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Effect of GYY4137 on tomcat epididymal spermatozoa stored at 5 °C during 7 days

Diploma Thesis

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Declaration

I declare that the Diploma Thesis “Effect of GYY4137 on tomcat epididymal spermatozoa stored at 5 °C during 7 days” is my own work and all the sources I cited in it are listed in Bibliography.

In Prague, 25. 4. 2021 _____

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Účinky GYY4137 na kocouří epididymální spermie uchované při 5 °C po dobu 7 dnů

Souhrn

Několik nedávných studií zkoumalo použití pomalu uvolňujícího H_2S donoru GYY4137 v *in vitro* podmínkách jakožto terapeutického činidla působícího proti oxidačnímu stresu. Většina těchto studií zpozorovala antioxidační účinky této sloučeniny poté, co byla použita na rozličné druhy buněk a tkání, které byly podrobené oxidačnímu stresu. Doposud však účinky této sloučeniny nebyly zkoumány na kocouřích spermiích. Na základě těchto pozorování byla hypotéza této práce založena na tom, že GYY4137 může mít antioxidační vlastnosti a může tak chránit spermie kocourů před oxidačním stresem, který často vzniká při skladování spermií. Cílem této práce bylo tedy otestovat účinky suplementace GYY4137 na parametry spermií jako je motilita, integrita plazmatické membrány, integrita akrozomu a celková antioxidační kapacita. Sperma sedmi kocourů bylo rozděleno na kontrolní a testovací skupinu, přičemž testovací skupina byla ošetřena dvěma různými koncentracemi GYY4137 (0,5 mM; 1 mM) a poté průběžně vyhodnocována po dobu 7 dní při skladovací teplotě 5 °C. V souladu s předchozími studiemi bylo potvrzeno, že skladování v chladu při 5 °C negativně ovlivnilo motilitu a akrozomální integritu spermií. Navíc, výsledky předložené diplomové práce poskytují důkaz, že GYY4137 vykazuje antioxidační vlastnosti po dobu 7 dní skladování spermií. Zároveň bylo prokázáno, že obě použité koncentrace (0,5 mM; 1 mM) GYY4137 nevykazovaly příznivé, ani nepříznivé účinky na parametry kočičích epididymálních spermií. Z této skutečnosti plyne, že při obou koncentracích GYY4137, které byly testovány v této práci, se neprokázal příznivý vliv na prodloužení životnosti kocouřích spermií skladovaných při teplotě 5 °C po dobu 7 dní.

Klíčová slova: oxidativní stress, spermie, kocour, ROS, sulfan, GYY4137

Effect of GYY4137 on tomcat epididymal spermatozoa stored at 5 °C during 7 days

Summary

Several recent studies investigated the usage of slow-releasing H_2S donor GYY4137 as *in vitro* therapeutic agent against oxidative stress. Most of those studies observed antioxidant properties of this compound when administered on different types of cells and tissues submitted to oxidative stress. However, to date, the effect of this compound on tomcat spermatozoa were not investigated. Based on those observations, the hypothesis of this thesis was that GYY4137 may possess antioxidant properties and thus may protect tomcat spermatozoa against oxidative stress, which frequently arises during sperm storage. Therefore, the aim of this thesis was to test the effect of GYY4137 supplementation on the sperm parameters such as motility, plasma membrane integrity, acrosomal integrity and total antioxidant capacity. The semen of 7 tomcats was treated with and without two different concentrations of GYY4137 (0.5 mM; 1 mM) and evaluated during 7 days at 5°C. Consistent with previous studies, cool storage at 5 °C negatively affects tomcat sperm motility and acrosome integrity. Moreover, the results from this thesis provide evidence that GYY4137 showed antioxidant properties after 7 days of sperm storage. However, both at 0.5 and 1 mM, GYY4137 did show neither beneficial nor detrimental effects on cat epididymal sperm parameters. In conclusion, at the concentrations tested in this study, GYY4137 did not prove to be beneficial in enhancing the lifespan of tomcat epididymal spermatozoa stored at 5 °C for 7 days.

Keywords: oxidative stress, spermatozoa, tomcat, ROS, hydrogen sulphide, GYY4137

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1 Introduction

Since the start of the 20th century, the average abundance of native species in most major land-based habitats has decreased by at least 20% (IPBES 2019). To date, the IUCN (2021) reported a total of 37,480 threatened species, of which 1,323 are mammals. Their future reproduction and survival is mostly relying on the assisted reproductive techniques (ART). This also applies for most of the wild felids. However, due to their specific reproductive biology it is difficult to obtain crucial information needed for successful application of ART in wild felids (Thongphakdee et al. 2017). This is also the reason why the domestic cat is used as a main model for obtaining the basic knowledge of reproductive anatomy and physiology of other felids.

To achieve the highest chance of fertilization under both natural and artificial conditions, the ejaculate/sperm sample must contain morphologically normal/undamaged spermatozoa. Unfortunately, in some species it is not always possible to obtain a mating partner or fresh sperm sample that can be used for fertilization. Due this reason, fresh samples are stored for later use. The most described methods of sperm storaging are cool storage and cryopreservation (Buranaamnuay 2017). However, according to Said et al. (2005) the spermatozoa stored this way have lower fertility potential in contrast to fresh sperm. On top of that, manipulation with sperm samples very often causes oxidative stress, which damages the spermatozoa. The oxidative stress is characterized by abnormally increased production of reactive oxygen species (ROS) (Henkel 2011). Whereas the small amounts of ROS play several important roles in organism (Bayr 2005), their abnormal production impairs most of the sperm parameters and thus lowers the chance of successful fertilization (Agarwal et al. 2014). According to Bayr (2005), this damage can be reversed or at least palliated by the administration of antioxidants.

One of the recently discussed gaseous molecules that affect the spermatozoa is hydrogen sulphide (H_2S). According to several studies the H_2S might have both positive (Wang et al. 2018) and negative (Zhang et al. 2017) effects. Several authors (e.g., Wang et al. 2018, Pintus et al. 2020) reported improvement of sperm parameters after the administration of H_2S to spermatozoa exposed to oxidative stress. However, according to Pintus et al. (2020) those effects are dependent on the dosage and the type of donor that releases H_2S. The H_2S donors can be divided according to their mechanism of release to fast-releasing (e.g., Na_2S, NaHS) and slow-releasing (e.g., GYY4137). Overall, the slow-releasing donors has proven to be better over the fast-releasing as they mimic the physiological conditions that are present in the

organism (Rose et al. 2015). The aim of this thesis was to investigate whether, in virtue of its antioxidant properties, the slow-releasing donor GYY4137 may increase the lifespan of tomcat epididymal spermatozoa stored at 5 °C during 7 days.

2 Scientific hypothesis and objectives of work

The hypothesis of this work was that GYY4137 may possess antioxidant properties and thus may protect tomcat spermatozoa against oxidative stress, which frequently arises during sperm storage.

The objective of this work was to test the effect of GYY4137 supplementation on tomcat epididymal spermatozoa stored at 5 °C during 7 days. Thus, the effects of GYY4137 were evaluated on sperm parameters such as motility, plasma membrane integrity, acrosomal integrity, and total antioxidant capacity.

3 Literature overview

3.1 Taxonomy

Taxonomy tree	
Kingdom	*Animalia*
Phylum	*Chordata*
Class	*Mammalia*
Order	*Carnivora*
Suborder	*Feliformia*
Family	*Felidae*

According to IUCN revised taxonomy of the Felidae family by Kitchener et al. (2017), the family can be divided into two subfamilies. These subfamilies are referred as “big cats” (*Pantherinae*) and “small cats” (*Felinae*). The big cats differ from the small cats by having an elastic ligament (hyoid) below the tongue, which allows them to roar, but not purr. The *Pantherinae* consist of only one lineage named Panthera, into which we can classify two genera - *Panthera* and *Neofelis.* The second subfamily *Felinae* includes another seven lineages. The lineages are leopard cat, puma (including genus *Acinonyx*), caracal, bay cat, ocelot, lynx and finally **domestic cat** lineage with genus *Felis*.

3.2 Biology

3.2.1 Distribution and lifespan

Felis catus appears to be on every inhabited continent. The only exception is Antarctica and some isolated islands. This mass distribution can be associated with the process of cat domestication by humans. Due to their relatively fast reproduction cycle and great ability to adapt, there is also a large global feral population around the world (Wilkins 2007). Cats in these feral populations are known for their high success of catching prey causing loss of local biodiversity.

Generally, the life of feral cats is considerably shorter than the life of domestic housekept cats. The average lifespan of feral cats is roughly 8 years due to risk of diseases and possibility of synanthropic incidents (e.g., car accidents, poisonings). On the other hand, the lifespan of housekept cat highly varies depending on nutrition, health and breed. The study of O'Neill et al. (2015) found out that some breeds of housekept cats often live longer than other breeds. On average, the crossbred cats showed greater lifespan (x̃ 14.1 years; the highest lifespan 26.7 years). The purebred cats varied substantially in their lifespan. The greatest lifespan was measured in Birman cats (x̃ 16.1 years; the highest lifespan 20.7). Greater lifespan was also measured in Burmese, Siamese and Persian cats (x̃ 14.3, 14.2, 14.1 years respectively; the highest lifespan 21 years on average). In this study, some other breeds showed much lower lifespan. Those are for example British shorthair, Maine Coon, Ragdoll, Abyssinian and Bengal cats (x̃ 11.8, 11.0, 10.1, 10.0, 7.3 years respectively; the highest lifespan 18.48 years on average).

3.2.2 Morphology and behaviour

Despite considerable variability across breeds, the morphology remains very similiar in domestic cats. The housekept cats are usually heavier and bigger than feral cats, also males are generally bigger than females. The average weight of housekept cats ranges from 4 to over 8 kg (Maine Coon breed). Due to the wild lifestyle the feral cats are slightly smaller and lighter (Queensland Government 2012). All felids are considered as a strictly carnivorous and thus their body is very well adapted for pursuing, capturing and killing their prey.

Being strictly carnivorous, all their senses are very well developed and adapted to hunting. Good example is a *tapetum lucidum* in their eyes. According to Ollivier et al. (2004) the *tapetum lucidum* is a biologic reflector system in the eyes of some vertebrates that normally functions at low light levels. It allows reflection of a light back into the eye, thereby enhancing visual sensitivity and vision in the dark. The tactile sense is enhanced by multiple vibrissae. Vibrissae are hairy outgrowths of the skin, which allow the cat to orientate in limited visibility or in complete darkness, based on the flow of air and objects around them. Thus, helping with food acqusition, overal locomotion, social communication and they also help blind kittens with orientation etc. (Gustafson et al. 1977; Ahl 1986). Adaptation of olfactory sense is the vomeronasal organ. The vomeronasal organ is a bilateral formation located in the inferior part of the nasal cavity. In cats most of the pheromones stimulate this

organ which leads to adjusting the reproductive function and behaviour (Døving & Trotier 1998).

Like most of the felids, the domestic cats have rounded heads and shortened faces. The mouth holds 28-30 teeth. Ones of the adaptations are canines - sharp, pointed teeth adapted to dispatching prey and tearing or cutting flesh (Smithers 1983). Other adaptation is represented by their limbs. Limb length in felids is a compromise between short and long length. The compromise in those lengths ensures maximum speed over distance with a precise leap and powerful grasp for holding the prey (Macdonald & Loveridge 2010). Also specialized claws help with capturing the prey. These claws are fully retractable and are protected in sheaths when at the rest. Another helpful bodypart is the tail. Tail helps the cat with balance either while climbing, leaping or balancing on narrow objects. The length of the tail varies among breeds (Kitchener 1991).

Despite the fact that the most felids are considered as solitary, the domestic cats are capable of living in social stable groups depending on many factors. One of those factors is the fact, if the cat is feral or housekept. The housekept cats usually form tolerant groups consisting of females, their offspring and in some cases also castrated males. In those groups, friendly social behaviour like grooming, greeting each other, playing, sleeping together etc. can be seen. Non-castrated males usually stay alone and socialize with the group only when mating (Borchelt & Voith 1981).

