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Cellular Basis and Optimization of Cryopreservation Survival of Bull Sperm

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To my family,

my parents' memory,

and the great hearts that prayed for me

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لا كنز انفع من العلم

علي بن ابي طالب

“What we know is a drop, what we don't know is an ocean”.

Isaac Newton

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ABBREVIATIONS AND ACRONYMS

AI: Artificial insemination

AICS: Artificial insemination using cryopreserved semen

ALH: Amplitude of the lateral movement of the head

AO: Acridine orange

AV: Asturiana de los Valles

BCF: Beat-cross frequency

BSA: Bovine serum albumin

CASA: Computer-Assisted Sperm Analysis

CAT: Catalase

DFI: DNA fragmentation index

DGC: Density gradient centrifugation

DLC: Double layer colloid centrifugation

FSC/SSC: forward/side scatter

GPx: Glutathione peroxidase

GSH-Px: Glutathione peroxidase

GSH: Reduced glutathione

GSSG: Glutathione disulphide or oxidised glutathione

H258: Hoechst 33258

H342: Hoechst 33342

HDS: Chromatin immaturity

LIN: Linearity index

LPO: Lipid peroxidation

M540: Merocyanine 540

MFI: Median fluorescence intensity

MOT: Total sperm motility

MT: MitoTracker deep red

PBS: Phosphate buffer-saline

PI: Propidium iodide

PNA-647: PNA Alexa Fluor 647

PROG: Progressive motility

PUFAs: Polyunsaturated fatty acids

PVP: Polyvinylpyrrolidone

R123: Rhodamine 123

ROS: Reactive oxygen species

SERIDA: Regional Service for Agrifood Research and Development

SCSA: Sperm Chromatin Structure Assay

SLC: Single layer colloid centrifugation

SOD: Superoxide dismutase

STR: Straightness index

VAP: Average speed

VCL: Curvilinear path velocity

VSL: Straight path velocity

WOB: Wobble index

SUMMARY

The agriculture/bio economics sector is responsible for producing the food needed for our society's daily survival. Cattle provide much of the protein and fat consumed in the world, both in meat and dairy products. In this context, artificial insemination using cryopreserved semen (AICS) is an indispensable tool in cattle management worldwide.

Although protocols for seminal cryopreservation in bulls are generally well established, further research and improvements are required to solve several problems that cause a decrease in bull semen quality and fertility post-thawing. Therefore, the development of new biotechnological tools and basic knowledge of sperm biology is required to improve AICS results. The need for these biotechnologies is more urgent for autochthonous or rare breeds. These breeds are essential to the societies in which they are present, yet there is a lack of research to improve the reproductive efficiency of these breeds. Thus, while the main aim of this thesis is to improve the cryopreservation protocols for bull semen, in several experiments, we used bulls from Asturiana de los Valles, a Spanish autochthonous cattle breed (Asturias, Spain), and also bulls from the Holstein breed.

The extension of the equilibration time may maintain or enhance the post-thawing bull semen quality, and this may depend on the semen freezing extender. Therefore, the first objective in this study was to test the impact of extending equilibration time from 4 h to 24 h using the semen extenders BIOXcell (in collaboration with SERIDA) and OPTIXcell (in collaboration with Xenética Fontao, Lugo) on post-thawing bull semen quality. The experiment in SERIDA also included adding 2 mM of GSH to the semen freezing extender. Two cattle breeds were used, Holstein-Friesian and Asturiana de los Valles. The extension of the equilibration time in both extenders reduced the post-thawing sperm motility while improving sperm viability. However, the differences between the equilibration times were small. GSH slightly decreased the mitochondrial superoxide.

In these experiments and those which follow, we used CASA to evaluate sperm motility and the kinematic parameters, while we used flow cytometry to assess sperm physiological parameters (viability, apoptosis, capacitation status, acrosomal damage, mitochondrial activity, production of cytoplasmic reactive oxygen species (ROS), production of mitochondrial $O_2^{\bullet-}$, %DFI and %HDS). In all experiments in this thesis, semen was evaluated directly post-thawing and after four or five hours of incubation.

Reactive oxygen species (ROS) increase due to the semen cryopreservation process. ROS may cause a depletion of the endogenous antioxidants in sperm and seminal plasma. This may expose sperm to oxidative stress, and consequently, reduce post-thawing semen quality and fertility. Thus, the second and third objectives of this thesis were to supplement the BIOXcell freezing extender with different concentrations of trehalose, and the antioxidants GSH, curcumin, and crocin to improve bull semen cryopreservation and post-thawing semen quality. Bulls from Holstein-Friesian and Asturiana de los Valles breeds were used.

In a first experiment, we supplemented BIOXcell freezing extender with trehalose (50 and 100 mM), and GSH (2 and 5 mM). The results showed a reduction in the post-thawing sperm quality when BIOXcell was supplemented with trehalose at 50, and especially, at 100 mM. Trehalose resulted in a slight antioxidant effect, reducing ROS. GSH at both concentrations decreased the production of the mitochondrial superoxide anion. However, GSH did not improve sperm quality after thawing.

In a second experiment, we supplemented BIOXcell with curcumin (0.05 and 0.1 mM), crocin (0.5 and 1.5 mM), and GSH (0.5 mM). Curcumin and crocin did not improve the post-thawing sperm quality. Interestingly, both curcumin concentrations increased cytoplasmic ROS production, while crocin led to a decrease of some sperm physiology parameters. GSH reduced the production of the mitochondrial superoxide anion with no improvement in overall sperm quality.

Our fourth objective was to test the effect of a pre-freezing single layer (SLC) and double-layer (DLC) colloid centrifugation on bull semen to improve post-thawing semen quality. The commercial BoviPure colloid was used for this purpose and the experiment was performed with semen from Holstein-Friesian breed bulls. The results showed that both pre-freezing SLC and DLC improved the motility and physiological parameters of sperm post-thawing, with similar results for both colloids. They also reduced sperm DNA fragmentation.

We concluded from the various experiments in this thesis that the extension of the equilibration time from 4 h to 24 h using BIOXcell or OPTIXcell resulted in small differences regarding post-thawing bull semen quality between the equilibration times. The supplementation of BIOXcell freezing extender with trehalose and GSH reduced the post-thaw bull sperm quality (trehalose), while it did not improve the quality (GSH). The pre-freezing supplementation of BIOXcell with curcumin and crocin reduced the post-thaw sperm quality. The pre-freezing use of SLC and DLC improved post-thawing bull semen quality.

RESUMEN

El sector de la agricultura (bioeconomía) es responsable de producir los alimentos necesarios para la supervivencia diaria de nuestra sociedad. El ganado proporciona gran parte de las proteínas y grasas consumidas en el mundo, en forma de carne y productos lácteos. En este contexto, la inseminación artificial con semen criopreservado (IASC) es una herramienta indispensable en el manejo del ganado en todo el mundo.

Aunque los protocolos para la criopreservación seminal en toros están bien establecidos, se requieren más investigaciones y mejoras para resolver varios problemas que causan una disminución en la calidad del semen y en su fertilidad después de la descongelación. Por lo tanto, se requiere el desarrollo de nuevas herramientas biotecnológicas y el avance en los conocimientos básicos de la biología espermática para mejorar los resultados de la IASC. La necesidad de estas biotecnologías es incluso más relevante para las razas autóctonas o raras. Estas razas son esenciales para las sociedades en las que están presentes, pero hay una especial falta de investigación para mejorar la eficiencia reproductiva de estas razas. Por lo tanto, mientras el objetivo principal de esta tesis es mejorar los protocolos de crioconservación para el semen de toro, en varios experimentos, utilizamos toros de Asturiana de los Valles, una raza de ganado autóctono español (Asturias, España) y también toros de la raza Holstein.

La extensión del tiempo de equilibrio puede mantener o mejorar la calidad del semen del toro después de la descongelación, y esto puede depender del diluyente de congelación del semen. Por lo tanto, el primer objetivo en este estudio fue evaluar el impacto de extender el tiempo de equilibrado de 4 h a 24 h utilizando los diluyentes BIOXcell (en colaboración con SERIDA) y OPTIXcell (en colaboración con Xenética Fontao, Lugo) sobre la calidad del semen de toro después de la descongelación. El experimento en SERIDA también incluyó la adición de 2 mM de GSH al diluyente de congelación de semen. Se utilizaron dos razas de ganado, Holstein-Friesian y la raza autóctona Asturiana de los Valles (Asturias, España). La extensión del tiempo de equilibrado en ambos diluyentes redujo la motilidad de los espermatozoides después de la descongelación, aunque mejoró su viabilidad. Sin embargo, las diferencias fueron pequeñas. GSH redujo ligeramente el superóxido mitocondrial.

En estos experimentos y en los siguientes, utilizamos CASA para evaluar la motilidad de los espermatozoides y los parámetros cinemáticos, mientras que utilizamos la citometría de flujo para evaluar los parámetros fisiológicos de los espermatozoides (viabilidad, apoptosis, estado de capacitación, daño acrosómico, actividad mitocondrial, producción de especies de

oxígeno reactivo citoplasmático (ROS), producción de mitocondriales $O_2^{\bullet -}$,% DFI y % HDS). En la mayoría de los experimentos, el semen se evaluó directamente después de la descongelación y después de cuatro o cinco horas de incubación.

Las especies reactivas de oxígeno (ROS) aumentan debido al proceso de criopreservación del semen. Las ROS pueden causar un agotamiento de los antioxidantes endógenos en los espermatozoides y el plasma seminal. Esto puede exponer a los espermatozoides al estrés oxidativo y reducir la calidad y fertilidad del semen después de la descongelación. Por lo tanto, el segundo y tercer objetivo de esta tesis fueron complementar el diluyente de congelación BIOXcell con diferentes concentraciones de trehalosa y los antioxidantes GSH, curcumina y crocina para mejorar la criopreservación del semen de toro y la calidad del semen después de la descongelación, utilizando semen de los toros de las razas Holstein-Friesian y Asturiana de los Valles.

En un primer experimento, suplementamos el diluyente de congelación BIOXcell con trehalosa (50 y 100 mM) y GSH (2 y 5 mM). Los resultados mostraron una reducción en la calidad del semen después de la descongelación cuando BIOXcell se suplementó con trehalosa a 50 mM y, especialmente, 100 mM. La trehalosa resultó en un ligero efecto antioxidante, reduciendo ROS. GSH a ambas concentraciones disminuyó la producción del anión superóxido mitocondrial. Sin embargo, GSH no mejoró la calidad del esperma después de la descongelación.

En un segundo experimento, suplementamos BIOXcell con curcumina (0.05 y 0.1 mM), crocina (0.5 y 1.5 mM) y GSH (0.5 mM). La curcumina y la crocina no mejoraron la calidad del semen después de la descongelación. Curiosamente, ambas concentraciones de curcumina aumentaron la producción de ROS citoplasmáticos, mientras que la crocina condujo a una disminución de algunos parámetros fisiológicos de los espermatozoides. GSH redujo la producción del anión superóxido mitocondrial sin mejorar la calidad general del esperma.

