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Effect of aminoguanidine supplementation on sperm cryopreservation in the domestic cat

Diploma Thesis

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Reprodukční biotechnologie

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Declaration

I declare that the Diploma Thesis “Effect of aminoguanidine supplementation on sperm cryopreservation in the domestic cat“ represents my own work and all the sources I used in the process of writing it are listed in the Bibliography.

In Prague, 14.4.2022 _____

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Summary

The effects of antioxidant supplementation of sperm samples intended for cool storage and cryopreservation have been investigated in multiple species during the past several decades. Various antioxidants have the ability to protect spermatozoa against oxidative stress by acting as free radical scavengers and oxidation inhibitors. The positive effect of aminoguanidine on sperm parameters such as motility and plasma membrane integrity has previously been documented in boar and rat. The present thesis is the first to evaluate the effect of aminoguanidine supplementation on sperm samples of the domestic cat (*Felis catus*). We hypothesized that supplementation of aminoguanidine would improve plasma membrane integrity, total and progressive motility and kinetic parameters in tomcat sperm samples subjected to cryopreservation and thawing. Sperm samples obtained from the epididymides after routine orchiectomies of 5 different domestic cats were supplemented with aminoguanidine in two concentrations- 1 mM and 5 mM, prior to cooling and cryopreservation. The samples were evaluated after 20- and 90- minutes of post-thaw incubation. The integrity of the sperm plasma membrane was evaluated with the use of a propidium iodide and carboxyfluorescein diacetate (PI/CFDA) staining solution. Total motility, progressive motility and kinetic parameters were evaluated using Computer Assisted Sperm Analysis (CASA). The age of the tomcats ranged from 9 to 11 months, the average body mass was 3,600 g. The freezing-thawing process negatively impacted all sperm parameters observed, which is in agreement with previous studies investigating the deleterious effects of cryopreservation. Progressive motility and plasma membrane integrity evaluated post-thaw showed higher values after supplementation of both concentrations of aminoguanidine. However, the difference between the CTR groups and the samples supplemented with aminoguanidine was not statistically significant. Supplementation of 1 mM and 5 mM of aminoguanidine did not have a significantly positive nor negative effect on total motility and kinetic parameters of tomcat sperm samples.

Keywords: antioxidants, free radicals, oxidative stress, sperm storage

Table of Contents

1. Introduction

Felids are among species most affected by loss of habitat and their population numbers are decreasing due to environmental changes and human action (Swanson et al. 2007). The domestic cat is a popular pet but also serves as an ambassador of its wild relatives. The biology and behaviour of felids make it difficult to obtain genetic material in the form of gametes from individuals living in the wild, so the domestic cat is often used as an animal model for research into Felid reproduction (Buranaamnuay 2018).

Assisted reproductive techniques (ARTs) are routinely used in breeding livestock and in treating fertility issues in humans (Periyasamy & Balaji 2010; Chambers et al. 2021). Although ARTs have the potential to help with managing reproduction in endangered species in zoos and in the wild, research into their use in specific species lags that of livestock and efficient protocols for individual species generally do not exist (Herrick 2019). The immediate use of sperm samples after collection is often not possible due to various reasons such as different location of individuals or the lack of appropriate mating partner. It is also important to store sperm samples for prolonged periods of time to preserve the individual's genetic material and potentially use it for evaluation or ARTs in the future.

Sperm samples can be collected *in vivo* or *post-mortem* and can be stored cooled for a short period of time or cryopreserved for longer term storage (Buranaamnuay 2017). Sperm intended for cryopreservation must be of sufficient quality since the freezing-thawing process causes damage to the cells. Cryopreservation can cause ice crystal formation and disturbances to the cell's osmotic pressure and is known to induce oxidative stress (Tiwari et al. 2022). Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) and the antioxidant capacity of the cell (Cilio et al. 2022). ROS are highly reactive metabolites that play an important role in cell signalling and processes such as hyperactivation, capacitation, and acrosome reaction (Bansal & Bilaspuri 2011). However, when their production exceeds the cell's antioxidant capacity, they can cause damage to proteins, nucleic acids, and lipids.

Sperm cells are particularly sensitive to oxidative stress due to their limited antioxidant defence. Spermatozoa are specialised cells whose purpose is to reach and fertilize an oocyte. They contain a small amount of cytoplasm, and their DNA is highly compacted which means

they are unable to synthesise new proteins (Angrimani et al. 2018). In addition, the relatively high amount of polyunsaturated fatty acids (PUFAs) in their plasmatic membrane renders sperm cells particularly sensitive to lipid peroxidation (Sanocka & Kurpisz 2004). The process of sperm collection and storage presents an additional source of potential oxidative damage to the sperm cell, so it is important to ensure optimal conditions throughout the whole process to minimize these risks. Sperm samples collected from the epididymides are especially susceptible to damage caused by oxidative stress since they contain higher levels of leukocytes and immature spermatozoa which act as an additional source of ROS (Wallach & Wolff 1995; Sharma et al. 2001, Khodamoradi et al. 2020).

Antioxidants can inhibit oxidation but also serve as free radical scavengers (Aziz et al. 2019). There is an effort to develop new ways to boost the sperm cell's antioxidant defence system by supplementing cooling and freezing extenders with antioxidants. Aminoguanidine serves as a scavenger of hydroxyl radicals and has proven effective in improving motility in spermatozoa subjected to oxidative stress (Abbasi et al. 2011; Alizadeh et al. 2016; Pintus et al. 2018).

2. Scientific hypothesis

The aim of this thesis was to determine whether the addition of different concentrations of aminoguanidine to the freezing medium may improve the quality of feline spermatozoa during cryopreservation and post-thaw incubation. The hypothesis of this study was that, in virtue of its antioxidant properties, aminoguanidine may reduce the deleterious effects of oxidative stress, which frequently arise as a consequence of freezing-thawing procedures.

The objective of this thesis was to evaluate the effect of aminoguanidine supplementation on cat sperm plasma membrane integrity, motility and kinetic parameters during post-thaw incubation.

3. Literature overview

3.1 Domestic cat (*Felis catus*)

The domestic cat (*Felis catus*) is a carnivore belonging to the family *Felidae*. The family Felidae consists of 41 species and 77 subspecies and is further divided into two subfamilies- "small cats" *Felinae* and "big cats" *Pantherinae*. The subfamily *Felinae* includes the domestic cat lineage (*genus Felis*) as well as six other lineages. Currently we recognise between four and six species in the *genus Felis*, one of which is the domestic cat (Kitchener et al. 2017). According to the International Union for Conservation of Nature (IUCN), most members of the *Felidae* family are among species greatly impacted by loss of habitat and overall decline in their population numbers and they face a serious threat to their taxa's genetic diversity. Felids are generally also affected by teratospermia which is a condition in which more than 60% of the individual's spermatozoa exhibit abnormal morphology (Pukazhenthi et al. 2001). Teratospermia negatively impacts the male's fertility especially due to poor motility of abnormal spermatozoa. According to Luvoni et al. (2003) the decrease of genetic variability caused by shrinking population sizes of most wild living felids may be to blame.

The domestic cat is a popular pet all around the world and is also the only member of the *Felidae* family that has been tamed and bred by humans for thousands of years. Human intervention and selective breeding of cats has resulted in the existence of a vast number of domestic cat breeds which differ not only morphologically or by lifespan but possess different behavioural traits too (Salonen et al. 2019). The International Cat Association currently recognizes 71 breeds of domestic cats.

3.1.1 General aspects of cat reproduction

The domestic cat is a seasonal breeder and both the female and male reproductive physiology is influenced by external factors such as the environment or photoperiod (Gobello 2022). Seasonal breeders go through cycles of reproductive activity and inactivity and their reproductive organs change accordingly throughout seasons. These changes are modulated by the hypothalamic-pituitary-gonadal axis reacting to the environment (Jiménez et al. 2015). The queen's ovulation is induced by mating or similar stimuli. However, there is now also

evidence of instances where queens ovulate spontaneously, especially if in contact with other female cats (Little 2012).

Since cats are polyoestrous long day breeders, the female oestrous cycles repeat during breeding season in healthy queens up until they fall pregnant, or the breeding season ends. Although cats can breed all year around their main mating season is associated with longer light exposure. The main factor influencing cat's seasonality is therefore photoperiod. Shorter days are associated with higher concentration of the hormone melatonin released by the pineal gland located in the brain (Little 2012). According to Faya et al. (2011) oestrous cycles are present throughout the entire year in queens subjected to temperate photoperiod, but they show higher reproductive activity during seasons with longer photoperiod.

Tsutsui et al. (2009) described that the tomcat's reproductive physiology is also affected by photoperiod. In their study, tomcats exhibited 2.5 times higher presence of immature spermatozoa during non-breeding season as well as lower semen volume and decreased sperm motility and viability compared to samples that were acquired during breeding season. This was in correlation with lower levels of both the luteinizing hormone (LH) and testosterone. According to Blottner and Jewgenow (2007) free roaming feral domestic cats in the northern hemisphere reproduce mainly between the months of January and July.

Another factor that plays a role in the domestic cat's reproductive success is age. The queen's prime reproductive years are between 1.5 and 8 years of age, younger and older queens tend to have irregular cycles (Griffin 2001). According to Diagone et al. (2012) tomcats between the age of two to four years exhibit larger size of Leydig cells than other age groups which could be related to this age group's better reproductive results. They have also found that there is a higher occurrence of spermatogonia irregularities in tomcats over the age of 6 years. However, according to Gobello (2022) the effects of age on testicular function are not well understood in the domestic cat.

The domestic cat presents a great model animal for research into reproductive health and physiology of Felids. Since the numbers of most of the wild living felids are decreasing, it is becoming progressively harder to develop protocols for assisted reproductive techniques (ARTs) for each species individually; the cat's role as a taxa ambassador is therefore very

important, even though there are apparent differences in weight, size, behaviour, and habitat (Swanson 2006).

Tomcat orchiectomy is a routinely performed surgical procedure often carried out to avoid unwanted breeding but also to curb behavioural issues such as aggression, urine marking or excessive roaming in search of a mate. It has also proven effective in reducing the prevalence of viral infections and sexually transmitted diseases among free roaming tomcats (Wongsaengchan & Mckeegan 2019). Castration can also be used to prevent orchitis or hormone-induced diseases such as testicular cancer (Oliveira et al. 2010). The domestic cat is therefore a great animal model for research into reproductive health of Felids since it is relatively easy to obtain their testes compared to their wild feline relatives. The domestic cat has also shown as a useful *in vitro* model for sperm capacitation (França & Godinho 2003).