The social behaviour of feral cats highly varies depending on their home ranges. According to Morris (1987), male cats living on farms tend to have up to 150 acres of territory. On the other hand, the territories of cats living in urban areas significantly decrease and very often overlap due to high concentration of individuals. Males generally tend to have territories that overlap territories of several females, which increases the possibility of potential matings. The smaller the home ranges are, the bigger is the possibility of creating larger social groups. This can be seen for example in the centre of Roma, where the cats form a „colony“ in which cats help each other with food acquisition, catching prey, raising kittens etc. (Natoli 1985; McDonald et al. 2018).

3.3 Tomcat's reproductive system

3.3.1 Anatomy of the reproductive system

The male gonads are testes. In the adult tomcat, the testes are situated outside from the abdominal cavity, in a pouch of furred skin called the scrotum. If there are no anatomical or physiological problems, the testes usually descent into this pouch during the first 3 months of tomcat's life. The scrotum is located ventrally to the anus, close to the ischial arch. The testicular temperature in scrotum is physiologically lower than the body temperature, causing the proper function of the testes. To guarantee these thermoregulation changes, several anatomical structures are needed. These structures are the dartos, the cremaster, and the pampiniform plexus. Cold temperatures cause contraction of the cremaster (and thus scrotum), helping the testes getting closer to the warm of the body. In the warm temperatures the cremaster shows opposite behaviour – it relaxes and pulls the testes further from the body which results in their cooling. The pampiniform plexus is located within the spermatic cord and consist of a network of small veins. These veins regulate the temperature of the testes by cooling the arterial blood entering the testis from the body and transferring its heat to the venous blood leaving the testis (Graça et al. 2012). The temperature of the testes is also affected by the dartos, which contracts the skin of scrotum.

The testicular parenchyma consists mostly of coiled seminiferous tubules lined by Sertoli cells and spermatogenic cells. Along dorso-lateral border of the testis lies long and coiled tube called epididymis. Epididymis is divided into three parts called *caput* (head), *corpus* (body) and *cauda* (tail) (Hamilton 1972). The tail is then connected to deferent duct, which passes out of the scrotum into the peritoneal cavity via the inguinal ring, where it joins urethra. The tract is shared from this point further by both the reproductive and urinary system. The tract is then supplied by the prostate gland lying near the bulbo-urethral glands and the neck of the bladder. Leaving the pelvic cavity, the urethra is surrounded by cavernous erectile tissue, consisting of connective tissue lined with endothelium. During any sexual excitement, the tissue is filled with blood causing penis to be erected. As other mammals like canids and ursids, tomcats have a penile bone, also named *os penis*. The end-part of the penis is called *glans penis*. The surface of this part is covered with backward-pointing barbs known as penile spines. The purpose of those spines is to stimulate ovulation in the queen during the coitus. Study of Aronson & Cooper (1967) found out, that the growth of the penile spines is related with the levels of androgens in tomcat. The tomcats with decreased volume of

androgens (caused for example by castration or illness) have their penile spines regressed. The last part of the reproductive system that can be seen is called the prepuce. In relaxed state, the prepuce covers the penis. Inside of the prepuce, there is a mucous membrane with lubricating glands, and the outside is covered with hairy skin (Aspinall 2011).

3.3.2 Physiology of the reproductive system

The tomcat is able to reproduce as soon as he reaches puberty. Broom and Fraser (2007) stated in their book, that puberty has been variously defined as a time period of individual's life, when necessary anatomical and physiological changes related to reproduction happen. These changes include full differentiation of the sexes, manifestation of the secondary sexual characteristics, activation of the neural tissues that mediate mating behaviour and mainly, the possibility of the individual to create gametes and thus reproduce. In the males, the gametes are created by the process called spermatogenesis.

The tomcat's puberty normally occurs in 8-10 months of age (Little 2012), but it might be influenced by many factors. The main factor influencing the onset of puberty is level of male's testosterone. Testosterone is produced by Leydig cells in testes. Thus, any anatomical or physiological problem affecting the testes (e.g., cryptorchidism, anorchism) might delay the onset of puberty (Wiebe & Howard 2009). Other factors like breed, malnutrition or photoperiod might also influence the onset of puberty (Little 2012). As written above, the puberty in males results in ability to create gametes through the spermatogenesis.

Spermatogenesis is a cyclic process of cellular differentiation that produces mature spermatozoa. According to Amann & Schanbacher (1983) approximately 4.5 cycles are needed to complete the whole process of differentiation in mammals. The total length of spermatogenesis in male domestic cat therefore takes 46.8 days (Franca & Godinho 2003). The process of spermatogenesis is divided into three stages: spermatocytogenesis, meiosis, and spermiogenesis. During the spermatocytogenesis, undifferentiated male germ cell called spermatogonia, undergo asymmetric and symmetric mitotic divisions. The asymmetric mitotic divisions maintain a self-renewing spermatogonia population. The symmetric mitotic divisions then follow, producing daughter cells that will go through a differentiation. This differentation results in primary (first) spermatocytes that proceed to the meiotic stage. In this stage, the primary spermatocytes go through two meiotic cell divisions, which results in haploid spermatids. The final stage is called spermiogenesis. During this stage, spermatids

differentiate into mature spermatozoa (Ramm & Schärer 2014). For better understanding the spermatocytogenesis, meiosis and spermiogenesis are shown in **Figure 1**.

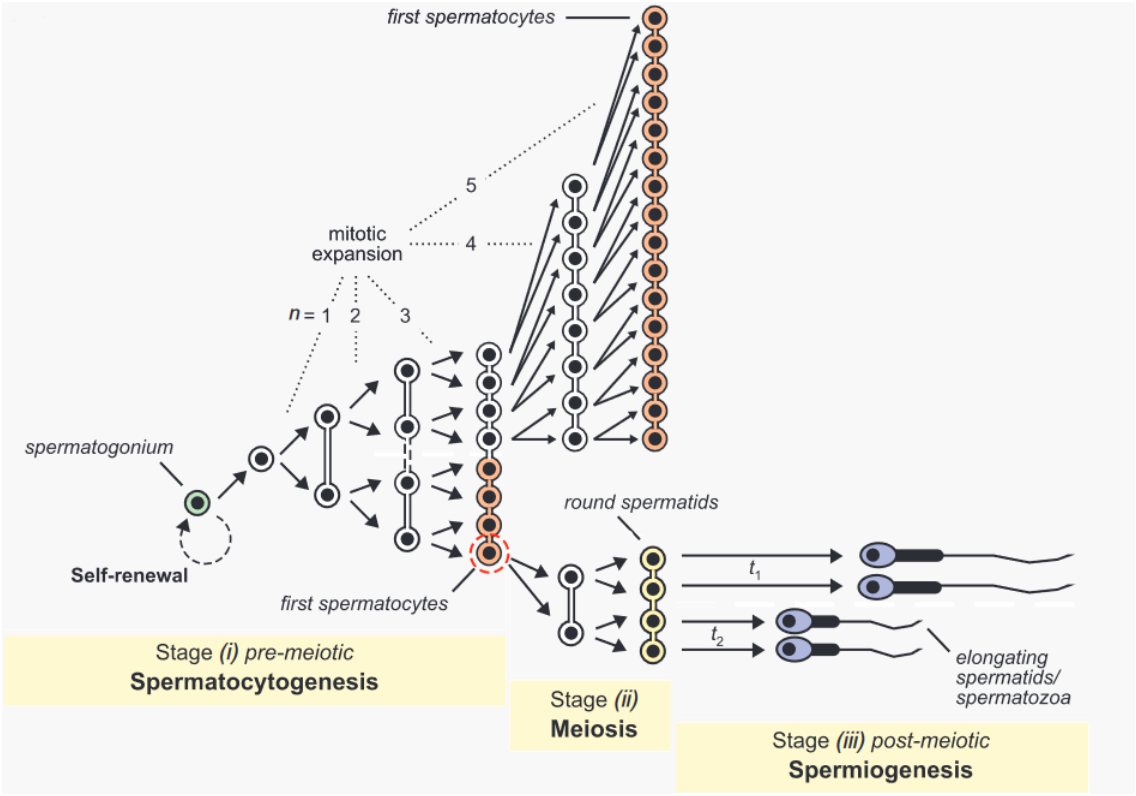

Figure 1. *Generalized scheme of sperm cell differentiation from spermatocytogenesis to spermiogenesis (Ramm & Schärer 2014).*

The maturation occurs to the sperm transit through the epididymis. This process is necessary for sperm to acquire its movement and is also associated with different morphological and physiological changes that spermatozoa undergo. The main morphological change, that can be observed, is migration of the cytoplasmatic droplet. The cytoplasmatic droplet migrates caudally from the sperm neck towards the annulus. Some species then lose this droplet upon ejaculation (Gervasi & Visconti 2017). According to Cooper (2010), the physiological significance of this migration has not been discovered yet, although according to Sutovsky & Lover-camp (2010), it has been observed that the retention of cytoplasmatic droplet in the spermatozoa of boar and bull has negative impact on fertility. Other biological changes and characteristics of spermatozoa in each part of the epididymis are described in the **Figure 2**. During the transit through the epididymis, a significant percentage of immature and abnormal spermatozoa is also removed (Axnér 2006). Mature spermatozoa are then ejaculated during coitus.

Ejaculation is a process in which seminal fluid is expulsed from the urethra. The process is caused by coordinated series of reflexes that are activated during two phases. Those phases are called emission and expulsion. The seminal emission includes secretion of seminal plasma from the accessory sexual glands and both epithelial cells of the urethra. Via contractions of the vas deferens the spermatozoa with seminal plasma are moved to the proximal urethra. In the meantime, urethral smooth muscles contract and thus close the neck of the bladder, preventing the ejaculate to pass the wrong way. The next phase - seminal expulsion then follows. The semen is rapidly pushed forward through the urethra and springs from the urethral opening. This expulsion is caused by the contraction of the muscles surrounding the urethra. Those muscles are called bulbocavernosus and the urethral sphincter (Lucio et al. 2011).

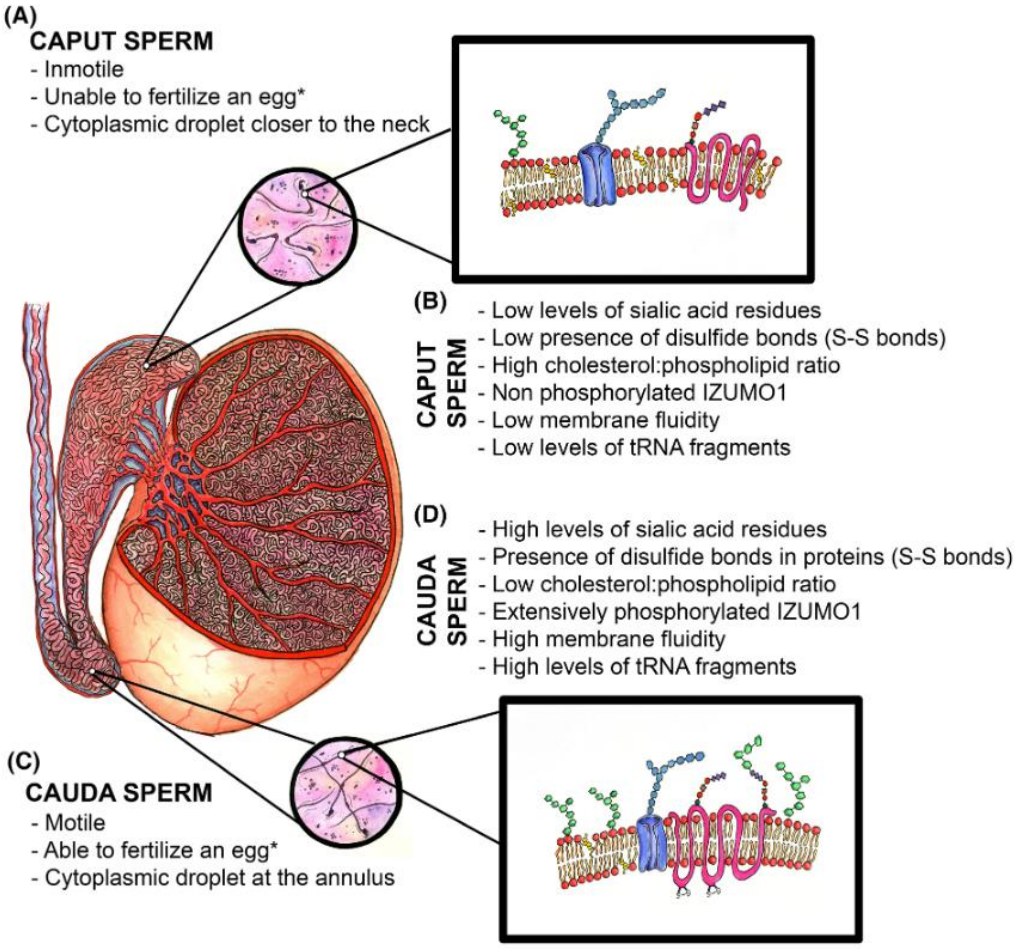

Figure 2. *Morphological and functional characteristics of both immature (A,B) and mature (C,D) spermatozoa during epididymal maturation (Gervasi & Visconti 2017).*