Nuestro cuarto objetivo fue probar el efecto de la centrifugación en coloide de capa única (SLC) y de capa doble (DLC), aplicado en precongelación, en semen de toro para mejorar la calidad tras la descongelación. Se utilizó el coloide comercial BoviPure con este fin y el experimento se realizó con semen de toros de la raza Holstein-Friesian en Xenética Fontao. Los resultados confirmaron que tanto el SLC previo a la congelación como el DLC mejoraron la motilidad y los parámetros fisiológicos de los espermatozoides después de la descongelación, con resultados similares. También redujeron la fragmentación del DNA espermático.

Con base en los diversos experimentos de esta tesis, concluimos que la extensión del tiempo de equilibrado de 4 a 24 horas usando BIOXcell u OPTIXcell resultó en pequeñas diferencias con respecto a la calidad del semen de toro después de la descongelación. La suplementación previa a la congelación de BIOXcell con trehalosa redujo la calidad del semen de toro después de la descongelación, mientras que con GSH no mejoró la calidad. La suplementación del diluyente BIOXcell previa a la congelación con curcumina y crocina redujo la calidad del semen después de la descongelación. La realización en precongelación de DGC (como SLC o DLC) con BoviPure mejoró la calidad del semen de toro después de la descongelación.

INTRODUCTION

2.1 Artificial insemination using cryopreserved semen in cattle

Dairy and beef production has experienced substantial enhancements on a worldwide basis since prolonged semen storage and transportation became a possibility. Prior to transport, semen should be carefully stored in a manner that preserves the sperm's viability and mobility for subsequent use in fertilization (Bailey et al., 2003).

In addition to short-term storage via cooling (Aurich, 2008; Wusiman et al., 2012), the spermatozoa can be stored indefinitely at -196 °C in liquid nitrogen (cryopreservation) for subsequent use in artificial insemination (AI). The use of artificial insemination with cryopreserved semen (AICS) is an essential biotechnology that has contributed to the improvement of modern production and genetics, particularly in dairy cattle (Foote, 2010). Indeed, AICS has multiple benefits: ejaculates from healthier, genetically superior sires that can be used to inseminate thousands of females around the world long after the sire's death; fewer costs in animal production due to less males in a farm; and, finally, improved health control and the prevention of the spread of sexually transmitted diseases, resulting from the lack of contact between males and females (Curry, 2000; Bailey et al., 2003).

AICS in modern cattle production has spread around the globe (Curry, 2000; Binyameen et al., 2019), especially for dairy cattle, due to the aforementioned advantages and good fertility results in this species. As an example, worldwide statistics from 2002 show that, at this time, there were 648 semen collection centers registered, 1,635 semen banks, more than 40,000 bulls housed in AI centers, and that 264 million doses of semen were produced (95% frozen doses: 50% in Europe, 27% in the Far East, and 16% in North America) (Thibier and Wanger, 2002). In Canada, about 75-80% of dairy cattle calves and 85-90% of piglets are produced through AI (cooled semen for pigs), and other industries, such as beef, sheep, goats, and fish, have also used this technique to optimize production (Bailey et al., 2003). Top sires can produce up to 60,000 doses of semen per year (Curry, 2000). Furthermore, dairy production has been improved by using AICS, because of the highly successful distribution of cryopreserved semen, and the development of improved protocols to preserve and apply bull semen (Hammerstedt et al., 1990; Curry, 2000; Thibier and Wagner, 2002), despite the slow progress of this technique over the past few decades (Grötter et al., 2019).

Although bull sperm is relatively resistant to cryopreservation, and there has been significant progress in AICS, the post-thaw viability and fertility of thawed spermatozoa is still reduced (Watson, 2000; Bailey et al., 2003). Eight to tenfold more frozen-thawed bull sperm,

compared to fresh sperm, are required to achieve equivalent fertility rates *in vivo*, which is a clear indicator to marked reduction in the fertilizing potential of the cryopreserved spermatozoa (Shannon, 1978; Shannon and Vishwanath, 1995). In most species, approximately 40-50% of the sperm population is damaged during the freezing/thawing process, as measured by indicators such as motility and membrane integrity, even when "optimized" cryopreservation protocols are used (Curry, 2000; Holt, 2000; Watson, 2000). These challenges motivated to produce a theoretical model and biotechnological applications for optimal sperm freezing regimes for each animal species (Curry, 2000; Barbas and Mascarenhas, 2009; Moore and Hasler, 2017).

Assisted reproductive techniques (such as gamete cryopreservation, artificial insemination (AI), embryo transfer, cloning, and *in vitro* fertilization) are not only used to enhance breeding, but also to maintain or preserve the genetic diversity of domestic breeds, with special attention to endangered and rare breeds (Comizzoli et al., 2000; Bailey et al., 2003; Barbas and Mascarenhas, 2009; Moore and Hasler, 2017). Whilst these technologies have been highly successful in commercial breeds, due to intense research and efforts in its application and coordination with genetics programs, the situation has been different for autochthonous or rare breeds. These breeds have very limited profitability, and, in many cases, have been displaced by the commercial ones. Fortunately, there has been a renewed interest in these breeds: many have characteristics (e.g., rusticity, adaptation to very specific environments) of great interest in a world facing environmental challenges, and also great cultural value (Evans and Yarwood, 2000; Solti et al., 2000; FAO, 2018). However, these breeds have a very different context and history than the commercial ones regarding genetics selection, inbreeding, farm modernization, and rearing methods. Considering semen cryopreservation and AI, the protocols adapted for commercial breeds might not be optimal in this situation. Therefore, they must be tested and improved.

The Asturiana de los Valles (AV) breed and the other Asturian autochthonous cattle breeds are essential for maintaining the local culture and economic activity. Therefore, in this thesis, we used bulls from the AV breed as an attempt to improve the sperm freezing protocols of this breed for use in AI centers. The results could also be useful for other rustic breeds with similar characteristics.

2.2 Sperm damage during the cryopreservation process

The diverse cell types have varying cryotolerance; therefore, the capacity of the cell to survive under an equivalent freezing regime differs, and each type has an ideal cooling rate (Mazur, 1963; Pegg, 2009). Regarding spermatozoa, the plasma membrane at physiological temperature is fluid. This fluidity is related to the content of phospholipids and cholesterol within it. Moreover, regions of fluid and gel phase coexist in the plasma membrane, being essential to maintain an efficient function (Elmoazzen et al., 2009; Lemma, 2011; Grötter et al., 2019). When membranes are cooled, the fluid phase of lipids is subjected to a transition to a crystalline liquid state, in which the chains of fatty acids scramble (Lemma, 2011). Near the lipids freezing point, the crystalline liquid state solidifies and transforms into a gel state, where the fatty acid chains are arranged in a parallel way, resulting in a rigid structure (Grötter et al., 2019). This lipid transition phase happens during high-speed cooling (cooling at rates higher than a few degrees per minute, above the freezing point of water). It is associated with the leakage of solutes across cell membranes, particularly potassium, and the uptake of calcium, which suddenly increases near the end of the phase transition (Drobnis et al., 1993). These changes in the lipid composition, which are combined by the leakage of solutes across the cell membrane, are associated with cellular injury, and this phenomenon is commonly known as “cold shock damage” (Drobnis et al., 1993; Gao and Crister, 2000; Holt, 2000; Grötter et al., 2019). This has a considerable effect on the other cellular structures and functions, such as a decrease in cellular metabolism, damage to intracellular organelles, alteration of the mitochondrial function, irreversible loss of motility, decreased energy production, and increasing numbers of non-fertile and dead sperm (De Leeuw et al., 1990; Drobnis et al., 1993; Curry, 2000; Leibo and Songsasen, 2002; Lemma, 2011).

The rearrangement of the membrane lipids during the cooling, freezing, and thawing process, which is associated with an increase in membrane permeability, also leads to the initiation of the premature capacitation process to a subpopulation of sperm. This phenomenon is called "cryocapacitation" (Bailey et al., 2000). Cryocapacition of the sperm happens due to the exit of cholesterol (Grötter et al., 2019) and the entrance of extracellular ions like calcium (Cormier et al., 1997), which increase membrane fluidity and explain the readiness of frozen/thawed spermatozoa to premature capacitation (Cormier et al., 1997).

The spermatozoa that undergoes cryocapacitation has a shorter lifespan, along with a reduction in its fertilizing capacity (Bailey et al., 2000).

The occurrence of the lipid transition phase shows species and individual dependence (Drobnis et al., 1993; Grötter et al., 2019). Generally, when the chains of fatty acids are longer, the temperature at which the lipid transition occurs is higher (Lemma, 2011). Abrupt lipid phase transition is associated with a low proportion of cholesterol in the cell membrane. Elevated cholesterol levels are thought to stabilize membranes during cooling (Drobnis et al., 1993; Holt, 2000). Human and rabbit sperm are highly resistant to cold shock, and a sudden lipid phase transition was not detected, because of a high sterol content in the plasma membrane of these sperm (Drobnis et al., 1993; Holt, 2000; Grötter et al., 2019). Bulls, horses, and boars produce sperm that is highly susceptible to cold shock (Grötter et al., 2019). Indeed, bull and boar spermatozoa undergo the lipid phase transition in a variety of temperatures, ranging from 38 °C to 0 °C (De Leeuw et al., 1990). The lipid transition phase happening during both freezing and thawing in the freeze-thaw cycle (De Leeuw et al., 1990; Holt, 2000).

Proteins are essential components of sperm membranes, and their interactions with lipids are critical for the membrane active function. When sperm are exposed to a freezing temperature below the transition phase temperature, leading to a change to the lipid gel state, this results in an interruption of the lipid-protein interactions. Therefore, the proteins no longer act as effectively as enzymes, receptors, or ion channels (Grötter et al., 2019). Lemma (2011) considered that these changes affect both plasma membrane integrity and motility, which can be regarded as indicators of sperm viability and metabolic activity. Sperm with low motility have minimal opportunity to reach the fertilization site *in vivo* and thus fertilize the oocyte (Grötter et al., 2019).

2.3 Influence of the cryoprotectant and equilibration time on the post-thawing semen quality

Cryopreservation aims to indefinitely maintain the viability and functionality of spermatozoa at freezing temperatures (sub-zero). However, it is currently unavoidable causing deleterious effects on cell structure during the freezing and thawing (Pegg, 2009; Woods et al., 2004). When cells cool to beneath freezing point, both ice production and osmotic imbalance will subject the plasma membrane components to irreversible changes, causing cellular damage – that is, cryoinjury (Woods et al., 2004).

During freezing, water must move through the cell membrane (Figure 2.1). When the cooling speed is low (slow freezing), most water will leave the cells, and the ice crystals will develop extracellularly (Meryman, 2007; Pegg, 2009). While ice forms extracellularly, the extracellular solute concentration of the unfrozen fraction will increase, leading to cell dehydration due to osmotic imbalance. Therefore, there will be an efflux of water due to the osmotic difference, leading to a reduction in cell size. The prolonged exposure of the cells to hyperosmotic conditions causes an increase in the concentration of electrolytes in the cytosol. Moreover, the plasma membrane could undergo injury, resulting in irreversible alterations on its permeability. Extracellular ice crystals can further penetrate the plasma membrane, growing intracellularly and causing mechanical damage to intracellular components. Conversely, fast cooling rates cause intracellular ice growth, since water does not have enough time to leave the cells, leading to damage to the cell organelles (Mazur, 1963; Mazur, 1970; Meryman, 2007; Pegg, 2009; Elmoazzen et al., 2009; Grötter et al., 2019). Intracellular ice could recrystallize during thawing, leading to further damage (Gao and Crister, 2000; Yeste, 2016).

