-thiocarboxyanhydride (Val-NTA)

The stock solution of Val-NTA 1 mM was prepared by dissolving the $H_2S$ donor into 10 % dimethyl sulfoxide (DMSO) in water. Serial dilutions were then prepared to reach the final concentrations of 100, 10, 1, and 0.1 µM in the sperm samples. The final DMSO concentration in all treatments was 0.1 %. After adding Val-NTA, the samples were stored at 17 °C and analysed at 0 h (control group only), 17 h, and 45 h. Before performing each analysis, samples were incubated for 20 minutes at 38 °C.

## 4.3 Sperm analysis

### 4.3.1 Evaluation of sperm head plasma membrane integrity

One aliquot (20 µL) of sperm sample was diluted in a staining solution containing 75 µL of PBS, 2 µL of carboxyfluorescein diacetate (CFDA), 2 µL of propidium iodide (PI) and 1 µL of 0.3 % formaldehyde in PBS. The sperm sample mixed with the staining solution was incubated at 38 °C for 10 minutes in the dark (Brito et al. 2003). Two hundred spermatozoa were evaluated under fluorescence microscopy (40× objective), the green heads were evaluated as intact, while the partially or entirely red heads as damaged.

### 4.3.2 Evaluation of acrosomal integrity

Acrosomal integrity was accessed by mixing the samples (45 µL) with 5 µL of 2.5 % glutaraldehyde solution and evaluated under phase-contrast microscopy (40× objective). Two hundred spermatozoa were examined to determine the percentage of cells with the normal apical ridge (Pursel & Johnson 1974).

### 4.3.3 Evaluation of sperm motility and kinetics

Sperm motility and kinetics were evaluated by loading 2 µL of the sample into a pre-warmed (38 °C) Leja chamber placed under phase-contrast microscopy (10× objective) connected with Computer Assisted Semen Analysis (CASA; Pintus et al. 2023). Total motility was determined as the percentage of spermatozoa with average path velocity (VAP) ≥10 µm/s, while progressive motility was determined as straightness (STR) ≥80%. The kinetics parameters were evaluated as the lateral amplitude of head movement (ALH; µm), beat cross frequency (BCF; Hz), linearity (LIN; %), STR (%), VAP (µm/s), straight linear velocity (VSL; µm/s) and curvilinear velocity (VCL; µm/s; Tremoen et al. 2010).

## 4.4 Statistical analysis

Data were analysed by the program IBM SPSS Statistics 28.0.0.0. A generalized linear model was used to analyse the impact of treatments on sperm parameters. Statistical significance was set at $p<0.05$. Data are shown as mean±SD. The experiment was replicated three times.

# 5 Results

## 5.1 Effect of Val-NTA on sperm head plasma membrane integrity during storage at 17 °C

Statistical analysis revealed that there was a significant difference observed in the control group between 0 and 45 hours ($p$<0.01), as shown in the Figure 6. At 17 hours, Val-NTA 100 µM showed a significantly lower percentage of spermatozoa with intact plasma membrane compared to the control group ($p$=0.028). There were no differences between the control group and the Val-NTA treatments at any of the other concentrations tested ($p$>0.05). Moreover, Val-NTA 100 µM showed significantly lower values of sperm plasma integrity than Val-NTA 1 and 10 µM ($p$<0.01). At 45 hours, no significant differences were found between the control group and the Val-NTA treatments ($p$>0.05, Figure 6).