3.2 Anatomy and physiology of tomcat reproductive system

The tomcat's gonads are testes. They are located outside of the male's body in the scrotum and are the site of sperm and hormone production. The location of the scrotum is ventral to the anus, and it is covered by fur (Raisi & Davoodi 2022). Testes are a paired organ and form during embryonic development, however, the mammalian gonad goes through an indifferent stage prior to developing into either testes or ovaries, depending on hormonal stimuli specific to X or Y chromosomes. The formation of gonads is therefore a result of gene expression. If the Y chromosome is present, testes form and secrete the Anti-Müllerian hormone (AMH) and testosterone. The role of AMH is to stop the formation of Müllerian ducts and stimulate the formation of the Wolffian ducts which develop into male reproductive organs; the epididymis, seminal vesicles, and vas deferens. Testosterone further stimulates the development of testes (Hannema & Hughes 2007).

Testes normally descent through the inguinal canal into the scrotum 19 to 41 days after birth (Zgoll & Wissforf 1998) in tomcats. Their descent is crucial in keeping them below the body temperature by several degrees Celsius which is an important evolutionary adaptation ensuring the male gametes stay viable (König & Liebich 2003). Testicular temperature is further regulated by muscles and blood vessels; the dartos and cremaster muscles and the pampiniform plexus. The pampiniform plexus is a network of small veins located in the spermatic cord and it ensures that the arterial blood entering the testes is cooled. The dartos

muscle is located underneath the skin of the scrotum, the cremaster covers both the spermatic cord and testes. The purpose of these muscles is to reduce the surface area of the scrotum by contracting and they also lower and lift the scrotum depending on the environmental temperature (Aspinall 2011). These mechanisms further ensure that the testes temperature stays optimal.

The parenchyma of the testes is composed of seminiferous tubules. The tubules are coiled and divided by interlobular tissue into pyramidal lobules. The epithelium of the seminiferous tubules consists of different developmental stages of cells undergoing spermatogenesis and Sertoli cells lining the walls of the tubules. Spermatogonia are located closer to the basal part of the seminiferous epithelium. As spermatogenesis progresses, spermatocytes, spermatids, and spermatozoa proceed further towards the lumen of the seminiferous tubules. Leydig cells are interspaced in the interstitial tissue of the seminiferous tubules alongside fibroblasts, arterioles, venules, capillaries, and lymphatic vessels (Heyn et al. 1997). In the domestic cat, Leydig cells can also be found in the interlobular tissue (König & Liebich 2003). The seminiferous tubules continue into rete testis, connecting them to the efferent ducts and the head of the epididymis.

Epididymides are a narrow, convoluted duct and their length varies between species. Morphologically, the epididymis is divided into three main segments with different functions: the head (caput), the body (corpus) and the tail (cauda) of the epididymis, as shown in **Figure 1.** These segments can, however, be further divided into smaller regions depending on their function and length among mammalian species (França et al. 2005). In the domestic cat, we distinguish between six different regions that vary by function. The first four regions play a role in spermatozoa maturation and the last two serve as the storage site for mature spermatozoa before ejaculation (Liman et al. 2019).

The epithelium lining the epididymal duct consists mainly of principal and basal cells as well as apical, halo, clear and narrow cells (Cornwall 2008). The ratio of these cells varies between the different segments of the duct in relation to their function, however the most prevalent cell type is the principal cell (Cornwall 2008). The most important function of these cells is secretion and absorption. This is crucial in creating the specific microenvironment necessary for sperm maturation and its further transport and storage in the epididymal cauda.

The epididymis continues into the deferent duct which leads out of the scrotum into the peritoneal cavity and joins the urethra. The urethra runs all the way to the tip of the penis and represents the end opening of both the reproductive and urinary system in males. It is supplied by the secretions of the prostate and the bulbourethral glands. These glands contain components important for sperm survival in the female reproductive tract, proteins that play a role in sperm-oviduct binding prior to fertilization, and proteins that serve as decapacitation factors (Henry et al. 2015). The opening of the urethra points caudally in tomcats and is ventral to the anus for behavioural reasons- the tomcat marks its territory by spraying urine upon surfaces at eye level (Aspinall 2011).

The tomcat's penis consists of the urethra surrounded by cavernous erectile tissue. The caverns fill up with blood during sexual arousal which causes the tissue to swell and become erect. *Os penis* is present and located within the erectile tissue in tomcats. When flaccid, the feline penis is covered by the prepuce. The surface of the tomcat's *glans penis* is covered with backward facing spines (Englar 2019). The penile spines are supposed to stimulate ovulation since the queen's ovulation is provoked (Aronson & Cooper 1967). The presence of these keratinized papillae is testosterone sensitive which means they are not prominent in tomcats that have undergone orchiectomy (Cooper & Aronson 1974).

3.3 Spermatogenesis, sperm maturation and ejaculation

Spermatogenesis can be defined as the production of spermatozoa from spermatogonia through division and differentiation (Gilbert 2000). It takes place in the epithelium of the seminiferous tubules within the testis and as it progresses, the more differentiated cells move closer to the lumen of the tubules (Kanatsu-Shinohara & Shinohara 2013). Spermatogenesis is a complex process that begins at puberty and continuously occurs throughout the male's life. This pattern can be seen mainly in non-seasonal and slightly seasonal species, whereas in species such as the red deer, spermatogenesis regresses at the end of the breeding season and recrudesces again with the start of the next breeding season (Lincoln 1992). Spermatogenesis first begins at around the age of 5 months in domestic cats and is induced by heightened levels of testosterone that stimulate the first meiotic processes. However, tomcats do not reach sexual maturity until the age of 7 to 12 months. Young tomcats under the age of 8 months generally produce significantly lower volumes of sperm cells (Axnér

2008). Spermatogenesis does not reach its full efficiency potential until after puberty and although tomcats can reach sexual maturity before the age of 1 year, their epididymal sperm concentration is lower compared to tomcats over the age of 1 year. However, there are no differences in any other sperm and testicular parameters (Pintus et al. 2021).

Spermatogenesis is regulated by the hypothalamic-pituitary-gonadal axis and the main hormones involved are the follicle stimulating hormone (FSH) released by the pituitary gland, androgen binding protein (ABP) produced by Sertoli cells and testosterone produced by Leydig cells in the testes (Diagone et al. 2012).

Spermatogenesis consists of three main stages- spermatocytogenesis, meiosis and spermiogenesis (Chocu et al. 2012). Throughout the whole process a portion of the cells goes down the route of apoptosis instead of continuing proliferation. In the domestic cat, this occurs especially during spermatocytogenesis and meiosis (Blanco-Rodríguez 2002). According to França and Godinho (2003) spermatogenesis takes approximately 46.8 days in the domestic cat, and it consists of 4.5 cycles of proliferation. The duration of spermatogenesis varies in mammalian species. The domestic cat is among domestic and laboratory animals with shorter duration of spermatogenesis. For example, in dogs, spermatogenesis takes on average 61.2 days (França et al. 2005). Giannakara et al. (2016) state that variation in spermatogenesis duration within the same species can be influenced by environmental conditions such as sperm competition.

The first stage of spermatogenesis; spermatocytogenesis is the process of proliferation of spermatogonia and it involves mitotic cell division (Gobello 2022). Spermatogonia are immature cells derived from primordial germ cells that form the seminiferous tubules. According to Gilbert (2000) we differentiate between several stages of spermatogonia. A1 spermatogonia are the result of primordial germ cell division during embryonic development. These cells begin their further division at puberty. This next round of mitotic division produces a population of new A1 spermatogonia capable of self-renewal as well as a population of A2 spermatogonia which undergo another round of mitotic division, creating A3 spermatogonia and A4 spermatogonia. All type A spermatogonia are said to possess the ability to undergo self-renewal. The A4 spermatogonia can therefore either produce more A4 spermatogonia as a result of their division or they can differentiate into the intermediate spermatogonium which

mitotically divides to form type B spermatogonia. Type B spermatogonia further mitotically divide to generate primary spermatocytes (Gilbert 2000).

At the next stage, primary spermatocytes undergo meiosis. The first meiotic division results in a pair of secondary spermatocytes. Finally, the second meiotic division generates four spermatids. Meiotic recombination during spermatogenesis is one of the key factors in ensuring genetic diversity (Wang et al. 2019). During meiosis, the cell's chromosome number reduces, the spermatids genome is therefore haploid (Kotaja 2014).

Spermiogenesis is the third stage of spermatogenesis. During spermiogenesis, spermatids differentiate into spermatozoa. After meiosis, spermatids appear round and lack the flagellum which renders them immobile. They must undergo further changes to gain motility and to be able to successfully fertilize an oocyte (Gilbert 2000). An important step is the formation of the acrosome derived from the Golgi apparatus. The acrosome is attached to the nucleus and forms an outer layer over its apical end (Kretser et al. 1998). On the other side of the cell, the distal centriole develops into a formation of microtubules- the axoneme. The axoneme forms the core structure of the flagellum. The mid-piece of the flagellum is composed of circularly arranged mitochondria wrapped around the axoneme of the flagellum. Finally, the chromatin of the cell rearranges, most of the histones in spermatids are replaced by protamines, and the nucleus condenses. The head elongates and becomes more compact, ejecting the surplus of the cytoplasm in the form of a cytoplasmic droplet (Zini & Agarwal 2011).

After spermiogenesis, sperm cells leaving the testis are immature and incapable of fertilizing an oocyte (França et al. 2005). Post-testicular maturation is a crucial part of the sperm cell development; however, its duration is not yet known in the domestic cat (Hassan et al. 2021). Before reaching the cauda of the epididymis, spermatozoa must undergo physiological and morphological changes. The shape and proportions of the sperm cell organelles and structures, as well as the composition of its cytoplasmic, acrosomal and membrane proteins change (Olson et al. 2002). As spermatozoa move through the epididymis, the proximal cytoplasmic droplet migrates further down the flagellum to a more distal position. In the cat, this occurs in the last region of the middle segment of the epididymis and although the effects of failed droplet migration on fertility have not been studied specifically in the domestic cat, studies in other mammals such as

bulls suggest a negative effect of the presence of proximal droplets on the sperm cell's ability to fertilize an oocyte (Axnér 2006).

The purpose of the last segment of the epididymis, the cauda, is storage of mature spermatozoa before ejaculation. The concentration of spermatozoa increases from the caput to the cauda of the epididymis which indicates absorption of fluid (Axnér et al. 1999). According to Axnér (2006) dehydration of sperm cells and their high concentration plays a role in keeping the spermatozoa in an inactive state and therefore prolongs their viability. Other factors such as low pH or low concentration of Na^+ and Ca^{2+} contribute to spermatozoa entering the quiescent state (Hamamah & Gatti 1998).