As such, it is necessary to adjust the cooling speed of the aqueous solution in which cryopreservation occurs, thus protecting cells from cryoinjuries and maximizing the post-thawing cell survival. For this reason, a major step in freezing protocols is the addition of cryoprotective substances to the freezing extender, termed cryoprotectants, to reduce cryodamage, both preventing ice nucleation and protecting cellular structures (Fuller, 2004; Pegg, 2009; Sieme et al., 2016; Grötter et al., 2019).

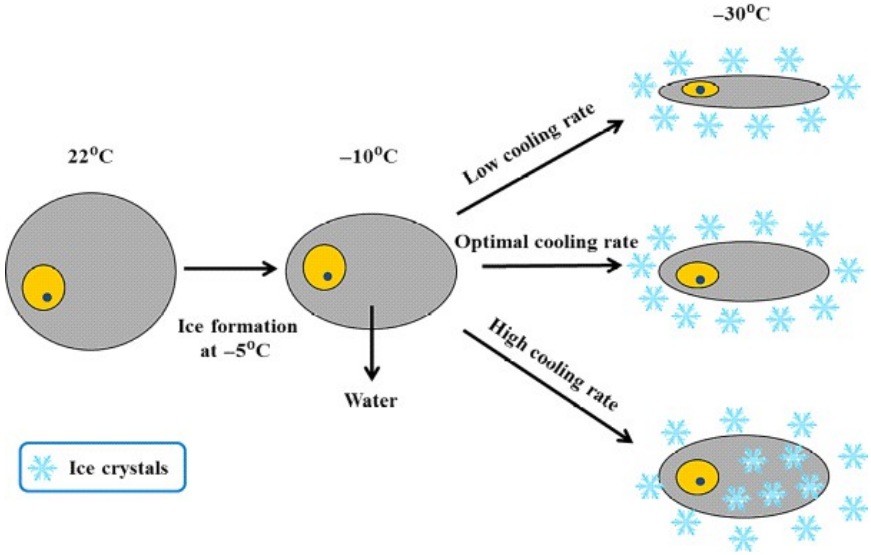

Figure 2.1. The events that occur in the cell during the freezing process. If the cooling rate is too slow, there will be no formation of intracellular ice crystals, but dehydration will be excessive (osmotic stress) If the cooling rate is too fast, intracellular ice nucleation will occur, which could cause damage to organelles and produce the phenomenon of recrystallisation during thawing (Grötter et al., 2019).

The freezing extenders are generally based on a buffered media with a range of components to protect the cells during the freezing/thawing process. An essential component in the semen extenders is a cell membrane penetrating cryoprotectant (glycerol, dimethyl sulfoxide, ethylene glycol, 1,2-propanediol, butanediol, methanol, and others), which are osmotically inactive and have an equal distribution inside and outside the cells. Glycerol was the first successful cryoprotectant for spermatozoa (Polge et al., 1949), and it has become the major component of most protocols for semen cryopreservation for many mammal species, including bull (Holt, 2000; Sieme et al., 2016). An important point to consider is that most of these permeable cryoprotectants are osmotically inactive. However, its presence in the freezing medium changes the osmotic balance inside and outside the cells: Water moves faster than glycerol across the cellular membrane, leading to an initial osmotic disturbance and shrinkage of the cells. The cryoprotectant quickly equilibrates, with cells retrieving their original volume (Sieme et al., 2016).

In many cases, extenders include non-permeating cryoprotectants (Sieme et al., 2016; Grötter et al., 2019), which help to dehydrate the cells and protect membranes, thus preventing the osmotic stress. They can be divided into osmotically active smaller molecules like salts and sugars (disaccharides, e. g. sucrose and trehalose) and osmotically inactive

macromolecules like polysaccharides (hydroxyethyl starch, maltodextrin, and other sugars), proteins (albumin, polyvinylpyrrolidone), lipids, and cholesterol and even ice blockers (Lv et al., 2019). Both permeating and non-permeating cryoprotectants will work together to lower the freezing point of the medium (the minimum temperature at which the solution remains in a liquid state) allowing increasing the fluid viscosity below the water freezing point. Ideally water turns into a glassy-like form (vitreous state), therefore preventing ice formation (Evans and Maxwell, 1987; Meryman, 2007; Sieme et al., 2016; Grötter et al., 2019). Nevertheless, this vitrification process has not achieved yet a commercial application for animal semen, being cryopreservation with ice formation the widespread method. The choice of extenders ranges from very simple (e.g., skimmed milk) to very complex, comprising permeable and non-permeable cryoprotectants (Sieme and Oldenhof, 2015). There are a wide range of commercial extenders (Vishwanath and Shannon, 2000; Lima-Verde et al., 2018; Lonergan, 2018; Miguel-Jimenez et al., 2020), mainly based in non-animal supplements such as soybean lecithin (Lima-Verde et al., 2018; Murphy et al., 2018). BIOXcell (IMV) is one of these extenders, which is based on soybean extract and has been extensively used for freezing bull semen (Layek et al., 2016). In this thesis, we used this extender and also OPTIXcell (IMV), the liposome-based extender, in several experiments to improve the freezing protocols of bull semen.

One of the essential steps in the process of semen cryopreservation is the equilibration phase. The equilibration period begins with the cooling of semen, where the temperature gradually decreases from room temperature to around 5 °C. The spermatozoa are subsequently left to equilibrate further, allowing enough time to interact with cryoprotectants, thereby giving cryoprotectant sufficient time to penetrate the cell membrane into the spermatozoa, in order to establish equilibrium between its intracellular and extracellular concentration (Salamon and Maxwell, 2000; Leite et al., 2010; Tirpan et al., 2017). Indeed, the equilibration was thought to be essential for glycerol equilibration. However, glycerol penetrates bull sperm rapidly, and no extended time is required for this reason; additionally, it can be added at any time during the cooling period (Berndtson and Foote, 1969; Coulter, 1992; Muino et al., 2007). The equilibration time also applies to other osmotically active components, which must distribute in and out of the cells (Tirpan et al., 2017).

Other authors (Mazur, 1984; Salamon and Maxwell, 2000; Bailey et al., 2000; Meryman, 2007) have pointed out that equilibration time allows the plasma membrane to adjust to the

new temperature range and adapt to the freezing step, also enabling a controlled decrease of the metabolic activity of spermatozoa. Leite et al. (2010) confirmed that the equilibration period during cryopreservation was essential for preserving total and progressive motility, plasma membrane and acrosomal integrity. In that study, the equilibration of bull semen (two and four hours) yielded significantly better results than no equilibration. However, there is no agreement on what period of equilibration is best for obtaining high-quality semen after thawing (Leite et al., 2010; Fleisch et al., 2017).

Conflicting reports and disagreements exist regarding the effect of the equilibration time on the quality and fertility of bovine spermatozoa. The extension of the equilibration time — from four to eighteen, twenty-four, and seventy-two hours— improved post-thawing bull semen quality in several studies, while not affecting field fertility (Foote and Kaproth, 2002; Fleisch et al., 2017). Similarly, Shahverdi et al. (2014) confirmed that the post-thawing semen quality of buffalo bull increased when the equilibration time was extended from two to sixteen hours, whilst equilibration time had no effect on DNA integrity. Shah et al. (2016) showed that the equilibration time at four hours was better than two hours and six hours regarding post-thawing buffalo bull semen quality. Herold et al. (2006) confirmed that the equilibration time had no effect on post-thawing buffalo semen quality. It is possible that the equilibration time depends on the type of extender (buffer and cryoprotectant) (Marshall, 1984; Foote and Kaproth, 2002; Muino et al., 2007; Leite et al., 2010; Shahverdi et al., 2014; Fleisch et al., 2017; Amal et al., 2019), and could interact with the freezing and thawing procedures (Marshall, 1984; Shah et al., 2016; Tirpan et al., 2017).

Additionally, commercial AI centers tend to optimize the production of semen doses, and the equilibration time is one of the factors involved in that optimization. centers processing high numbers of ejaculates find it challenging to handle all of the semen collected within the same working day. In these conditions, it would be more practical to collect the samples, then freeze them the following morning (Foote and Kaproth, 2002; Muino et al., 2007; Shahverdi et al., 2014; Fleisch et al., 2017). Similarly, extending equilibration time is practically appropriate for semen collected in the field, which is far away from the freezing facilities (Foote and Kaproth, 2002).

Most studies approaching prolonged equilibration periods were carried out using egg yolk or milk-based extenders (Foote and Kaproth, 2002; Almeida et al., 2017; Shah et al., 2016), although a few used egg yolk-free extenders (Fleisch et a., 2017). In SERIDA (Regional

Service for Agrifood Research and Development), where we conducted some studies for this thesis, they use BIOXcell extender in bull semen freezing. We studied the potential improving effect of extending equilibration time to twenty-four hours whilst using this extender on post-thawing bull semen quality. To the best of our knowledge, no study has used BIOXcell as bull semen extender for prolonging equilibration time, and there is only one study in existence that was conducted on buffalos (Shahverdi et al., 2014). In another study in this thesis, conducted in the Xenética Fontao artificial insemination center, we assessed the impact of extending equilibration time to twenty-four hours on post-thawing bull semen quality using OPTIXcell extender.

2.4 Impact of reactive oxygen species (ROS) on semen quality, and the role of antioxidants as supplements to the semen extender

The univalent reduction of oxygen leads to the production of reactive intermediate products called reactive oxygen species (ROS). ROS includes superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$), all of which are considered to be harmful to the cells (Hammond and Hess, 1985). ROS are oxidizing agents generated as by-products of oxygen metabolism behaving as highly reactive molecules (Henkel, 2011). If ROS levels exceed the ability of the cells to remove them, oxidative stress results, which may cause damage and could result in cell death (Hammond and Hess, 1985; Schieber and Chandel, 2014).

In spermatozoa, the major sources of endogenous ROS are dead and abnormal, or immature, spermatozoa, as well as the leukocytes that can pass to the ejaculate (Shannon and Curson, 1972; Aitken and Clarkson, 1987; Agarwal et al., 2006). ROS accumulation in the semen is limited by a combination of enzymatic and non-enzymatic antioxidants in the seminal plasma and in sperm membranes and cytoplasm. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are major enzymatic antioxidants (Ball et al., 2000; Baumber et al., 2000; Baumber et al., 2005; Baumber and Ball, 2005). Reduced glutathione (GSH), α-tocopherol, ascorbic acid, carotenoids, ubiquinones, taurine, vitamin E, hypotaurine, and many others, are non-enzymatic antioxidants (Alvarez and Storey, 1983; Kefer et al., 2009; Agarwal et al., 2014).