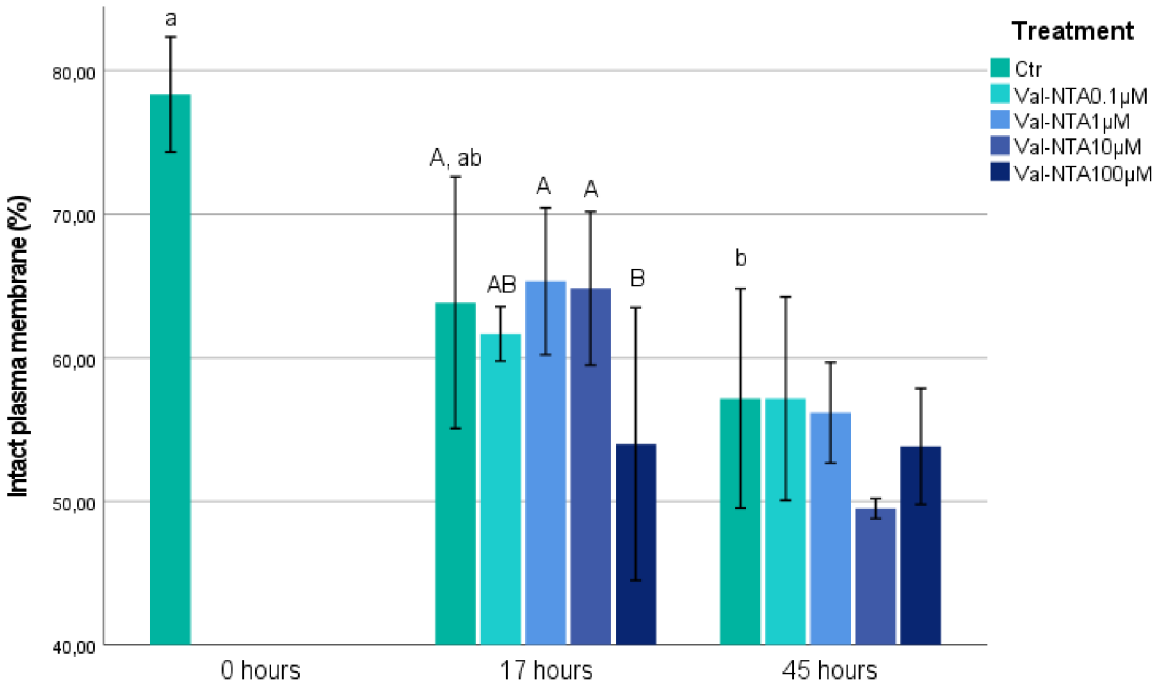


**Figure 6** – Intact plasma membrane (%). Different capital letters indicate a significant differences among each treatment at each time points (p<0.05). Different small letters indicate a significant difference among control group at each storage time (p<0.05). Data are shown as mean±SD.

## 5.2 Effect of Val-NTA on sperm acrosome integrity during storage at 17 °C

Statistical analysis revealed that there was a significant difference in the control group between 0 and both 17 and 45 hours ($p$<0.001), as shown in the Figure 7. At 17 hours, there were no differences between the control group and Val-NTA treatments at any of the concentrations tested ($p$>0.05, Figure 7). At 45 hours, however, both Val-NTA 0.1 and 100 µM showed a significantly lower percentage of spermatozoa with intact acrosome compared to the control group and Val-NTA 1 µM ($p$<0.05, Figure 7).