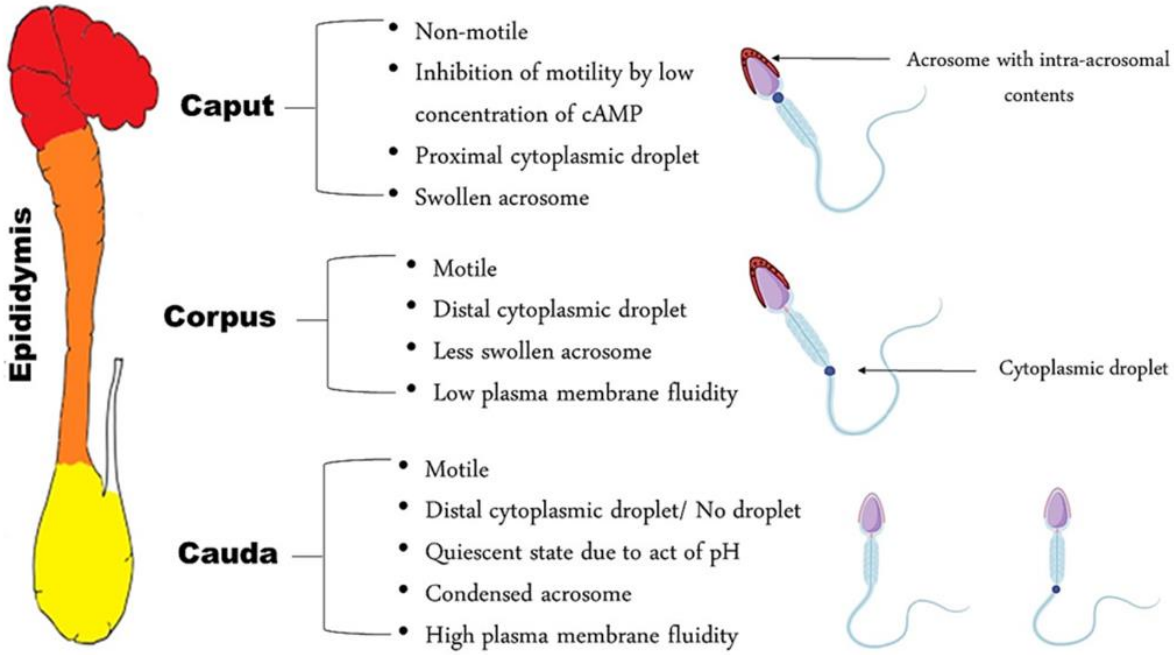

Figure 1. Schematic representation of spermatozoa characteristics in each epididymal segment (Hassan et al. 2021).

During ejaculation, the secretion of the prostate and bulbourethral glands is released into the urethra. Seminal plasma contains enzymes and other proteins as well as lipids and elements such as Na^+, K^+, P, Mg^{2+}, Cl and Ca^{2+} (Zambelli et al. 2010). The pH of the seminal plasma is higher than that of the epididymal fluid which further supports the claim that lower pH levels play a role in inducing the quiescent state in spermatozoa within the cauda of the epididymis.

The secretion of seminal fluid and spermatozoa during ejaculation consists of two phases. The phase during which seminal plasma and spermatozoa from the cauda of the epididymis secrete into the urethra is called emission. Emission is followed by expulsion, the process during which the ejaculate rapidly advances forward through the urethra and exits the male's body through the tip of the penis. These processes are regulated by a series of reflexes that include parasympathetic and sympathetic actions and muscle contractions (Lucio 2011).

3.4 Sperm cell structure and function

Spermatozoa are highly specialized cells containing genetic material, and their role is to transport it into the next generation by fertilizing an oocyte. The sperm cell is one of the smallest cells in the mammalian body, it is smaller than the oocyte and is also the only cell that must leave the individual's body to fulfil its mission. Compared to other mammalian cells, the spermatozoon is highly modest: it lacks certain organelles and most of the head is occupied by its genetic material. The purpose of this selective elimination of some organelles and the adaptation of others during spermatogenesis is to compliment the organelles of the oocyte, which is, contrary to the spermatozoon, rich in cytoplasm and possesses a stockpile of mRNAs and proteins. These molecules are very scarce in the spermatozoon in order to maintain the cell's smaller size and motility (De Jonge & Barratt 2017).

All mammalian spermatozoa can be divided into two main parts: the sperm head, and the flagellum, also known as the tail. The head contains the nucleus carrying the gamete's haploid genetic information in the form of DNA arranged in chromosomes. Another organelle located in the head of the sperm is the acrosome which is a specialized secretory vesicle containing enzymes that play a crucial part in penetrating the oocyte's zona pellucida. Some of these enzymes also catalyse the acrosome reaction (Alberts et al. 2002). Other important structures are the equatorial segment which forms a cuff around the middle part of the spermatozoon during and after the acrosome reaction and the post acrosomal region which plays an important role in binding of the sperm with the oocyte (Temple-Smith et al. 2018). The head is connected to the flagellum by the neck or connecting piece.

The flagellum is the structure responsible for the sperm cell's propulsion. It can be divided into three distinct parts; the midpiece, principal piece, and the end piece (Temple-

Smith et al. 2018). The mammalian flagellum consists of nine outer and two central microtubule doublets as well as accessory proteins that comprise the axoneme. There are also nine outer dense fibres that are paired with the nine outer microtubule doublets. These dense fibres are anchored in the connecting piece of the flagellum. Mitochondria are elongated and tightly packed around the outer layer of the dense fibres of the midpiece and form the mitochondrial sheath. Mitochondria take part in generating energy needed for sperm movement. The surface of the principal piece is covered by a fibrous sheath whose role is to maintain the integrity of the axoneme whilst movement occurs (Lindemann & Lesich 2016). The end piece of the flagellum contains the axoneme surrounded by the plasma membrane, the fibrous sheath and outer dense fibres are not present (Lehti & Sironen 2017).

The morphology of spermatozoa is specific to the given species, the shape of the head and the length of the flagellum are rather diverse in mammals. Barbosa et al. (2019) note that the spermatozoon of the domestic cat is an elongated ovoid shaped cell and the total size of the cat spermatozoa evaluated in their study was approximately 59 µm. The tail of the cat sperm cell takes up ¾ of the total length of the cell, the shortest part of the spermatozoon is the head, with the midpiece coming in second.

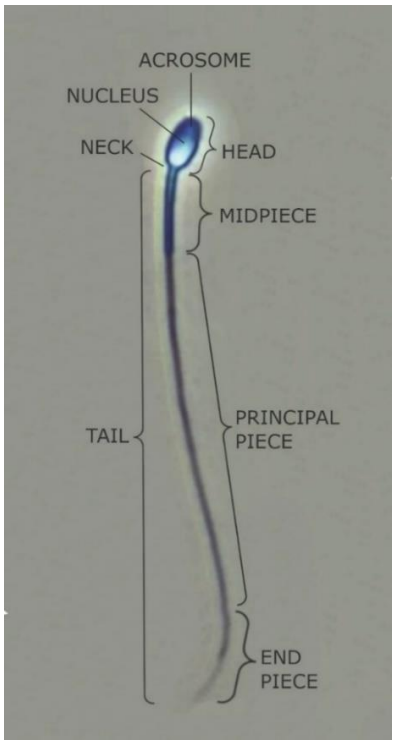

Figure 2. Sperm morphology in the tomcat (Pintus et al. 2021).

3.5 Oxidative stress

Oxidation is defined as the loss of an electron, while reduction is the gain of an electron. Oxygen is a highly electronegative atom and therefore tends to take an electron from other molecules. Reactive oxygen species (ROS) are metabolites derived from the reduction of oxygen and possess an unpaired electron which renders them more inclined to pair with other electrons and makes them considerably reactive (Peña et al. 2019). This group of metabolites includes both free radicals such as, for example, the superoxide anion ($O_2^{-\bullet}$), the hydroxyl radical ($OH^{\bullet}$), the lipid peroxide radical ($LOO^{\bullet}$) and non-free radicals including hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2) which serves as a precursor of $HO^{\bullet}$. Reactive oxygen species also include alkoxyl radicals ($RO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$) and organic hydroperoxides (ROOH) as well as free nitrogen radicals which are considered a subclass of ROS. Nitric oxide ($NO^{\bullet}$) is one of the free radicals belonging to this subclass (Sutovsky et al. 2006, Bansal & Bilaspuri 2011).

Oxidative stress is caused by imbalances between the production of ROS and the cell's or tissue's ability to break these products down (Pizzino et al. 2017). An excess of ROS can cause oxidative damage to proteins, nucleic acids and lipids that finally leads to the cell's death. DNA fragmentation and lipid peroxidation are frequent subjects of research focused on oxidative damage. The level of lipid peroxide content of human spermatozoa was in fact one of the first indicators of oxidative stress associated with a decrease in sperm motility (Jones et al. 1979).

Free radicals and other oxidants can be derived from both endogenous and exogenous sources and can have adverse effects on many different biological molecules- nucleic acids, proteins, and lipids. Endogenous sources of ROS include cellular organs such as mitochondria, peroxisomes, and endoplasmic reticulum. These are all organelles with high oxygen consumption (Phaniendra et al. 2015). Most intracellular ROS originate in mitochondria which are the site of cellular respiration. Cellular respiration generates adenosine triphosphate (ATP) from electrochemical energy. A loss of an electron of the mitochondrial transport chain may occur during this process (Peña et al. 2019). Exogenous inducers of oxidative stress include pollution, alcohol and tobacco consumption, heavy metals, pesticides and various drugs and medications (Phaniendra et al. 2015).

The main factors contributing to accumulation of high levels of ROS in spermatozoa *in vitro* are the exposure of the cells to an inadequate environment as well as various techniques used in manipulating with, evaluating, and storing spermatozoa. The cell also often lacks the ability to defend itself through its endogenous mechanisms (Baldi et al. 2020). The latter is particularly prevalent in individuals with spermatogenesis abnormalities and infections associated with inadequate antioxidant defence mechanisms (Bansal & Bilaspuri. 2011).

Oxidative stress plays a role in the metabolism of all different kinds of cells, not just spermatozoa. It can therefore impact not only sperm cells but also other cells of the male reproductive system and can further influence the efficiency of spermatogenesis and other physiological processes.

3.5.1 Nitric oxide (NO•)

Nitric oxide is a highly reactive free radical generated by nitric oxide synthases (NOSs) which convert L-arginine to L-citrulline. We distinguish between three isoforms of NOS that are involved in producing NO•: the neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Phaniendra et al. 2015). In the domestic cat, the expression of all three isoforms of NOS in sperm cells has been documented (Liman & Alan 2016).