However, ROS have a very important physiological role in cells. They participate in signaling pathways, for they function as signaling molecules (Khan and Wilson, 1995; Schieber and Chandel, 2014; Gao et al., 2017). In spermatozoa, ROS are responsible for maintaining and activating important physiological functions, such as motility, hyperactivation, capacitation, and acrosome reaction, as well as the zona binding process and fertilization, by promoting tyrosine phosphorylation (Kodama et al., 1996; O'flaherty et al., 1997; de Lamirande et al., 1997; O'Flaherty et al., 1999; Gao et al., 2017). Therefore, the balance between the generation of ROS and the scavenging capacity of the endogenous antioxidants must be precisely controlled, in order to protect the spermatozoa, whilst maintaining their fertilizing ability (Baumber et al., 2000; Agarwal et al., 2014).

The sperm plasma membrane is rich in polyunsaturated fatty acids (PUFAs), rendering them extremely susceptible to oxygen-induced damage, and hence lipid peroxidation (LPO).

The large number of mitochondria, low volume and antioxidants in the cytoplasm are other factors rendering spermatozoa more susceptible to oxidative stress (Aitken et al., 1989; Ball, 2008).

High levels of ROS in spermatozoa and seminal plasma result in the initiation of LPO of membranes, oxidative damage to proteins and enzyme inactivation, depletion of antioxidants (Agarwal et al., 2014; Harchegani et al., 2019), increased percentage of apoptotic cells (Simões et al., 2013), reduced sperm motility, viability, mitochondrial activity (Baumber et al., 2000; Tvrda et al., 2016a; Tvrda et al., 2019), and DNA integrity. This results in reduced sperm fertility and embryo quality (Simões et al., 2013; Harchegani et al., 2019). The lack of enzyme repair systems, and low antioxidants in spermatozoa, results in the inability to recover the damage caused by ROS and LPO (Peris et al., 2007; Agarwal et al., 2014). (Figure 2.2).

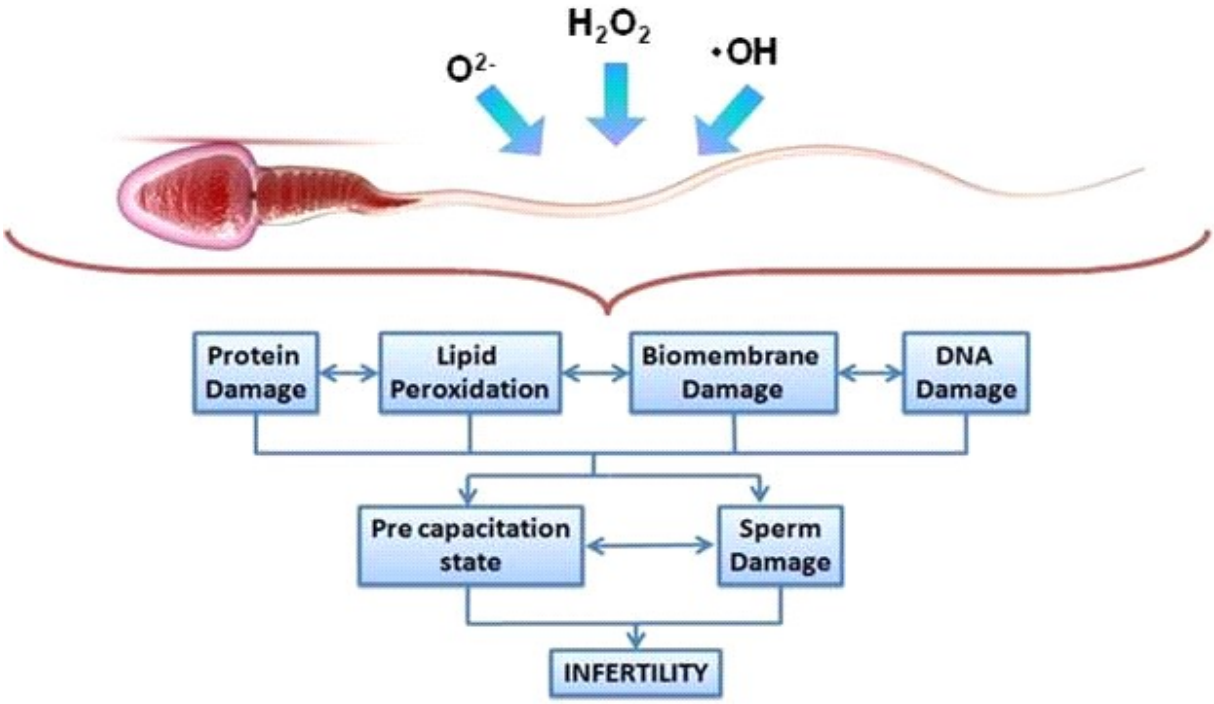

Figure 2.2. Effect of ROS on sperm physiology. The increase in extracellular ROS levels after ejaculation induces several changes in sperm physiology, including protein damage, lipid peroxidation, membrane and DNA damage a premature capacitation state, and a decrease in the sperm lifespan (Cuevas et al., 2013).

During semen cryopreservation, the generation of high levels of ROS by several sources remains one of the leading causes of sperm cryoinjury, resulting in low cryopreserved sperm quality and fertility post-thawing (Mazzilli et al., 1995; Chatterjee and Gagnon, 2001). Several authors have shown that LPO in the plasma membrane of spermatozoa increased after the cryopreservation (Chatterjee and Gagnon, 2001; Gao et al., 2017), and it appears to be associated with a decrease in sperm membrane fluidity (Giraud et al., 2000; Chatterjee and Gagnon, 2001; Gao et al., 2017). The freezing/thawing process of sperm either leads to a reduction in endogenous antioxidant levels, or a decrease in their activity to metabolize ROS toxicity, which may affect the sperm function (Lasso et al., 1994; Bilodeau et al., 2000; Gadea et al., 2011; Gao et al., 2017). Therefore, several experiments in this thesis have focused in the use of several molecules as antioxidants for improving bull semen cryopreservation.

2.4.1 Trehalose and reduced glutathione (GSH) as supplements to semen freezing extenders

Trehalose is a disaccharide and a non-permeant cryoprotectant. It has a protective role against the osmotic effect that may happen during the freezing-thawing process. Trehalose causes cellular osmotic dehydration before freezing, therefore decreasing the amount of water inside the sperm, thus protecting spermatozoa from cryoinjury by ice crystallization (Chen et al., 1993; Molinia et al., 1994; Aboagla and Terada, 2003).

Trehalose is a non-reducing sugar; thus, it is not an antioxidant. However, many studies have reported antioxidant effects (Chhillar et al., 2012; Kumar et al., 2013; Iqbal et al., 2018). The antioxidant properties of trehalose may be related to its effectiveness in sperm membrane stabilization (Aisen et al., 2005; Hu et al., 2010), or that the supplementation of semen extender with trehalose has an enhancing effect on the activity of different antioxidants in semen, thus decreasing the oxidative stress and improving cryopreserved semen quality post-thawing (Hu et al., 2010; Iqbal et al., 2016; Wang and Dong, 2017).

Several authors have reported the enhanced effect of the supplementation of freezing extender with trehalose on sperm motility and different sperm physiological parameters, such as viability, membrane integrity, acrosome integrity, mitochondrial activity, and intact DNA, as well as decreasing the percentage of cryocapacitated and abnormality in bovine sperm post-thawing (Hu et al., 2010; Chhillar et al., 2012; Oh et al., 2012; Kumar et al., 2013; Büyükleblebici et al., 2014; Ozturk et al., 2017). Adding trehalose to the semen freezing extender reduced ROS production and decreased lipid peroxidation, thus improving bull

sperm quality post-thawing (Chhillar et al., 2012). The same results have also been reported in different species, such as buffalo (Kumar et al., 2013; Iqbal et al., 2016, Iqbal et al., 2018), goats (Aboagla and Terada, 2003), red deer (Wang and Dong, 2017), and sheep (Matsuoka et al., 2006).

In this thesis, we have proposed using trehalose for improving the post-thawing quality of semen frozen with BIOXcell. Previous studies, including supplementation of semen freezing extender with trehalose, have been conducted by using egg yolk or milk-based extenders. To our knowledge, there are no reports that include using egg yolk-free extenders such as BIOXcell, in bull, which make our study the first in this regards.

Another supplement that is potentially beneficial to semen cryopreservation is reduced glutathione (GSH), a non-protein thiol composed of three amino acids: cysteine, glycine, and glutamate. It is ubiquitous in living beings (Lu, 2009; Lu, 2013). GSH has a strong electron-donating character; thus, it has a high ability to neutralize free radicals in the cells by bonding its extra electron to the reactive oxygen species. When the GSH molecule loses its electron, it becomes oxidized. Two oxidized GSH become linked by a disulphide bridge to form glutathione disulphide or oxidized glutathione (GSSG), a reaction catalized by glutathione peroxidase (GSH-Px). This linkage is reconverted back to reduced glutathione by the glutathione disulphide reductase, which uses reducing equivalents from NADPH, creating a dynamic balance between GSH synthesis, its utilization, and its recycling from GSSG. Therefore, both GSH and GSSG are considered as essential antioxidants in the cells (Brown et al., 1977; Meister, 1994). (Figure 2.3).

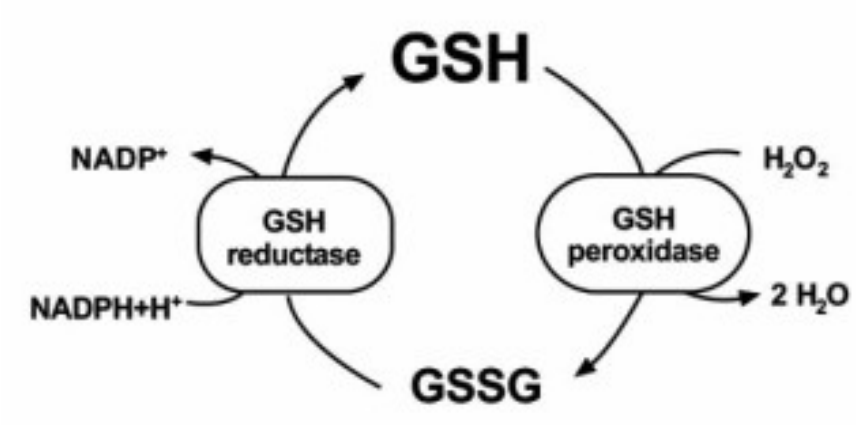

Figure 2.3. GSH and its recycling from GSSG (Russell et al., 2008).

GSH can react with many ROS directly, or as a cofactor for GSH-Px, an enzyme that detoxifies H_2O_2 and peroxides generated from the attack of oxygen radicals to the spermatozoon (Bilodeau et al., 2001). In the case of sperm cryopreservation, the high levels of ROS produced due to the process leads to a steep decline in sperm GSH levels, as reported in bull (Bilodeau et al., 2000), boar (Gadea et al., 2004), and humans (Gadea et al., 2011), being associated with a decrease in sperm quality.

Supplementation of GSH as an antioxidant to the semen extender pre- or post-thawing has been reported in several studies. For example, Shah et al. (2017a) showed that GSH could be effectively used as an additive in bull semen freezing extender, in order to reduce cryocapacitation, apoptosis-like changes, fragmented DNA, and to increase the percentage of spermatozoa having high transmembrane mitochondrial potential. Adding GSH and GGSG to the BIOXcell freezing extender led to a lower portion of acrosome damage and total abnormalities on post-thawing bull sperm (Sariözkan et al., 2009). Ansari et al. (2012) indicated that GSH, which was supplemented to the pre-freezing extender, increased sperm motility, plasma membrane integrity, viability, and sperm DNA integrity, as well as the *in vivo* fertility rate of post-thawing buffalo sperm. However, Foote et al. (2002) and Sariözkan et al. (2009) did not find an effect of supplementing the freezing extender with different concentrations of GSH on the field fertility of thawed bull semen.