3.3.3 Sperm cell structure and characteristics

Already in the year 1975, the mammalian sperm cell has been described and also divided into two main parts by Fawcett – the sperm head and tail. The head, from its tip, is mostly covered by acrosomal cap in the most mammals. Under the acrosomal cap is hidden the acrosome and nucleus. The acrosome is a bag-like organelle, containing enzymes, that helps in the process of "sperm-egg" binding during the fertilization (Abou-Haila & Tulsiani 2000). The nucleus contains the paternal genome of the species. In here, the genome is protected from ongoing protamination and remains functionally inert (Johnson et al. 2011). The nucleus of each species acquires a characteristic shape, due to several processes in the development of the sperm. For example, the shape of the sperm head in rodents resembles a hook (falciform), in the carnivores, ungulates and primates, the sperm heads are spatula-shaped (spatulate) (Sutovsky & Manandhar 2006). Detail of the tomcat spermatozoon (*Felis catus*) is pictured in the **Figure 3.**

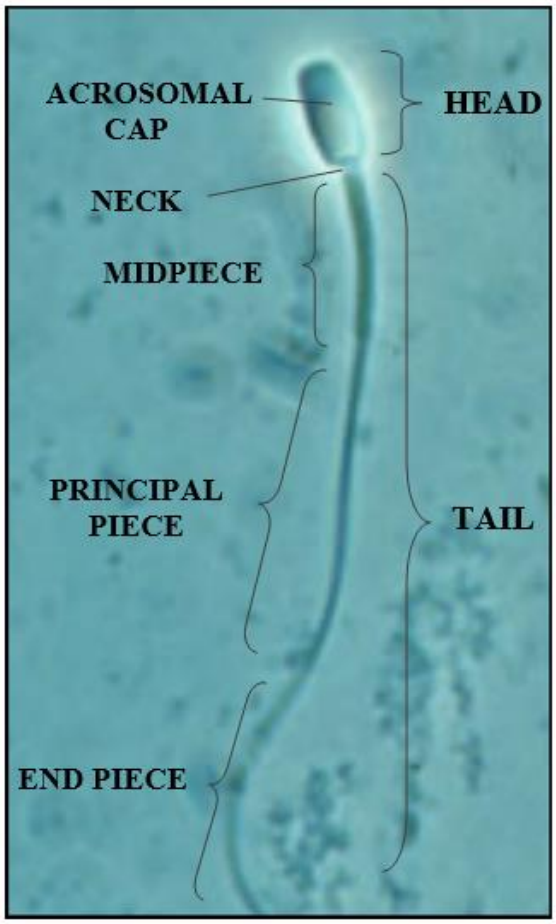

Figure 3. *Detail of tomcat (Felis catus) spermatozoon (Axnér & Forsberg 2007).*

The tail of the sperm (or flagellum) provides the movement to sperm. The movement is secured by the system of microtubules called axoneme. Axonome ensures the flexibility and the strenght of the tail at the same time. Although the inner structure of the whole tail is mostly same, the external structure differs in several parts, which gives the possibility to topologically divide the sperm tail into four main segments. Starting after the head, the segments are: connecting piece, midpiece, principal piece and end piece (Sutovsky & Manandhar 2006). The connecting piece, sometimes called the neck of the sperm (Fawcett 1975; Avidor-Reiss et al. 2020), as the name suggest, connects the sperm tail with its head piece (Sutovsky & Manandhar 2006). This connection is mantained by sperm centriole and another two specialized cytoskeletal structures called the capitulum and the segmented columns (Avidor-Reiss et al. 2020). The next segment (midpiece) is fully covered by the mitochondrial sheat. This sheat mediate the energy for the movement of the sperm. Every mitochondrion in this sheat also carries copies of the paternal mitochondrial genome (Sutovsky & Manandhar 2006). At the distal end of the mitochondrial sheath, there is a ring called annulus, which is separating the mid piece and the principal piece. From this part, the principal piece is covered in fibrous sheath that consists of a series of transverse ribs and two longitudinal columns which run along opposite sites of the sheath (Fawcett 1975; Sutovsky & Manandhar 2006). This sheath serves as a support for the sperm axoneme and according to Eddy et al. (2003) it also plays role in the process of sperm capacitation and hyperactivation. The termination of the fibrous sheath marks the beginning of the end piece. This segment is typical by absence of any sheathing, leaving the axoneme uncovered (Fawcett 1975; Sutovsky & Manandhar 2006).

3.4 Reactive oxygen species (ROS) and sperm function

Under ordinary conditions, there are antioxidants and pro-oxidants in a mammal body which remains in balance. In a healthy male, the spermatozoa contain an antioxidant defence mechanism protecting sperm cells from oxidative damage. However, in some cases, the pro-oxidants known as reactive oxygen species, exceeds the capacity of antioxidants causing the oxidative stress (Henkel 2011). Saalu (2010) and Hampl et al. (2012) stated oxidative stress as “a condition that reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system’s ability to readily detoxify (antioxidant defenses) the reactive intermediates or to repair the resulting damage”.

It is known that oxidative stress is one of the reasons of sperm dysfunction and infertility in males (mammals).

The imbalance of oxidants and antioxidants is caused by high levels of mentioned reactive oxygen species – ROS. Reactive oxygen species is a term into which we can include free radicals, primarily oxygen radicals and nonradical oxidizing agents. Halliwell (1989) defined free radicals as any species that contains one or more unpaired electrons. Those free radicals act as oxidizing agents formed as byproducts from the oxygen metabolism. Oxygen radicals include for example superoxide ($O_2^{\bullet}$), hydroxyl ($^{\bullet}OH$), peroxyl ($RO_2^{\bullet}$), and hydroperoxyl ($HO_2^{\bullet}$) radicals (Bayr 2005). The most known nonradical oxidazing agents are hydrogen peroxide (H_2O_2), hypochlor-ous acid (HOCl), and ozone (O_3) (Bayr 2005). When these nonradical agents come into a contact with free (highly reactive) radicals, they can be easily converted into radicals (Halliwell 1989).

3.4.1 Physiological and pathological role of ROS on sperm function

ROS might be also produced naturally during metabolism and are included in many processes like gene expression, enzymatic reactions, activation of nuclear transcription factors, mitochondrial electron transport and many others (Bayr 2005).

In the male body, the ROS play important role in mature spermatozoa, where they are involved especially in sperm motility, sperm capacitation and acrosome reaction (Ramadan & Agarwal 2002). They can also operate as signaling molecules (Tafuri et al. 2015) to exert their physiological function (Wang & Patterson 2015). The signaling occurs on chemical basis, where the ROS operate through chemical reactions with specific atoms of target proteins, resulting in several modifications of covalent proteins (D'Autréaux & Toledano 2007). On the other hand, higher levels of the free radicals can directly damage DNA by attacking the purine and pyrimidine bases (Geva et al. 1998), also lipids can be damaged by oxidation of the sperm plasma membrane (Kothari et al. 2010) and proteins, which all may lead to sperm dysfunctions or even cell death and thus male subfertility or infertilty (Agarwal et al. 2014). Whilst the small amounts of ROS are needed for the proper sperm function, according to Agarwal et al. (2014) the higher levels may have bionegative effects or may be even toxic (Vertuani 2004). Vertuaní (2004) also claims that ROS at sub-toxic levels may act as biological signal molecules. The ROS production on subtoxic level can lead to changes in cellular/extracellular redox state, which signal changes in cell function.

Apart from ROS, reactive nitrogen species (RNS) (Kothari et al. 2010) and reactive sulfur species (RSS) (Jacob et al. 2004) are also involved in several cellular processes and some of them are associated with sperm processes. The most discussed RNS affecting the sperm is gasotransmitter NO. Nitric oxide plays important role in sperm processes prior to fertilization, such as capacitation, acrosome reaction, hyperactivation, and zona pellucida binding. The NO has also positive effects on semen handling and storage (Jovičić et al. 2018). According to study of Lewis et al. (1996), low concentrations of NO have positive effect on sperm motility. On the other hand, high levels may have opposite effect due to inhibiton of sperm respiration (Rosselli et al. 1995). The study of Herrero and Gagnon (2001) proved that NO affects sperm capacitation because the NO-releasing compound has the ability to accelerate capacitation process. On the other hand, the study also proved that increased production of NO leads to premature sperm hyperactivation which complicates the sperm movement through female reproductive tract. Besides NO, H_2S is another gasotransmitter that plays an important role in the organism, while under certain circumstances it is also able to generate ROS. H_2S is mentioned in the later chapter *3.5 Role of H2S in sperm biology*.

The overproduction of ROS may originate either by endogenous or by exogenous sources (Wang & Patterson 2015). The exogenous sources are associated with the environmental factors such as exposure to radiaton, toxins, poisons and excessive smoking and alcohol consumption in human. All of which elevate ROS levels in seminal plasma (Bardaweel et al. 2017). The main endogenous sources are considered leukocytes (macrophages, neutrophils) and immature spermatozoa. The leukocytes are considered to be the major source of physiological ROS (Garrido et al. 2004). When the leukocytes are activated by any stimuli (e.g., inflamation, infection), their levels can raise up to 100 times more ROS than normal (Agarwal et al. 2003, Lavranos et al. 2012). At the same time, the proinflammatory cytokines are raised and the antioxidants are decreased, causing oxidative stress (Agarwal et al. 2014). Lu et al. (2010) confirmed that abnormally high concentrations of seminal leukocytes inflict sperm damage caused by oxidative stress.

Another endogenous source of ROS is considered to be immature, dead and abnormal spermatozoa. Two mechanisms of ROS production in spermatozoa are described. One at the plasma membrane level caused by NADPH oxidase system and second at the mitochondria level caused by NADH-dependent oxidoreductase reaction (Agarwal et al. 2014). The first one happens during spermatogenesis. The immature spermatozoa expel excess cytoplasm around them. This state is known as excess residual cytoplasm (ERC). ERC then activates the NADPH system, which generates ROS resulting in oxidative stress (Rengan et al. 2012). The

second mechanism is affected by the dead or abnormal spermatozoa contained in semen. Damaged spermatozoa considerably increase the production of ROS, which impair sperm mitochondria, resulting in loss of motility. Due to high content of mitochondria that are present in the spermatic cells, the second mechanism appears to be the main source of ROS in spermatozoa (Agarwal et al. 2014).

