Doctoral Thesis

Study on negative effects of ROS on fresh and frozen bull sperm with antioxidants treatment

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The author

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GENERAL INTRODUCTION
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General Introduction

Reproduction involves producing a new generation resembling its predecessors, with sexual reproduction combining sperm and oocyte to form a diploid zygote (Flesch & Gadella, 2000) and the process of spermatogenesis involves the multiplication of spermatogonia, meiosis of spermatocytes, and the differentiation of spermatids into sperm capable of fertilizing an egg. In mammals, spermiogenesis includes four phases: Golgi, cap, acrosome, and maturation (Abou-Haila and Tulsiani, 2000; Clermont and Leblond, 1955; Bedford and Nicander, 1971; Breed, 2004). Spermatogonia in the basal compartment are classified into type A pale (mitotic replication), type A dark (testicular stem cells), and type B (Schlatt and Ehmcke, 2014; Ehmcke and Schlatt, 2006). However, maturation of bull spermatid includes eight spermatogenic stages.

Sperm ejaculation is a complex process. After spermiogenesis in the seminiferous tubules, spermatogonia undergoes mitosis and meiosis to become spermatocytes, then spermatids, and finally mature into nonmotile spermatozoa. These spermatozoa are transported passively through testicular fluid to the epididymis, gaining motility. The vas deferens connects the ejaculatory ducts to the epididymis, which in bulls is about 40 meters long, serving for sperm transport, storage, and maturation. Sperm transport through the epididymis varies by species: about 14 days in bulls, 3 to 5 days in mice, and 11 days in rats (Fournier-Delpech and Thibault, 1993). Spermatocytogenesis in bulls takes approximately 21 days (Johnson et al., 2000).

Sperm is a specialized cell with a unique structure: the head, midpiece, and flagellum (Publicover et al., 2007). The head contains paternal DNA, minimal cytoplasm, and enzymes to penetrate the oocyte's cumulus cells and zona pellucida (Evenson et al., 2002). During maturation, 85% of histones in the nucleus are replaced by protamines, which are arginine-rich proteins linked

by disulfide bridges (Ward, 2018; Ribas-Maynou et al., 2022). The sperm head's primary role is delivering a haploid chromosome set to the female gamete (Mortimer, 1997). Maintaining the integrity of the sperm head membranes is crucial for fertilization, as only sperm with intact membranes can undergo capacitation and the acrosome reaction (Yanagimachi, 1981).

The acrosome is a specialized organelle unique to sperm, absent in somatic cells. Originating from the Golgi apparatus, it forms a cap-like structure over two-thirds of the nucleus, with inner and outer membranes (Abou-Haila and Tulsiani, 2000; Toshimori and Iti, 2003). This cap, 0.2 to 0.3 μm thick, is rich in polyunsaturated fatty acids (Saacke & Almquist, 1964) and contains hydrolytic enzymes such as protease, glycosidase, acrosin, and hyaluronidase. These enzymes facilitate sperm penetration through the zona pellucida and fusion with the oocyte (Yanagimachi, 1994; Grootegoed et al., 2000; Breitbart, 2002; Florman, 1994; Hirohashi and Yanagimachi, 2018; Dan, 1952, 1954; Darszon et al., 2011). Acrosin, synthesized as inactive proacrosin, is activated during acrosomal exocytosis (Vazquez-Levin et al., 2007). Hyaluronidase helps depolymerize the matrix between cumulus oophorus cells (Hirayama et al., 1989).

Acrosome biogenesis begins during meiosis or early spermiogenesis (Anakwe and Gerton, 1989; Escalier et al., 1991; Moreno et al., 2000) and is divided into four phases: Golgi, cap, acrosome, and maturation. The Golgi phase involves the Golgi apparatus producing glycoproteins and forming proacrosomal vesicles necessary for acrosome formation (Leblond and Clermont, 1952; Hess, 1990; Russell et al., 1993). In the cap phase, the acrosomal granule enlarges, flattens, and spreads over the nucleus to form a cap. The acrosome phase features the marginal ring associating with the growing edge of the acrosome. During the maturation phase, the acrosome condenses and attaches to the inner acrosomal membrane. Although the acrosome's basic structure

is similar across mammals, its shape and size can vary among species, including paddle, hook, and spatula-like forms (Eddy and O'Brien, 1994; Bedford, 2014).

Defects in the sperm head and abnormal acrosomes impair sperm's ability to bind to the zona pellucida and undergo the acrosome reaction (Silber, 1989; Menkveld et al., 2011; Abu Hassan Abu et al., 2012). This reaction typically occurs in the female reproductive tract (Boatman and Robbins, 1991) as sperm reach the cumulus cell-oocyte complexes.

The midpiece of a spermatozoon, located between the head and tail, contains a mitochondrial sheath coiled around the axoneme, generating ATP through oxidative phosphorylation (Bahr and Engler, 1970; Gaffney et al., 2011; Hirata et al., 2002). It is separated from the principal piece by the annulus and typically houses 70–80 mitochondria (Rajender et al., 2010; Garcia-Rincon et al., 2016; Nakata et al., 2015; Ankel-Simons and Cummins, 1996). Mitochondria are crucial for energy production, redox and calcium homeostasis, steroid hormone biosynthesis, and apoptosis regulation (Zorov et al., 1997; Boguenet et al., 2021). The size of the midpiece and mitochondrial count can vary significantly among species (Favard and Andre, 1970; Ramalho-Santos et al., 2007).

The sperm flagellum consists of four parts: the neck with two centrioles, the mid-piece with mitochondria providing energy, the principal piece with fibrous elements, and the end-piece. In bull sperm, the flagellum measures about 54 μm, though it's longer in species like rats and mice, enhancing force generation (Katz and Drobnis, 1990). The axoneme, the core structure of the flagellum, features microtubules (Tubulin α and β) arranged in a 9+2 pattern (Afzelius, 1959; Eddy et al., 2003; Kalthoff, 2001; Inaba, 2003; Link, 2001; Turner, 2003), with two central microtubules and nine outer dense fibers linked by dynein arms and radial spokes (Gibbons and Grimstone,

1960; Hopkins, 1970). Dynein, a high molecular weight ATPase, converts ATP hydrolysis into mechanical energy, crucial for flagellar motion. Dynein arms are categorized into inner and outer types; inner arms generate sliding forces between microtubules, promoting flagellar bending, while outer arms increase sliding velocity and beat frequency (Toshimori and Eddy, 2014). The outer dynein arms consist of an intermediate–light chain complex, and heavy chain structures that include an ATPase-associated ring (head), a stalk, and a stem (Schmidt and Carter, 2016). The axoneme also contains calcium-binding proteins: calmodulin, centrin/caltractin, and a light chain of the outer dynein arms (Smith, 2002). Calcium influences the axoneme by modulating flagellar bend curvature and hyperactivated motility in spermatozoa (Ho et al., 2002). Proper axoneme formation is essential for effective sperm motility and fertility.

Sperm morphology strongly correlates with sperm function and varies significantly across species. For example, sperm lengths range from 50 to 70 μm in humans, boars, bulls, goats, and rabbits, while mouse sperm measures about 150 μm, and rat sperm is even longer (Brotherton, 1975). The sperm head shape also varies round in humans and other mammals but sickle-shaped in mice and rats. This morphology affects sperm velocity, which is influenced by flagellum length and mitochondrial volume. Research shows a positive correlation between mitochondrial volume and flagellum length, indicating that sperm motility depends on mitochondrial size and number (Ruiz-Pesini et al., 1998; Cardullo and Baltz, 1991). However, longer midpieces can slow sperm movement, and sperm with longer heads and shorter midpieces swim faster compared to those with longer midpieces and shorter heads (Malo et al., 2006). Additionally, studies suggest a connection between the head and midpiece affecting sperm motility (Gage, 1998; Piasecka and Kawiak, 2003). Tail length also impacts sperm motility, as observed in primates, rodents (Froman and Feltmann, 1998), and red Iberian deer (Malo et al., 2006). Variations in sperm length are

mainly attributed to tail length differences (Cummins and Woodall, 1985). Morphological variations in the flagellum, midpiece, and principal piece contribute to adaptations for sperm survival and travel in the female reproductive tract (Maree, 2011). Defects in the midpiece and tail, which are crucial for energy production and motility, significantly impair sperm function and are key indicators of reduced fertility in humans (Kruger et al., 1988), stallions (Jasko et al., 1990), bulls (Sekoni and Gustafsson, 1987), goats, and rams (DeJarnette et al., 1992; Gravance et al., 1995).

Spermatozoa have a high and fluctuating energy demand, which they meet through glycolysis and oxidative phosphorylation (Wallimann et al., 1992; Du Plessis et al., 2015). Glycolysis primarily occurs in the sperm head and principal piece of the flagellum, while oxidative phosphorylation takes place in the mitochondria (Freitas et al., 2017; Amaral, 2022). Both pathways generate ATP, essential for sperm functions such as motility, capacitation, hyperactivation, and the acrosome reaction (Miki, 2007; Mannowetz et al., 2012; Mukai and Travis, 2012). Despite glycolysis producing fewer ATP molecules than oxidative phosphorylation, energy production varies by species. For example, human, mouse, and boar spermatozoa predominantly rely on glycolysis (Miki et al., 2004; Williams and Ford, 2001; Rodríguez-Gil and Bonet, 2016), while bull, stallion, and ram spermatozoa mainly depend on oxidative phosphorylation (Gibb et al., 2020; Gibb et al., 2014). Sperm cells switch metabolic pathways based on oxygen availability and substrate concentration, such as glucose, pyruvate, lactate, sorbitol, glycerol, and fructose (Cao et al., 2009; Williams and Ford, 2001; Hereng et al., 2011; Jones et al., 1992; Rigau et al., 2001). This adaptability ensures sustained motility throughout their journey from the epididymis through the female reproductive tract to the oocyte. Sperm flagellum compartments are crucial for compartmentalizing these metabolic pathways. Additionally, some

spermatozoa, including those from bulls, rams, dogs, and cockerels, use short-chain fatty acids as an energy source in the presence of oxygen, with species-specific differences noted (Minassian and Terner, 1966; Scott and Dawson, 1968; Poulos and White, 1973; Scott, 1973; Howarth, 1978; Mita et al., 1995). Several glycolytic enzymes, such as hexokinase, lactate dehydrogenase, and glyceraldehyde 3-phosphate dehydrogenase, are found in the head membrane, fibrous sheath, and mitochondria (Bradley et al., 1996; Westhoff and Kamp, 1997; Bunch et al., 1998; Mori et al., 1998).

Oviductal fluid is rich in substrates and cofactors crucial for oocyte maturation, fertilization, and early embryo development. It includes glucose, arginine, serum albumin, transferrin, glycoproteins, galactose, immunoglobulins, lactate, pyruvate, bicarbonate, cytokines, growth factors, amino acids, enzymes, hormones, and extracellular vesicles (Beier, 1974; Binelli et al., 2018; Rodríguez-Alonso et al., 2020; Saint-Dizier et al., 2020; Engle and Foley, 1975; Kamp et al., 2003; Mannowetz et al., 2012).