Regarding the supplementation of GSH to the bull semen thawing medium, Gadea et al. (2008) pointed out that it resulted in a higher number of non-capacitated viable sperm, a reduction in ROS generation, lower DNA fragmentation, as well as improving the fertilizing capacity *in vitro*. These researchers further did not find an effect on motility parameters. GSH supplementation to the freezing extender increased the activity of some antioxidants, like superoxide dismutase (SOD) and glutathione peroxidase (GPx), which accompanies enhanced semen quality post-thawing (Wang and Dong, 2017).

A combination of GSH with other antioxidants, such as vitamin C, to the freezing extender resulted in increased individual sperm motility, viability, plasma membrane integrity, and acrosome integrity in post-thawing bull sperm (Eidan, 2016), whilst a combination of GSH with SOD improved the total and progressive motility and GPx activities post-thawing (Karaji et al., 2014).

An improvement in semen quality, fertility, and a decrease in ROS levels, resulting from the supplementation of freezing extender with GSH, was also reported in buck (Razliqi et al.,

2015), boar (Yeste et al., 2014), red deer (Wang and Dong, 2017), buffalo (Ansari et al., 2012), and humans (Gadea et al., 2011).

Only one other study has used egg yolk-free extenders supplemented with GSH in bull (Sariözkan et al., 2009). In that study, only one concentration of GSH (2 mM) was used. In this thesis, we use supplementation of different concentrations of GSH to the BIOXcell freezing extender, with the aim of improving post-thawing bull semen quality.

2.4.2 Curcumin and crocin as supplements to the semen freezing extender

There is an interest on the use of natural compounds as antioxidant additives in freezing or thawing semen extender, in order to reduce the harmful effects of ROS on sperm. In this thesis, we were interested in studying the possible benefits of the natural antioxidants curcumin and crocin as supplemented to the bull semen freezing extender.

Curcumin (diferuloylmethane) is a natural radical-trapping antioxidant (Valgimigli and Pratt, 2012). It is a yellow polyphenolic compound from the spice turmeric, isolated from the herb *Curcuma longa* by crushing the herb roots into powder (Tvrda et al., 2016a; Cousins et al., 2007). Turmeric has also been used as a cosmetic and medical treatment, in addition to its use as a food ingredient (Cousins et al., 2007). Moreover, curcumin has anti-inflammatory and anti-cancer properties (Menon and Sudheer, 2007). It is a scavenger to different ROS (Reddy and Lokesh, 1994; Unnikrishnan and Rao, 1995; Zhang et al., 2017). Tvrda et al. (2016a) pointed out that curcumin behaves as a potent antioxidant, preventing oxidative damage to spermatozoa. Thus, it preserves the motility, viability, and mitochondrial activity, as well as enzymatic and non-enzymatic antioxidants, in bull sperm subjected to induced oxidative stress. Curcumin supplementation to the semen extender led to a decrease in the percentage of the total sperm abnormalities and an enhancement of the sperm viability post-thawing in bull (Bucak et al., 2012) and goats (Bucak et al., 2010). Shah et al. (2017b) emphasized that the supplementation of the semen extender with curcumin led to improved sperm quality and DNA integrity post-thawing, as well as increasing the total antioxidant contents and decreasing lipid peroxidation at pre-freezing and post-thawing in buffalo bull. Similar findings were found in boar (Chanapiwat and Kaeoket, 2015), and in goats (Bucak et al., 2010).

Crocin is a glucosyl ester of crocetin, a water-soluble carotenoid extracted from the perennial herb saffron (*Crocus sativus*). These carotenoids act as antioxidants, with a high ability to scavenge the free radicals (Bors et al., 1982; Rios et al., 1996; Bakhtiary et al., 2014;

Sapanidou et al., 2016; Esposito et al., 2019), especially superoxide anion (Galano et al., 2010). The supplementation of bull semen with 1 mM of crocin post-thawing improved the motility, viability, and acrosomal integrity of sperm, reducing the ROS levels and lowering lipid peroxidation (Sapanidou et al., 2015; Tsantarliotou et al., 2016). Cyclophosphamide-treated Mice that received an intraperitoneal dose of crocin showed improved sperm quality (Bakhtiary et al., 2014).

To the best of our knowledge, there are no previous studies of the use of curcumin or crocin for freezing bull spermatozoa in BIOXcell extender. Thus, in our study, we supplement BIOXcell with different concentrations of curcumin and crocin, to test the potential, improving the impact of that on post-thawing sperm. This is the first report in this regards.

2.5 Application of sperm selection procedures on cryopreserved semen quality

There are various criteria on the concept of sperm quality (Morrell and Rodriguez-Martinez, 2009). Colenbrander et al. (2003) considered that sperm quality could be described regarding sperm number, motility, and morphological normality. Other authors have added additional parameters, such as sperm membrane integrity and chromatin integrity (Graham, 2001; Rodriguez-Martinez, 2006). Generally, it is supposed that *in vivo* fertilization should be carried out by spermatozoa with high motility, normal morphology, intact membranes, and intact chromatin (Morrell and Rodriguez-Martinez, 2009). However, spermatozoa must overcome more stringent challenges before fertilization of the oocyte *in vivo* than *in vitro* (Thys et al., 2009). *In vivo*, spermatozoa quickly move away from seminal plasma when semen is deposited, and make their way to the location of fertilization in a limited period (Bedford, 2004). Some parts of the female reproductive tract represent a barrier to the crossing of sperm; thus, poorly viable spermatozoa cannot go forward (Suarez, 2006). According to the site of semen deposition during natural mating, or when using AI, some parts of the female reproductive tract (e.g., the cervix and utero-tubal junction) present the first impediment to sperm progress for filtering out poorly viable spermatozoa (Hunter and Rodriguez-Martinez, 2004). During their way through the female reproductive tract, spermatozoa undergo a series of changes (capacitation and acrosome reaction) to prepare the plasma membrane for the final binding to the zona pellucida (Bedford, 2004). Several mechanisms have been proposed *in vitro* that could be used to mimic the selection of high-quality sperm in the female

reproductive tract and to filter spermatozoa from the seminal plasma, such as colloid centrifugation (Morrell and Rodriguez-Martinez, 2009).

Although seminal plasma is useful to sperm function, it is also harmful to sperm survival (Morrell and Rodriguez-Martinez, 2009). Seminal plasma contains decapacitation proteins that help to maintain sperm function (Perez-Pe et al., 2001), which must dissociate before reaching the zona pellucida of the oocyte (Morrell and Rodriguez-Martinez, 2009). Moreover, depending on the species, seminal plasma contains motility inhibiting factors that curbs sperm motility *in vitro* (Kordan et al., 1998), and it can contain ROS (Hammadeh et al., 2008). Moreover, seminal plasma could be a via of transmission for a variety of pathogens to the female reproductive tract (Morrell, 2006). Sperm survival during cool storage and cryopreservation can be improved by removing most of the seminal plasma in stallion (Aurich, 2005; Moore et al., 2005), and boar (Roca et al., 2006).

Colloid centrifugation is a technique in which extended semen is centrifuged through layers of colloid, in order to separate spermatozoa from the seminal plasma and to select spermatozoa with high-quality and chromatin integrity, which are deposited as a pellet. Different centrifugation conditions (e.g., force and time), and various physical properties of colloids, can be used in this process (Morrell and Rodriguez-Martinez, 2009).

Density gradient centrifugation (DGC, figures 2.4a and 2.4b) is a technique of colloid centrifugation in which there are at least two layers of colloid, of different densities. This technique separates motile, morphologically normal, chromatin-intact spermatozoa from the rest of the ejaculate (Morrell, 2006). Moreover, spermatozoa can be separated from bacteria (Nicholson et al., 2000) and viruses (Englert et al., 2004; Morrell and Geraghty, 2006), which may be found in the ejaculate. Brahem et al. (2011) confirmed that DGC enabled selecting spermatozoa with higher DNA integrity. A limitation is that it is only possible to process small volumes of ejaculate at once (Edmond et al., 2008).

A new method, so-called Single Layer Centrifugation (SLC), was developed at the Swedish University for Agricultural Sciences (SLU), as a simplification of DGC. In this way, only one layer of colloid can be used, also enabling it to process a larger volume of sample (maybe whole ejaculates) than DGC (Thys et al., 2009). The seminal plasma is kept above the colloid, whilst the spermatozoa moves to the bottom of the conical centrifuge tube during centrifugation (Morrell and Rodriguez-Martinez, 2009) (Figure 2.4c). The duration of the preparation of SLC is shorter, and the process is less complicated than the DGC. Morrell et al.

(2008) showed that the species-specific SLC formulation enabled it to produce spermatozoa of comparable quality rather than DGC using fresh stallion semen. Similarly, Thys et al. (2009) confirmed that SLC offers an alternative method to DGC, with equivalent results of semen quality and *in vitro* fertility rate of frozen-thawed bull spermatozoa. Moreover, the removal of pathogens contained in the semen sample is an additional advantage of using SLC (Morrell and Rodriguez-Martinez, 2009).

Percoll™ was the colloid most commonly used for animal semen. It consists of polyvinylpyrrolidone (PVP)-coated silica particles in a saline solution, used in DGC (Morrell and Rodriguez-Martinez, 2009). By late 90s and early 2000s, Percoll™ was replaced by silane-coated silica colloids and as species-specific formulations for use in semen samples of different animal species (Morrell, 2006; Morrell and Geraghty, 2006; Samardzija et al., 2006; Morrell and Rodriguez-Martinez, 2009; Thys et al., 2009; Martinez-Alborcia et al., 2013; Šterbenc et al., 2019), and for humans (Brahem et al., 2011).

BoviPure™ is a colloid specifically formulated for bull semen (Samardzija et al., 2006; Valeanu et al., 2015). It is produced by the Nidacon International AB, at Gothenburg, Sweden (Morrell and Rodriguez-Martinez, 2009). Valeanu et al. (2015) showed that the use of the BoviPure gradient as SLC after thawing improved bull sperm quality and decreased DNA fragmentation, both at 0 hours and after a six-hour incubation period. Samardzija et al. (2006) showed that post-thawed bull sperm separated by BoviPure showed higher quality and embryonic developmental rate after IVF than those separated by Percoll. However, Arias et al. (2017) indicated that the post-thawing use of both Percoll and BoviPure as SLC increased the proportion of bull spermatozoa with high $\Delta\Psi_m$ (mitochondrial membrane potential).

Using SLC with Bovicoll on pre-freezing bull semen increased the post-thawing proportion of live spermatozoa with high $\Delta\Psi_m$; however, it also increased superoxide-positive spermatozoa, while there was no effect on membrane or chromatin integrity (Nongbua et al., 2017). SLC was successfully used for obtaining high-quality spermatozoa from frozen-thawed stallion semen (García et al., 2009; Johannisson et al., 2009), cooled stored stallion semen (Morrell et al., 2009), and frozen-thawed ram semen (Šterbenc et al., 2019).