According to Wildt (1994), 28 species of the family Felidae can be classified as teratospermic, including domestic cat. Teratospermia is a condition, in which ejaculate contain more than 60% of abnormal/damaged spermatozoa, probably caused by inbreeding and/or poor nutrition (Pukazhenthi et al. 2001; 2006). The relationship amongst teratozoospermia and male infertility was investigated in the study of Agarwal et al. (2014) on teratospermic men. The results of this study proved, that the semen of teratospermic donors contain significantly more ROS (145.4 RLU/sec/10^6 sperm), compared to the semen of normospermic donors (64.8 RLU/sec/10^6 sperm). Another study of Pukazhenthi et al. (1999), compared the sperm cells viability of both normospermic and teratospermic tomcats of Felis catus after a sperm storage (freezing/thawing). The study reported that in contrast with normospermic donors, the sperm of teratospermic donors showed decreased parameters such as motility and acrosomal integrity. If several previous studies (Lavranos et al. 2012; Agarwal et al. 2014) proved, that damaged/abnormal spermatozoa generate ROS, then this study suggests that teratospermia is closely linked with generation of ROS.

3.4.2 Oxidative stress induced by sperm storage

The most common methods of sperm storaging are cool storage and cryopreservation (Buranaamnuay 2017). According to Bucak et al. (2008) both those storaging methods and also thawing processes cause the generation of reactive oxygen species (ROS). In the sperm cell, the protective antioxidant system is mostly formed of cytoplasmic origin. During the terminal stages of sperm cell differentiation, most of the cell cytoplasm is excreted, thus leaving the sperm with insufficient antioxidant capacity to prevent the negative effects of abnormal amounts of ROS (Bansal & Bilaspuri 2011). During the methods mentioned above, the sperm is also exposed to cold shock and usually also to atmospheric oxygen, which leaves the sperm more susceptible to lipid peroxidation due to higher production of ROS in sperm samples (Bucak et al. 2008).

The storage cooling at 5°C will be mainly discussed as it is the method used in this thesis. The study of Angrimani et al. (2017) investigated the effects of different cooling times on epididymal spermatozoa of *Felis catus*. The samples were cooled at 5°C and stored for 24,

48 and 72 hours. Then several sperm analyses were performed (motility, plasma membrane integrity, morphology, oxidative status). Angrimani et al. reported decrease in total and progressive motility after 72 h of storaging compared to 48 h. A decrease in sperm membrane integrity was observed between the 48 and 72 hours of storaging. The longest cooling time (72 h) also significantly increased the spermatozoa with both minor and total defects compared to the storaging for 24 and 48 h. However, no damage caused by oxidative stress was observed during the cooling period of 72 hours. Similar study by Gañán et al. (2009) also investigated impact of cool storage (5°C) and cryopreservation on parameters of tomcat spermatozoa collected from epididymides previously stored at 5 °C during 72 hours. Significant decrease in motility was observed after 48 and 72 hours of cool storage. In contrast, other sperm parameters such as morphology and acrosome integrity were similar in all groups during the 72 hours of storage. On the other hand, sperm collected from epididymides stored at 5 °C for 72 hours and then cryopreserved showed a significant decrease in motility and acrosome integrity compared to those cryopreserved at 0 hour of storage. Thus, in contrast to cryopreservation, which generates excessive amounts of ROS during the whole process (Lucio et al. 2016), both studies (Angrimani et al. 2017, Gañán et al. 2009) suggest that the cool storaging is more favorable in terms of preserving the sperm parameters during the first 72 hours of storage. Bansal & Bilaspuri (2011) claims that excessive production of ROS during cryopreservation is associated with decreased sperm characteristics such as reduced motility, viability, fertility, antioxidant status, membrane integrity and other sperm disfunctions. This was also confirmed in the study by Reddy et al. (2010), where the cryopreserved (thawed) buffalo semen shows lower motility and lower success rate of *in vitro* fertilisation by 10%-20% compared to fresh semen samples. In some cases, the damage caused by the oxidative stress may be bypassed by the application of antioxidants prior to freezing processes (Bucak et al. 2008).

3.5 Role of H_2S in sperm biology

3.5.1 Production and function of H_2S

H_2S (hydrogen sulfide) is an endogenous gas that is produced naturally by various cells and tissues such as brain (Diwakar & Ravindranath 2007), aorta/heart (Geng et al. 2004; Shibuya et al. 2009), liver and pancreatic islets (Kaneko et al. 2006). The production of H_2S is especially secured by cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS), and 3-

mercaptopyruvate sulfurtransferase (3-MST). The 3-MST is an enzyme that can be found in the mitochondria, and the CSE with CBS can be found in the cytosol of the several cells (Kolluru 2017). The most of CBS is primarily distributed in the brain and the nervous system (Robert et al. 2003), while the CSE has been observed in the heart (Geng et al. 2004) and in the cells of smooth muscles (Zhao et al. 2001). H_2S is mainly derived from L-cysteine and L-homocysteine, that can be obtained directly from the diet or by the methionine transulfuration pathway (Olson 2016). Both CSE and CSB were observed in the male reproductive system. The study by Sugiura et al. (2005) reported that both enzymes are expressed in the rat testes. The CBS was observed in the Leydig and Sertoli cells, whereas CSE only in Sertoli cells and spermatids. The study by Gao et al. (2019) proved the presence of both enzymes in the epididymes of rats. Both enzymes were also observed in the human corpus cavernosum (d'Emmanuele di Villa Bianca et al. 2009) and spermatozoa (Wang et al. 2018). All those observations indicate that H_2S is involved in the sperm physiology.

In a human body, H_2S acts primarily as a vasorelaxant, by causing hyperpolarization and vasorelaxation of vascular endothelium and smooth muscle cells (Yang et al. 2008). Many studies also proved that H_2S has the ability to regulate/inhibit the expression of some proinflammatory factors (Oh et al. 2006; Hu et al. 2007; Pan et al. 2011). Secondarily, as a reducing agent and antioxidant, H_2S is suppressing the damage caused by oxidative stress in several tissues such as gastric epithelium (Yonezawa et al. 2007), epithelium cells in lungs (Han et al. 2011), neurones (Kimura & Kimura 2004) and **spermatic cells** (Pintus et al. 2020). In the study of Wang et al. (2018), semen of infertile and subfertile men showed reduced levels of H_2S in the seminal plasma compared to fertile men, proving that H_2S might play an important role in male fertility.

Several studies also found out that, H_2S can cause both positive (Wang et al. 2018; Lorian et al. 2019; Pintus et al. 2020) and negative (Wiliński et al. 2015; Zhao et al. 2016; Zhang et al. 2017) effects on sperm parameters. Positive effects were reported in the study of Wang et al. (2018). The authors observed an improved sperm motility in asthenospermic men with a reduced H_2S concentration in the seminal plasma after a supply of exogenous H_2S (GYY4137 - H_2S donor, 2.5 μM). In the same study, mice sperm parameters were impaired by LPS and then treated with GYY4137. The treatment with GYY4137 restored all the sperm parameters to normal values. Recent study of Lorian et al. (2019) investigated the protective effects of NaHS on rat sperm parameters, previously damaged by induced varicocele (i.e. pathological enlargement of pampiniform venous plexus). The results of this investigation claimed that NaHS administration improved the sperm motility, viability and preserved

normal morphology, compared to the varicocele group. Pintus et al. reported that GYY4137 preserve boar sperm motility and protect the acrosomal status against the induced oxidative stress. The antioxidant properties of GYY4137 are discussed in detail at chapter *3.5.4.* On the other hand, as mentioned, several studies reported negative effects of H_2S on spermatozoa. Wiliński et al. observed an attenuating effect of NaHS (H_2S donor) medium on the motility of mice spermatozoa. Similar study on boars was done by Zhao et al. (2016), but with different H_2S donor – Na_2S. According to this study, Na_2S treatments (25, 50, 100 μM) significantly decreased spermatozoa motility. Zhao et al. also reported increased ROS generation (H_2O_2) by 20–40% after 24 h incubation (50 μM). In this study, mice spermatozoa were treated by Na_2S, receiving the similar results with both decreased motility and increased ROS generation. Another study by Zhang et al. (2017) confirmed decreased sperm parameters in mice after five-week treatments with Na_2S (10 mg/kg; 50 mg/kg) administered *in vivo*. Both treatments decreased the sperm concentration and motility. Moreover, it impaired the membrane integrity and increased the total number of abnormal spermatozoa. Despite different results from several authors, it is clear that H_2S plays a critical role in male (in)fertility, and therefore should be the subject of further research.

3.5.2 H_2S donors

It is possible to divide H_2S donors in two categories based on their release mechanism - the fast-releasing and slow-releasing agents. The most researched fast-releasing H_2S donors are probably sodium hydrosulphide (NaHS) and the inorganic salts sodium sulphide (Na_2S). Unfortunately, increasing number of experimental studies prove that H_2S levels released by the fast-releasing H_2S donors might not be representative of the physiological H_2S levels in tissues and cells (Rose et al. 2015). The H_2S released by the slow-releasing H_2S donors is released at slow, continuous pace and at smaller levels. Thereby the slow-releasing H_2S donors are more similar to the physiological conditions that are present in the organism (Rose et al. 2015). One of the slow-releasing H_2S donors is the phos-phorodithioate derivative GYY4137.

3.5.3 GYY4137 as a H_2S donor

GYY4137 (morpholin-4-ium-4-methoxyphenyl(morpholino) phosphinodithioate) is a slow-releasing H_2S compound that effectively mimics the time course of H_2S release *in vivo* (Liu et al. 2013). The compound is soluble in water and its melting point ranges between 159.8°C to 164.0°C. As a slow-releasing donor, the increase in H_2S concentration was reported to last up to 180 minutes (Zhao et al. 2014). However, Pintus et al. (2020) observed that the total antioxidant capacity of GYY4137 tended to increase even over 3 hours of incubation. According to Zhao et al. (2014), this release seems to be pH and temperature dependent *in vitro.* The most enhanced H_2S generation, was achieved under acidic conditions especially around pH 3. On the other hand, limited release was observed at lower temperatures (on ice) around 4°C. In a contrast with other H_2S donors (e.g., NaHS), the GYY4137 does not cause any damage to the target cell. In the case of NaHS, which is the fast release H_2S donor, the same concentrations and time course as in GYY4137 caused the apoptosis of cultured smooth muscle cells and fibroblasts (Yang et al. 2004; Baskar et al. 2007). Another described ability of this compound is to dilate blood vessels – act as vasorelaxant (Li et al. 2008). In this study on rats, the GYY4137 developed slow and persistent relaxation of precontracted rat aortic rings, by opening of vascular smooth muscle. Moreover, in long term, the GYY4137 treatment resulted in a lowering of blood pressure in both normotensive and hypertensive rats. The blood pressure lowering might be connected with another ability of GYY4137 described in the study of Grambow et al. (2014). According to this study on mice, the GYY4137 has also antithrombotic effect. The GYY4137 inhibits thrombin adhesion molecule expression ssociated platelet activation, causing prolongation of venular thrombus formation and tail-vein bleeding time which might also lower the blood pressure. Pintus et al. (2020) also mentioned **the antioxidant properties** of GYY4137 on the boar sperm cells.

3.5.4 Antioxidant properties of GYY4137

The experimental study of Pintus et al. (2020) investigated antioxidant properties of GYY4137 on the boar sperm under induced oxidative stress. A solution composed of 0.1 mM $FeSO_4$ and 0.5 mM sodium ascorbate (Fe^{2+}/ascorbate) was added to the sperm samples to induce the oxidative stress. These samples were treated with GYY4137 at three different concentrations. After the treatment several sperm parameters such as sperm motility, sperm

mitochondrial status, acrosomal status, sperm plasma membrane integrity and lipid peroxidation were evaluated. The results of this study prove that GYY4137 preserve sperm motility and protect the acrosomal status against the oxidative stress. On the other hand, no significant effect on mitochondrial status, plasma membrane integrity and lipid peroxidation were observed. In addition, this study found out, that the effects of used H_2S donors (GYY4137, Na_2S) are dependent on their dosage and the type of donor.