NO• is a soluble gas and according to Farhad et al. (2011) may act as a pro-inflammatory and anti-inflammatory mediator in various types of tissue. One of the sources of NO• are macrophages which play a role in the organism's primary immune defence. The iNOS isoform is expressed in response to inflammation in activated leukocytes which are one of the main sources of ROS in semen (Gulcin 2020).

NO• is a small hydrophobic molecule and has a half-life of just a few seconds. It can diffuse through membranes and serves as a signalling compound between cells (Herrero et al. 2003). It causes smooth muscles in the walls of blood vessel to relax so its presence consequently lowers blood pressure. NO• also influences sperm motility and processes such as capacitation, hyperactivation and acrosome reaction (Herrero et al. 1994, Herrero et al. 1999). Low concentrations of NO• and NO-releasing compounds stimulate these processes and have a positive impact on sperm motility. However, high concentrations of NO• have the opposite effect (Staicu & Parra 2017). Higher concentrations of both nitric oxide (NO•) and

peroxynitrite ($ONOO^-$) have also been found in enlarged testicular veins and may play a role in causing oxidative stress in individuals suffering with varicocele (Alizadeh et al. 2016).

3.5.2 Effects of oxidative stress on spermatozoa

Oxidative stress is associated with a decrease in the sperm fertilizing ability. However, certain levels of free radicals generated as a by-product of the respiratory chain reactions is necessary for the physiological function of sperm cells, especially during hyperactivation, capacitation and acrosome reaction, as well as during sperm-oocyte binding and fusion leading up to fertilization (Rivlin et al. 2004, Chi et al. 2008). When produced in small quantities, ROS promote processes such as cell signalling which is involved in cell differentiation, proliferation, and migration (Bansal & Bilaspuri 2011).

Ejaculate consists mostly of mature spermatozoa but may contain immature sperm cells, round cells from different stages of spermatogenesis, epithelial cells and cells that are involved in the immune response- leukocytes. The presence of these cells should however be rare in healthy individuals. Activated leukocytes and immature spermatozoa are thought to be the main endogenous sources of overproduction of ROS leading to oxidative stress. Fertilization capacity and viability of spermatozoa both decrease with higher concentration of granulocytes and macrophages (Sharma et al. 2001). These activated leukocytes cause an increase in the production of proinflammatory cytokines and decrease the antioxidant capacity of the cell which causes an imbalance and leads to oxidative stress (Agarwal et al. 2014).

Immature, abnormal, and damaged spermatozoa are another source of oxidative stress. During spermatogenesis, spermatozoa eject most of their cytoplasm. However, damaged spermatozoa fail to do so. This excess residual cytoplasm affects the sperm's motility and fertilization ability by activating the nicotinamide adenine dinucleotide (NADPH) system which in effect generates ROS (Agarwal et al. 2014). According to Peña et al. (2019) a frequently overlooked source of ROS are dead spermatozoa.

Agarwal et al. (2014) state that the two main physiological culprits involved in ROS generation are the NADPH oxidase system at the membrane level and the nicotinamide adenine dinucleotide (NADH)-dependent oxido-reductase reaction which takes place in mitochondria. Spermatozoa produce adenosine triphosphate (ATP) as a source of energy

through glycolysis and oxidative phosphorylation, the latter during mitochondrial respiration. Seminal plasma is rich in sugars such as glucose and fructose and is therefore the main source of ATP supporting the cell's motility (Sutovsky et al. 2006). The production of ATP in spermatozoa is key in ensuring the cell's motility.

One of the detrimental effects of excess ROS production is the alteration of the molecular composition of spermatozoa organelle structures, especially the plasma membrane. The plasma membrane of mammalian spermatozoa is very high in phospholipids, sterols, and saturated and polyunsaturated fatty acids (PUFAs). Lipids are responsible for the fluidity of the membrane and change their composition throughout spermatogenesis, epididymal maturation and physiological processes which take place in the female reproductive tract, one of which is capacitation. The high content of these molecules renders spermatozoa particularly susceptible to ROS induced damage (Sanocka & Kurpisz 2004). Spermatozoa are especially vulnerable to lipid peroxidation. The double bonds of PUFAs cause them to be more likely to react with ROS and start a cascade of chemical reactions that change the fluidity, composition, and structure of the cell's membrane. By-products of lipid peroxidation can also disrupt proteins of the electron transport chain in mitochondria and decrease the mitochondrial membrane potential. This leads to a decline in ATP production. These changes can lead to loss of motility and overall reduction of fertilizing potential of the sperm cell (Takeshima et al. 2021).

Another way oxidative stress negatively impacts spermatozoa is by causing damage to the genetic information of the gamete- its DNA. Oxidative stress can damage DNA directly or indirectly through sperm caspase or endonuclease activation (Takeshima et al. 2021). The spermatozoa's chromatin structure is different from that of somatic cells. The sperm chromatin undergoes structural remodelling during spermatogenesis and histones and other chromosomal proteins associated with DNA are replaced by protamines. These alterations also ensure the cell is in a transcriptionally inactive state leading up to fertilization and protect its DNA from damage (Bennetts & Aitken 2005). However, even with these mechanisms in place, the sperm cell's DNA is not exempt from fragmentation. Since the DNA has a double-helix structure, fragmentation can occur on either just one strand or on both strands simultaneously. Once the spermatozoa's nucleus condenses during epididymal maturation

the cell loses the ability to repair any DNA damage. The oocyte possesses mechanisms to repair the breaks in sperm DNA during early embryonic development, but this ability decreases with maternal age (Takeshima et al. 2021).

The haploid paternal genome combines with that of the oocyte after fertilization and has a further role in regulating the early embryo's development since it also carries important epigenetic information (Peña et al. 2019). According to Toor and Sikka (2019) free radicals can cause epigenetic alterations of DNA through changes to the methylation pathways which plays a role in regulating the expression of a multitude of genes.

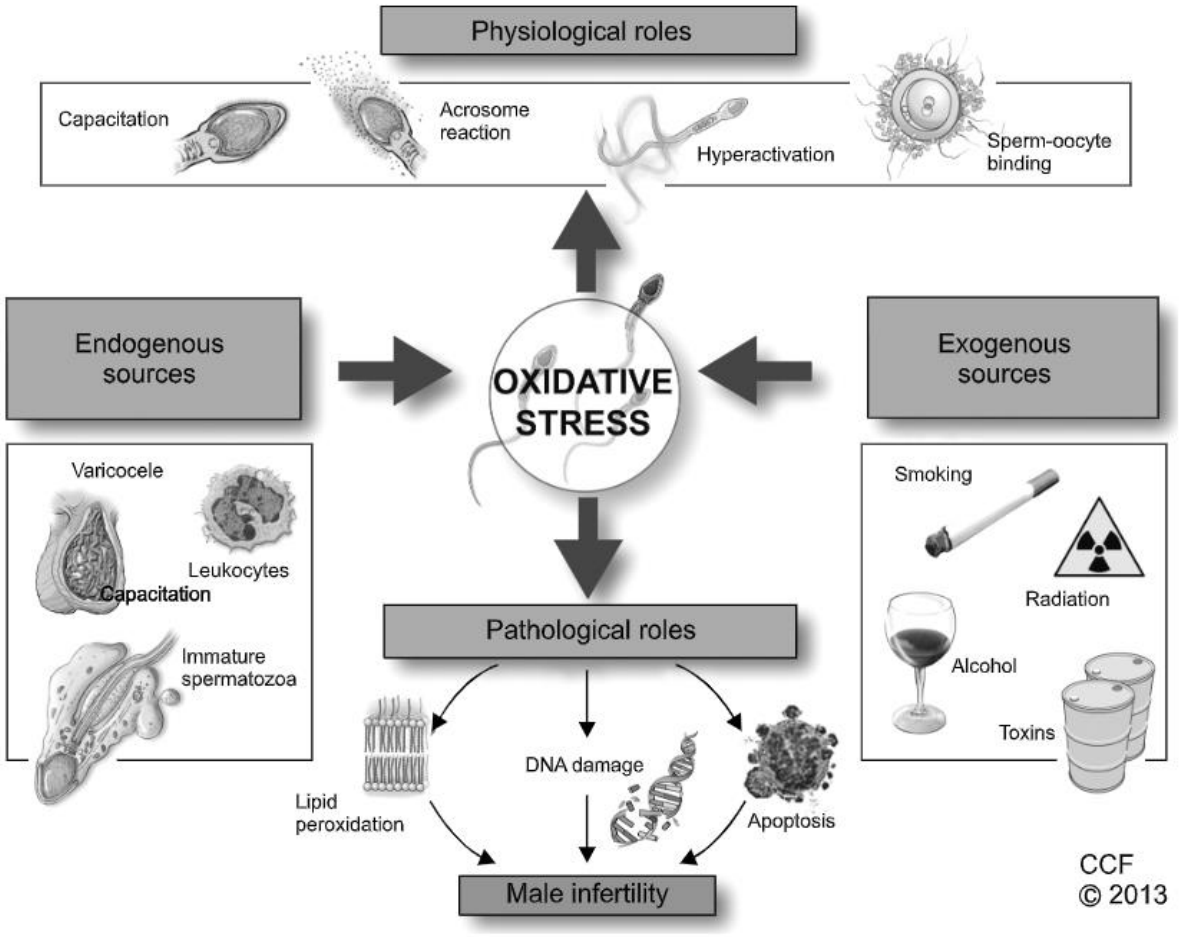

Figure 3. Oxidative stress in male reproduction (Agarwal et al. 2014)

3.6 Sperm antioxidant defences

Spermatozoa's unique structure and biochemical composition make them especially vulnerable to oxidative stress. Their survival and quality depend on the presence of antioxidants originating from both the male and female reproductive tract (Aitken & Drevet 2020). These various molecules possess the ability to protect cells and organisms against oxidative deterioration and are a subject of interest for researchers in different fields, including reproduction. Antioxidants inhibit oxidation of different substances, but they also act as free radical scavengers by reacting with these molecules. They can do so through accepting or donating electrons to cancel out the unpaired status of the free radicals (Aziz et al. 2019).

According to Aziz et al. (2019) antioxidants can be classified for example according to their solubility, size, or kinetics. Based on their activity, we distinguish between enzymatic and nonenzymatic antioxidants. Enzymatic antioxidants can break down free radicals in several steps in the presence of molecules containing elements such as copper (Cu), manganese (Mn), iron (Fe), or zinc (Zn). This group includes antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). Nonenzymatic antioxidants interrupt the free radical's reaction chains and include, among others, ascorbic acid (vitamin C), α tocopherol (vitamin E), coenzyme Q10, L-carnitine, carotenoids, and glutathione (Takeshima et al. 2020).