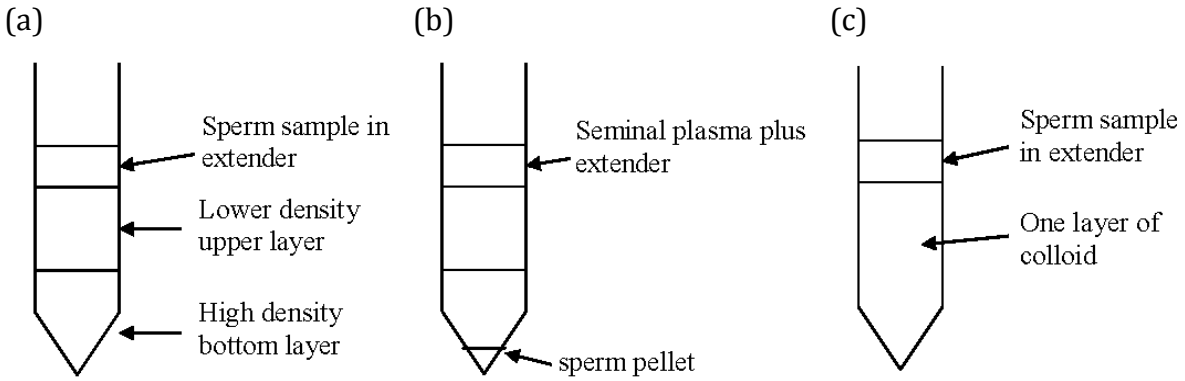

Figure 2.4. Colloid centrifugation: (a) density gradient before centrifugation (double layer centrifugation, DLC); (b) density gradient after centrifugation; (c) single layer centrifugation before centrifugation (modified from Morrell and Rodriguez-Martinez, 2009).

The positive results after the freezing of selected sperm by SLC or DGC may not solely be due to the direct effect of the withdrawal of dead or abnormal sperm from the rest of the ejaculate. These spermatozoa could harm the entire sample by producing toxic substances or free radicals, possibly exacerbated in freezing/thawing (Shannon and Curson, 1972; Aitken and Clarkson, 1987; Agarwal et al., 2006). Therefore, their elimination would prevent the exposure of the other sperm to these substances and improve the quality and fertilizing ability of spermatozoa surviving the cryopreservation (Martinez-Alborcia et al., 2013).

Gloria et al. (2016), in a study on bull semen, showed that both SLC and double layer centrifugation (DLC) using iodixanol gradient before semen cryopreservation improved sperm characteristics in poor quality samples post-thawing, although there was also a reduction in the number of spermatozoa recovered.

We performed a collaboration with the breeding center Xenética Fontao (Xunta de Galicia, Lugo, Spain), for evaluating both SLC and DLC using the BoviPure colloid to improve freezability and post-thawing bull semen quality. This included semen samples of different post-thawing quality to improve specifically samples from difficult but genetically valuable sires. To our knowledge, this is the first study to use the BoviPure colloid for sperm selection before freezing in bull.

2.6 Sperm function tests to assess sperm quality post-thawing

Freezing-thawing affects sperm functionality, with even sublethal damage abolishing sperm fertility (Holt, 2000). Sperm should be motile and with functional, competent plasma membrane, intact acrosome, active mitochondria, and intact DNA to reach and fertilize the oocyte (Gillan et al., 2005).

Several techniques allow sperm function testing, with an increased focus on combining the techniques, in order to assess different function aspects at the same time (Gillan et al., 2005; Farah et al., 2013). Advanced technologies, such as computer-assisted sperm analysis (CASA), fluorescence microscopy, flow cytometry, and computer analysis system, have been used to evaluate sperm quality (Holt, 2000).

2.6.1 Motility assessment by computer-assisted sperm analysis (CASA)

CASA systems provide image analysis on microscopic images or recordings of spermatozoa (Amann and Waberski, 2014). These systems couple a microscope with a camera and a computer with specific software. In the case of motility assessment, the CASA analyzes a series of images from real-time or pre-recorded sperm samples, rendering and measuring the sperm trajectories. As a result, these systems provide objective measurements not only of sperm motility (proportion of motile spermatozoa) g but also of kinematic parameters such as velocity, linearity, hyperactivation, progressivity, etc.

Moreover, CASA can provide individual information from each tracked spermatozoa. This allows researchers to carry out multiparametric analyses, allowing to identify different sperm subpopulations (Martínez-Pastor et al., 2011). The study of these individual subpopulations enhance the information obtained from semen samples, allowing to better establishing the physiological condition of the sample (Ramón and Martínez-Pastor, 2018).

2.6.2 Flow cytometry analysis

Flow cytometry and microscopy, coupled with fluorescent staining, are widely applied to evaluate sperm characteristics, with increasing use of flow cytometry as an alternative to microscopy, even if the latter is still essential for examining details of sperm structure (Holt, 2000; Gillan et al., 2005). The discovery of a variety of fluorochromes and compounds conjugated to fluorescent probes has allowed for a more comprehensive analysis of sperm

quality at the level of biochemistry, ultrastructural, and functionalism (Magistrini et al., 1997; Gillan et al., 2005). There are many fluorescent probes (fluorochromes) used to label spermatozoa., providing information on different cellular and molecular components. Fluorochromes absorb photons from laser radiation, causing the redistribution of electrons in that fluorochrome. When these electrons return to their normal state, they emit energy (fluorescence) at a longer wavelength. The different fluorescence intensities that result are collected by special detectors, allowing a multiparameter analysis of the cells. Furthermore, the use of fluorescents with a different emission spectra allows us to analyze several sperm markers simultaneously.

Flow cytometry is a biophysical technique that allows the rapid analysis of individual cell characteristics, such as cell size, subcellular components, cell count, and more. Cells are labeled with fluorescent markers, and carried in a laminar flow to an interrogation cell. The cells are interrogated by the laser, and identified by light scattering and fluorochrome excitation. Flow cytometry makes it possible to assess thousands of cells in seconds, and has been effectively used to describe changes in sperm structure and function (Graham, 2001).

We have used flow cytometry extensively in this thesis, allowing us to determine a large number of sperm physiological variables at once.

Evaluation of sperm viability

The plasma membrane surrounds the sperm cell, and, because of being selectively permeable, maintains the chemical gradient of ions and other soluble components (Silva and Gadella, 2006). Plasma membrane integrity is considered as an indirect indicator of sperm viability (Magistrini et al., 1997).

Many staining protocols can be used to evaluate sperm plasma membrane integrity (Magistrini et al., 1997), mainly with two categories of dyes. One category comprises membrane-impermeable dyes that can pass through the damaged plasma membrane and are excluded by the intact membrane, such as ethidium homodimer, propidium iodide (Martínez-Pastor et al., 2010; Farah et al., 2013), and Hoechst 33258 (Silva and Gadella, 2006; Farah et al., 2013). The other category of viability dyes can be either the non-fluorescent ester of a fluorochrome (e.g. carboxyfluorescein diacetate, calcein), which can pass the membrane and turn into fluorescent substances intracellularly in functional cells (Holt, 2000), or a membrane-permeable fluorescent compound that has affinity for DNA, like SYBR-14 (Silva and Gadella, 2006; Martínez-Pastor et al., 2010; Farah et al., 2013). The combination of two

groups of dyes can be implemented, such as SYBR-14/PI (Holt, 2000; Martínez-Pastor et al., 2010), in which SYBR-14 stains the nuclei of living sperm bright green, whilst PI only stains non-living sperm (Garner and Johnson, 1995).

Evaluation of the acrosome integrity

The acrosome is an acidic secretory organelle filled with hydrolytic enzymes that assist the sperm in penetrating the zona pellucida. Initial sperm-zona binding will elicit the acrosome reaction, resulting in the release and activation of acrosomal enzymes (Silva and Gadella, 2006). Acrosome should remain intact before and during transit of the spermatozoon through the female reproductive tract and until zona binding occurs (Silva and Gadella, 2006). Thus, an early acrosome reaction renders the spermatozoon infertile (Glenn et al., 2007). Freezing induces physical damage in some spermatozoa. Therefore, the proportion of undamaged acrosomes is typically estimated. Since acrosomal integrity does not necessarily reflect plasma membrane integrity, the combination of the two tests provides a more accurate assessment (Holt, 2000).

Acrosomal integrity can be evaluated by using probes that recognize targets inside the acrosome. Anti-CD46 is an antibody that binds to the acrosomal matrix and has been utilized in studies with human sperm (Tao et al., 1993; Martínez-Pastor et al., 2010; Zoppino et al., 2012). Lectins, which are proteins that bind to glycosidic residues, have been used for a similar reason in many animal species (Magistrini et al., 1997; Silva and Gadella, 2006; Martínez-Pastor et al., 2010). The most commonly used lectin conjugates are *Pisum sativum* (pea) agglutinin (PSA), and *Arachis hypogaea* (peanut) agglutinin (PNA) (Tao et al., 1993; Silva and Gadella, 2006). For human sperm, concanavalin A lectins (conA) have been used with the same purpose (Silva and Gadella, 2006). For boar, equine, and canine sperm, PNA labelling was specific for the outer acrosomal membrane, whereas PSA labels acrosomal matrix glycoproteins (Silva and Gadella, 2006). Since PSA and PNA cannot penetrate an intact acrosomal membrane, only damaged or reacted acrosomes spermatozoa are stained (Gillan et al., 2005). However, some techniques first permeabilize the acrosome (mainly for microscopic evaluation), resulting in the labelling of acrosome-intact spermatozoa. Acrosomal probes are generally conjugated with the green fluorochrome fluorescein isothiocyanate (FITC) and combined with viability dyes, such as PI (Silva and Gadella, 2006; Martínez-Pastor et al., 2010; Alvarez et al., 2012).

Assessment of mitochondrial activity

The mitochondrial activity is an indicator of sperm function. Mitochondria are located in the mid-piece region of the sperm flagellum, and they produce ATP to support sperm motility and other functions (Amaral et al., 2013). Rhodamine 123 (R123) was one of the first specific probes, and it is commonly used to evaluate the mitochondrial activity of spermatozoa (Garner et al., 1997). R123 has been displaced by improved probes, like JC-1 (5,5´, 6,6´ tetrachloro-1,1´,3,3´-tetraethylbenzimidazolylcarbocyanine iodide), which has been widely used for sperm analysis (Martínez-Pastor et al., 2010). Another group of highly specific dyes that stain the active mitochondria with a wide range of emission fluorescence is the group of MitoTracker® dyes (Invitrogen, Life Technologies, Carlsbad, California, USA). They have been used to evaluate spermatozoa in multicolor studies in different animal species (Martínez-Pastor et al., 2010). MitoTracker® Deep Red FM is one of the dyes that can be excited with a red laser (635 nm), allowing to combine it with other probes that are excited with a blue laser (488 nm), thus reducing the risk of fluorescence spill-over (Martínez-Pastor et al., 2010).