In the similar study of Wang et al. (2017) with mice, the sperm parameters were firstly lowered by LPS (lipopolysaccharide). The administration of LPS caused reduced sperm count (59%), reduced motility (29.3%) and progressive motility (9.5%). The GYY4137 was then applicated restoring all the sperm parameters to normal values. The GYY4137 also improved the histological structure of the seminiferous tubules and restored ongoing H_2S biosynthesis in the testes. Thus, the study provides evidence that GYY4137 has positive effect against sperm dysfunction and oxidative stress in mice. Another study on rats done by Xia et al. (2019), also proved that GYY4137 may reduce the sperm damage and epididymis injury. The rats underwent several surgical procedures to induce varicocele. Varicocele results in the decrease of sperm parameters, histological damage and higher levels of oxidative stress. One group was treated with GYY4137. Several sperm parameters such as sperm concentration, sperm motility and viability were then evaluated. Xia et al. (2019) reported that GYY4137 treatment effectively improved all mentioned sperm parameters. Moreover, compared to the non-treated group, the treatment inhibited the apoptosis of spermatic cells.

4 Materials and methods

4.1 Animals

Semen samples were collected from the testis and epididymides of 8 tomcats after routine castration performed at the veterinary clinic Červený vrch. Six of the tomcats were European breed, one tomcat was Ragdoll breed and the last one was mix of unknown breeds. The tomcats's body mass ranged from 3.1 kg to 4.6 kg. They aged between 7 to 30 months.

4.2 Sperm collection and conservation

As mentioned before, the sperm samples used in this study were collected from 8 tomcats after routine castration. Tomcats old enough to show complete spermatogenesis and containing spermatozoa in their epididymis were used for this thesis. After the castration, the testes, epididymides and a part of the deferens ducts were placed in physiological solution, consisting of NaCl (0.9%) supplied with 50 μg/mL of gentamicin. These samples were then transported at room temperature and processed within 4 hours after castration. At the laboratory, the samples were dissected from connective tissues and visible blood vessels. After that, the epididymides were separated from the testes and individually weighed to the nearest 0.01 g (HM-120, A&D Company, Tokyo, Japan). These weights are shown in the **Table 2**. To release the spermatozoa, both epididymal tails were placed in a glass Petri dish and then carefully minced into 800 μl of tris-citrate-fructose (3.025% Tris, 1.7% citric acid, 1.25% fructose, 0.06% Sodium Benzyl penicillin, 0.1% streptomycin sulphate) (Thuwanut et al. 2008). The samples were left to incubate at room temperature for 10 minutes, which helped with the release of present spermatozoa. The sperm aliquot was then fixed in 0.3% formaldehyde solution, allowing determining the sperm concentration using a Bürker chamber. Then, tris-citrate-fructose was used to dilute the rest of the sperm samples, until the final dilution of approximately 10–15 × 10^6 spermatozoa/mL. Then, the samples were randomly split into three groups, which were supplemented with vehicle only (control group, CTR) or GYY4137 at 1 mM and 0.5 mM in phosphate-buffered saline (PBS) solution. Samples were then cooled at 5°C in the fridge. Sperm analyses were perfomed at 0 hour (CTR only), and at 3 and 7 days of storage at 5°C. Before performing sperm analyses, the samples were incubated in a water bath at 38°C for 20 minutes.

4.3 Sperm evaluation

4.3.1 Sperm motility

The sperm motility was evaluated subjectively. To evaluate the sperm motility an aliquot of each semen sample was placed into a Makler counting chamber (Sefi-Medical instruments, Haifa, Israel; chamber depth: 10 μm) at 38°C. The phase contrast microscopy (10× objective) was used for the sperm evaluation. The motility was reported as the percentage of motile spermatozoa to the nearest 5%. The 0-5 scale test (Platz & Seager 1978; Axnér et al. 2004; Ros-Santaella & Pintus 2017) was used to evaluate the quality of sperm movement, whilst the highest number was 5 (progressive and vigorous movements) and the lowest was 0 (no motility).

4.3.2 Evaluation of the acrosomal integrity

Acrosome integrity was evaluated according to the protocol described by Thuwanut et al. (2008) with minor modifications. Briefly, an aliquot (10 μl) of sperm sample was gently spread on a microscope slide, air-dried, and fixed with 95% ethanol for 30 seconds. Then, 20 μl of a solution composed of 50 μL of PBS (Phosphate Buffered Saline), 50 μL of PNA-FITC (Peanut agglutinin fluorescein isothiocyanate; 200 μg/ml) and 5 μL of propidium iodide (500 μg/ml) was laid on the smear and incubated in a moist chamber at 4 °C for 15 minutes in the dark. After gently removing the coverslip, the samples were washed with distilled water and air-dried again for 15 minutes. Then, a drop of DAKO (fluorescence mounting medium) was laid on the smear and covered with the coverslip. The sperm was then evaluated under the epifluorescent microscope (Nikon Eclipse E600, Nikon, Tokyo, Japan; 40× objective). Two hundred sperm were counted on each slide and classified into two categories: intact (green fluorescence over the entire acrosome region) and non-intact acrosome (disrupted or no green fluorescence over the acrosome region).

4.3.3 Evaluation of the plasma membrane integrity

The evaluation of the plasma membrane integrity was done by firstly mixing 10 μL of sperm sample with a staining solution. The staining solution consisted of 2 μL propidium iodide (0.5 mg/mL in phosphate buffered saline solution, PBS), 2 μL carboxyfluorescein diacetate (0.46 mg/mL in dimethyl sulfoxide, DMSO), 1 μL formaldehyde (0.3%, *v/v* in PBS)

and finally 85 µL of phosphate buffered saline solution (PBS). The sample was then incubated in the dark for 10 minutes at 38 °C (Harrison et al. 1990). After that, an aliquot of 10 µL was placed on a microscope slide and covered with a coverslip. This aliquot was then evaluated under the epifluorescence microscope (Nikon Eclipse E600, Nikon, Tokyo, Japan; 40× objective). The membrane integrity was evaluated by the color of the sperm heads. Sperm heads that were partially or entirely red, were classified as damaged, whilst the ones with the entirely green heads were classified as intact. Two hundred sperm were evaluated this way for every used sample.

4.3.4 Evaluation of the total antioxidant capacity

The total antioxidant capacity (TAC) was determined spectrophotometrically (Libra S22, Biochrom, Harvard Bioscience Company, Cambourne, United Kingdom) as described by Ozcan (2004). Briefly, the total antioxidant capacity assay is based on the decolourization of the 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) by antioxidants according to their concentration and antioxidant capacities. It was not possible to acquire the full sample from only one tomcat, therefore the size of sample used in this analysis is 6. Due to the limited volume of sample available, this analysis was performed only at day 7. At day 7 of semen storage, samples were centrifuged at 16,000 *g* during 10 minutes. Then 20 µl of supernatant from CTR, GYY4137 1 mM, or GYY4137 0.5 mM groups was added to 800 µl of reagent 1 (acetate buffer 0.4 M, pH 5.8) and 80 µl of reagent 2 ($ABTS^{\cdot+}$ in acetate buffer 30 mM, pH 3.6) and carefully mixed. After 5 minutes, the absorbance was measured at 660nm. The total antioxidant capacity was expressed as percentage relative to the CTR group (assuming CTR is equal to 100%).

4.3.5 Statistical analysis

The SPSS 20.0 statistical software (IBM Inc., Chicago, IL, USA) was used to perform all the statistical analyses. Shapiro-Wilk's and Levene's tests were used to analyse the normal distribution and the variance homogeneity of the data, respectively. Generalized linear model was used to analyse the impact of treatments on sperm parameters (motility, acrosomal integrity, plasma membrane integrity). To evaluate the total antioxidant capacity of GYY4137, the ANOVA and Games-Howell post-hoc test was performed. Data are shown as mean ± SD. Statistical significance was set at $p < 0.05$.

5 Results

Overall, the weight of tomcats ranged from 3.1 to 4.6 kg (**Table 1.**). All tomcats showed spermatozoa in their epididymal caudae. However, the age of the tomcat seems to have impact on sperm concentration. On average, higher sperm concentrations were observed in the sperm samples of older tomcats. Due to the lack of breed diversity and little number of tested tomcats, the influence of breed on spermatozoa was not assessed in this thesis.

Table 1. – *Several chosen characteristics of castrated tomcats*

Cat code	Age (months)	Body mass (kg)	Sperm concentration*	Breed
G1D	8	4.6	23.63	European
G2D	9	3.5	12.25	European
G3D	7	3.3	21.38	European
G5D	10	3.1	31.75	European
G6D	10	4	13.00	European
G52M	7	4.2	23.88	Mix of breeds
G53M	30	4	86.25	Ragdoll
$\bar{x}$	11.57 ± 3.1	3.81 ± 0.2	30.31 ± 9.66	-

**(millions/ml)*

Table 2. – *The weights of the testes, epididymides (rounded to nearest 0.01 g) and body mass of tested tomcats (rounded to nearest 0.1 kg)*

Cat code	Mean testes mass (g)	Mean epididymides mass (g)	Body mass (kg)
G1D	1.39	0.22	4.6
G2D	0.91	0.16	3.5
G3D	1.13	0.20	3.3
G5D	1.25	0.24	3.1
G6D	1.16	0.27	4
G52M	0.72	0.23	4.2
G53M	1.46	0.25	4
$\bar{x}$	1.15 ± 0.1	0.22 ± 0.01	3.81 ± 0.2

Individual mass of testes and epididymides together with body mass for each tomcat are shown in the **Table 2**. It seems that the testes and epididymides of the tomcats with higher body mass were slightly bigger in contrast to the ones with the lower body mass.

5.1 Subjective evaluation of sperm motility

There were no differences between CTR group at days 0 and 3 of storage, whereas a significant decrease in the percentage of motile sperm were observed at day 7 (*p<0.001*, **Figure 4**). There were no differences between treatments, although there was a tendency for higher percentage of motile sperm in G0.05mM at day 7 compared to the CTR group (*p>0.05*, **Figure 4**). Overall, G1mM shows the lowest values of sperm motility at any day of storage.