In spermatozoa, glucose is metabolized through glycolysis and the pentose phosphate pathway (PPP), which occurs in the principal piece and midpiece of the flagellum, respectively (Urner and Sakkas, 1999; Zimmermann and Geyer, 1981). Under anaerobic conditions, pyruvate is converted into lactate, generating NAD+ for glycolysis (Boguenet et al., 2021). In aerobic conditions, pyruvate enters mitochondria, is oxidized to acetyl CoA, and enters the Krebs cycle (Hildyard and Halestrap, 2003; Ferramosca and Zara, 2014). Respiratory substrates like malate and pyruvate stimulate sperm motility, while inhibitors like rotenone and cyanide impede it (Grootegoed et al., 1984; Nakamura et al., 1984; Ramió-Lluch et al., 2014; Rogers et al., 1977). Approximately 70% of ATP produced drives axonemal dynein for motility, while 30% supports substrate cycling and ionic gradients (Bohnensack and Halangk, 1986). ATP is vital for

capacitation and the acrosome reaction, essential for fertilization (Austin, 1952; Chang, 1984; Yanagimachi, 1989; Tulsiani et al., 2007).

Linear motility is essential for sperm migration from the cervix to the oviduct, features low lateral amplitude and high straight-line velocity (Shalgi et al., 1992; Ishijima, 2015). During fertilization, hyperactivation involves high curvilinear velocity and increased lateral amplitude (Suarez, 2008; Stauss et al., 1995). Spermatozoa use ATP for motility and hyperactivation while producing reactive oxygen species (ROS) through mitochondrial electron leakage.

Spermatozoa experience oxidative stress when reactive oxygen species (ROS) production exceeds normal levels or when antioxidant levels are reduced (Agarwal and Sengupta, 2020; Pereira et al., 2022). Many studies have shown that antioxidants in cryopreservation media can mitigate oxidative stress in sperm cells. It also is believed that low molecular weight non-enzymatic antioxidants play a more significant role than high molecular weight enzymatic molecules (Kovalski et al., 1992). However, it is not well understood which type of oxidative stress has the most detrimental effect on sperm motility or how antioxidants specifically reduce cytoplasmic or mitochondrial oxidative stress to maintain sperm motility.

Therefore, the purpose of this study was to use fresh bull semen samples treated with hydrogen peroxide and antimycin to elucidate the efficiency of antioxidants against both endogenous and exogenous oxidative stress. These findings could have important implications for improving the survivability of frozen-thawed bull semen.

INTRODUCTION
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Introduction

Oxidative stress exerts a profoundly detrimental effect on several vital components of spermatozoa, including the axon of the tail, the acrosome, and the midpiece. These structures are critical for the sperm's ability to move, penetrate the egg, and carry out fertilization. The tail's axon is central to the sperm's motility, the acrosome contains enzymes essential for penetrating the egg, and the midpiece houses mitochondria, which produce the energy needed for sperm motility. When oxidative stress disrupts these components, it hampers the sperm's ability to function properly.

Specifically, oxidative stress disrupts the metabolic processes that drive ATP production within the mitochondria and cytoplasm, which is critical for the energy-dependent processes in sperm. The production of ATP in sperm cells is essential for their motility and overall viability, as it fuels the movement of the flagellum and other cellular activities necessary for fertilization. When oxidative stress impairs these processes, the sperm's motility is reduced, which can lead to decreased fertilization potential (Aitken et al., 1998; Moazamian et al., 2015).

Moreover, oxidative stress triggers the apoptotic pathway within sperm cells, a process that leads to programmed cell death. This is particularly concerning because apoptosis in sperm cells results in DNA fragmentation, which can significantly impair the genetic integrity of the sperm. DNA fragmentation is a critical issue as it can lead to the failure of the sperm to fertilize the egg, and even if fertilization occurs, it may result in chromosomal abnormalities in the developing embryo. The activation of apoptosis through oxidative stress underscores the importance of maintaining a balance in ROS levels to ensure the viability and genetic integrity of sperm (Lewis and Aitken 2005; Aitken and Baker 2006; Bansal and Bilaspuri 2011; Agarwal et al., 2014b).

ROS, or reactive oxygen species, are chemically reactive molecules containing oxygen, and they are generated by both endogenous (internal) and exogenous (external) factors. Within sperm cells, ROS can oxidize lipids, proteins, nucleic acids and essential biomolecules that are critical for maintaining the structural integrity and function of sperm. Lipid oxidation can disrupt the integrity of sperm membranes, protein oxidation can impair enzymatic functions and structural proteins, and nucleic acid oxidation can lead to mutations or breaks in DNA strands. Collectively, these oxidative processes contribute to what is termed oxidative stress, which is the imbalance between the production of ROS and the body's ability to neutralize and detoxify them (Morielli and O'Flaherty 2015; Selvam et al., 2020).

Endogenously, ROS are primarily produced as by-products of ATP synthesis in the mitochondrial electron transport chain. During normal metabolic activities, these ROS are neutralized by cellular antioxidant systems, such as the glutathione reduction pathway, which helps maintain a balance. However, under conditions where there is a rapid increase in ATP production, the generation of ROS can exceed the neutralizing capacity of the cell's antioxidant systems, leading to an accumulation of oxidative stress. This is particularly detrimental within the mitochondria, where excessive ROS can lead to mitochondrial dysfunction, further exacerbating oxidative stress and reducing ATP production. Since sperm motility is heavily dependent on ATP produced by mitochondria, any impairment in mitochondrial function can significantly reduce sperm motility, leading to compromised fertilization capability (Aitken et al., 2016; Bulkeley et al., 2021).

Furthermore, mitochondrial damage is not only caused by an imbalance in ROS production but also by an excessive influx of calcium into the mitochondria. This disrupts the electron transport chain, leading to further ROS production and intensifying oxidative stress within the cell.

While temporary boosts in motility can be achieved by adding substrates like glucose and fatty acids, which supply additional fuel for ATP production, prolonged ATP overproduction without adequate ROS management can worsen oxidative stress, ultimately leading to diminished sperm motility and viability (Zhu et al., 2019; Islam et al., 2021).

Exogenous factors, such as environmental stressors or pathological conditions, can also induce oxidative stress in sperm cells. For instance, ROS produced by leukocytes-immune cells that are activated in response to bacterial infections in semen-are intended to eliminate pathogens but can inadvertently damage sperm cells in the process. This leukocyte-derived ROS can oxidize sperm components, leading to reduced motility and viability, thus impairing fertilization potential (Agarwal and Said 2005; Agarwal et al., 2014a; Fraczek et al., 2016).

Cryopreservation, a common practice in reproductive technologies for preserving sperm, also poses a significant risk of oxidative stress. The process involves rapid changes in osmotic pressure and temperature, which can generate ROS both within and outside of sperm cells. These ROS can lead to the oxidation of sperm lipids and proteins, resulting in structural and functional damage. The process of freezing and thawing can cause organelle damage, particularly in the mitochondria, leading to the release of ROS into the cytoplasm, which exacerbates lipid peroxidation and further impairs sperm motility, viability, and overall function (Morris et al., 2012; Pini et al., 2018; Chatterjee and Gagnon 2001; Chatterjee et al., 2001).

To combat oxidative stress, the body relies on antioxidants that are naturally present in seminal plasma. These antioxidants are crucial in neutralizing free radicals and protecting sperm from oxidative damage. However, in the context of frozen-thawed semen, the antioxidant defense may be compromised due to the dilution of semen or the removal of seminal plasma during the

cryopreservation process. This reduction in antioxidant activity necessitates the addition of exogenous antioxidants to counteract the increased oxidative stress (Lewis et al., 1997; Bansal and Bilaspuri 2011; Sharma et al., 2013).

Various antioxidants can be employed to protect sperm from oxidative damage. Small molecule antioxidants, such as Vitamin C, Vitamin E, glutathione, ubiquinol, coenzyme Q10, Pyrroloquinoline quinone (PQQ), and peroxiredoxins (PRDXs), directly neutralize ROS and protect cellular components from oxidative damage. On the other hand, large molecule antioxidants, including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and albumin, typically function by converting harmful ROS into less reactive molecules or by stabilizing free radicals to prevent oxidative damage. These antioxidants operate through different mechanisms, making it essential to identify which antioxidants are most effective at counteracting specific oxidative stresses to ensure the maintenance of sperm functionality (O'Flaherty and Scarlata 2022).

Given the importance of mitigating oxidative stress in sperm, this study aims to assess the effectiveness of various antioxidants in protecting against both endogenous and exogenous oxidative stress. The study will focus on using fresh bull semen samples treated with hydrogen peroxide (H_2O_2) and antimycin to induce oxidative stress. By evaluating the response of sperm to these treatments, the study seeks to identify antioxidants that can improve sperm viability, particularly in the context of frozen-thawed semen, where oxidative stress is a significant challenge. The findings from this study could have important implications for enhancing the quality and viability of sperm used in artificial insemination and other reproductive technologies.

MATERIALS AND METHODS
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Materials and methods

Semen collection and incubation of sperm

Livestock Improvement Association of Japan, INC (Tokyo, Japan) kindly provided fresh semen from Japanese breeding bulls that were collected by an artificial vagina and diluted with TRIS-egg yolk extender. The fresh semen tube was placed in thermos bottles and transported to the laboratory within 4 hours at 4°C. The fresh semen was washed twice via centrifugation (400 × g, 3 min) with the HTF medium (Umehara et al., 2020). After centrifugation, the sperm pellet was resuspended in mHTF medium containing PQQ disodium salt (BioPQQ®; Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan), ergothioneine (14905, Cayman, Ann Arbor, Michigan, USA) or Vitamin C (A5960, Sigma–Aldrich, St Louis, MO, USA), and the sperm was used for all analyses. Some samples were treated with H_2O_2 (20779-65, Nacalai tesque, Osaka, Japan) or antimycin (A8674, Sigma) to examine the effects of induced ROS. The samples were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Frozen bull semen was also gifted from Livestock Improvement Association of Japan, INC. The 0.5 ml straw of frozen semen was thawed in water at 37°C for 30 s and then immediately diluted with 6 ml of first thawing mHTF medium. In the first thawing medium, 1 μM PQQ, 100 μM ergothioneine or 1000 μM Vitamin C was added to the mHTF medium. The first thawing medium containing frozen-thawed sperm was centrifuged at 300*g* (5 min, 37°C), and then the sperm pellet was washed twice with each medium. After centrifugation, the sperm pellet was resuspended in each medium and the sperm was used for all analyses. The samples were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Assessment of Sperm Motility

Sperm motility was evaluated using a computer-assisted sperm analysis (CASA), as described in our previous study (Islam et al., 2021). A volume of 3 µL of the sample was placed in a pre-warmed counting chamber for the CASA reading after incubation of sperm at different time intervals. Sperm tracks (0.5 s, 45 frames) were captured at 60 Hz according to our previous study using a CASA system (HT CASA-CerosII; Hamilton Thorne, Beverly, MA, USA). Amplitude of Lateral Head Displacement (ALH) refers to the measure of the side-to-side movement of the head of a spermatozoon during its forward progression. Straight-Line Velocity (VSL) is the average speed of a sperm cell along a straight line from its starting point to its ending point. Curvilinear Velocity (VCL) is the average speed of a sperm cell along its actual curvilinear (non-linear) path over a given time. The measuring unit of these kinematic parameters was µms−1. At least 200 spermatozoa were assessed in each CASA analysis. Motile single sperm was calculated by multiplying motility by total concentration.