Both seminal plasma and the cytoplasm of spermatozoa contain endogenously produced antioxidants that protect spermatozoa from oxidative stress via prevention, interception, and repair (Takeshima et al. 2020). These molecules include enzymatic antioxidants such as CAT, SOD or GPX (Tavilani et al. 2008).

Antioxidants inhibit the activity of free radical producing enzymes such as NADPH oxidase and xanthine oxidase (XO) (Aziz et al. 2019). Since increased amounts of ROS are detrimental to spermatozoa and influence their ability to fertilize an oocyte there is an effort to develop additional ways of boosting the cell's natural antioxidant capacity with supplementation. In recent years there has also been an effort to identify alternative natural sources of antioxidants, for example of plant origin (Gulcin 2020). The effects of oral

antioxidant supplementation on human pregnancy outcomes are being studied especially in the context of in vitro fertilization (IVF) an intracytoplasmic sperm injection (ICSI). Gharagozloo and Aitken (2011) as well as Li et al. (2022) note that although a positive impact of antioxidant supplementation has been recorded in management of oxidative stress in spermatozoa, further studies into pregnancy and miscarriage rates are needed. Li et al. (2022) also state that different antioxidants vary in their effects on sperm parameters and should therefore be used accordingly.

3.6.1 Aminoguanidine

Aminoguanidine (AG) is a specific inhibitor of the inducible nitric oxide synthase (iNOS), an isoforms of nitric oxide synthase (NOS), and it has the ability to reduce $NO^{\bullet}$ content in various tissues (Abbasi et al. 2011). Aminoguanidine also serves as a free radical scavenger. Among these free radicals are H_2O_2, HClO and $ONOO^-$ (Yildiz et al. 1998).

In a study from 2018, Pintus et al. concluded that boar spermatozoa exposed to induced oxidative stress and supplemented with aminoguanidine showed an increase in sperm velocity, acrosome integrity and plasma membrane integrity as well as reduced lipid peroxidation levels compared to the control group. Their results indicate that aminoguanidine proves effective in protecting boar spermatozoa against oxidative stress *in vitro* and does not show signs of cytotoxic effects.

Abbasi et al. (2011) state that aminoguanidine directly scavenges hydroxyl radicals and inhibits DNA damage. They conducted a study involving varicocelized rats and concluded that individuals which have been administered with intraperitoneal injections of aminoguanidine showed increased DNA repair *in vivo*. Alizadeh et al. (2016) have conducted a similar study in which they subjected rats with high levels of oxidative stress caused by varicocele to injections of aminoguanidine. The supplementation of aminoguanidine prior to sperm evaluation appeared to have a positive effect on improving motility and other parameters of the rat's spermatozoa. Although there are several studies focusing on rat and boar spermatozoa, the effect of aminoguanidine supplementation has not yet been researched in the cat.

3.7 Sperm collection and storage

Artificial reproductive techniques (ARTs) are becoming more widely used not only in management of livestock reproduction but also in the field of wild animal reproduction. ARTs are a useful tool within conservation programs of endangered species (Swanson 2006). Sperm cells represent one of the biological materials used as a genetic resource. It is important to store genetic material of endangered species both in the form of sperm and oocytes and the domestic cat is used as an animal model for improving old and developing new methods of collection and storage of wild felid sperm (Buranaamnuay 2018).

Sperm collection in felids is slightly complicated by their biology and behaviour. The use of an artificial vagina is only possible in tomcats that have been previously trained for it and requires a teaser female (Prochowska et al. 2015). The use of electroejaculation has certain ethical implications, and some researchers note that the composition and quality of sperm obtained via electroejaculation may be different than that of sperm obtained by natural ejaculation (Zambelli et al. 2010). Another disadvantage is the need of anaesthesia and the cost of the equipment. However, electroejaculation has proven practical especially in males that are not trained for the artificial vagina and in wild felids. Retrieval of epididymal spermatozoa by slicing can be time consuming since the sample does get contaminated by cells and debris and its limitations also include the fact that this method can only be used once on each male since it requires surgical removal of the testes by orchiectomy. Other methods include the flushing of the epididymis, vas deferens and epididymal duct which can also only be done after the testes are removed during castration or *post-mortem* (Hori et al. 2015). One of the more recently introduced methods of sperm collection is urethral catheterization (Zambelli et al. 2006). Jelinkova et al. (2018) note that semen collected by electroejaculation and epididymal slicing after orchiectomy is of better quality than sperm collected by urethral catheterization after medetomidine administration. However, Prochowska et al. (2016) state that spermatozoa collected by urethral catheterization are not ejaculated but expelled into the urethra which may cause contact with urine and debris. Both studies emphasize the importance of further research into the effects of different collection methods on the quality of sperm.

The sperm cell's viability outside of the male's body can be prolonged using cool storage or cryopreservation. Cool storage is commonly used for samples that are intended for evaluation or further use within a short period of time from collection whereas cryopreservation can be used for storing sperm cells for prolonged periods of time. Gañán et al. (2009) state that cat spermatozoa collected from epididymides can be stored at 5°C for up to 3 days and can still be successfully cryopreserved after. Angrimani et al. (2018) note that to ensure high quality of the sample, sperm collected from tomcat epididymides can be stored at 5°C for only 48 hours, spermatozoa stored at 5°C for 72 hours exhibit lower progressive motility. According to Harris et al. (2001) 50% of spermatozoa in cat sperm stored at 4°C for more than 14 days remain motile. Sperm intended for cryopreservation should not be subjected to prolonged cooling periods prior to freezing (Gañán et al. 2009). The freezing-thawing process further influences motility and other sperm parameters so it is important to ensure spermatozoa used for cryopreservation are of sufficient quality (Angrimani et al. 2018).

The most commonly used method of cryopreservation involves the use of liquid nitrogen (LN_2) (Buranaamnuay 2018). Different freezing protocols are used depending on further intent with the sample and on availability. We distinguish between fast freezing, slow freezing and cryopreservation using a programmable freezer. Slow freezing consists of cooling the sample from room temperature to 5°C followed by gradual freezing. The major downsides of slow freezing are the possible ice crystal formation in the case of quick temperature changes and cell shrinking caused by the cooling rate being too slow (Sharma et al. 2015). An optimal cooling rate is therefore essential for the method's success. During rapid freezing, samples are subjected to an equilibration period at 4-5°C and exposed to nitrogen vapours first before being fully submerged in liquid nitrogen itself at -196°C. Programmable freezers have the advantage of being more precise, however, they can be less efficient than manual methods since the samples release heat once they are placed onto the plate in the freezer. This can influence the cooling rate (Holt 2000).

Prior to cooling and cryopreservation, sperm is diluted and in the case of cryopreservation, cryoprotectants are added. The use of cryoprotectants during cryopreservation is crucial in preventing ice formation in sperm cells and in ensuring the cell's osmotic pressure and pH remain optimal. The most frequently used permeable cryoprotectant is glycerol, routinely combined with egg yolk (Buranaamnuay 2017). Luvoni et al. (2003) note

that since spermatozoa are sensitive to high concentration of glycerol, the most commonly used concentration is only 4%. The addition of saccharides such as glucose or lactose provides extracellular energy to sperm (Luvoni et al. 2003). Freezing extenders can also contain antibiotics to prevent any bacterial contamination (Sharma et al. 2015).

3.7.1 Use of antioxidants during cool storage and cryopreservation

Cryopreservation exposes spermatozoa to damage caused by formation of intracellular ice crystals and by high osmotic and thermal stress (Tiwari et al. 2022). The process of cryopreservation and thawing also promotes excessive generation of ROS (Lucio et al. 2016). The sperm cell's cytoplasm is reduced and contains limited amounts of antioxidants (Angrimani et al. 2018). A rich source of antioxidants is seminal plasma. It contains antioxidants such as L-carnitine and taurine, however, according to Luvoni et al. (2003) the presence of seminal plasma during storage can negatively affect the sperm fertilizing ability in the cat. Human seminal plasma contains motility inhibitors derived from semenogelin I and II (Yoshida et al. 2003). Cat seminal plasma contains semenogelin II (Mogielnicka-Brzozowska et al. 2020). Prolonged exposure of spermatozoa to seminal plasma can be detrimental to the cell's motility and viability, although Chi et al. (2008) note that removing seminal plasma entirely is counterproductive since it does contain endogenous antioxidants.

The addition of antioxidants into freezing media has recently been explored as part of an effort to develop new cryopreservation protocols that insure better protection against the adverse effects of oxidative stress. Positive effects of various antioxidants on spermatozoa have been documented in a variety of species. Luvoni et al. (2002) evaluated the effect of taurine supplementation on cat sperm parameters and concluded that sperm motility post-thaw was better preserved in spermatozoa subjected to taurine. Sperm from the flat-headed cat (*Prionailurus planiceps*) subjected to glutathione peroxidase (GPX) had higher motility and greater mitochondrial membrane potential compared to the control group in a study conducted by Thuwanut et al. (2011). Taylor et al. (2009) described a positive effect of vitamin E supplementation on human post-thaw sperm motility. Vitamin E supplementation and cysteine supplementation also improved cat sperm motility in a study conducted in 2008 by Thuwanut et al. However, not all antioxidants proved effective in improving sperm parameters

post-thaw. Supplementation of catalase (CAT) and superoxide dismutase (SOD) proved to have no positive effect on sperm parameters such as viability, acrosome integrity or motility in cats (Thiangtum et al. 2009). The benefits of antioxidant supplementation are, in many cases, inconclusive and there should be an effort to further explore the option of antioxidant supplementation in cryopreservation extenders, especially to establish antioxidant combinations and doses for individual species.

4. Material and methods

4.1Animals

Sperm samples were collected from the epididymides of 10 healthy privately-owned tomcats after orchiectomies performed at the veterinary clinic Tervet in Řevnice. We began collecting the samples at the start of March 2021, the last sample was collected at the start of July 2021. Although a total of 10 orchiectomies were performed, only samples with ≥13 $\times 10^6$/ml sperm concentration and/or ≥60% total motility were used for the experiments and are shown in **Table 1**. There was also an issue with two of the samples during cryopreservation so a total of 5 tomcats were included in the study. The average age of the tomcats was 10.2 months with the youngest being 9 months and the oldest 11 months of age.

4.2Sample collection and preparation

The tomcat body mass was measured to the nearest 1 g using a Kruuse 250 weight scale prior to administering anaesthesia. Testes and epididymides enclosed by the tunica vaginalis of tomcats were removed at the clinic by bilateral orchiectomy under general anaesthesia (Medeson 1 mg/ml- 0.1 ml/kg; Nalgosed 10 mg/ml- 0.05 ml/kg). Samples were submerged into tubes with physiological solution consisting of 0.9% (w/v) NaCl and 10 µg/ml gentamicin immediately after the procedure. The samples were then kept at room temperature up until their arrival at the laboratory. The samples were processed within a 3-hour period after castration.