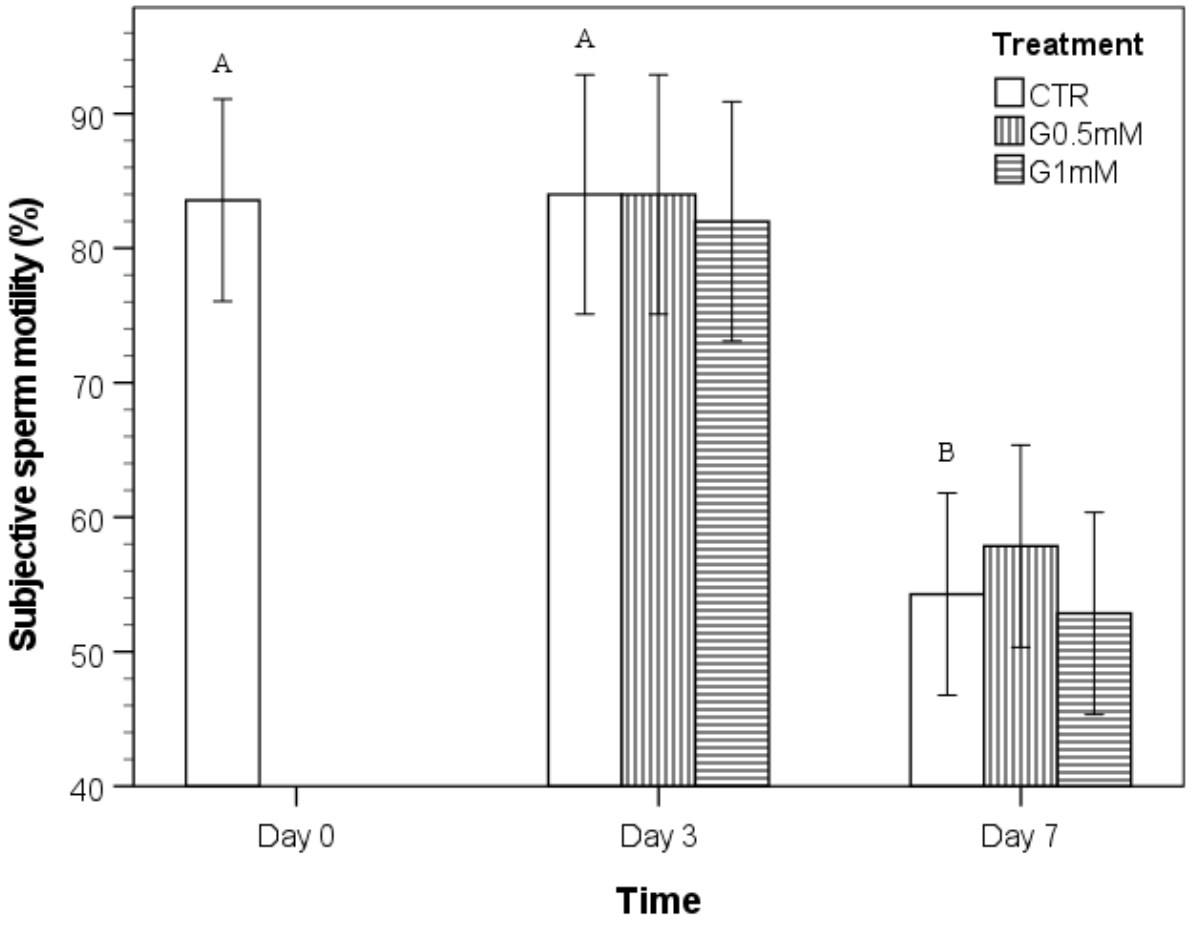

Figure 4. *Subjective sperm motility in tomcat sperm samples without (CTR) or with GYY4137 treatment (G0.5mM; G1mM) during the 7 days of storage at 5°C. CTR = control group; G0.5mM treatment = GYY4137 0.5 mM; G1mM treatment = GYY4137 1 mM. Different capital letters indicate significant differences among days for the CTR group (p<0.05). There were no differences among treatments at each day of storage. Data are shown as mean ± SD.*

In the terms of sperm movement quality, no significant difference was observed between CTR group at days 0 and 3 of cool storage. At the day 7, the CTR group showed significant decrease quality of sperm movement (*p<0.001*, **Figure 5**). Neither of the treatments showed any difference during 7 days when compared to the control group. It is worth to mention that the G0.5mM treatment presented higher movement quality than the CTR group on day 7, although the difference was not statistically significant (*p>0.05*, **Figure 5**). The G1mM group did not differ from the CTR group at the day 7 (*p>0.05*, **Figure 5**).

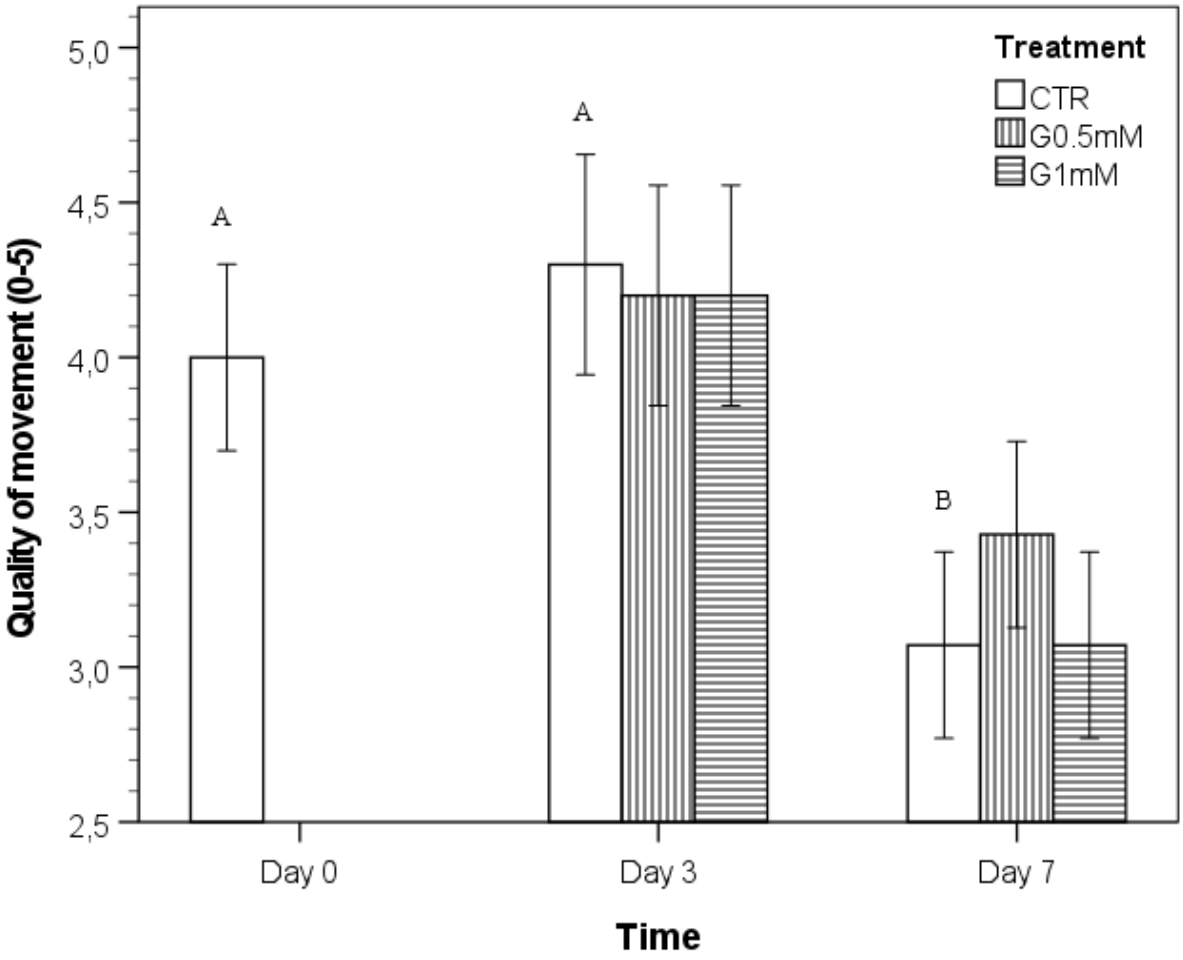

Figure 5. *Quality of movement in tomcat sperm samples without (CTR) or with GYY4137 treatment (G0.5mM; G1mM) on day 0, 3, 7. CTR = control group; G0.5mM treatment = GYY4137 0.5 mM; G1mM treatment = GYY4137 1 mM. Different capital letters indicate significant differences among days for the CTR group (p<0.05). There were no differences among treatments at each day of storage. Data are shown as mean ± SD.*

The only significant difference in the sperm motility index (SMI) of CTR groups was observed on the day 7. On this day, the CTR group showed lower SMI in comparison to previous days (*p<0.05*, **Figure 6**). No significant differences among treatments at days 3 and 7 were observed in the SMI. Also, at day 7, in spite of higher sperm motility index in the G0.5mM group, it did not statistically differ from the CTR group (*p=0.193*, **Figure 6**).

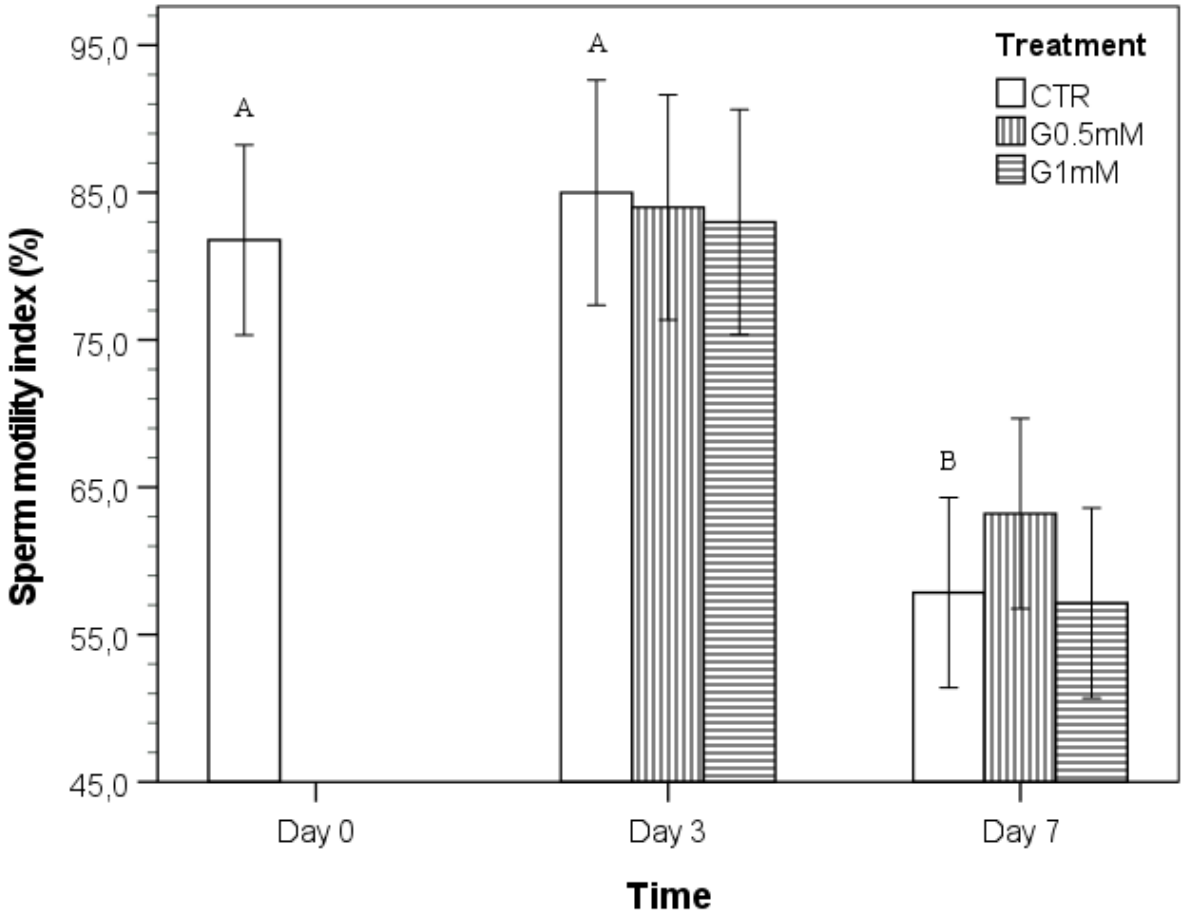

Figure 6. *Sperm motility index in tomcat sperm samples without (CTR) or with GYY4137 treatment (G0.5mM; G1mM) during the 7 days of storage at 5°C. CTR = control group; G0.5mM treatment = GYY4137 0.5 mM; G1mM treatment = GYY4137 1 mM. Different capital letters indicate significant differences among days for the CTR group (p<0.05). There were no differences among treatments at each day of storage. Data are shown as mean ± SD*

5.2 Evaluation of acrosomal integrity

The CTR group at day 0 differ from CTR group at days 3 and 7 ($p<0.05$). On the storage days 3 and 7, the percentage of spermatozoa with intact acrosome in CTR groups did not significantly differ from other treatments ($p>0.05$, **Figure 7**). No significant difference between both treated groups was observed at any day of storage ($p>0.05$, **Figure 7**).