Detection of plasma membrane fluidity

An increase in the plasma membrane fluidity is considered an indicator of premature capacitation. These changes in the plasma membrane resemble changes occurring during physiological capacitation, and they increase during freezing and thawing (Cormier et al., 1997). The fluorescent lipophilic dye, merocyanine 540 (M540), has been used to evaluate the degree of lipid disorder or fluidity in the plasma membrane of spermatozoa (Peña et al., 2004; Hallap et al., 2006). M540 can be used in combination with membrane-impermeable DNA-binding dyes, such as YO-PRO-1, to distinguish between live and dead ("apoptotic"-dead) sperm. Thus, M540 detects sperm subpopulations with higher membrane fluidity (Peña et al., 2004; Hallap et al., 2006).

Detection of oxidative stress

The detection of reactive oxygen species (ROS) and other free radicals can be carried out by reagents that accumulate intracellularly and become fluorescent upon oxidation (Martínez-Pastor et al., 2010). The 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) has been used to detect the intracellular ROS in spermatozoa. It penetrates the plasmalemma and emits green fluorescent upon oxidation (Martínez-Pastor et al., 2010). An improved variant, CM-H_2DCFDA

(5-(and 6-)chloromethyl-2,7-dichloro-dihydrofluorescein diacetate, acetyl ester), was tested by Domínguez-Rebolledo et al. (2010) in red deer. These probes can be combined with viability dyes to remove the non-viable population from the analysis (Domínguez-Rebolledo et al., 2010; Martínez-Pastor et al., 2010). Whilst CM-H_2DCFDA is non-specific for different kinds of ROS, there are some probes that may detect specific oxidant species (Martínez-Pastor et al., 2010). The MitoSOX™ Red is one such probe, selectively detecting superoxide anion radical ($O_2^{\bullet-}$) produced in the mitochondria (Koppers et al., 2008).

2.6.3 Assessment of DNA integrity

DNA damage occurs in spermatozoa for different reasons. One of the most common causes that affects sperm DNA integrity is the exposure to oxidative stress (Simoes et al., 2013; Harchegani et al., 2019). Cryopreservation can cause sperm DNA damage due to several mechanisms: Increased ROS levels causing strand breaks, deterioration of DNA repair enzymes, and cell shrinkage, which may cause mechanical stress of the nucleus and DNA molecules (Ugur et al., 2019). While the damaged sperm may have the ability to fertilize the oocyte, the embryonic development can be arrested due to DNA damage (Rybar et al., 2004; Castro et al., 2016).

There are many methods for evaluating the sperm DNA damage. The Sperm Chromatin Structure Assay (SCSA) is the first specific method for spermatozoa, described by Evenson et al. (1980), and widely used since (Evenson, 2016). The principle of the method is that fragmented DNA has a higher susceptibility to acid denaturation *in situ* compared to the intact sperm. The denatured DNA is stained by acridine orange (AO). Depending if the AO is bound to single (denaturated) or double (intact) stranded DNA, it emits red or green fluorescence, respectively, which can be detected by flow cytometry. A DNA fragmentation index can be calculated as the ratio between the red fluorescence and the total fluorescence (red + green) (Rybar et al., 2004; Küçük, 2018).

About twelve years after the introduction of the SCSA test, the Terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP Nick End Labelling (TUNEL) test was introduced as another flow cytometric assay for sperm DNA fragmentation (Evenson, 2016; Hassanen et al., 2019). Other methods are available, although they do not use flow cytometry for assessment.

2.7 Justification of the Study

Overall, there has been considerable progress in the cryopreservation protocols for bull semen. However, there are still many challenges preventing excellent results post-thawing. Therefore, the main aim of this thesis was to advance science on bull semen cryopreservation. We worked with Asturiana de los Valles, a Spanish autochthonous cattle breed, and also Holstein breed, due to the availability and collaborations with SERIDA, and also with the Xenética Fontao artificial insemination center in Lugo.

We attempted to advance in this direction by applying different techniques. The prolongation of the equilibration time is one of these strategies, also aiming to improve the logistics at breeding centers. Many studies have confirmed that it would be possible to maintain and even enhance post-thawing quality by extending the equilibration time (Foote and Kaproth, 2002; Shahverdi et al., 2014; Fleisch et al., 2017). Many authors also confirmed that these results might depend on the extender used (Fleisch et al., 2017; Amal et al., 2019). Thus, we tested the effect of extending the equilibration time in two experiments. At SERIDA, we used the BIOXcell extender, and at the Xenética Fontao artificial insemination center, we used the OPTIXcell extender.

Another interest was improving sperm cryopreservation by using non-enzymatic antioxidants. Sperm cryopreservation reduces the endogenous antioxidant levels in spermatozoa and seminal plasma or decreases their activity (Bilodeau et al., 2000; Gadea et al., 2011; Gao et al., 2017), and also causes an increase on reactive oxygen species (ROS) and other harmful molecules. This situation leads to oxidative stress, contributing to lower sperm quality and fertility post-thawing (Mazzilli et al., 1995; Chatterjee and Gagnon, 2001). Thus, we tested the supplementation of the freezing extender with trehalose (a cryoprotectant with some antioxidant properties), reduced glutathione (GSH), curcumin, and crocin. These substances may contribute to removing ROS or protecting the spermatozoa against oxidative stress, hence increasing the post-thawing semen quality (Hu et al., 2010; Tsantarliotou et al., 2016; Shah et al., 2017a; Shah et al., 2017b). Whereas there are some studies about using these antioxidants with bull spermatozoa, there is a lack of reports related to their use for supplementing the BIOXcell extender.

Finally, resulting from our collaboration with Xenética Fontao, we directed our attention towards the application of sperm selection to improve post-thawing results. There is evidence of the improvement of the post-thawing quality of bull semen by using colloid centrifugation

prior to freezing (Gloria et al., 2016; Nongbua et al., 2017). Indeed, several authors (Samardzija et al., 2006a; Samardzija et al., 2006b; Valeanu et al., 2015; Arias et al., 2017) confirmed that the BoviPure colloid led to an enhancement when applied to post-thawed semen. Thus, we decided to test the possible improving effect of BoviPure on pre-freezing bull semen for improving post-thawing results. As an added novelty, we decided to test single-layer centrifugation (SLC), a simpler and more practical approach, versus the commonly used double-layer centrifugation (DLC).

OBJECTIVES

The main aim of the present study is to contribute to improving the cryopreservation protocols for bull semen. Based on the bibliographic background and in our hypothesis, the specific objectives defined for this thesis were:

1. To test the effect of extending equilibration time on post-thawing bull semen quality, to improve sperm cryopreservation and the management of the breeding centers.

2. To improve bull semen cryopreservation by supplementing the freezing extender with trehalose and GSH.

3. To improve bull semen cryopreservation by supplementing the freezing extender with the antioxidants curcumin and crocin.

4. To test pre-freezing single and double-layer colloid centrifugation to select an optimized bull sperm subpopulation for higher post-thawing quality.

MATERIALS AND METHODS

4.1 Experimental design

4.1.1 Experiment 1. Extending equilibration time and supplementing the semen freezing extender with GSH

We tested the effect of extending the equilibration time and adding of GSH to the freezing extender on bull semen post-thawing quality. In the experiment, which was conducted in SERIDA, we used twelve bulls (8 Holstein and 4 AV). The extended semen (BIOXcell) was equilibrated at 5 °C (after cooling from room temperature to 5 °C within ninety minutes) for four and twenty-four hours. Each dose was also split for assessing the effect of supplementation of GSH 2 mM to the freezing extender on post-thawing semen, and possibly the interaction with the extended equilibration time.

This experiment was continued in a collaboration with Xenética Fontao (Lugo), testing the same equilibration times with twelve Holstein bulls and the OPTIXcell extender.

Samples were tested after thawing and after four (Xenética Fontao doses) or five hours (SERIDA experiment) of incubation at 38 °C. In both experiments, the motility and several sperm physiology parameters were estimated using CASA and flow cytometry, immediately after thawing.

4.1.2 Experiment 2. Supplementation of trehalose and GSH to the semen freezing extender

We conducted this experiment to assess the potential improving effect of supplements (trehalose and GSH) to the semen freezing extender on post-thawed semen quality. This experiment was conducted in SERIDA-Cenero. We used twelve bulls (8 Holstein and 4 AV). The ejaculates (in BIOXcell) were split into five parts: Control (without supplementation), trehalose at 50 and 100 mM, and at GSH 2 and 5 mM, and then the semen was frozen. The thawed semen was evaluated by CASA and flow cytometry, directly after thawing and after five hours of incubation at 38 °C.

4.1.3 Experiment 3. Supplementation of curcumin, crocin, and GSH to the semen freezing extender

We tested the antioxidants curcumin, crocin, and GSH as supplements to the freezing extender for improving the post-thawing bull semen quality. Eight Holstein bulls were used in this experiment. The bulls were allocated at the Artificial Insemination center (Centro de Selección y Reproducción Bovina de Cenero, SERIDA), where the experiment was conducted. We split the ejaculates into the control (without supplementation) and supplemented the extender with curcumin 0,05 and 0,1 mM, crocin 0,5 and 1,5 mM, and GSH 0.5 mM, and then the semen was frozen. The post-thawing semen was evaluated by CASA and flow cytometry immediately after thawing and after five hours of incubation at 38 °C.

4.1.4 Experiment 4. Semen selection by single layer (SLC) and double layer (DLC) colloid centrifugation

We tested the suitability of the BoviPure colloid for carrying out pre-freezing SLC and DLC to improve the post-thawing bull semen quality and the freezability of selected males. Semen samples from twelve Holstein bulls were used. Each ejaculate was divided into three aliquots: control (without processing), SLC and DLC. The pellets from SLC, and DLC were resuspended and the three kinds of samples were cryopreserved in OPTIXcell. The post-thawing semen quality was evaluated by CASA and flow cytometry, immediately after thawing and after four hours of incubation at 38 °C.

4.2 Reagents and solutions

General reagents, and when no stated, were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Freezing extenders BIOXcell, and OPTIXcell were obtained from IMV (L'Aigle, France). The colloidal solution BoviPure and its companion washing medium BoviWash were purchased from Nidacon (Gothenburg, Sweden).

We used PBS (phosphate buffer-saline) for extending bull semen prior to studying the motility, and for preparing the staining solutions for flow cytometry analyses. We prepared a stock of PBS 10X as displayed in Table 4.1, diluting it ten times with milli-Q water prior to use. The final pH was adjusted to 7.4.

Consumables and solutions for flow cytometry were purchased from Beckman Coulter (Brea, CA, USA) and Thermo Fisher (Waltham, MA, USA). Fluorescent probes for flow cytometry were purchased from Sigma-Aldrich or Thermo Fisher, and are described in the corresponding section.

Table 4.1. PBS 10X for sperm work. The reagents were diluted with milli-Q water and the solution was autoclaved.

	mM	g/L	g/100 mL
NaCl	1400	81,8	8,18
KCl	150	11,2	1,12
Na_2HPO_4	70	9,9	0,99
KH_2PO_4	15	2	0,2

4.3 Animals and sample collection

The researchers involved in the experiments did not contact with any animal in any case. Semen was obtained by artificial vagina (42 °C to 46 °C, non-spermicidal lubricant) from trained animals subjected to a routine collection for semen freezing and AI (Bhattacharyya et al., 2009). The collection was carried out by trained personnel from SERIDA or Xenética Fontao, and the aliquots were handed to the researchers. All these centers are qualified and certified for their activity as breeding centers, following Spanish and European regulations (Reales Decretos 2256/1994, 2129/2008, 841/2011).