The first step in preparing the samples for further processing was to separate the epididymides from the testes using a sterile scalpel blade. Both the epididymides and the testes were weighed to the nearest 0.001 g (HM-120, A&D Company, Tokyo, Japan) separately before further analysis. Then the testes were discarded. Both epididymal caudae were placed in a glass Petri dish and carefully minced with a sterile scalpel blade into 800µl of tris-citrate-fructose (TCF; 3.025% Tris, 1.7% citric acid, 1.25% fructose, 0.06% sodium benzyl penicillin, 0.1% streptomycin sulphate, w/v) and incubated for 10 minutes at room temperature to ensure spermatozoa release into the solution (Hermansson & Axnér 2007). At the end of the incubation, 10 µl of the sample was collected in order to assess the sperm concentration using

a Bürker chamber. Another small aliquot of the sperm sample (approximately 50 µl) was diluted into TCF to a final concentration of 10-15 x 10^6 spermatozoa/ml and incubated for 20 minutes in a water bath at 38 °C in order to evaluate the sperm motility and plasma membrane integrity in the fresh sample (only control group, CTR). The rest of the sample was further diluted 1:1 into TCF supplemented with 8% glycerol. The final sperm and glycerol concentration was 10-40 x 10^6 spermatozoa/ ml and 4%, respectively. The sample was subsequently divided into three groups at random: the control group (CTR), the sample supplemented with 1 mM of aminoguanidine, and the sample supplemented with 5 mM of aminoguanidine. The aminoguanidine solution was prepared by dissolving aminoguanidine hydrochloride into a phosphate buffer saline (PBS) solution immediately before usage. The CTR group was supplemented with PBS only.

4.3Sperm cryopreservation and thawing

Prior to cooling and freezing, the samples were individually packaged in 0.25 ml straws and subjected to a 90-minute cooling and equilibration period at 5°C degrees. The straws reached the temperature of 5°C within one hour but were kept in for an additional 30 minutes to complete the equilibration period. A representative cooling curve is shown in **Figure 4**. The samples were then exposed to liquid nitrogen vapours for 10 minutes before being immersed in liquid nitrogen for storage. The samples were kept vertically submerged in liquid nitrogen at -196°C for at least one month before thawing.

To initiate the thawing process, the straws were removed from liquid nitrogen and immediately placed in a warm water bath at 38°C for 30 seconds. After that, the surface of the straws was wiped dry, and they were emptied into a microcentrifuge tube.

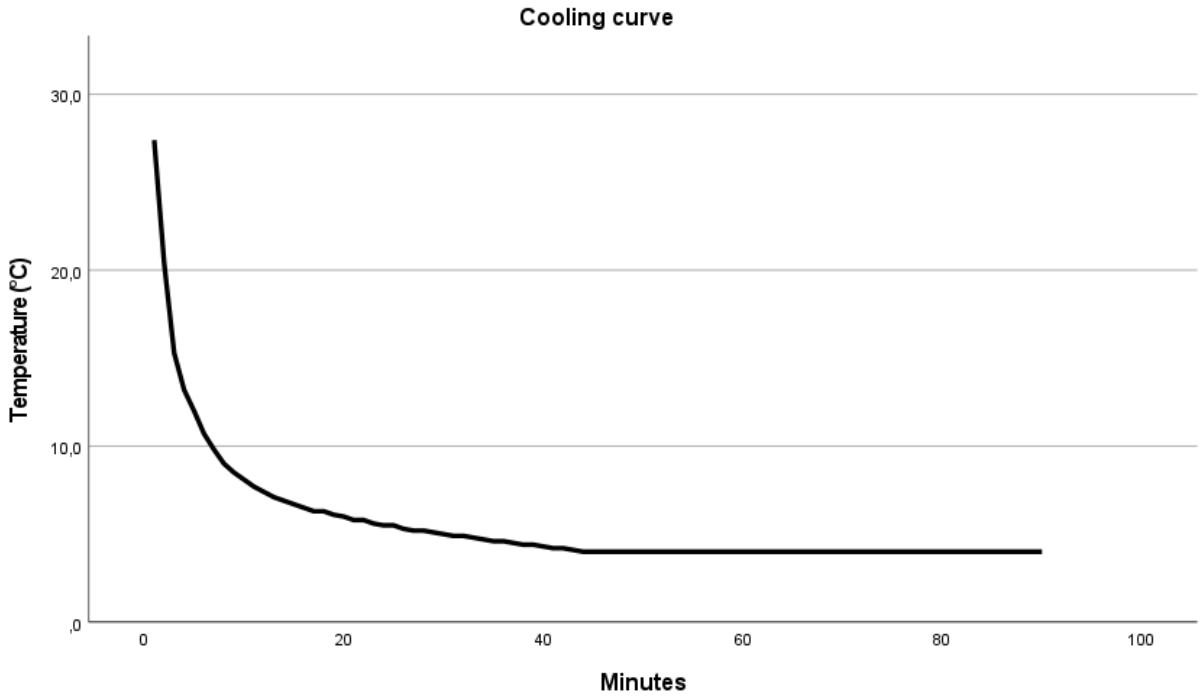

Figure 4. Representative cooling curve of cat sperm sample.

4.4Evaluation of sperm parameters

All parameters were evaluated before and after cryopreservation. Only the CTR group was used for evaluation prior to cryopreservation. The times are marked as followed: 0 mins for samples evaluated prior to freezing, 20 mins for samples evaluated 20 minutes after thawing and 90 mins for samples evaluated 90 minutes after thawing. Both pre- and post-thaw samples were incubated in a water bath at 38°C.

4.5 Plasma membrane integrity (PI/CFDA)

Integrity of the plasma membrane was evaluated by placing 10 µl of the sample into a staining solution composed of 2 µl of propidium iodide (PI 0.5 mg/ml, w/v in PBS), 2 µl of carboxyfluorescein diacetate (CFDA, 0.46 mg/ml, w/v in dimethyl sulfoxide), 1 µl formaldehyde (0.3%, v/v), and 85 µl of PBS. The sample was incubated in the dark for 10 minutes at 38°C. After the incubation period, 10 µL of the prepared mixture containing spermatozoa and the staining solution were placed onto a microscope slide and covered with a coverslip to be evaluated under an Eclipse E600 epifluorescence microscope (Nikon, Tokyo, Japan) under a magnification power of 400×. Spermatozoa showing green fluorescence over

the entire head were considered to have their plasma membrane intact, while those that showed partially, or fully red fluorescence were considered to have a damaged plasma membrane. A total of 200 sperm cells from each sample were evaluated.

4.6 Sperm motility and kinetics

The total motility, progressive motility and sperm kinetics were assessed using data obtained by Computer Assisted Sperm Analysis (CASA; NIS-Elements, Nikon, Tokyo, Japan and Laboratory Imaging, Prague, Czech Republic), which consisted of an Eclipse E600 tri-ocular phase contrast microscope (Nikon, Tokyo, Japan), equipped with a warming stage set at 38°C (Tokai Hit, Shizuoka, Japan), and a DMK23UMO21 digital camera (The Imaging Source, Bremen, Germany). At least 200 sperm cells were assessed per each sample. Contaminating cells and debris were removed manually prior to analysis to prevent them from influencing the data. The CASA was set to 60 frames per second and a minimum of 31 frames.

Total motility was calculated as the percentage of cells with the average path velocity (VAP) ≥ 10 µm/s, while progressive motility was calculated as the percentage of cells with straightness ≥ 80%.

A total of eight sperm kinetics parameters were evaluated by analysing 6 fields in each sample. Amplitude of lateral head displacement (ALH) in µm, beat-cross frequency (BCF) in Hz, linearity (LIN) in %, straightness (STR) in %, average path velocity (VAP) in µm/s, curvilinear velocity (VCL) in µm/s, straight linear velocity (VSL) in µm/s, and wobble (WOB) in % were evaluated. A representative diagram of sperm kinetic parameters (WOB excluded is shown in **Figure 5**.

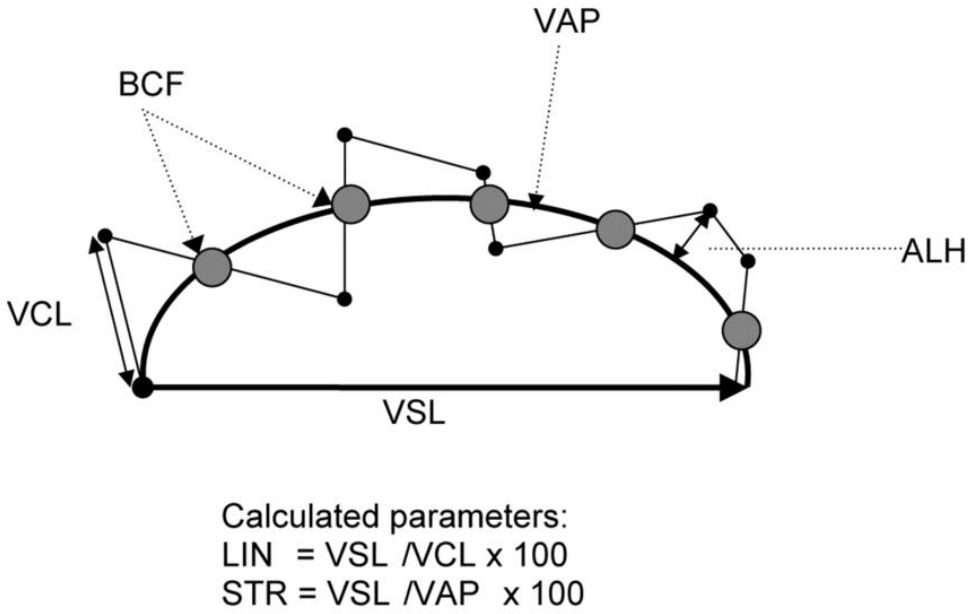

Figure 5. Sperm kinetic parameters assessed by Computer Assisted Sperm Analysis (CASA) (Agarwal et al. 2014).

4.7 Statistical analysis

The statistical analyses were performed using the SPSS 20.0 statistical software package (IBM Inc, Chicago, IL, USA). We used a generalized linear model to analyse the effect of aminoguanidine supplementation and post-thaw incubation time on plasma membrane integrity, sperm motility and kinetic parameters. Data are shown as mean ± SD. Statistical significance was set at $p < 0.05$.