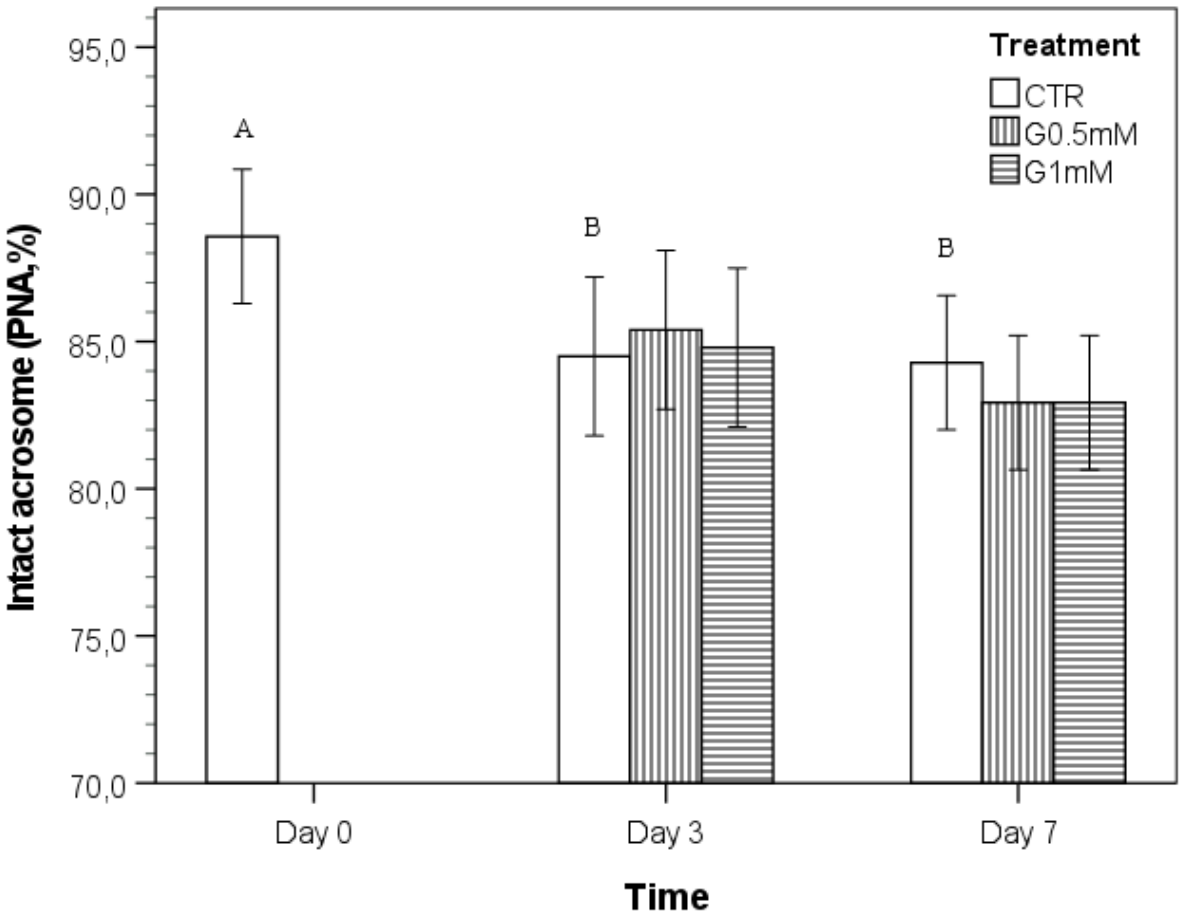

Figure 7. *Sperm acrosomal integrity in tomcat sperm samples without (CTR) or with GYY4137 treatment (G0.5mM; G1mM) during the 7 days of storage at 5°C. CTR = control group; G0.5mM treatment = GYY4137 0.5 mM; G1mM treatment = GYY4137 1 mM. Different capital letters indicate significant differences among days for the CTR group ($p<0.05$). There were no differences among treatments at each day of storage. Data are shown as mean ± SD.*

5.3 Evaluation of plasma membrane integrity

As shown in **Figure 8**, the sperm membrane integrity did not significantly differ in the CTR groups during the 7 days of storage (*p>0.05*). Compared to the CTR group at day 0, the CTR group at day 7 showed lower percentage of spermatozoa with intact plasma membrane, although differences were not statistically significant (*p=0.082*, **Figure 8**). It is worth to highlight that both treatments presented higher percentage of intact plasma membrane than the CTR group at day 7, however the statistical difference was not significant (*p>0.05*, **Figure 8**).

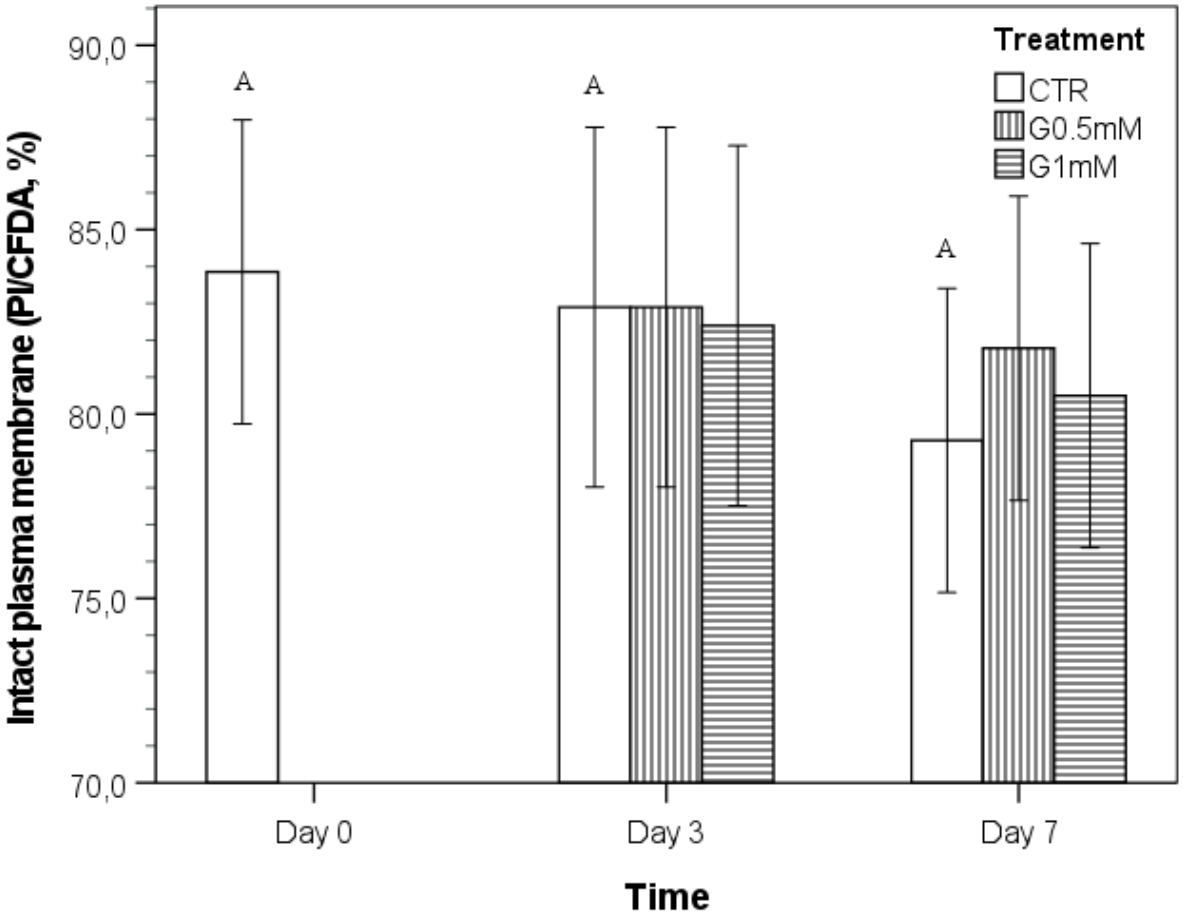

Figure 8. *Sperm membrane integrity in tomcat sperm samples without (CTR) or with GYY4137 treatment (G0.5mM; G1mM) during the 7 days of storage at 5°C. CTR = control group; G0.5mM treatment = GYY4137 0.5 mM; G1mM treatment = GYY4137 1 mM. Different capital letters indicate significant differences among days for the CTR group (p<0.05). There were no differences among treatments at each day of storage. Data are shown as mean ± SD.*

5.4 Evaluation of the total antioxidant capacity

The GYY4137 1 mM and 0.5 mM showed 179.22% and 133.55% total antioxidant capacity (TAC) over the CTR group, respectively (assuming the CTR group was equal to 100%). The TAC of GYY4137 1 mM was significantly higher than GYY4137 0.5 mM and CTR group (*p<0.01*, **Figure 9**). The TAC of GYY4137 0.5 mM was also significantly higher than that of the CTR group (*p<0.01*, **Figure 9**).

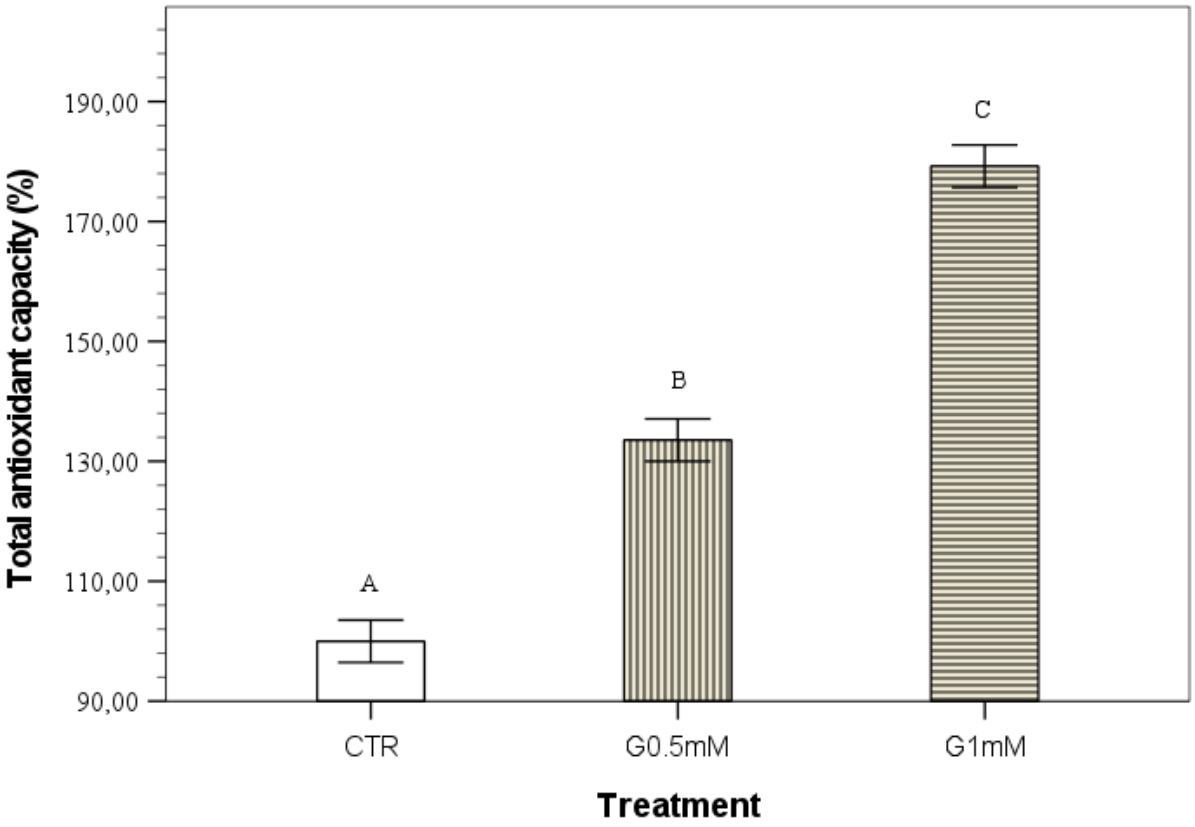

Figure 9. *Total antioxidant capacity of each used treatment. CTR = control group; G0.5mM = GYY4137 0.5 mM; G1mM = GYY4137 1 mM. Different capital letters indicate significant differences among each treatment (p<0.05). Data are shown as mean ± SD.*

6 Discussion

Despite firstly considered poisonous and harmful for organism, several recent studies (Wang et al. 2018; Gao et al. 2019; Pintus et al. 2020) discovered that H_2S plays an important role in male fertility, by affecting the sperm parameters. However, according to several authors (Wang et al. 2018; Pintus et al. 2020) the effects of H_2S on spermatozoa may be either positive or negative depending on its dosage and type of donor. The H_2S donors can be divided by the mechanism of release into fast-releasing and slow-releasing donors. According to Rose et al. (2015) the amounts of H_2S released by the fast-releasing donors (e.g., NaHS, Na_2S) are not similar to the physiological amounts present in the cells. On the other hand, the slow-releasing donors, such as GYY4137, release H_2S at slow, continuous pace and at smaller levels, which are more similar to the physiological conditions that are found in the organism (Rose et al. 2015). In spite of its high antioxidant capacity, the results of this thesis demonstrate that GYY4137, both at 1 and 0.5 mM, did not have neither beneficial nor detrimental effects on cat epididymal spermatozoa stored at 5 °C during 7 days. However, it is worth to highlight that, at seven days of storage, there was a tendency for higher sperm motility and plasma membrane integrity in samples treated with GYY4137 0.5 mM than at GYY4137 1 mM and control group.