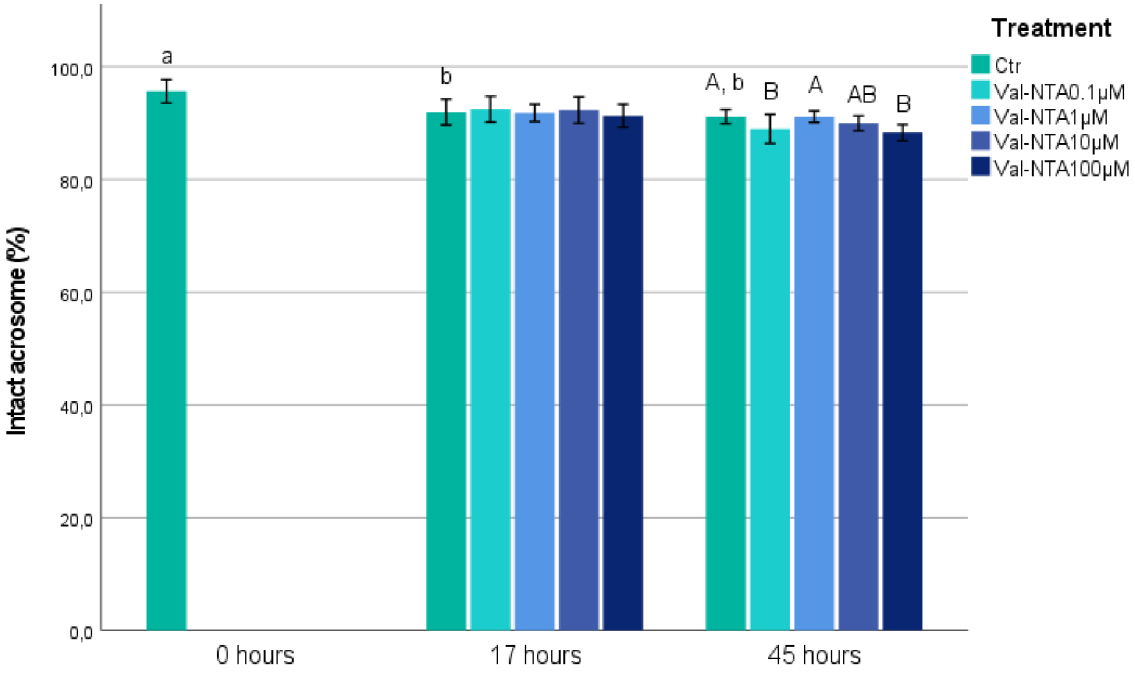


**Figure 7** Intact acrosome (%). Different capital letters indicate significant differences among each treatment at each time point (p<0.05). Different small letters indicate a significant difference among control group at each storage time (p<0.05). Data are shown as mean±SD.

## 5.3 Effect of Val-NTA on sperm motility and kinetics during storage at 17 °C

### 5.3.1 Sperm motility

In the evaluation of the effect of Val-NTA on sperm motility, as shown in Table 1, statistical analysis revealed significant differences between the control group at 0 hours and both 17 ($p$<0.05) and 45 hours ($p$<0.001). At 17 and 45 hours, there were no differences between the control group and Val-NTA treatments 0.1, 1, and 10 μM ($p$>0.05, Table 1). In contrast, Val-NTA 100 μM showed a significantly lower value of total motility than that of the control group and any other Val-NTA treatment at each storage time ($p$≤0.001).

In the evaluation of progressive motility, as shown in Table 1, statistical analysis revealed no differences between the control group among the storage times ($p$>0.05). At 17 hours there were no differences between the control group and Val-NTA at all the concentrations ($p$>0.05), although Val-NTA 10 μM showed slightly higher values at 17 hours compared to the control group ($p$>0.05, Table 1). However, there was a significant difference between Val-NTA 0.1 and 10 μM ($p$=0.020). At 45 hours there were no differences between treatments ($p$>0.05).

**Table 1** Sperm total motility (%) and progressive motility (%).

| Time (h) | Treatment | Total motility (%) | Progressive motility (%) |
|---|---|---|---|
| 0 | Ctr | 74.21±5.78$^{a}$ | 40.84±6.69 |
| 17 | Ctr | 58.81±2.34$^{A,b}$ | 50.18±12.25$^{AB}$ |
| | Val-NTA0.1μM | 52.36±12.53$^{A}$ | 38.56±3.00$^{A}$ |
| | Val-NTA1μM | 60.71±9.65$^{A}$ | 50.49±10.12$^{AB}$ |
| | Val-NTA10μM | 57.04±11.41$^{A}$ | 58.04±0.58$^{B}$ |
| | Val-NTA100μM | 29.92±3.88$^{B}$ | 43.55±18.19$^{AB}$ |
| 45 | Ctr | 55.13±6.54$^{A,b}$ | 48.91±8.62 |
| | Val-NTA0.1μM | 53.11±13.91$^{A}$ | 44.31±6.89 |
| | Val-NTA1μM | 58.32±14.58$^{A}$ | 45.85±11.88 |
| | Val-NTA10μM | 58.06±16.25$^{A}$ | 53.83±9.47 |
| | Val-NTA100μM | 27.20±8.48$^{B}$ | 43.81±8.98 |

Different capital letters indicate significant differences among each treatment at each time point ($p$<0.05). Different small letters indicate a significant difference among the control group at each storage time ($p$<0.05). Data are shown as mean±SD.