5. Results

The age of the tomcats used in our study ranged from 9 to 11 months and the average tomcat weight was 3,600 g. Spermatozoa were present in the caudae of the epididymides in all tomcats, however as mentioned before, only those with sperm concentration ≥13 $\times 10^6$/ml and/or total motility ≥60% were used. The breed of all tomcats included in the study was European shorthair. An overview of chosen characteristics of tomcats, testes mass, body mass and gonadosomatic index are shown is shown in **Table 1.** The gonadosomatic index (%) represents the testes mass in proportion to the total body mass.

Table 1. Tomcat age, body mass (rounded to the nearest 1 g), sperm concentration (rounded to the nearest 0.001 × 10^6/ml), mean testes mass, mean epididymides mass (rounded to the nearest 0.001 g) and gonadosomatic index (rounded to the nearest 0.001 %).

Cat code	Age (months)	Body mass (g)	Sperm concentration (× 10^6/ml)	Mean testes mass (g)	Mean epididymides mass (g)	Gonadosomatic index (%)
G1A	11	3,500	75.625	1.326	0.236	0.038
G3A	10	3,700	29.750	1.231	0.223	0.033
G4A	11	3,500	39.375	1.490	0.272	0.042
G8A	10	3,500	32.440	1.016	0.187	0.029
G9A	9	3,800	13.000	0.536	0.146	0.014
$\bar{x}$ ± SD	10.2 ± 0.75	3,600 ± 126.49	38.04 ± 20.7	1.12 ± 0.33	0.21 ± 0.04	0.03 ± 0.01

5.1 Evaluation of plasma membrane integrity (PI/CFDA)

There were significant differences between sperm samples from the CTR group evaluated prior to freezing and post-thaw both at 20- and 90- minutes of post-thaw incubation. The CTR group evaluated prior to cryopreservation showed higher percentage of spermatozoa with intact plasma membrane in comparison with the CTR group at both 20- and 90- minutes post-thaw (*p<0.05*). There was a slight tendency for treatment of both 1 mM and 5 mM of aminoguanidine to have a positive effect on plasma membrane integrity at 20- as well as 90- minutes post-thaw, however the differences were not statistically significant (*p>0.05).* Supplementation of 1 mM of aminoguanidine and 5 mM of aminoguanidine did not have a statistically significant impact on the plasma membrane integrity (*p>0.05*).

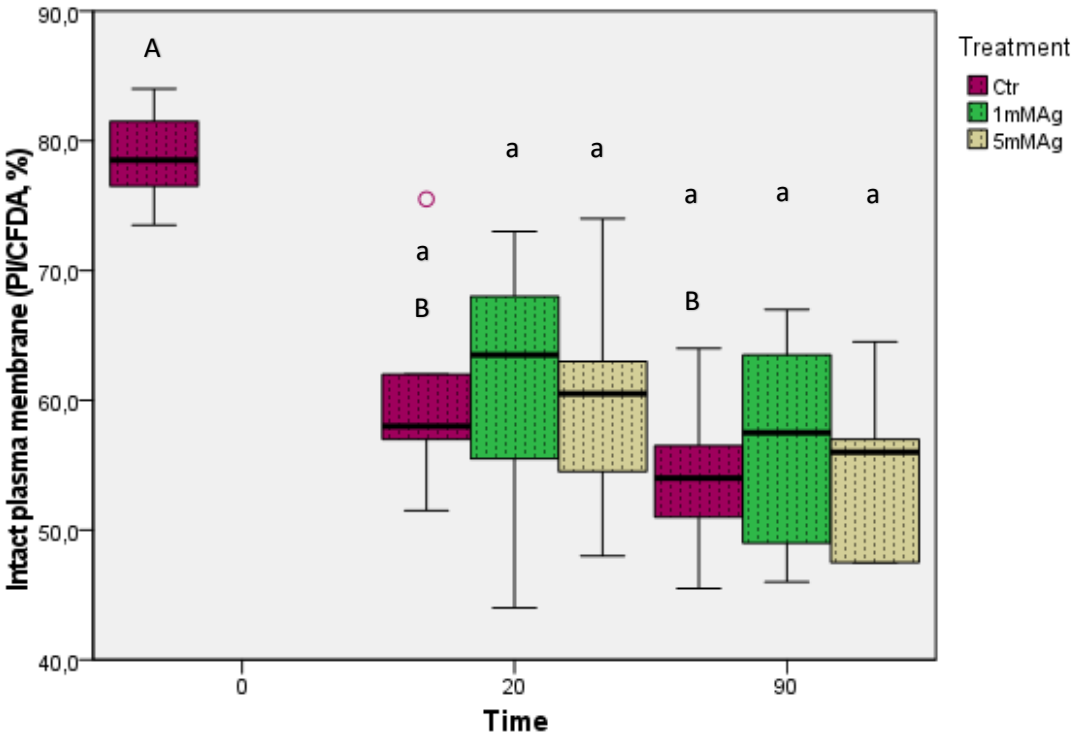

Figure 6. Sperm plasma membrane integrity (PI/CFDA) in tomcat sperm samples without treatment (CTR) and with aminoguanidine treatment of 1 mM of aminoguanidine (1 mM Ag) and 5 mM of aminoguanidine (5 mM Ag) before cryopreservation (0 min, CTR group only) and 20- and 90-minutest post-thaw. Each box contains the central 50% of the observations, the whiskers contain the central 95% of observations. Different capital letters indicate significant differences (p<0.05) between CTR

groups (A representing the highest value). Different lower-case letters indicate significant differences ($p<0.05$) among the CTR group and treatments within each incubation time (a representing the highest value).

5.2 Evaluation of sperm motility and kinetic parameters

There were significant differences in total motility of the CTR group evaluated prior to freezing and the CTR groups evaluated at both 20- and 90- minutes post-thaw (*p<0.05*). The CTR group evaluated prior to cryopreservation showed higher values of total motility in comparison with the CTR group at both 20- and 90- minutes post-thaw (*p<0.05*). Progressive motility of sperm samples in the CTR group evaluated prior to freezing did not significantly differ from progressive motility of sperm samples in the CTR group evaluated after 20 minutes of post-thaw incubation (*p>0.05)*. However, there was a decrease in progressive motility of sperm samples evaluated 90 minutes after thawing compared to the CTR group evaluated before freezing (*p<0.05*). Kinetic parameters of the CTR group were significantly lower post-thaw compared to fresh samples (*p<0.05*).

Progressive motility evaluated at 20- and 90- minutes of post-thaw incubation was higher in samples supplemented with both 1 mM and 5 mM of aminoguanidine. However, the difference between samples subjected to treatment and the CTR group was not statistically significant (*p>0.05*). Overall, the supplementation of 1 mM of aminoguanidine and 5 mM of aminoguanidine did not improve progressive and total motility nor kinetic parameters at 20 or 90- minutes post-thaw (*p>0.05*).

Table 2. Tomcat spermatozoa motility and kinetic parameters without treatment (CTR) and with aminoguanidine treatment of 1 mM of aminoguanidine (1 mM Ag) and 5 mM of aminoguanidine (5 mM Ag) before cryopreservation (0 min, CTR group only) and 20- and 90-minutes (min) post- thaw.

Treatment	Time (min)	Total motility (%)	Progressive motility (%)	ALH (µm/s)	BCF (Hz)	LIN (%)	STR (%)	VAP (µm/s)	VCL (µm/s)	VSL (µm/s)	WOB (%)
CTR	0	76.67 ± 9.5^{A}	60.38 ± 9.46^{A}	5.57 ± 0.19^{A}	17.59 ± 1.15^{A}	53.86 ± 4.89^{A}	84.08 ± 3.19^{B}	101.41 ± 5.57^{A}	156.76 ± 3.58^{A}	88.18 ± 5.92^{A}	62.6 ± 4.61^{A}
CTR	20	46.28 ± 8.62^{Ba}	45.21 ± 14.36^{ABa}	4.58 ± 0.29^{Ba}	17.62 ± 0.58^{Aa}	54.84 ± 1.33^{Aab}	88.598 ± 1.309^{Aa}	85.79 ± 6.42^{Ba}	135.8 ± 13.5^{Ba}	77.52 ± 6.54^{Ba}	61.16 ± 2.08^{Aa}
1 mM Ag	20	34.75 ± 11.34^{b}	38.68 ± 5.07^{a}	4.18 ± 0.55^{a}	16.43 ± 1.41^{a}	51.49 ± 4.25^{b}	88.198 ± 0.521^{a}	74.82 ± 12.72^{b}	124.38 ± 15.07^{a}	68.11 ± 11.57^{b}	57.26 ± 4.94^{a}
5 mM Ag	20	45.12 ± 10.42^{a}	49.84 ± 10.17^{a}	4.54 ± 0.42^{a}	17.37 ± 1.16^{a}	55.38 ± 2.46^{a}	89.583 ± 1.039^{a}	80.86 ± 5.88^{ab}	128.85 ± 13.45^{a}	73.75 ± 4.94^{ab}	61.1 ± 2.73^{a}
CTR	90	16.84 ± 3.83^{Ca}	34.79 ± 14.87^{Ba}	3.57 ± 0.25^{Ca}	13.62 ± 0.68^{Ba}	45.57 ± 1.33^{Ba}	89.977 ± 1.245^{Aa}	61.11 ± 4.934^{Ca}	116.09 ± 9.35^{Ca}	56.66 ± 4.05^{Ca}	49.82 ± 1.11^{Ba}
1 mM Ag	90	15.05 ± 4.11^{a}	44.67 ± 19.37^{a}	3.58 ± 0.47^{a}	13.62 ± 0.87^{a}	44.33 ± 1.49^{a}	89.489 ± 0.565^{a}	61.92 ± 9.398^{a}	119.76 ± 16.68^{a}	57.28 ± 8.63^{a}	48.62 ± 1.71^{a}
5 mM Ag	90	17.52 ± 4.37^{a}	43.86 ± 18.55^{a}	3.55 ± 0.43^{a}	13.25 ± 1.77^{a}	46.14 ± 4.42^{a}	90.69 ± 2.35^{a}	61.65 ± 8.89^{a}	119.03 ± 11.94^{a}	57.51 ± 8.68^{a}	50.13 ± 3.82^{a}

Different capital letters indicate significant differences ($p<0.05$) between CTR groups (A representing the highest value). Different lower-case letters indicate significant differences ($p<0.05$) among the CTR group and treatments within each incubation time (a representing the highest value). Data are shown as mean ± SD.

ALH= amplitude of lateral head displacement; BCF= beat cross frequency; LIN= linearity; STR= straightness; VAP= average path velocity; VCL= curvilinear velocity; VSL= straight linear velocity; WOB= wobble.