Determination of ROS by Flow Cytometry

Photo-oxidation Resistant DCFH-DA is a total ROS (H_2O_2, superoxide anion, and hydroxyl radical) probe (R253, DOJINDO LABOLATORIES Co., Ltd., Kumamoto, Japan). According to manufacturer's protocols, sperm was pretreated with a photo-oxidation-resistant DCFH-DA working solution and incubated for 30 minutes at 37°C in a humidified atmosphere of 5% CO_2 in the air. The sperm was washed twice with mHTF. After removing the supernatant, the sperm was mixed with mHTF containing antioxidants (PQQ, ergothioneine or Vitamin C) and/or inducers of oxidative stress (H_2O_2 or antimycin). After 0, 1, 2, 3 and 4-hours incubation, the samples were analyzed using a flow cytometer (Attune®NxT Acoustic Focusing Cytometer,

Thermo Fisher Scientific Inc., Waltham, MA, USA). Green fluorescence (DCF) was evaluated using a 488 nm laser and a 530/30 nm bandwidth filter. Data were expressed as the percentage of fluorescent-positive sperm. The cutoff values were set using the unstained sample. The gating strategy is shown in Fig. 1. The localization of fluorescence in sperm was determined using an APX100 Digital Imaging System (EVIDENT Co., Ltd., Tokyo, Japan).

Mitosox deep red assay

The generation of mitochondrial superoxide anion was investigated according to MitoSOX™ deep Red Assay Kit (MT-14, DOJINDO LABORATORIES). The sperm was pretreated with MitoSOX™ deep red working solution, incubated at 37°c for 30 minutes, and washed twice with mHTF. After removing the supernatant, the sperm was mixed with mHTF containing antioxidants (PQQ, ergothioneine or Vitamin C) and/or inducers of oxidative stress (H_2O_2 or antimycin). The samples were analyzed using the flow cytometer after 0, 1, 2, 3 and 4-hours incubation. The fluorescence was evaluated using a 638 nm laser and a 570/14 nm bandwidth filter. Data were expressed as the percentage of fluorescent-positive sperm. The cutoff values were set using the unstained sample. The gating strategy is shown in Fig. 1. The localization of fluorescence in sperm was determined using a Nikon AX confocal microscope (Nikon Solutions Co., Ltd., Tokyo, Japan).

Mitochondrial activity

Mitochondrial activity of sperm was measured using a MitoPT® JC-1 Assay Kit (911, Immuno Chemistry Technologies, LLC, Bloomington, MN, USA) according to our previous study. Briefly, sperm were incubated with 200 µL of working solution containing 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) dye at 37 °C for 30 min in the dark.

The sperm suspension was centrifuged and washed twice with the mHTF medium. After washing, the sperm pellet was resuspended in the mHTF medium and analyzed with the flow cytometer using a 488 nm laser and filters with a bandwidth of 530/30 and 574/26 nm. Data were expressed as the percentage of fluorescent-positive sperm. The gating strategy is shown in Fig. 1. A total of 50,000 sperm events were analyzed.

Immunofluorescence

Sperm was mounted on glass slides, air-dried, fixed with 4% paraformaldehyde for 30 min and then permeabilized with 0.3% (v/v) Triton X-100 with PBS for 30 min at room temperature. After washing with PBS, the samples were incubated with the blocking solution from MOM Kit (MKB-2213, Vector Laboratories, Newark, CA, USA) at 25 ℃ for 30 min to block nonspecific sites. The samples were then incubated at 4 ℃ overnight with primary rabbit antibodies: anti-4 hydroxynonenal antibody (4-HNE; 1:100; ab48506; Abcam, Cambridge, UK). After washing with PBS, the antigens were visualized using Cy3-conjugated sheep anti-mouse IgG (1:100; C2181, Sigma). Digital images were captured using an APX100 Digital Imaging System.

Statistical analysis

In the fresh semen study, at least three animals were used, and experiments were replicated at least three times in each group. In the frozen semen study, five animals were used. Quantitative data were presented as means ± SEM. Percentage data were subjected to arcsine transformation before statistical analysis. Motile single sperm data were analyzed in a paired t-test with p-value correction using the Bonferroni method for multiple tests. Differences between groups were assessed by one-way analysis of variance (ANOVA). When ANOVA was significant, differences among values were analyzed by Tukey's Honest Significant Difference test for multiple

comparisons. Dunnett's test was used to analyze the ROS inducer/antioxidant combination experiment (Figure 5) and the frozen sperm experiment (Figure 6). Comparison controls were listed in the figure captions. R (version 4.3.1) was used for statistical analysis. A value of $p<0.05$ was defined as a significant difference.

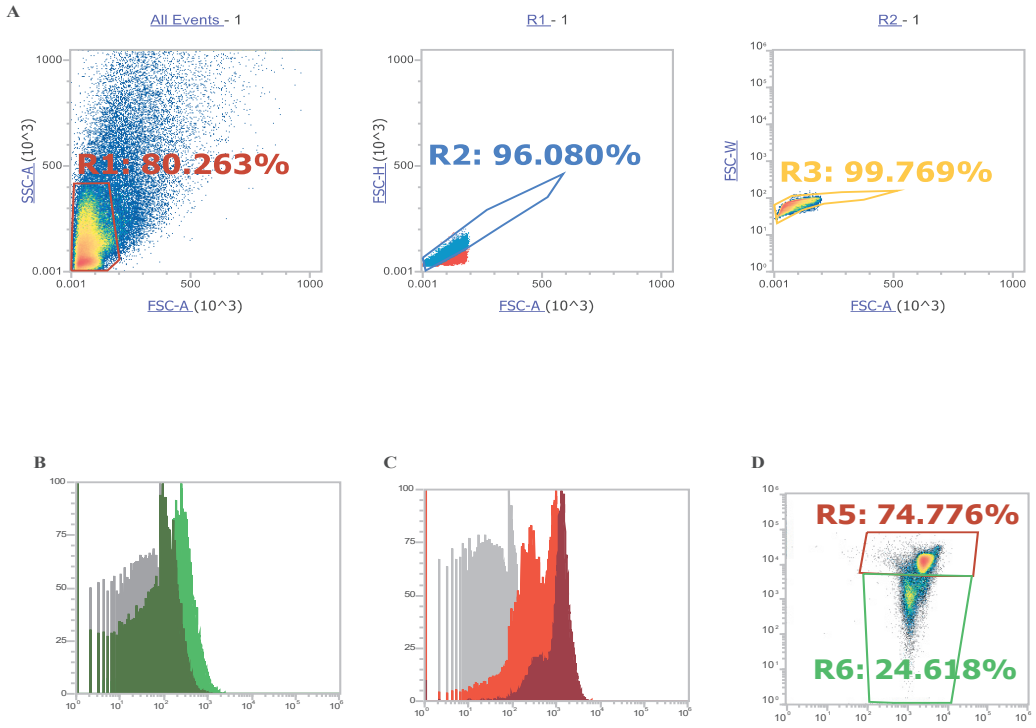

Figure 1: Gating strategy of flow cytometry. (A) Gating strategy for the selection of single sperm. Using forward scatter (FSC)-A and side scatter (SSC)-A dot plots, cells of similar size and complexity were first selected (R1). In FSC-A and FSC-H dot plots and FSC-A and FSC-W dot plots, similar-size cells were accumulated near the area; thus, using these plots, again similar-size cells were selected (R2, R3). The cells in R3 were used for the below analysis. (B) Histograms of DCFH-DA staining. (C) Histograms of mtSOX deep red staining. (D) The dot plots of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) green (x-axis) and red (y-axis). The percentage of JC-1 red-positive sperm (R5) was used for the analysis.

RESULTS
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Results

Changes in motility and intracytoplasmic and mitochondrial ROS levels of fresh sperm with incubation time

The number of motile single sperm and ALH (μm) were significantly decreased from 0 to 4 hours of incubation periods (Fig.2A, B). The VCL (μm/sec) did not differ from 0 to 4 hours of incubation periods (Fig.2B). However, in VSL (μm/sec) highly significant differences was found from 0 to 4 hours of incubation periods (Fig.2B). The ROS level was measured in the cytoplasm and mitochondria of fresh by using two different probes (DCFH-DA and mtSOX deep red). The total ROS level was significantly increased in a time-dependent manner in fresh sperm from 0 to 4 hours of incubation periods (Fig.2C). On the other hand, mitochondrial ROS levels rose sharply up to 1 hour of incubation and remained significantly higher levels for up to 4 hours. Therefore, it was shown that both total ROS levels and mitochondrial ROS levels increased in different patterns by incubation, and viability was greatly reduced by incubation; however, the relationship among them has been uncleared.