### 5.3.2 Sperm kinetics

Statistical analysis revealed significant differences between the control group at 0 hours, and both 17 and 45 hours ($p$<0.05) in VSL, as shown in Table 2. There were differences at 17 hours in ALH, VAP, VCL, and VSL between Val-NTA 100 μM and all other Val-NTA treatments and the control group ($p$<0.001, Table 2). However, no significant differences were found in these parameters between the control group and Val-NTA treatments (0.1, 1, 10 μM) or between these Val-NTA treatments themselves ($p$>0.05). As for BCF, significant differences were not found between any Val-NTA or control treatment ($p$>0.05, Table 2). STR and LIN were significantly higher in Val-NTA 100 μM than in the control group ($p$<0.001) and Val-NTA 0.1 and 1 μM ($p$<0.01), but they did not differ from Val-NTA 10 μM ($p$>0.05, Table 2). At 45 hours, ALH, LIN, STR, VAP, VCL, and VSL showed significant differences between Val-NTA 100 μM and all other Val-NTA treatments (0.1, 1, 10 μM) and the control group ($p$<0.001, Table 2). However, no differences were found between the control group and Val-NTA treatments (0.1, 1, 10 μM) or between these Val-NTA treatments themselves ($p$>0.05). As for BCF, there were no differences between the control group and Val-NTA treatments at any of the concentrations tested ($p$>0.05).

**Table 2** Sperm kinetics.

| Time (h) | Treatment | ALH (μm) | BCF (Hz) | LIN (%) | STR (%) | VAP (μm/s) | VCL (μm/s) | VSL (μm/s) |
|---|---|---|---|---|---|---|---|---|
| **0** | **Ctr** | 5.27±0.27 | 13.41±0.48 | 42.09±4.03 | 70.70±5.38 | 72.26±2.52 | 127.27±12.18 | 51.63±1.88$^{a}$ |
| **17** | **Ctr** | 6.11±1.13$^{A}$ | 14.10±2.35 | 49.33±11.44$^{A}$ | 78.16±11.18$^{A}$ | 85.69±0.89$^{A}$ | 142.01±15.54$^{A}$ | 68.02±8.10$^{A,b}$ |
| | **Val-NTA0.1μM** | 5.48±0.63$^{A}$ | 13.88±1.52 | 52.14±7.61$^{A}$ | 81.13±10.13$^{A}$ | 77.03±4.03$^{A}$ | 125.08±9.05$^{A}$ | 63.07±7.65$^{A}$ |
| | **Val-NTA1μM** | 5.61±1.05$^{A}$ | 14.46±1.80 | 52.88 ±2.36$^{A}$ | 82.88±1.60$^{A}$ | 80.10±10.10$^{A}$ | 129.54±10.25$^{A}$ | 67.64±8.49$^{A}$ |
| | **Val-NTA10μM** | 5.78±1.57$^{A}$ | 15.54±1.73 | 58.57±1.37$^{AB}$ | 85.45±1.49$^{AB}$ | 87.07±14.72$^{A}$ | 130.91±24.13$^{A}$ | 75.6±12.52$^{A}$ |
| | **Val-NTA100μM** | 3.76±0.98$^{B}$ | 15.69±2.02 | 68.27 ±4.50$^{B}$ | 93.40±2.55$^{B}$ | 53.88±11.76$^{B}$ | 77.85±20.69$^{B}$ | 50.16±9.45$^{B}$ |
| **45** | **Ctr** | 5.80±1.21$^{A}$ | 14.23±2.09 | 55.57±6.51$^{A}$ | 84.31±5.50$^{A}$ | 79.97±12.15$^{A}$ | 126.66±22.03$^{A}$ | 68.51±7.96$^{A,b}$ |
| | **Val-NTA0.1μM** | 5.63±0.26$^{A}$ | 14.28±1.45 | 52.51±4.88$^{A}$ | 82.13±6.45$^{A}$ | 81.07±9.85$^{A}$ | 131.42±15.00$^{A}$ | 67.42±6.52$^{A}$ |
| | **Val-NTA1μM** | 5.64±0.75$^{A}$ | 14.60±1.40 | 52.02±1.91$^{A}$ | 80.96±0.62$^{A}$ | 82.33±6.86$^{A}$ | 134.15± 6.86$^{A}$ | 67.30±5.51$^{A}$ |
| | **Val-NTA10μM** | 5.78±0.81$^{A}$ | 15.00±2.20 | 57.25±3.03$^{A}$ | 85.04±3.04$^{A}$ | 84.89±4.81$^{A}$ | 131.33±10.74$^{A}$ | 73.47±3.13$^{A}$ |
| | **Val-NTA100μM** | 3.40±0.61$^{B}$ | 16.15±1.08 | 72.00±1.69$^{B}$ | 94.65±1.45$^{B}$ | 50.16±8.15$^{B}$ | 69.95±12.19$^{B}$ | 47.58±6.72$^{B}$ |

Different capital letters indicate significant differences among each treatment at each time point *(*$p<0.05$*)*. Different small letters indicate a significant difference among the control group at each storage time *(*$p<0.05$*)*. Data are shown as mean±SD.