6. Discussion

A significant proportion of spermatozoa sustain damage to their plasmatic membrane during *in vitro* manipulation and lose their fertilizing ability (Pukazhenti et al. 1999). This is especially prominent in felids since the majority of felid species is affected by teratospermia (Pukazhenthi et al. 2001). Teratozoospermic samples exhibit lower viability and patterns such as retained cytoplasmic droplet, acrosomal defects and abnormalities in sperm chromatin and tend to be more susceptible to oxidative stress (Luvoni 2006).

Cryopreservation is a method routinely used to store livestock and human spermatozoa and there is now also a growing interest in developing freezing protocols for pets and endangered animal species. The domestic cat serves as a model animal in research into wild felids reproduction (Prochowska et al. 2015). The process of cryopreservation and thawing exposes sperm to oxidative stress which has a negative impact on the spermatozoa's fertilizing capacity due to the decrease in motility and plasma membrane integrity (Thiangtum et al. 2009; Tiwari et al. 2022). Excessive production of reactive oxygen species (ROS) can be offset by enzymatic and non-enzymatic antioxidants that can inhibit oxidation or serve as free radical scavengers (Cheuquemán et al. 2018; Aziz et al. 2019). The sperm cell possesses endogenous antioxidant defence mechanisms; however, these might be impaired during *in vitro* manipulation and storage (Bensal & Bilaspuri 2011).

Several recent studies have explored the possibility of antioxidant supplementation to prevent oxidative stress during sperm cooling and cryopreservation (Luvoni et al. 2002; Pintus et al. 2018; Tiwari et al. 2022). Supplementation of freezing extenders with antioxidants minimises the negative effects of ROS and has proven effective in improving the quality of tomcat spermatozoa after thawing (Luvoni 2006; Cheuquemán et al. 2018). Certain antioxidants such as vitamin A can be toxic and harmful to cells in higher concentration (Salehi et al. 2018). Another such example is hydrogen sulfide (H_2S) which is a potentially toxic gas that also possesses antioxidant properties and is effective in preventing oxidative stress in spermatozoa (Pintus et al. 2020). It appears that the benefit of these molecules depends on the dosage (Salehi et al. 2018). Silvestre et al. (2021) note that one of the factors influencing the outcome of antioxidant supplementation is the antioxidant concentration. Excessively

high antioxidant concentration may have a detrimental effect on sperm parameters. It could be beneficial to access the oxidation level of sperm samples prior to freezing and at different times of post-thaw incubation before antioxidant supplementation.

Aminoguanidine inhibits the activity of inducible nitric oxide synthase (iNOS) and serves as a ROS scavenger. iNOS is one of the isoforms responsible for generating nitric oxide ($NO^{\bullet}$) in a range of cells including spermatozoa and its activity is induced by exposure to cytokines and bacterial products as part of an immune response in leukocytes (Dixit & Parvizi 2001). Therefore, leukocytes present an additional source of ROS, and their presence has a negative impact on sperm quality. Leukocytes are more prevalent in samples obtained from the epididymis since the mincing of the epididymal caudae often causes cuts to capillaries (Khodamoradi et al. 2020). Samples collected from the epididymides are more likely to contain excess amounts of ROS. Supplementation of iNOS inhibitors such as aminoguanidine could be a way to prevent additional damage caused by oxidative stress in epididymal samples especially if they are intended for cooling or cryopreservation.

According to Sannomiya et al. (1997) aminoguanidine has the ability to prevent leukocyte-endothelial interactions in rats with diabetes mellitus. Several recent studies also focused on the potential benefits of aminoguanidine therapy in patients undergoing radiotherapy (Ekici et al. 2016). Aminoguanidine proved effective in reducing oxidative stress in rat kidney and testis cell lines subjected to radiation. Aminoguanidine also served as an iNOS inhibitor in rat hepatocytes and was able to scavenge hydroxyl and peroxyl radicals in the lungs of rats (Ekici et al. 2016; Saadat et al. 2019). It has also been deemed beneficial in ameliorating cardiovascular function in rats subjected to inflammation (Beheshti et al. 2020).

The protective antioxidant properties of aminoguanidine have been studied in the context of reproduction in boar and rat (Abbasi et al. 2011; Alizadeh et al. 2016; Pintus et al. 2018). Aminoguanidine administered subcutaneously had a positive effect on sperm viability, mitochondrial membrane potential and motility in varicocelized rats (Alizadeh et al. 2016). Varicocele causes mitochondrial membrane dysfunction which results in excess generation of ROS. Increased levels of $NO^{\bullet}$ in testicular veins are especially prevalent in human patients suffering with varicocele (Mitropoulos et al. 1996).

Supplementation of aminoguanidine proved to be effective in preserving sperm motility, acrosome integrity and plasma membrane integrity in boar spermatozoa *in vitro* (Pintus et al. 2018). However, authors also note that higher concentrations of aminoguanidine result in a decrease in progressive and linear motility. As previously mentioned, spermatozoa require the presence of ROS to maintain normal function especially in relation to cell signalling involved in processes such as hyperactivation. A decrease in kinetic parameters could in this case be explained by a reduction in ROS production.

The use of antioxidant supplementation in felid reproduction has previously been explored by multiple authors and the results vary (Thuwanut et al. 2008; Thuwanut et al. 2010; Thuwanut et al. 2011; Macente et al. 2018;). One of the antioxidants investigated was vitamin E. Thuwanut et al. (2008) found vitamin E to have a positive effect on progressive motility and membrane integrity of spermatozoa post-thaw. Their samples were supplemented with 5 mM of water-soluble vitamin E analogue (Trolox). In a study done by Macente et al. (2018) sperm samples supplemented with 0.3 mM, 0.6 mM, and 0.9 mM of vitamin E analogue (α-tocopherol) did not exhibit an improvement in progressive motility post-thaw. Macente et al. (2018) note that one possible explanation could be the variability between media used for cryopreservation as well as a difference between isoforms of vitamin E and the concentration used for the treatment.

The effects of aminoguanidine in the context of reproduction have not previously been studied in Felid species. The interspecies variability of sperm characteristics such as membrane composition and endogenous antioxidant content makes it hard to apply findings from rat and boar focused studies to an entirely different species. In the present study, we investigated the effect of *in vitro* aminoguanidine supplementation on tomcat sperm parameters post-thaw. We evaluated the plasma membrane integrity, total and progressive motility, and the following sperm kinetic parameters: ALH, BCF, LIN, STR, VAP, VCL, VSL and WOB. We found that freezing and thawing lead to a decrease in total and progressive motility and negatively impacted the majority of sperm kinetic parameters. The negative effect of cryopreservation on tomcat sperm parameters and viability were previously reported by Buranaamnuay et al. (2017). Samples from the CTR group evaluated at 90 minutes of post-thaw incubation showed higher STR than samples evaluated prior to freezing which could indicate a decrease in movement speed. The freezing and thawing process also negatively

impacted the integrity of the sperm plasma membrane which is in agreement with a study conducted by Chaquemán et al. (2018). The values significantly decreased post-thaw in both the CTR group at 20- and at 90- minutes. The plasma membrane integrity of the CTR group at both 20- and 90- minutes did not significantly differ from sample groups subjected to 1 mM of aminoguanidine and 5 mM of aminoguanidine. However, there was a slight tendency for improvement. Samples subjected to both 1 mM and 5 mM of aminoguanidine showed higher values of plasma membrane integrity compared to the CTR group at both 20- and 90- minutes. The supplementation of 1 mM and 5 mM of aminoguanidine did not significantly impact total motility nor kinetic parameters. Progressive motility evaluated post-thaw improved with supplementation of both concentrations of aminoguanidine, although the difference between samples subjected to aminoguanidine supplementation and the CTR group was not statistically significant.

Breed, age, weight, and timing of sample collection in relation to seasonality and sexual activity all potentially influence sperm concentration and sperm parameters such as motility (Axnér & Forsberg 2007). Sperm samples used in the present study were all obtained from tomcats of the same breed; therefore, the influence of this factor cannot be assessed. The influence of age is apparent since the youngest tomcat (G9A) in the study also exhibited the lowest sperm concentration of only 13×10^6/ml at 9 months. The sample with the highest concentration of 75.625×10^6/ml was obtained from tomcat G1A at 11 months. The variability of sperm concentration between samples potentially influenced the results of our study. For future evaluation of effects of various treatments including antioxidant supplementation, it would be beneficial to eliminate the high variation of sperm concentration between samples and to include a higher number of tomcats to assemble a more representative sample group.

7. Conclusion

The freezing-thawing process had a negative impact on the sperm parameters evaluated in our study. These findings are in agreement with previous studies investigating the effect of cryopreservation on tomcat sperm parameters. The supplementation of 1 mM and 5 mM of aminoguanidine did not significantly improve the integrity of tomcat sperm plasma membrane (PI/CFDA) at 20- or 90- minutes of post-thaw incubation. The total and progressive motility as well as kinetic parameters did not significantly improve with supplementation of 1 mM and 5 mM of aminoguanidine. However, the plasma membrane integrity and progressive motility were slightly higher in samples supplemented with aminoguanidine compared to the CTR group. The results from the present study indicate that supplementation of aminoguanidine does not significantly improve plasma membrane integrity, motility, or kinetic parameters of tomcat sperm. These findings are not consistent with other studies evaluating the effect of aminoguanidine on sperm quality in rat and boar. This could be due to interspecies differences in sperm parameters and sensitivity to aminoguanidine. The results were also possibly influenced by the choice of aminoguanidine concentration and by high variation between sperm sample concentration.

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9. List of abbreviations

ABP	Androgen binding protein
AG	Aminoguanidine
ALH	Amplitude of lateral head displacement
AMH	Anti-Müllerian hormone
ARTS	Assisted reproductive techniques
ATP	Adenosine triphosphate
BFC	Beat-cross frequency
CASA	Computer Assisted Sperm Analysis
CAT	Catalase
CFDA	Carboxyfluorescein diacetate
CTR	Control group
DNA	Deoxyribonucleic Acid
eNOS	Endothelial nitric oxide synthase
FSH	Follicle stimulating hormone
GPX	Glutathione peroxidase
ICSI	Intracytoplasmic sperm injection
iNOS	Inducible nitric oxide synthase
IUCN	Union for Conservation of Nature
IVF	In vitro fertilization
LH	Luteinizing hormone
LIN	Linearity
LN_2	Liquid nitrogen

mRNA	Messenger ribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
nNOS	Neuronal nitric oxide synthase
NOS	Nitric oxide synthase
PBS	Phosphate buffer saline
PI	Propidium iodide
PI/CFDA	Propidium iodide and carboxyfluorescein diacetate
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
STR	Straightness
TCF	Tris- citrate- fructose
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight linear velocity
WOB	Wobble
XO	Xanthine oxidase