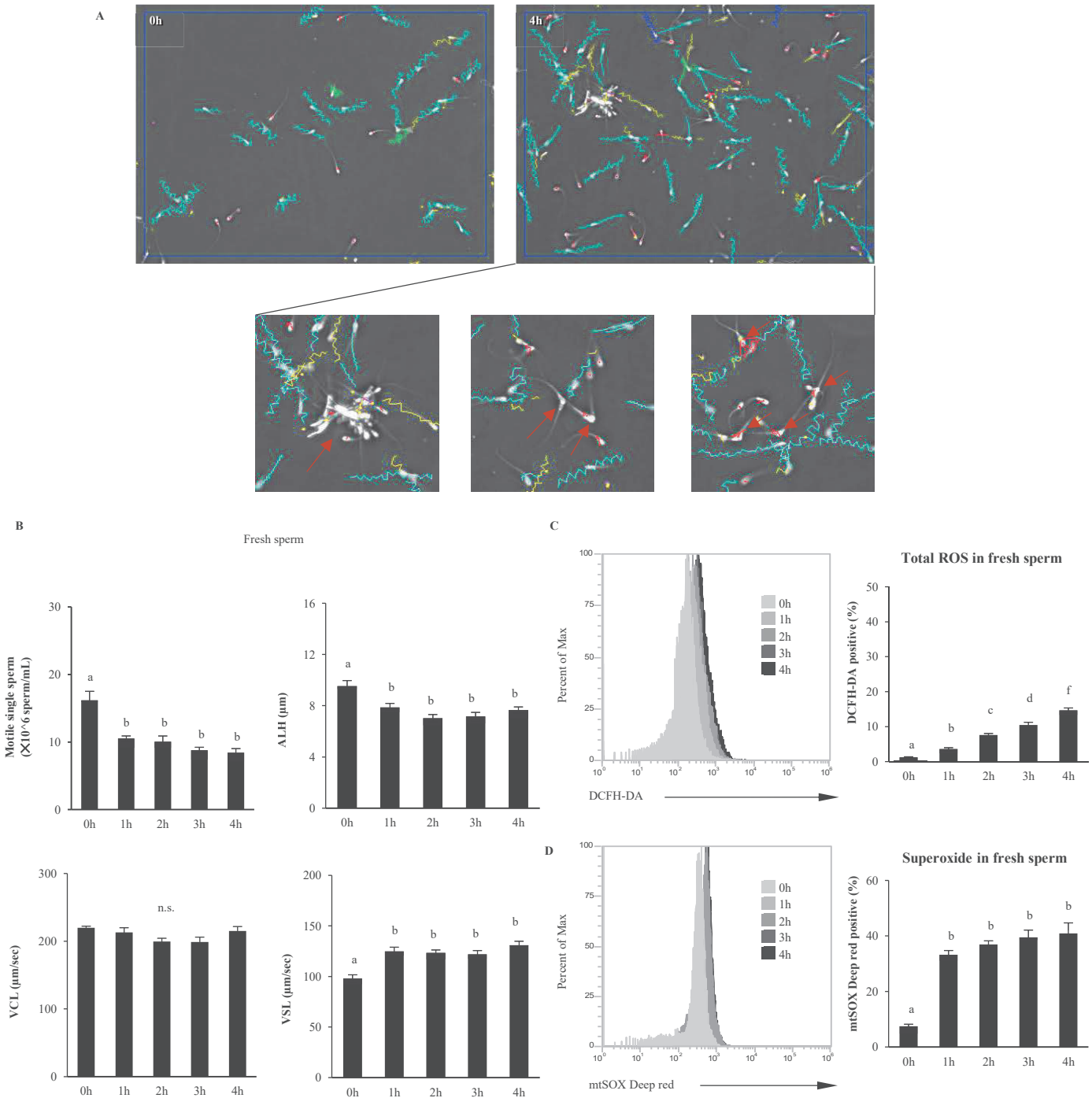


Figure 2. The significant decrease in viability and an increase in ROS levels of fresh bull sperm during the incubation period. (A) Tracks of sperm incubated at 0 and 4 hours were determined using the CASA system. Red arrows indicate the aggregated sperm, which is the cause

of overestimating CASA parameters because it lowers the number of dead sperm. (B) Changes in fresh sperm motile single counts implying sperm viability and in kinetics patterns with incubation time. (C, D) Time-dependent changes in total ROS in the cytoplasm or mitochondrial superoxide level of fresh bull sperm detected by DCFH-DA or mtSOX deep Red, respectively. Flow cytometry patterns and percentage of positive sperm were shown. Different letters represent significantly different groups ($p < 0.05$).

Induced the oxidative stress in fresh semen by H_2O_2 and antimycin.

To elucidate the relationship between the three factors that were unknown in the above experiments, bull fresh sperm was treated with either two different oxidative stress inducers, hydrogen peroxide or antimycin (Fig.3A). After one hour of incubation, the sperm motility and ROS levels by using DCFH-DA and mtSOX Deep probe were examined. Percent of positive sperm stained by DCFH-DA (total ROS positive) differed significantly by the treatment of more than 50 μM of H_2O_2 from control group (Fig.3B-D). However, the motile single sperm percentage was significantly reduced by more than 10 μM H_2O_2 treatment (Fig.3E). On the other hand, the motion speeds (VSL and VCL) were significantly reduced at concentrations of 50 μM or more (Fig.3E). The percentage of mtSOX Deep Red positive sperm was significantly increased by the treatment with antimycin in a dose-dependent manner (Fig.3F, G). Although the motile single sperm ration was slightly decreased by the treatment, the VCL (μm/sec) and VSL (μm/sec) were significantly reduced in a dose-dependent manner (Fig.3H). In other words, the viability of sperm was sensitively reduced by hydrogen peroxide treatment, while the motility rate of sperm was reduced by the increase in endogenous ROS in the mitochondria in fresh bull sperm.

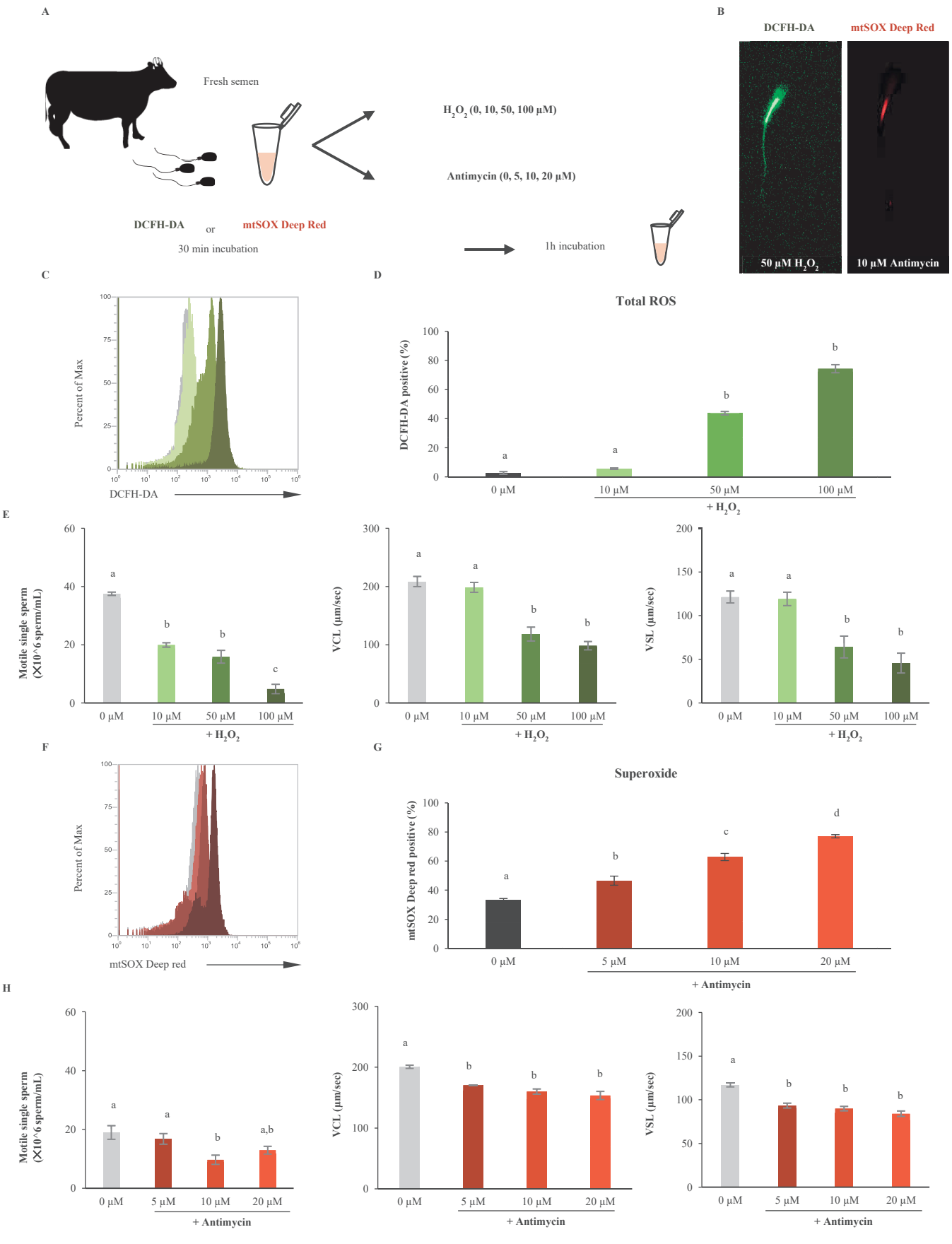

A
Fresh semen
DCFH-DA or mtSOX Deep Red
30 min incubation
H2O2 (0, 10, 50, 100 µM)
Antimycin (0, 5, 10, 20 µM)
1h incubation
B
DCFH-DA
mtSOX Deep Red
50 µM H2O2
10 µM Antimycin
C
Percent of Max
DCFH-DA
D
Total ROS
DCFH-DA positive (%)
0 µM
10 µM
50 µM
100 µM
+ H2O2
E
Motile single sperm (X10^6 sperm/mL)
VCL (µm/sec)
VSL (µm/sec)
F
Percent of Max
mtSOX Deep red
G
Superoxide
mtSOX Deep red positive (%)
5 µM
10 µM
20 µM
+ Antimycin
H
Motile single sperm (X10^6 sperm/mL)
VCL (µm/sec)
VSL (µm/sec)

Figure 3. The impact of oxidative stress inducers, H_2O_2 and antimycin, on fresh bull sperm motility and ROS levels. (A) Experimental design to investigate the effect of the oxidative stress inducers on fresh bull sperm. (B) Representative images of the sperm fluorescence signal with DCFH-DA and mtSOX deep Red. Left: DCFH-DA fluorescence image of sperm treated with H_2O_2. Right: mtSOX deep red fluorescence image of sperm treated with antimycin. (C) Flow cytometry patterns of DCFH-DA. (D) Percentages of DCFH-DA positive sperm. (E) Sperm kinetics changed by H_2O_2. (F) Flow cytometry patterns of mtSOX deep Red. (G) Percentages of mtSOX deep Red positive sperm. (H) Sperm kinetics changed by antimycin**.** Different letters represent significantly different groups ($p < 0.05$).

Effect of different antioxidants on total ROS and mitochondrial ROS levels under the presence of different oxidative stress inducers

The effects of antioxidant factors, PQQ, ergothioneine and Vitamin C on extracellular oxidative stress stimulation (H_2O_2) and mitochondrial oxidative stress were investigated using fresh bull sperm (Fig.4A). The percentage of sperm with an increase in total ROS by H_2O_2 treatment did not change significantly with PQQ, but a significant dose-dependent decrease was observed with ergothioneine and Vitamin C (Fig.4.B, C). PQQ and vitamin C dramatically reduced the percentage of mtSOX Deep Red positive sperm (ROS generated in mitochondria by antimycin treatment) in a dose-dependent manner (Fig.4D, E). On the other hand, there was no dose-dependent change in ergothioneine (Fig.4D, E).