Overall, our evaluation of sperm motility showed no significant difference between treatments and the CTR groups in all evaluated parameters (i.e., subjective motility, quality of sperm movement, SMI), which suggests that GYY4137 had no effect on the sperm motility during 7 days of cool storage. However, the positive effect of GYY4137 on sperm motility was observed in other similar studies (Wang et al. 2018; Xia et al. 2019; Pintus et al. 2020). The study by Wang et al. (2018) investigated the effects of GYY4137 on asthenospermic men. The administration of GYY4137 (2.5 μM) significantly improved the motility of spermatozoa obtained from ejaculates that show decreased H_2S concentration in the seminal plasma. On the other hand, the same treatment did not show any effect on samples with a normal seminal H_2S concentration. Xia et al. (2019) tested the GYY4137 treatment (20 mg/kg; intraperitoneally) on rats submitted to experimentally-induced varicocele. The induced varicocele caused a decrease in sperm parameters such as viability, concentration and also motility. Authors reported that the treatment with GYY4137 significantly improved sperm motility in varicocele-induced rats. Moreover, the authors also reported that the GYY4137 inhibits the oxidative stress previously induced by varicocele. Other study by

Pintus et al. (2020) tested the effect of three concentrations (3, 30, and 300 μM) of GYY4137 on boar spermatozoa submitted to oxidative stress. The spermatozoa treated with 3 and 30 μM of showed higher percentages of total motility compared to the CTR group submitted to oxidative stress, proving that both concentrations preserve the motility of boar spermatozoa. All those studies came to same conclusion that GYY4137 preserves the motility of spermatozoa submitted to oxidative stress. Nevertheless, the ability of GYY4137 to preserve the sperm motility in this thesis is not in agreement with those studies. Such discrepancy might be caused by the fact that those studies did not test the *in vitro* effect of GYY4137 for the longer time periods (7 days) compared to this thesis.

In the present study, the evaluation acrosomal integrity did not reveal any significant difference between treated groups at each day of storage. No difference was also observed between the treated groups and CTR group during the time of evaluation. Divergent results were reported in the mentioned study by Pintus et al. (2020) on boar spermatozoa that were submitted to oxidative stress. Three different concentrations of GYY4137 (3, 30, and 300 μM) were tested and each evaluated after 210 minutes of incubation time in this study. The results of this evaluation reported that in comparison to the control group, the treatment with GYY4137 enhanced the acrosomal integrity in samples under artificially-induced oxidative stress. Moreover, the authors found that the treatment partly reduced the damage induced by high ROS production on acrosomal status by decreasing the percentage of acrosome-lost spermatozoa.

The membrane integrity evaluation in this thesis demonstrated no effect of GYY4137 on spermatozoa. Following the results of our evaluation, we could not state that the GYY4137 can preserve the sperm plasma membrane integrity during cool storage. Nevertheless, our results agree with the results reported in the mentioned study by Pintus et al. (2020). Three different concentrations of GYY4137 were tested on boar spermatozoa under oxidative stress. Neither of the three concentrations used showed any significant effect on the plasma membrane integrity. According to the study of Pintus et al. (2020) and results of this thesis, I may suggest that the GYY4137 has no particular effect on the integrity of plasma membrane. On the other hand, the study by Xia et al. (2019) reported an improvement in the sperm viability of varicocele-induced rats after the administration of GYY4137 (20 mg/kg). However, the difference in results could have been affected by several factors such as concentration and different species used in this thesis.

Some studies observed the antioxidant properties of GYY4137 on several tissues and cells such as neurones (Kimura & Kimura 2004), pulmonary epithelial cells (Han et al. 2011), gastric epithelial cells (Yonezawa et al. 2007) and spermatic cells (Wang et al. 2017; Xia et al. 2019; Pintus et al. 2020). Wang et al. (2017) reported that administration of GYY4137 had positive effect on mice spermatozoa submitted to the oxidative stress induced by the application of LPS. The treatment alleviated the sperm parameters (concentration, motility) damaged by the oxidative stress. Xia et al. (2019) induced the oxidative stress on rat spermatozoa by performing experimental varicocele. Authors reported that varicocele impaired the sperm parameters such as concentration, motility and viability. The varicocele-induced rats were injected with GYY4137 (20 mg/kg), which effectively improved all mentioned parameters. Other study by Pintus et al. (2020) evaluated the total antioxidant capacities of GYY4137 (150, 300, 600, 1200, and 2400 μM) at 20, 120, and 210 minutes. The study reported that GYY4137 showed increased levels of total antioxidant capacity at all concentrations tested. Moreover, the levels of GYY4137 increased during the time period and were significantly higher after 210 minutes than after 20 minutes of incubation at the highest concentrations tested. According to the observed increase over time, the authors suggested that the total antioxidant capacity of GYY4137 might increase even after the 210 minutes of incubation. In this thesis, this hypothesis was tested. However, due to the limited volume of sample available in this thesis, the antioxidant capacity of GYY4137 was evaluated only at day 7. Overall, both treated groups of GYY4137 (0.5, 1 mM) showed higher antioxidant capacity over the non-treated CTR group, which supports what was previously found by Pintus et al. (2020). The results also indicate that the highest concentration of GYY4137 (1 mM) shows higher antioxidant capacity over to the lowest concentration (0.5 mM).

Several studies have previously investigated the effect of cool storage on tomcat sperm quality (Angrimani et al. 2017, Gañán et al. 2009, Tittarelli et al. 2006). According to those studies, the semen quality (in terms of sperm parameters) decreases over the time of storage. In this thesis, the tomcat spermatozoa were stored in cool storage at 5°C during 7 days. The results of this thesis agree with what was found in previously mentioned studies.

The evaluation of the sperm motility in this thesis resulted in significant decrease at day 7 in all subsequent evaluations (subjective motility, movement quality, SMI). Similar results were reported in the studies by Angrimani et al. (2017) and Gañán et al. (2009). Both studies reported decreased total and progressive motility after three days (72 hours) of incubation. Moreover, Gañán et al. (2009) reported significant decrease in the quality of movement and SMI at 72 hours of incubation. However, our evaluation did not reveal any significant

decrease at day 3 (72 hours), but rather suggests that the decrease began between day 3 and 7, which differs with the results of Gañán et al. (2009). The decrease in motility after 48 hours of cool storage was observed in the study by Tittarelli et al. (2006). The effects of cool storage after 48 hours were not investigated in this thesis, as the first evaluation was performed at day 3 (72 hours), however previously mentioned studies suggest that the motility might decrease even before 72 hours of incubation.

The effect of cool storage on acrosomal integrity was investigated by several studies (Perez-Marin et al. 2017, Angrimani et al. 2017, Gañán et al. 2009). None of those studies observed any significant effect of cool storage on acrosomal integrity of tomcat spermatozoa. However, in contrast to those studies, our evaluation resulted in significant decrease of acrosomal integrity in CTR groups at day 3 and 7 compared to day 0. The study by Perez-Marin et al. (2017) stored the tomcat epididymides at 4 °C for 12 hours. The authors reported that at the time of evaluation, the cool storage did not affect the acrosomal integrity of epididymal spermatozoa. According to Angrimani et al. (2017), the cool storage at 5°C also did not significantly affect the acrosome integrity of tomcat epididymal spermatozoa during the 72 hours of storage. Similar investigation done by Gañán et al. (2009) reported no significant effect of cool storage at the same temperature (5°C) on tomcat spermatozoa during 72 hours. Filliers et al. (2008) also investigated the effect of cool storage on parameters of tomcat spermatozoa, but compared to other studies, the sperm samples were stored at 4°C for 10 days. The acrosomal integrity was evaluated for each day of this study, however no significant difference was observed. The results of those studies suggest that the cool storage has no effect on acrosomal integrity of tomcat spermatozoa.

The evaluation of plasma membrane integrity in this thesis did not reveal any significant differences among CTR groups during the 7 days of incubation. Following our results, we might suggest that the cool storage does not significantly impair the sperm membrane integrity for at least 7 days. Those results are in agreement with other studies in cats (Tittarelli et al. 2006; Filliers et al. 2008; Angrimani et al. 2017). Whereas the study by Tittarelli et al. (2006) investigated the effects of cool storage for three days (72 hours), the study by Filliers et al. (2008) continued with the evaluation to the day 10. Despite the difference in storaging time, none of these studies observed any difference in the plasma membrane integrity at any day. Angrimani et al. (2017) observed significant decrease in sperm membrane integrity between 48 and 72 hours. The decrease in plasma membrane integrity between 48 and 72 hours could be caused by maintaining the epididymides in the

refrigerator until processed, compared to other mentioned studies, where the epididymes were processed as soon as possible.

7 Conclusion

The findings from this thesis provides evidence that GYY4137 at both concentrations tested (0.5 mM, 1 mM) shows antioxidant properties after 7 days of sperm storage at 5 °C. However, both concentrations have neither beneficial nor detrimental effects on motility, plasma membrane and acrosome integrity of tomcat epididymal spermatozoa stored at 5 °C during 7 days. It is worth to note that there was a tendency for higher sperm motility and acrosome integrity in samples supplemented with GYY4137 at 0.5 mM compared to GYY4137 1 mM and control group. Moreover, some of the findings of this thesis (e.g., motility, acrosomal integrity) are not consistent with other studies investigating the effects of GYY4137 on sperm function. Those differences in results could be caused by different concentration, species or storage conditions used. The findings from this thesis also confirm that the the cool storage negatively affects the sperm motility (day 7) and acrosomal integrity (day 3 and 7). In contrast the integrity of sperm plasma membrane is not affected by cool storage. Overall, the results of this thesis confirm the antioxidant properties of GYY4137 and suggest that, at least at the concentrations 0.5 and 1 mM, this slow-releasing H_2S donor does not enhance sperm lifespan of tomcat epididymal spermatozoa stored at 5 °C up to seven days.

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9 List of abbreviations

3-MST	3-mercaptopyruvate sulfurtransferase
ART	Assisted Reproductive Techniques
CBS	Cystathionine β-synthase
CSE	Cystathionine γ-lyase
ERC	Excess Residual Cytoplasm
GYY4137	Morpholin-4-ium-4-methoxyphenyl(morpholino) Phosphinodithioate
IPBES	Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services
IUCN	International Union for Conservation of Nature
LPS	Lipopolysaccharide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
PBS	Phosphate buffered saline solution
PNA-FITC	Peanut Agglutinin-fluorescein Isothiocyanate
RLU	Relative Light Unit
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RSS	Reactive Sulfur Species
SMI	Sperm Motility Index
TAC	Total Antioxidant Capacity