# 6 Discussion

The primary objective of the diploma thesis was to evaluate the effects of $H_2S$ on sperm quality. To achieve this, one of the novel $H_2S$ donor NTA was used, specifically Val-NTA, in various concentrations. Notably, the mechanism of $H_2S$ release by, NTAs, differs significantly from that of other $H_2S$ donors due to the release of COS, which is then converted to $H_2S$ by the metalloenzyme CA (Powell et al 2016). The natural occurrence of the CA enzyme in porcine spermatozoa and seminal plasma allows for the use of NTAs as suitable $H_2S$ donors for sperm cells, and further exploration of the potential impact of $H_2S$ on sperm quality (Pintus et al. 2023).

Our study demonstrated a decline in various sperm parameters during storage, namely in the integrity of the sperm plasma membrane, acrosomal integrity, and total motility. These findings are consistent with previous research, which has also reported a decrease in sperm parameters over time (De Leeuw et al. 1990; Henning et al. 2012). Henning et al. (2022) recently reported that a temperature range of 15-20 °C appears to be optimal for the long-term storage of boar spermatozoa. Notably, our study is consistent with their findings as we stored the sperm at 17 °C. The deterioration of sperm parameters during prolonged storage can be attributed to oxidative stress, which is often provoked by exposure of cells to low temperatures during the storage of spermatozoa (Bucak et al. 2008). However, $H_2S$ has been identified as a potent scavenger of ROS due to its antioxidant properties, which have been found in sperm cells as well (Pintus et al. 2020). Studies have shown that $H_2S$ can upregulate the expression of various antioxidant enzymes, such as SOD and GPx, which effectively clears ROS and protects the cells against oxidative stress (Li et al. 2015; Shefa et al. 2018).

Our experimental results revealed that Val-NTA did not exhibit any positive influence on the sperm plasma membrane integrity, acrosomal integrity, motility, or kinetics. Moreover, the application of Val-NTA 100 µM resulted in a considerably negative effect. Our findings are in agreement with previous studies. Wang et al. (2011) found that men with asthenozoospermia had lower levels of $H_2S$ in their seminal plasma. The application of an $H_2S$ donor (NaHS) improved the motility of sperm. However, the fast release of $H_2S$ at a high concentration (5 µM) had a negative effect on sperm motility and hypermotility. Similarly, Zhao et al. (2016) found that $H_2S$ donor ($Na_2S$) showed a negative influence on boar sperm cells at high concentrations (25-100 µM), and controversially, high concentrations, such as 50 µM of $H_2S$ donor, even caused oxidative stress. High concentrations of $H_2S$ may be toxic because it binds

to and inhibits cytochrome *c* oxidase, a key enzyme involved in the oxidative phosphorylation process of energy production in the mitochondria of cells. This inhibition disrupts cellular respiration, leading to a decrease in ATP production and possible cell death (Szabo et al. 2014).

Unexpectedly, we did not find any positive effect on the plasma membrane of spermatozoa consistently with previous studies that investigated the effect of $H_2S$ donors in boar semen (Pintus et al. 2020; Pintus et al. 2023). In a study by Pintus et al. (2020), the application of $Na_2S$ (300 uM) demonstrated a negative effect on the sperm plasma membrane and lipid peroxidation. This experiment was conducted under artificially-induced oxidative stress, which is distinct from the experimental environment used in this study. Environmental conditions, such as the length of incubation, temperature, oxidative stress or variation of CA levels in the porcine ejaculates, could significantly influence the impact of Val-NTA on the plasma membrane of sperm cells. Therefore, different environmental conditions may have different effects on the potential impact of Val-NTA on sperm plasma membrane integrity.