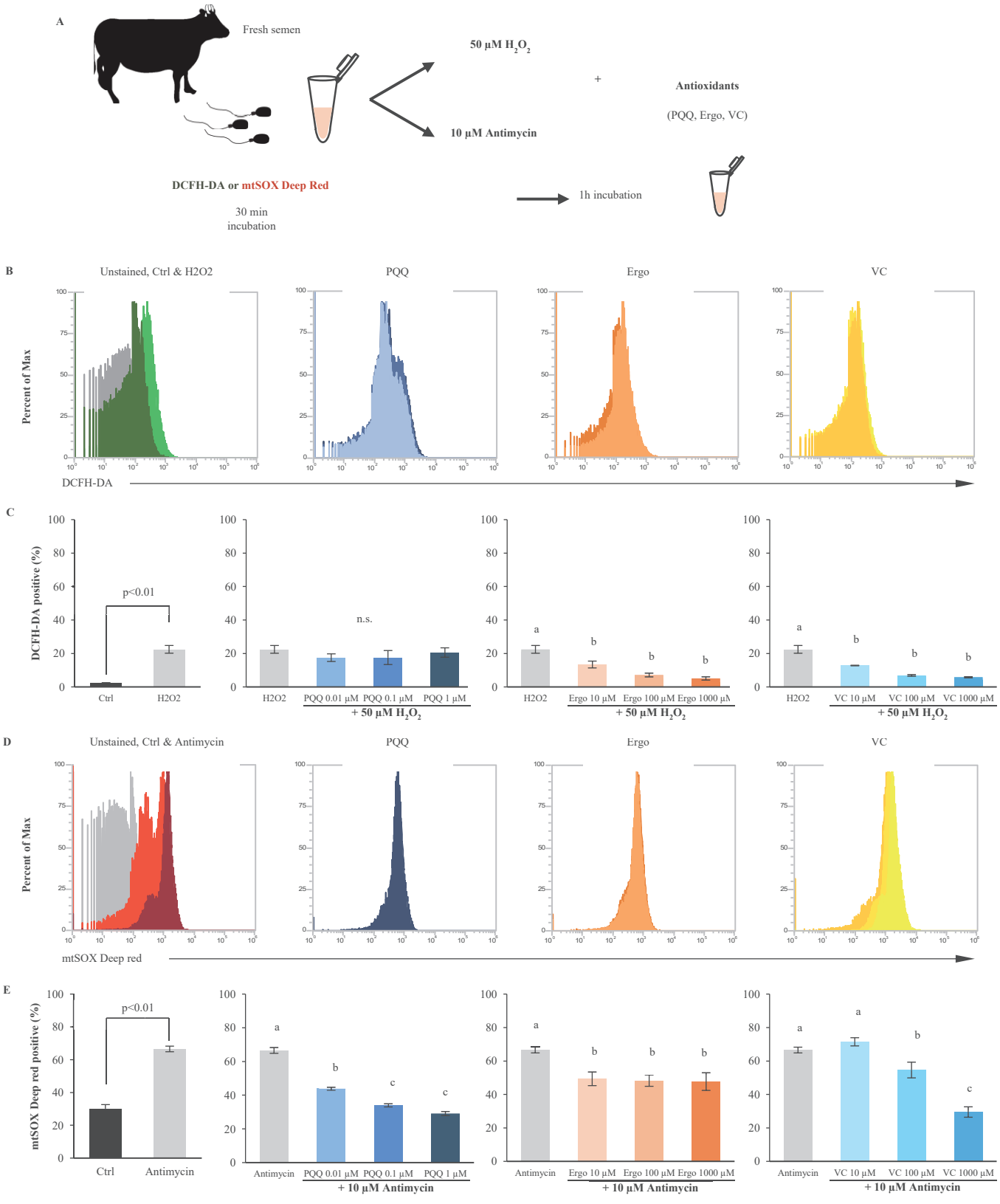
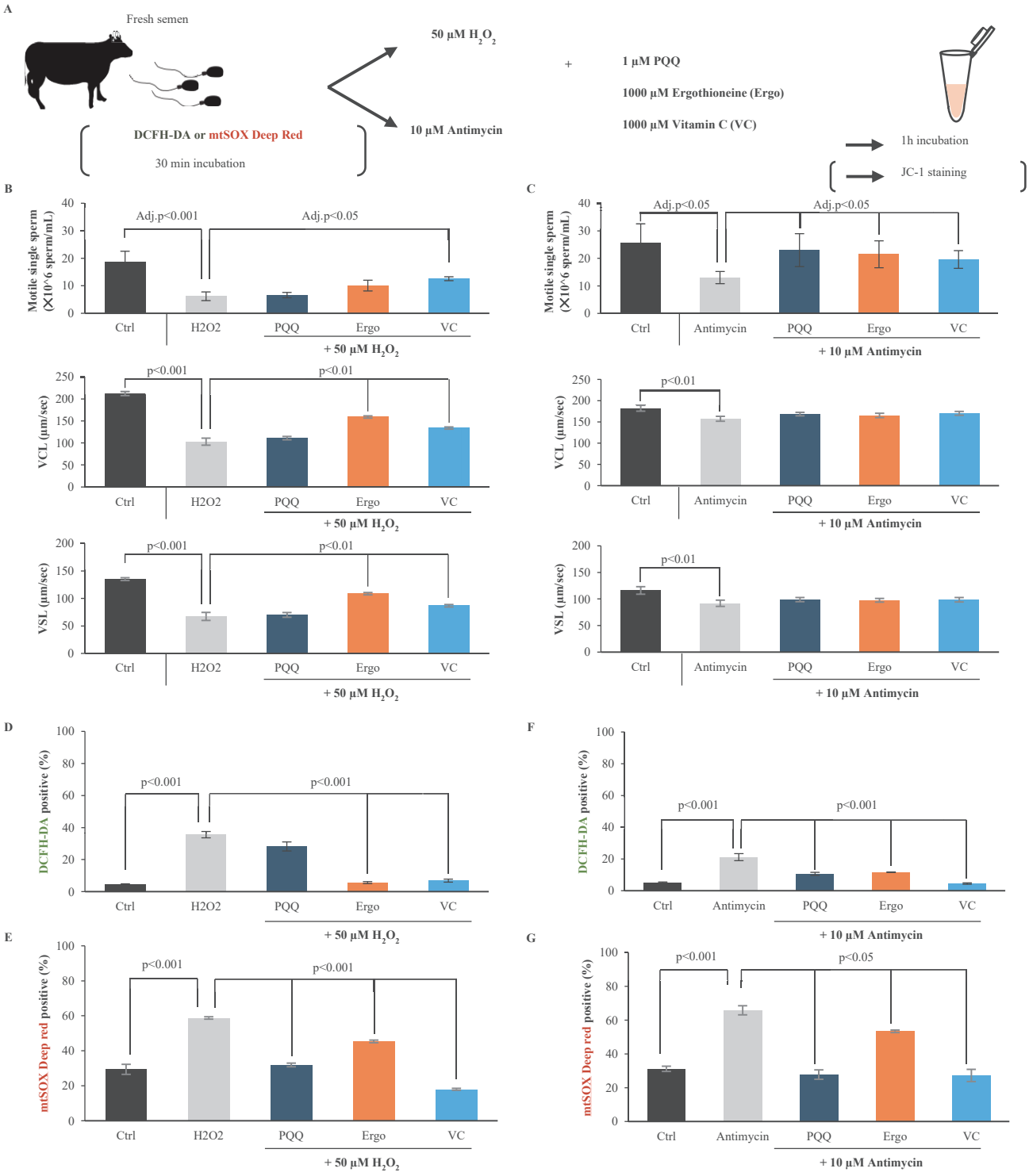

A
Fresh semen
50 µM H2O2
+
Antioxidants
(PQQ, Ergo, VC)
10 µM Antimycin
DCFH-DA or mtSOX Deep Red
30 min
incubation
1h incubation
B
Unstained, Ctrl & H2O2
PQQ
Ergo
VC
Percent of Max
DCFH-DA
C
DCFH-DA positive (%)
p<0.01
n.s.
Ctrl
H2O2
PQQ 0.01 µM
PQQ 0.1 µM
PQQ 1 µM
Ergo 10 µM
Ergo 100 µM
Ergo 1000 µM
VC 10 µM
VC 100 µM
VC 1000 µM
+ 50 µM H2O2
D
Unstained, Ctrl & Antimycin
mtSOX Deep red
E
mtSOX Deep red positive (%)
Antimycin
+ 10 µM Antimycin

Figure 4. The impact of antioxidants on total ROS and mitochondrial ROS levels in fresh bull sperm under oxidative stress inducers. (A) Experimental design to investigate the effect of three antioxidants on induced reactive oxygen species in fresh bull sperm. (B) Flow cytometry patterns of H_2O_2 and antioxidant-treated sperm stained with DCFH-DA. (C) Percentages of DCFH-DA positive sperm. (D) Flow cytometry patterns of antimycin and antioxidant-treated sperm stained with mtSOX deep Red. (E) Percentages of mtSOX deep Red positive sperm. Different letters represent significantly different groups ($p < 0.05$).

Effect of different antioxidants on sperm motility and ROS level under the presence of different oxidative stress inducers

The negative effect of H_2O_2 on sperm motility was not improved by PQQ or ergothioneine, and significant improvement was observed only with Vitamin C (Fig.5B). However, ergothioneine, like Vitamin C, significantly increased the motility rate of sperm suppressed by H_2O_2 (Fig.5B). On the other hand, the negative effect of antimycin on sperm motility was improved by any antioxidant factors (Fig.5B). However, none of the antioxidant factors affected the effect of antimycin on exercise speed of sperm (Fig.5C). Interestingly, H_2O_2 treatment not only increased the percentage of total ROS-positive sperm, but also increased the percentage of the mitochondrial ROS positive sperm (Fig.5D, E). Ergothioneine and Vitamin C significantly reduced the percentage of total ROS-positive cells, while the mitochondrial ROS-positive sperm was significantly reduced by all of antioxidant factors (Fig.5D, E). The effect of antimycin significantly increased not only the percentage of ROS-positive sperm in the mitochondria but also the percentage of total ROS-positive sperm, and total ROS-positive sperm was significantly reduced by all of antioxidant factors (Fig.5F, G). In measuring mitochondrial membrane potential using JC-1, H_2O_2 did not

affect the high mitochondrial membrane potential (hMMP; Fig.5H, I). On the other hand, antimycin significantly reduced the percentage of spermatozoa with hMMPs, but they were rescued by all antioxidant factors (Fig.5J).



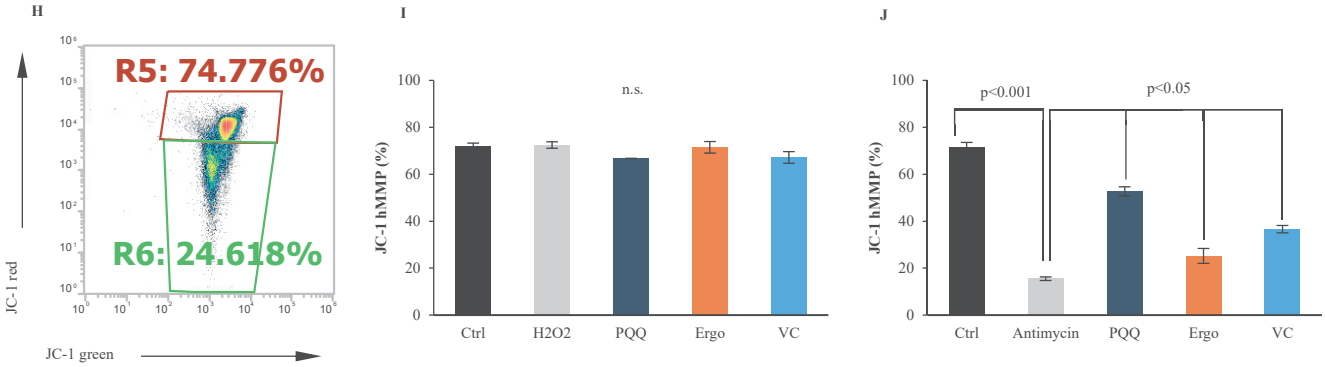


Figure 5: The impact of various antioxidants on fresh bull sperm motility and reactive oxygen species (ROS) levels when exposed to different oxidative stress triggers. (A) Experimental design to examine the impact of various antioxidants on sperm motility and levels of reactive oxygen species (ROS) under the two oxidative stress inducers. (B, C) Changes in sperm viability and motility under the H_2O_2 (B) or the antimycin (C). (D-G) Effect of antioxidants on the percentages of DCFH-DA and mtSOX deep Red positive sperm under the H_2O_2 or the antimycin. (H-J) Measurement of mitochondrial activity using JC-1 kit. (H) Representative flow cytometry pattern of Ctrl sperm stained with JC-1. Effect of antioxidants on the percentages of high mitochondrial membrane potential (hMMP) under the treatment of H_2O_2 (I) and antimycin (J). H_2O_2 or antimycin groups were used as the control for statistical analysis ($p < 0.05$).

Effect of antioxidants on frozen-thawed bull sperm motility and ROS levels

The effects of PQQ which had the characteristic of reducing ROS generated in mitochondria, Vitamin C which reduced both total ROS and ROS in mitochondria and ergothioneine which reduced total level of ROS generated by H_2O_2 on frozen-thawed bull sperm

were examined (Fig. 6A). The percentage of total ROS-positive sperm was significantly reduced by Vitamin C and ergothioneine, although PQQ did not decrease it (Fig.6B). On the other hand, mitochondrial ROS-positive sperm was significantly reduced by any antioxidant factors (Fig.6C). Thus, when frozen-thawed sperm was incubated, the oxidative stress was elevated in both cytoplasm and mitochondria. When both or either inductions were suppressed by PQQ, Vitamin C or ergothioneine, sperm motility was significantly increased by all of antioxidants (Fig.6D). In addition, a significant increase in total mobility speed (VCL) was observed, suggesting that the increase in reactive oxygen species due to both the mitochondrial abnormalities and the thawing process in frozen-thawed sperm caused a decrease in sperm motility and mobility speed (Fig.6D).

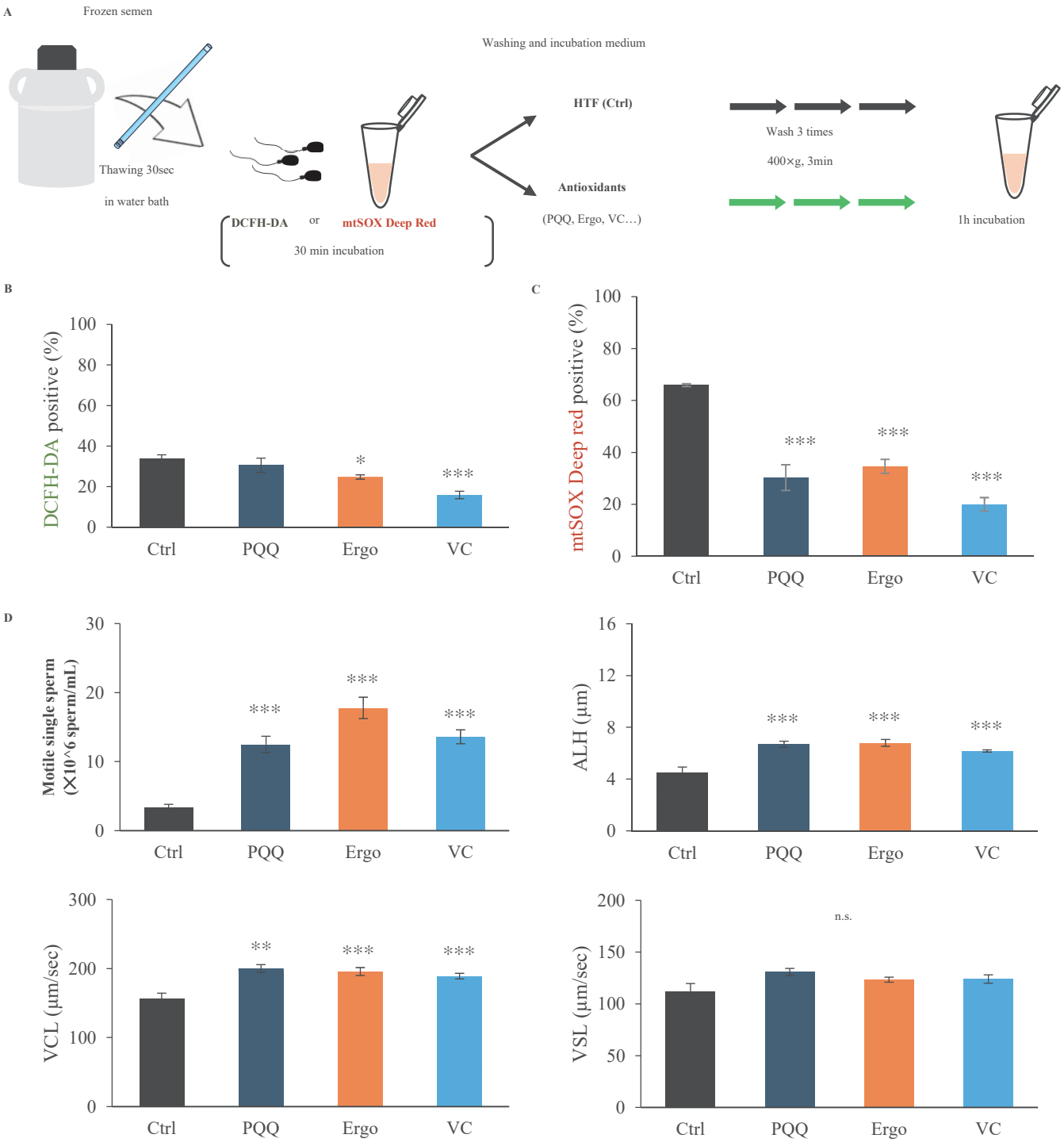


Figure 6: The effect of various antioxidants on frozen-thawed bull sperm motility and ROS levels. (A) Experimental design to examine the impact of PQQ, Vitamin C and ergothioneine on frozen-thawed bull sperm motility and ROS levels. Various antioxidants are included in all

thawing, washing and incubation processes using HTF medium as the basic medium. (B, C) Effect of antioxidants on the percentages of DCFH-DA (B) and mtSOX deep Red (C) positive sperm. (D) Changes in frozen-thawed sperm viability and kinetics patterns with antioxidants. Asterisks represent significant differences from the control (Ctrl; ✻: $p < 0.05$, ✻✻: $p < 0.01$, ✻✻✻: $p < 0.001$).

Localization of oxidized lipids and the effects of antioxidant factors in fresh or frozen-thawed bull sperm

Localization of oxidized lipids was detected by immunostaining using anti-4-HNE antibodies. As a result, positive 4-HNE signals were strongly detected not only in the middle piece by antimycine treatment, but also in the sperm head. These positive signals were attenuated by any antioxidant factors. Similarly, the positive signals of 4-HNE were detected in the sperm head and midpiece by H_2O_2 treatment. The 4-HNE positive signals were not abated by PQQ but were suppressed by ergothioneine and Vitamin C (Fig.7A). These results showed the same trend as the results of detecting ROS-positive sperm. Furthermore, because of experiments with frozen-thawed sperm, strong signals were detected in the midpiece, which were reduced by PQQ and Vitamin C, and these were also the same as in the detection experiment of ROS-positive sperm (Fig.7B).

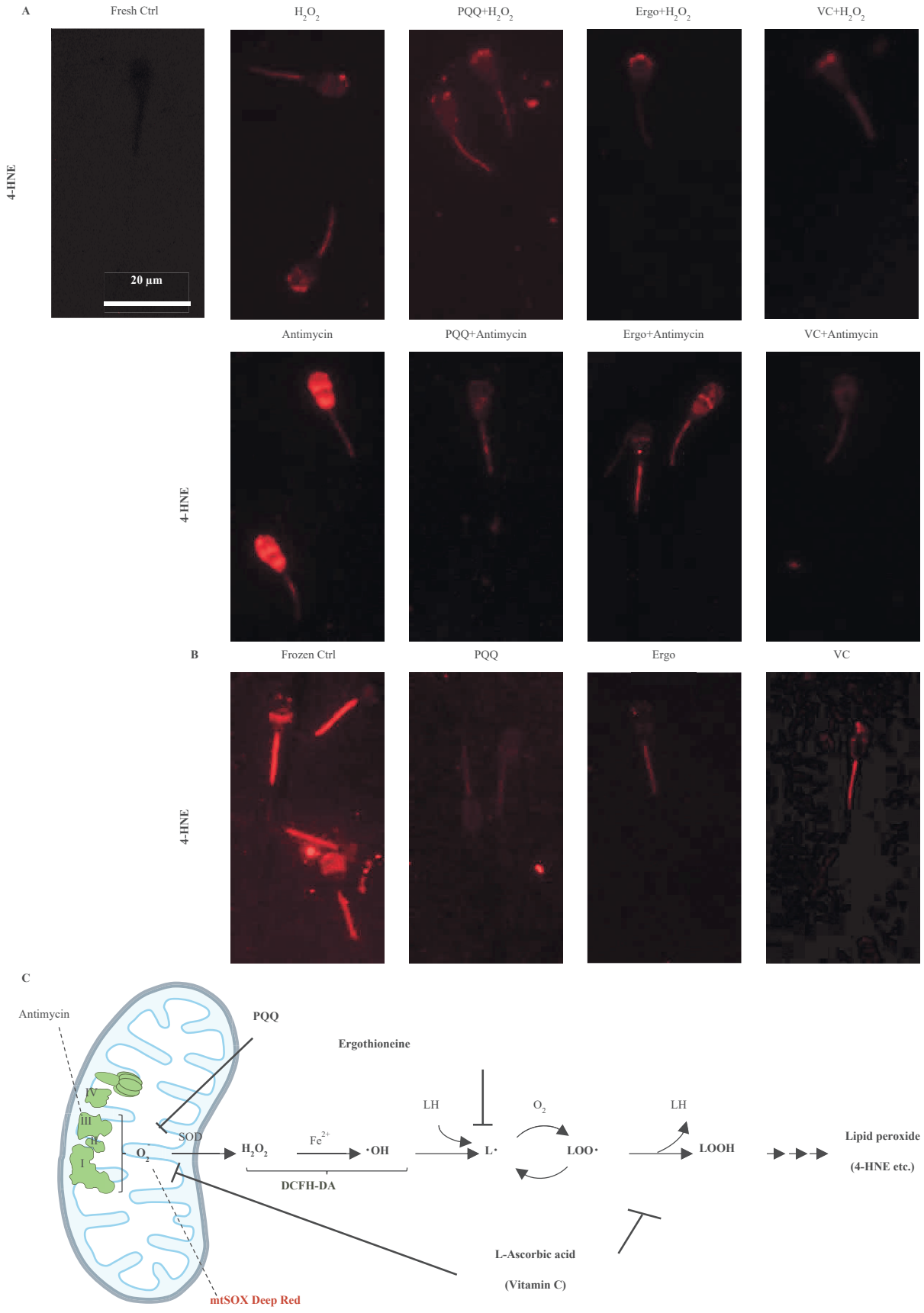

A
Fresh Ctrl
H_2O_2
PQQ+H_2O_2
Ergo+H_2O_2
VC+H_2O_2
4-HNE
20 μm
Antimycin
PQQ+Antimycin
Ergo+Antimycin
VC+Antimycin
4-HNE
B
Frozen Ctrl
PQQ
Ergo
VC
4-HNE
C
Antimycin
PQQ
Ergothioneine
IV
III
II
I
SOD
O_2
H_2O_2
Fe^{2+}
·OH
DCFH-DA
LH
L·
O_2
LOO·
LH
LOOH
Lipid peroxide
(4-HNE etc.)
L-Ascorbic acid
(Vitamin C)
mtSOX Deep Red

Figure 7: Localization of oxidized lipids in fresh or frozen-thawed bull sperm. (A, B) Representative images of the sperm fluorescence signal with 4-HNE. (A) The ROS inducer/antioxidant combination experiment in fresh sperm. (B) The effect of various antioxidants on frozen-thawed sperm. The scale bar represents 20 μm. (C) This graphical image shows the ROS production cascade in sperm beginning at the mitochondria. It also shows the site of action of the antioxidants used in this experiment.