The results of the study indicated that the evaluation of acrosome integrity did not yield any positive findings. Moreover, after 45 hours of sperm storage, the Val-NTA 0.1 and 100 μM showed significantly lower levels than the control group. Pintus et al. (2020), on the other hand, showed that $H_2S$ donors, such as GYY4137 and $Na_2S$, have the potential in preserving acrosome integrity under the induced-oxidative stress. However, Pintus et al. (2023) reported similar findings to our study, indicating that Gly-NTA and Leu-NTA did not exert any beneficial effects on spermatozoa acrosomal integrity (concentration 1 and 10 nM, storage time 1 and 3.5 h). While the results of the present study did not indicate a significant impact of Val-NTA on acrosome integrity, it is important to consider that other $H_2S$ donors may hold promise in this regard. Further research is warranted to determine the potential of Val-NTA and other $H_2S$ donors in preserving male fertility potential through acrosomal integrity.

The evaluation of sperm motility showed that Val-NTA 100 μM had a negative effect on the total motility and most of the kinetics parameters. On the other hand, the kinetics parameters LIN and STR of Val-100 μM showed considerably high values compared to the control group. This finding might be explained by the fact that the sperm trajectories tend to become more rectilinear when the sperm motility and kinetics decrease because the tail movement becomes less coordinated and the forward propulsion of the sperm is weakened. The tail of the sperm is responsible for generating the force required for movement, and when its function is compromised, the sperm's movement becomes more erratic and less efficient (Briz & Fàbrega 2013). However, many previous studies showed a positive effect of $H_2S$ donors on sperm motility (Wang et al. 2017; Pintus et al. 2020; Pintus et al. 2023). In a study conducted by Wang

et al. (2017), the levels of $H_2S$ in seminal plasma were examined. The results showed that men with lower levels of $H_2S$ in seminal plasma had poorer sperm motility, indicating that $H_2S$ may indeed have a role to play in this process. Similarly, Pintus et al. (2020) reported that GYY4137 at concentrations of 3 and 30 μM significantly enhanced sperm motility under artificially-induced oxidative stress. Pintus et al. (2023) studied the effect of Gly-NTA and Leu-NTA (1 and 10 nM) on mitochondrial activity and results showed that these $H_2S$ donors increased the sperm progressive motility after 3.5 h of incubation. Similarly, Val-NTA 10 μM showed slightly higher values of progressive motility than that of the control group at both 17 and 45 hours of sperm storage. Pintus et al. (2023) showed that both Gly- and Leu-NTA enhance the activity of the mitochondria, which can be described as $H_2S$ enhancing mitochondrial function in mammalian cells by increasing the activity of ATP synthase and other enzymes. Recent evidence showed that porcine sperm cells heavily rely on oxidative phosphorylation for ATP production needed for flagellar movements (Nesci et al. 2020). $H_2S$ can stimulate cell bioenergetics and may increase sperm survival during long-term storage, but sperm mitochondria are also involved in sperm fertilization events like hyperactivation (Gupta et al. 2018), which can be used in future applications for ART.

The study was conducted by replicating the experiment thrice during the summer months, potentially affecting the study's outcomes due to lower sperm parameters observed in boars during this season (Berger et al. 1980; Mauget & Boissin 1987). Despite this, the evaluation of sperm morphology and motility did not reveal any significant reductions. To ensure the relevance of results, future studies should include a larger number of replicates conducted during various seasons.

Val-NTA is a chemical compound that has been shown to exert varying effects on sperm function, depending on the concentration and specific parameters measured. At certain concentrations, Val-NTA has been demonstrated to not affect several parameters of sperm motility, such as velocity and progression, which are critical factors in determining fertility (Tremoen et al. 2010). However, at a concentration of 100 μM, Val-NTA significantly increased sperm LIN and STR. Although the mechanisms responsible for the effects of Val-NTA on sperm function are not yet fully elucidated, we can speculate that the compound may modulate ion channels and signalling pathways critical for sperm capacitation and acrosome reaction, such as the $Ca^{2+}$ and $K^+$ channels (Kumar et al. 2019). Alternatively, Val-NTA may regulate $K^+$ channels, which in turn, promote sperm hyperactivity and ATP generation by the mitochondria (Gupta et al. 2018; Nesci et al. 2020). However, the effects of Val-NTA on sperm function may be influenced by a range of factors, such as levels of CA in the porcine ejaculate,

concentrations of treatment, or cytotoxicity. Kaur et al. (2021) found that slow-releasing NTAs (Val-NTA) were effective growth inhibitors in cancer cells over 72 h, suggesting that slow $H_2S$ delivery may inhibit cancer cell growth. The NTAs exhibited negligible cytotoxicity in cancer cells over 1.5 h, indicating that COS is not acutely toxic. These C-substituted NTAs based on α-amino acids enable molecular-level control over $H_2S$ release rates, making them potentially useful donors to help understand the physiological effects of COS and/or $H_2S$. Overall, further research is necessary to fully understand the mechanisms and clinical implications of Val-NTA on sperm function.

# 7 Conclusion

We can conclude that no evidence was found to support the hypothesis that Val-NTA has a beneficial effect on the sperm plasma membrane, acrosomal integrity, motility, and kinetics during 45 h of semen storage at 17 °C. Moreover, the results of the study indicate that Val-NTA, at high concentration exerts a detrimental effect on boar spermatozoa, suggesting that caution should be taken when considering its potential use in reproductive medicine. This finding is consistent with previous studies and our hypothesis. At lower concentrations, Val-NTA did not exhibit any beneficial effects on sperm parameters, potentially attributable to the variation of CA levels in the porcine ejaculates. Thus, further investigations are warranted to examine the effects of Val-NTA in greater detail, with a focus on its potential impact on mitochondrial activity.

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# 9 List of abbreviations

| | |
|---|---|
| β-Ala-NTA | *N*-thiocarboxyanhydride derived from amino acid β-alanine |
| 3-MST | 3-mercapto pyruvate sulfur transferase |
| AI | artificial insemination |
| Aib-NTA | *N*-thiocarboxyanhydride derived from amino acid 2-aminoisobutyric acid |
| Ala-NTA | *N*-thiocarboxyanhydride derived from amino acid alanine |
| ALH | amplitude of head movement |
| ART | assisted reproduction technology |
| ATP | adenosine-3-phosphate |
| BCF | beat cross frequency |
| BPS | phosphate-buffered saline |
| BTS | Beltsville Thawing Solution |
| CA | carbonic anhydrase |
| $Ca^{2+}$ | calcium ions |
| CASA | Computer-Assisted Semen Analysis |
| CatSper | cation channels of sperm |
| CBS | cystathionine β-synthase |
| CFDA | carboxyfluorescein diacetate |
| COS | carbonyl sulfide |
| CSE | cystathionine γ-lyase |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DQH | sperm surface protein |
| Gly-NTA | *N*-thiocarboxyanhydride derived from amino acid glycine |
| GPx | glutathione peroxidase |
| GYY4137 | morpholine-4-ium-4-methoxyphenyl phosphinodithioate |
| $H_2S$ | hydrogen sulfide |
| HT-29 cells | colon cancer cell line |
| $IC_{50}$ | half-maximal inhibitory concentration |
| Ile-NTA | *N*-thiocarboxyanhydride derived from amino acid L-isoleucine |
| $K^+$ | potassium ions |
| Leu-NTA | *N*-thiocarboxyanhydride derived from amino acid leucine |

| | |
|---|---|
| LIN | linearity |
| MAPK | mitogen-activated protein kinases |
| MCF-7 cells | breast cancer cell line |
| $Na_2S$ | sodium sulfide |
| NaHS | sodium hydrosulfide |
| NTA | *N*-thiocarboxyanhydride |
| Phe-NTA | *N*-thiocarboxyanhydride derived from amino acid phenylalanine |
| PI | propidium iodide |
| PI3K/Akt | phosphatidylinositol 3' –kinase signalling pathway |
| Pro-NTA | *N*-thiocarboxyanhydride derived from amino acid proline |
| PSP | prostatic secretory protein |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| SQR | sulfide quinone oxidoreductase |
| SOD | superoxide dismutase |
| STR | straightness |
| Val-NTA | *N*-thiocarboxyanhydride derived from amino acid valine |
| VAP | average path velocity |
| VCL | curvilinear velocity |
| VSL | straight linear velocity |