DISCUSSION
(Page No. 31-34)

Discussion

The findings of this study indicate that oxidative stress significantly inhibits sperm function, affecting both the cytoplasm and mitochondria. It was observed that sperm viability decreased sharply due to oxidative stress caused primarily by H_2O_2 originating outside the cell, rather than by mitochondrial sources. However, elevated levels of ROS within the mitochondria also contributed to reduced sperm viability. Mitochondrial dysfunction may result in the release of substantial amounts of cytochrome C and calcium, which could negatively impact sperm survival. Additionally, oxidative stress within the mitochondria was found to decrease sperm motility even at levels that did not significantly affect overall sperm viability.

In experiments investigating lipid oxidation, strong oxidative signals were detected in the sperm head region following antimycin treatment, aligning with previous studies that link the integrity of the acrosome cap to sperm survival (Nagy et al., 2004; Fayyaz et al., 2017; Guidobaldi et al., 2017; Islam et al., 2023). A decline in sperm motility has been reported to result from the oxidation of mitochondrial DNA, mitochondrial transcription factors, and mitochondrial RNA polymerase (Amaral et al., 2007; Zhu et al., 2019). In this study, a decrease in sperm motility was observed at concentrations of antimycin that did not significantly reduce sperm viability, suggesting that the mechanism behind this effect could be the reduction in mitochondrial ATP synthesis capacity. In essence, the study demonstrated that oxidative stress from external sources rapidly diminishes sperm viability, while oxidative stress within the mitochondria impairs sperm motility and further reduces sperm survival under severe stress conditions.

The study also detected strong signals of lipid oxidation in the midpiece region of frozen-thawed sperm, which were mitigated by the antioxidants PQQ and Vitamin C. This finding

suggests that excessive oxidative stress, mainly driven by mitochondrial dysfunction, significantly compromises the viability of frozen-thawed sperm. Numerous studies have reported abnormal mitochondrial membrane potentials in frozen-thawed spermatozoa, as well as structural abnormalities in mitochondria observed through electron microscopy (Khalil et al., 2018). The functional and structural disruptions of mitochondria caused by the freezing and thawing processes are thought to exacerbate oxidative stress. Conversely, ergothioneine was found to improve the viability of frozen-thawed sperm and counteract the decrease in sperm viability induced by oxidative stress in the cytoplasm and cell membrane caused by H_2O_2 treatment. This suggests that the rapid increase in temperature and osmotic pressure during the thawing process is a significant factor contributing to the reduced viability of frozen-thawed sperm. Various studies have documented abnormalities in the sperm cell membrane of frozen-thawed spermatozoa using cell membrane normality tests with fluorescent staining reagents, which typically cannot pass through intact cell membranes (Peña et al., 2003; Partyka et al., 2010). In summary, in frozen-thawed sperm, both oxidative stress in the cell membrane during the thawing process and excessive ROS production due to mitochondrial damage during incubation can significantly decrease sperm viability.

PQQ has been reported to directly affect mitochondria by crossing cell membranes to maintain mitochondrial function (Zhu et al., 2006; Rucker et al., 2000; Hoque et al., 2021). Indeed, studies have shown that PQQ not only enhances the stability of mitochondrial DNA in boar sperm but also promotes sustained ATP synthesis by improving gene expression from mitochondrial DNA, thereby maintaining increased motility for extended periods (Zhu et al., 2019).

Vitamin C, a water-soluble antioxidant, penetrates cell membranes and exhibits ROS-scavenging abilities in both the cytoplasm and mitochondria (Sönmez et al., 2005; Hu et al., 2010).

Similarly, MitoQ and CoQ10 are antioxidants that are expected to act on mitochondria, and although their specific effects have not been fully analyzed, they have been reported to have positive effects on sperm function (Zhu et al., 2019; Arjun et al., 2022).

Ergothioneine, despite being a water-soluble antioxidant, exerts its effects not only outside the cell but also within the cell by crossing the blood-brain barrier and being taken up via the transporter OCTN1. It has been reported that OCTN1 is expressed in human spermatozoa, although its exact localization within the cell has not been fully analyzed (Xuan et al., 2003). In this study, while ergothioneine could not completely counteract the effects of ROS on mitochondrial membrane potential caused by antimycin, it did improve sperm survivability. This suggests that ergothioneine was absorbed into the sperm cytoplasm in bull sperm but may not have efficiently penetrated the mitochondria, instead exerting its antioxidant effects in the cytoplasm.

Antioxidant factors play a critical role in maintaining sperm motility and protecting sperm quality during handling, as well as in enhancing sperm motility after the freezing and thawing processes. However, when adding antioxidant factors, it is crucial to consider whether these compounds are taken up by the sperm. For instance, membrane-impermeable antioxidants such as glutathione have been reported to improve sperm motility, but their direct effect on sperm function is unlikely (Sławeta and Laskowska 1987; Chatterjee et al., 2001; Ansari et al., 2012). Another important consideration is the stability of antioxidant factors. CoQ10, for example, is known to be easily oxidized in solution, and Vitamin C also readily oxidizes in solution, which diminishes its antioxidant functions. Simplicity is essential in reproductive techniques for animal production, such as artificial insemination, but antioxidant factors may need to be mixed immediately before use to ensure their stability. This stability should be verified alongside cell membrane permeability.

In conclusion, to prevent the qualitative deterioration of sperm in bulls, it is essential to carefully select and combine appropriate antioxidant factors according to the specific oxidative stress encountered in different compartments of the sperm. The results of this study offer valuable insights into the use of antioxidants in the preparation of semen for artificial insemination, highlighting the importance of targeted antioxidant strategies to preserve sperm viability and functionality.

GENERAL DISCUSSION
(Page No. 35-40)

General Discussion

Oxidative stress has negative impacts on sperm fertilization capacity by impairing sperm motility and reducing sperm viability. Moreover, damage or fragmented sperm DNA can result in early embryonic arrest, abnormal cell division, and impaired developmental competence. Oxidative stress in sperm cells can result from infections, genital tract inflammation, animal aging, high environmental temperatures, and assisted reproductive technologies like sperm cryopreservation and sperm sexing.

The optimal thermal comfort range for most animals is 4-25°C. Temperatures above 25°C cause heat stress, disrupting homeostasis (Hanse, 2004; Marai and Haeeb, 2010). With global temperatures projected to rise by 1-7°C by the end of the century, heat stress poses a global threat to livestock production efficiency, impacting both tropical and temperate regions (Baumgard and Rhoads, 2013; Knight and Harrison, 2012; New et al., 2011; Renaudeau et al., 2012; Takahashi, 2012; Das et al., 2016; Hansen, 2009).

Bulls possess natural defense systems comprising antioxidants that scavenge and neutralize ROS. However, during spermatogenesis, sperm cells shed most of their cytoplasmic contents, resulting in a significantly reduced intracellular antioxidant capacity. Consequently, sperm protection against ROS primarily relies on the antioxidant capacity of seminal plasma. However, heat stress diminishes accessory gland secretions, lowering ejaculate volume (Suhair and Abdalla, 2013), and alters seminal plasma composition, including reduced testosterone (Chella et al., 2017; Moura et al., 2019) and changed electrolyte and protein levels (Rahman et al., 2018). Therefore, heat stress particularly affects spermatogenic precursor cells (Phillips and McKenzie, 1934), impairs testicular blood flow, and reduces delivery of oxygen and nutrients, leading to poor

spermatogenesis, damage mature sperm in epididymis (Jones and Dacheux, 2007), alters sperm morphology (Dada et al., 2003; Hansen, 2009; Zhang et al., 2015), reduced fertility (Rizzoto and Kastelic, 2020), lowers conception rates, impairs embryo development (De Rensis and Scaramuzzi, 2003; Marai et al., 2002; Yaeram et al., 2006).

Heat stress affects sperm by triggering endoplasmic reticulum stress (Kim et al., 2016), increasing lipid peroxidation, and elevating heat shock protein expression (Ananthan et al., 1986; Mujahid et al., 2007; Tan et al., 2010). Heat stress also accelerates iron release from ferritin, forming superoxide anions and hydrogen peroxide, which lead to oxidative chain reactions (Freeman et al., 1990; Powers et al., 1992). Superoxide, a precursor to most reactive oxygen species (ROS), is produced by NADPH oxidase during NADPH to NADP+ conversion (Segal and Abo, 1993). Heat stress activates NADPH oxidase, increasing the NADP+/NADPH ratio (Moon et al., 2010). Mitochondrial ATP synthesis is impaired due to electron transport chain dysfunction, increasing ROS in the liver (Yang et al., 2010) and skeletal muscle (Mujahid et al., 2009). Heat stress also upregulates heme oxygenase 1 and antioxidant enzymes like glutathione peroxidase 1 (Tyler, 2002; Shahat et al., 2020).

To counteract the adverse effects of heat stress during spermatogenesis and maturation, supplementing an antioxidant-enriched diet can be an effective strategy to enhance natural defense mechanisms against oxidative stress as because this study unveils that antioxidant such as Vitamin C, PQQ and Ergothioneine can neutralize reactive oxygen species (ROS), protecting sperm cells from endogenous and exogenous oxidative damage. In addition to antioxidants, supplementing fatty acids, particularly docosahexaenoic acid (DHA, C22:6 ω3), is crucial for overall sperm health. Animals lack the desaturases and elongases needed to synthesize essential fatty acids, making dietary supplementation necessary (Aitken et al., 2006). DHA is a major long-chain

polyunsaturated fatty acid (PUFA) found in the phospholipids of mammalian spermatozoa (Poulos et al., 1986). It is vital for maintaining sperm membrane integrity, fluidity, and viability (Robinson et al., 2016). Adequate DHA levels ensure the structural and functional integrity of sperm membranes, supporting sperm motility and fertility. Thus, combining an antioxidant-enriched diet with DHA supplementation can effectively mitigate the oxidative stress induced by heat, enhancing sperm quality and fertility in heat-stressed animals.

Sperm sexing, particularly in cattle, is used to enhance production and reproductive outcomes by enabling the selection of desired offspring sex (O'Brien et al., 2009). Flow cytometry is a common method for sperm sexing, especially in bovines (Garner and Seidel, 2008). However, this technique induces molecular changes such as increased reactive oxygen species (Tvrda et al., 2016), heightened membrane permeability, and reduced intracellular ATP levels, which negatively impact sperm motility, viability, and longevity (Carvalho et al., 2010; Holden et al., 2017). The sperm chromatin structure assay shows that sex-sorted bovine sperm have more DNA damage and lower chromatin homogeneity compared to conventionally sorted sperm (Boe-Hansen et al., 2005). Ram sperm exhibit reduced progressive motility and increased capacitation post-sorting (Hollinshead et al., 2003). Additionally, sorted bull and ram sperm show higher acrosome reaction rates after thawing (Hollinshead et al., 2003; Moce et al., 2006). In vitro studies reveal that sexing impairs cattle sperm binding to tubal explants, affecting gamete-maternal interactions (de Oliveira Carvalho et al., 2018). Therefore, based on the result from this study, it can be stated that the addition of antioxidant (PQQ, Vitamin C, and Ergothioneine) before or after sorting can enhance sperm sexing technology by reducing the oxidative stress, which may improve sperm viability, and motility. After all, may increase success rates in artificial insemination.

Sperm treatment in assisted reproductive technology (ART) increases oxidative stress. Cryopreservation, a key ART for livestock breeding, is vital for preserving male fertility in mammals (Anger et al., 2003). During cryopreservation, mitochondria suffer direct DNA damage and indirect damage due to loss of genetic coding capacity affecting mitochondrial function (Kurland and Andersson, 2000). Freeze-thaw cycles deplete natural antioxidants in bovine semen (Bilodeau et al., 2000) and significantly elevate reactive oxygen species (ROS) levels (Peris et al., 2007), reducing sperm motility. Studies with frozen-thawed semen show lower conception rates (Bhosrekar et al., 2001). Moreover, the results of this study also reveal that the thawing of frozen semen generates reactive oxygen species (ROS) in both sperm cytoplasm and mitochondria. Cytoplasmic ROS, originating from enzymes and the plasma membrane, acts as signaling molecules in processes like capacitation and acrosome reaction but cause oxidative damage at high levels. Mitochondrial ROS, produced by the electron transport chain and specific enzymes, regulate mitochondrial function; excessive levels impair ATP production, reduce sperm motility, and may lead to apoptosis.

Furthermore, Artificial Insemination (AI) is a key assisted reproductive technology that has transformed livestock farming, enhancing cattle production and genetic progress through high-quality frozen sperm (Yanez-Ortiz et al., 2021; Medeiros et al., 2002). Therefore, adding antioxidants such as PQQ, Vitamin C, and Ergothioneine to the thawing medium is crucial for protecting sperm from oxidative stress. These antioxidants work by neutralizing reactive oxygen species (ROS) that can damage sperm cells during the thawing process. Conversely, by incorporating these antioxidants in optimized concentrations and combinations, alongside standardized thawing protocols, the risk of oxidative damage will be minimized. Therefore, this

approach will significantly enhance sperm viability and function, leading to improved quality and fertility outcomes after thawing.

The average motility in the frozen thawed control group was found 54.4% whereas in the antioxidant treated group was 73.77%. Therefore, the results of this study also show that antioxidant-enriched thawing media can improve freeze-thawed sperm motility by about 20% compared to controls, likely by protecting fatty acid components of sperm cell membranes from free radical damage. This will allow for a potential reduction of 20% in sperm concentration for artificial insemination (AI). If 0.5 ml straw contains 25 million sperm then, therefore, based on the result from this study, 5 million sperm can be reduced per straw. Also, if consider, the average volume of semen per ejaculation is about 8.0 ml and the concentration is 1.5 billion per ml. Then the sperm in the ejaculate will be 12.0 billion per ml and the number of the straw will be 600 compared to 480 straws when the sperm concentration was 25 million per straw. Consequently, lowering the sperm concentration enables the production of an additional 120 frozen straws from a single bull ejaculation. If consider the value of each straw is about 50 dollars, potentially increasing its economic value about six thousand dollars. Although, the price of semen straws varies based on factors such as breed, demand, supply, and geographic location.

However, findings of this study suggest that using antioxidants in thawing media can enhance AI success rates in livestock by improving frozen sperm quality and this approach can reduce the need for repeated inseminations, saving on semen doses, labor, and veterinary services. Improved sperm quality supports sustainable livestock farming by maximizing output per breeding cycle. Addressing ROS effects with antioxidants will enhance reproductive success, cost-efficiency, and sustainability, contributing to a more productive and resilient livestock sector. The FAO predicts that global meat and milk production must double by 2050 to meet growing demands

due to population increases, rising incomes, and urbanization (Thornton, 2010). This "livestock revolution" offers both opportunities and challenges, transforming the sector into a more organized industry with extensive market chains. Livestock support over 1.3 billion people worldwide, including 600 million smallholder farmers in developing countries (Thornton, 2010). They are vital to livelihoods across diverse climates (Salem, 2010; Silanikove et al., 2010) and contribute to 58% of the Sustainable Development Goals and impacts 16% of their targets (Mehrabi et al., 2020).

However, *in vitro* studies shed light on the impact of antioxidants on sperm quality. Moreover, conducting field studies through AI of cows will provide important data for determining the most effective antioxidant treatment.

CONCLUSIONS
(Page No. 40-42)

Conclusions

Maintaining the quality of bull sperm, particularly in the context of artificial insemination and other reproductive technologies, requires a strategic and well-informed approach to managing oxidative stress. Sperm cells are highly sensitive to oxidative damage, which can arise from various sources, including environmental factors, metabolic processes within the cell, and procedures like cryopreservation. Oxidative stress can impair critical functions of sperm, such as motility, viability, and the ability to fertilize an egg, by damaging key cellular components like the plasma membrane, mitochondria, and DNA.

To effectively counteract oxidative stress and preserve sperm quality, it is essential to use a combination of antioxidants that are specifically tailored to address the oxidative challenges present in different compartments of the sperm. Each compartment-the cytoplasm, mitochondria, and plasma membrane-faces distinct oxidative threats, and not all antioxidants are equally effective in every cellular environment. For example, some antioxidants are more efficient at neutralizing ROS within the mitochondria, where energy production occurs, while others are better suited to protecting the plasma membrane or cytoplasm from oxidative damage.

The findings of this study emphasize the importance of selecting the appropriate antioxidants based on their ability to target specific sites of oxidative stress within the sperm. By understanding how different antioxidants interact with various sperm compartments, reproductive specialists can optimize the preparation of semen for artificial insemination, ensuring that sperm quality is maintained throughout the process. This is particularly important when dealing with frozen-thawed sperm, which is more vulnerable to oxidative stress due to the physical and chemical changes that occur during freezing and thawing.

Incorporating the right combination of antioxidants can significantly improve sperm viability, motility, and overall functionality, thereby enhancing the chances of successful fertilization. These insights provide valuable guidance for developing antioxidant strategies in semen preparation, helping to safeguard the reproductive potential of bull sperm and improve outcomes in animal breeding programs. As research continues to explore the specific mechanisms by which antioxidants protect sperm, the application of these findings will become increasingly refined, leading to more effective and reliable methods for preserving sperm quality in artificial insemination and other reproductive technologies.

SUMMARY
(Page No. 43-45)

SUMMARY OF THE THESIS

The process of mammalian fertilization involves the migration of sperm through the female reproductive tract, where they must travel from the uterus to the oviduct, ultimately reaching the ampullary-isthmic junction, the site of fertilization. This journey is complex and requires sperm to maintain a linear motility pattern, which is critical for successful fertilization. For sperm to sustain this motility, they require a continuous and sufficient supply of energy, which is primarily generated through mitochondrial ATP production.

However, the quality of sperm can significantly deteriorate during cryopreservation, a common method used for sperm storage in artificial insemination. Cryopreservation exposes sperm to stressful conditions that lead to the excessive production of reactive oxygen species (ROS). These ROS are highly reactive molecules that can cause oxidative damage to various cellular components, including lipids, proteins, and DNA. Elevated levels of ROS during cryopreservation compromise sperm membrane integrity, acrosomal integrity (which is essential for fertilization), and mitochondrial function, all of which are critical for maintaining sperm motility and overall fertility.

Oxidative stress, caused by both internal (endogenous) and external (exogenous) factors, plays a central role in this deterioration. It damages essential components of sperm cells, such as the plasma membrane, mitochondria, and acrosome, leading to reduced metabolic activity and, ultimately, diminished fertility. Given the importance of preserving sperm quality for reproductive success, this study aimed to investigate the specific effects of oxidative stress on bull sperm and to evaluate the efficacy of targeted antioxidants in mitigating these harmful effects.

In the study, fresh bull semen samples were subjected to treatments with hydrogen peroxide (H_2O_2) and antimycin, both of which are known to induce oxidative stress. H_2O_2 primarily acts as an external source of oxidative stress, while antimycin disrupts mitochondrial function, leading to increased production of mitochondrial ROS. The antioxidants Pyrroloquinoline quinone (PQQ), ergothioneine, and vitamin C were then applied to assess their ability to counteract the induced oxidative stress.

To evaluate the impact of these treatments, various parameters of sperm health were measured, including sperm motility, viability, and ROS levels in both the cytoplasm and mitochondria. Advanced techniques such as computer-assisted sperm analysis (CASA) and flow cytometry were used to provide precise measurements of these parameters. The results revealed that H2O2 rapidly decreased sperm viability, indicating that external oxidative stress is particularly detrimental to sperm survival. On the other hand, antimycin-induced mitochondrial ROS primarily affected sperm motility, underscoring the critical role of mitochondria in sustaining sperm movement.

Among the antioxidants tested, PQQ and vitamin C were found to be particularly effective in reducing mitochondrial ROS, while ergothioneine and vitamin C were more successful in reducing cytosolic ROS. This suggests that different antioxidants may be better suited for targeting specific sources of oxidative stress within the sperm cell. In the context of freeze-thawed sperm, which are more susceptible to oxidative damage due to the physical stresses of freezing and thawing, all three antioxidants showed a positive effect by improving sperm motility and inhibiting ROS production.

Additionally, the study employed immunofluorescence techniques to detect the localization of oxidized lipids, such as 4-hydroxynonenal, within the sperm. This analysis revealed that oxidative stress impacts not only the mid-piece of the sperm, where mitochondria are concentrated, but also the sperm head, which is crucial for successful fertilization.

These findings highlight the significant potential of targeted antioxidants in mitigating the harmful effects of oxidative stress on bull sperm. By incorporating antioxidants into semen preparation and insemination media, it may be possible to reduce the negative impact of oxidative stress, thereby enhancing sperm motility within the female reproductive tract and increasing the likelihood of successful fertilization.

Furthermore, the application of these findings to the thawing process of frozen sperm represents a promising approach to improve conception rates in livestock. By optimizing the use of antioxidants, the overall production of livestock could be enhanced, which would have significant economic benefits. Increased livestock production can contribute to poverty reduction, boost income for farmers, and support the achievement of the United Nations Sustainable Development Goals (SDGs), particularly those related to ending hunger, promoting economic growth, and ensuring sustainable agriculture.

In conclusion, this study provides valuable insights into the role of oxidative stress in sperm quality deterioration and the potential of antioxidants to counteract these effects. The strategic use of antioxidants could lead to significant advancements in artificial insemination practices, ultimately improving reproductive outcomes and contributing to global food security and economic development.

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