In the first experiment, we used bulls kept at SERIDA and Xenética Fontao breeding centers. In the part conducted at the SERIDA, we used twelve bulls (four Asturiana de los Valles and eight Holstein-Friesian). In the part conducted at Xenética Fontao, we used twelve Holstein-Friesian bulls. All of the animals were in regular AI service and were housed in half-open barns (one bull in each separate barn) under natural environments. They were in good health and met the EU requirements for bulls used in artificial insemination centers. Semen was collected from these bulls (one ejaculate per day) on different days (at least, two days between collections), totaling three replicates for each bull conducted within a one-month period as much.

In the second experiment, we used the same twelve bulls at SERIDA, as in the first experiment, totaling three replicates for each bull conducted within a one-month period as much. The animal conditions and collection methodology was the same.

In the third experiment, we used eight Holstein-Friesian bulls that were kept in SERIDA. Semen collection was carried out as described for experiments 1 and 2, with one replicate per bull.

In the fourth experiment, we used the same bulls at Xenética Fontao, as in the first experiment, with the same number of replicates and ejaculates per day.

4.4 Semen processing

After semen collection, the ejaculate volume was estimated directly from the graduated tubes, whilst the concentration was determined by absorbance with an adapted photometer calibrated for bull semen (Accucell, IMV Technologies, L'Aigle, France).

In all the experiments, the sperm doses (0.25-ml straws) were frozen in liquid nitrogen vapors using a programmable freezer (Digit-cool; IMV Technologies). The freezing followed a standard curve for bovine semen, as described by Muiño et al. (2008): -5 °C/min from +4 °C, to -10 °C; -40 °C/min from -10 °C, to -100 °C, and -20 °C/min from -100 °C, to -140 °C, and then stored in liquid nitrogen. Thawing was completed in a water bath at 37 °C for thirty seconds. Two straws were thawed in each case, and semen was pooled in pre-warmed (at 25 °C) 1.5-ml tubes. The quality of the cryopreserved samples was checked by CASA and flow cytometry as described in the corresponding sections, directly after thawing and post-incubation.

4.4.1 Experiment 1

In the first experiment, two equilibration times were applied prior to freezing the semen. Half from each extended semen sample (with BIOXcell extender in SERIDA, and with OPTIXcell extender in the Xenética Fontao) was equilibrated at 5 °C for four hours (semen was left to cool to 5 °C for ninety minutes); for the other half, the equilibration time was extended to twenty-four hours. After each equilibration time, semen was packaged in 0.25 ml French-straws and cryopreserved.

In the part conducted in SERIDA for experiment 1, GSH 4 mM was prepared at room temperature in BIOXcell. Semen pre-diluted with BIOXcell (184×10^6/ml) was split into two aliquots in 15-ml tubes. The Control tube received another volume of BIOXcell, whereas the GSH tube received another volume of the BIOXcell with 4 mM GSH. Therefore, the final concentration of GSH would be 2 mM in the supplemented tube, whilst the semen concentration in each tube would be 92×10^6/ml. After this step, the aliquots were treated as indicated above for achieving a factorial arrangement of 2 equilibration times × 2 supplementation treatments. After each equilibration time, semen was packaged in 0.25 ml French-straws and cryopreserved.

4.4.2 Experiment 2

In the second experiment, two concentrations of trehalose (100 mM, 200 mM) and two concentrations of GSH (4 mM, 10 mM) were prepared at room temperature using BIOXcell. Semen pre-diluted with BIOXcell (184×10^6/ml) was split into five aliquots in 15-ml tubes. We added another volume of BIOXcell (Control) or supplemented BIOXcell to the corresponding tubes, achieving a sperm concentration of 92×10^6/m and trehalose 50 mM and 100 mM, and GSH, 2 mM and 5 mM (tubes T50, T100, G2, G5) Afterwards, semen was left to cool to 5 °C for ninety minutes, then to equilibrate in a cooler at 5 °C for four hours. Subsequently, it was packaged in 0.25 ml French-straws and cryopreserved.

4.4.3 Experiment 3

In the third experiment, two concentrations of curcumin (100 μM and 200 μM), two concentrations of crocin (1 mM and 3 mM), and one concentration of GSH (1 mM) were prepared using BIOXcell at room temperature. The semen extension and supplementation was carried out as for experiments one and two, with a final sperm concentration of 92×10^6/ml and six 15-ml tubes: Control, 0.05 mM (CU0.05) and 0.1 mM (CU0.1) curcumin, 0.5 mM (CR0.5) and 1.5 mM (CR1.5) crocin, and 0.5 mM (GSH0.5) GSH. These tubes were left to cool at 5 °C for ninety minutes, then to equilibrate at 5 °C for four hours. After this step, semen was packaged in 0.25 ml French-straws and cryopreserved.

4.4.4 Experiment 4

In the fourth experiment, each ejaculate was divided at room temperature into three aliquots: Control (without colloid processing), SLC, and DLC. SLC and DLC were prepared in 15 ml tubes at room temperature:

- SLC: 10 ml of BoviPure™ colloid 80%.
- DLC: 5 ml of BoviPure™ 40% and 5 ml of BoviPure™ 80%. It was prepared by pipetting the higher density layer into the bottom of the tube, then carefully layering 5 ml of the lower density layer on top.

The procedure for SLC and DLC colloid centrifugation was as follows: 1 ml of semen was pipetted in each of two tubes (15 ml each), then diluted slowly with BoviWash™ at room temperature. Two milliliters of BoviWash were added to the one milliliter of semen so that the final sperm concentration was lower than 250×10^6/ml. Afterwards, the diluted semen was

pipetted slowly on top of the upper layer of each column of BoviPure (for SLC and DLC). The colloid tubes were centrifuged for thirty minutes at 300^{x}g, without break. The supernatant was carefully removed, and 1 ml of BoviWash™ was added to the pellets. Then, the semen was extended with OPTIXcell to the semen concentration (around 100×10^{6}/ml), cooled to 5 °C for ninety minutes, kept to equilibrate for four hours at 5 °C, and, finally, packaged in 0.25 ml French-straws for cryopreservation.

4.5 Determination of sperm motility by CASA (Computer Assisted Sperm Analysis)

CASA (Computer Assisted Sperm Analysis) allows analyzing the sperm motility with precision and repeatability (Amann and Waberski, 2014). The CASA system further enables the analysis of the spermatic kinematics generated in a microscopic field by capturing and digitizing the images of sperm, which is identified according to the size, shape, and brightness of their heads.

To analyze the motility and motility parameters in our studies, we put 5 µl of each sample diluted in PBS-0,5% BSA (prepared from PBS 10X diluted 10 times with ddH_2O; then adding 5 mg/mL of bovine serum albumin) with a semen concentration around 30 to 50×10^{6}/ml into a 10-µm deep Makler® sperm counting chamber (Sefi-Medical Instruments, Haifa, Israel) that was previously tempered at 37 °C. It was immediately examined under a microscope (Nikon E400, Tokyo, Japan), with a ×10 negative phase contrast objective, which was attached to a video camera (Basler A312fc, Basler Vision Components, Ahrensburg, Germany). At least three fields were captured, with around two hundred sperm/field. Motility parameters were determined using the ISAS® V.1.019 system (Proiser R+D, Paterna, Valencia, Spain), establishing the total head area to be between 25 and 80 μm^2 and the image acquisition speed at 53 s^{-1}. The parameters analyzed for sperm motility using the ISAS computerized system are shown below:

- **Total sperm motility** (MOT): Proportion of sperm with VCL>10 µm/s.

- **Progressive motility** (PROG): Proportion of sperm with progressive displacement, defined as VCL>10 µm/s and STR> 80%.

- **Curvilinear path velocity** (VCL): Speed with respect to the real trajectory, expressed in µm/s.

- **Straight path velocity** (VSL): Speed with respect to the straight or linear path,

expressed in μm/s.

- **Average speed** (VAP): Average path velocity according to the average smoothed path, expressed in μm/s.

- **Linearity index** (LIN): Relationship between VSL and VCL, expressed as a percentage.

- **Straightness index** (STR): Relationship between VSL and VAP, expressed as a percentage.

- **Wobble index** (WOB): Ratio between VAP and VCL, expressed as a percentage. Provides an estimate of the winding of the actual path.

- **Amplitude of the lateral movement of the head** (ALH): Amplitude of variations of the actual sperm head trajectory along its average trajectory (ALH mean of the trajectory), expressed in μm.

- **Beat-cross frequency** (BCF): Beat-cross frequency: the time-average rate at which the actual sperm trajectory crosses its average path trajectory, expressed in Hz.

The data obtained by the CASA system from the movement of each spermatozoon were summarized by extracting the median values for each sample. A sperm subpopulation analysis was performed by applying the two-step AGNES cluster algorithm procedure (Ledesma et al., 2017). This analysis allows a grouping of the motility data to classify the sperm in groups characterized by distinctive motility patterns.

4.6 Flow cytometry analysis

We used several fluorescent probes in the same solution to stain the spermatozoa. We combined the probes according to their fluorescence emission spectra. A summary of the probes used and their emission maxima are shown in Table 4.2. We prepared two combinations of fluorescent probes to enable the assessment of different physiological variables, as detailed below:

The first stain group contained:

- **Hoechst 33342** (H342): Used to discard residues in the sample. It stains the DNA of all cells with a nucleus since it is able to cross intact membranes. It is excited with an ultraviolet or violet laser, emitting blue fluorescence.

- **Propidium iodide** (PI): This probe only enters cells when compromised plasma membrane, binding to the DNA; thus, sperm stained with PI (red-fluorescent nucleus) is considered non-viable or dead. It is excited with an ultraviolet or blue to green laser, emitting red fluorescence.

- **YO-PRO-1**: The membrane-impermeable DNA-binding probe. It can only enter cells that have started the apoptosis process, altering the permeability of the plasma membrane through the opening of pannexin channels. It is excited with a blue laser, emitting green fluorescence.

- **Merocyanine 540** (M540): This probe indicates the degree of lipid disorder of the cell membrane. It binds to membranes with high levels of a lipid disorder. It is used to evaluate the degree of plasma membrane fluidity, which is associated with sperm capacitation. It is excited with a blue laser, emitting orange fluorescence.

- **MitoTracker deep red** (MT): This probe accumulates inside the mitochondria and emits red fluorescence if they have high membrane potential (active mitochondria). It is excited with a red laser, emitting red fluorescence.

The second stain group contained:

- **Hoechst 33258** (H258): This membrane-impermeant probe only stains the DNA of cells with damaged membranes. H258+ spermatozoa are considered non-viable or dead. It is excited with an ultraviolet or violet laser, emitting blue fluorescence.

- **MitoSOX**: This probe targets the mitochondria and selectively detects superoxide anion radical ($O_2^{\bullet -}$) produced in the mitochondria. It emits red fluorescence when oxidized with the superoxide anion.

- **CM-H_2DCFDA**: This probe penetrates the plasma membrane, is hydrolyzed to a membrane-impermeant form by cytoplasm esterases, and emits green fluorescent after oxidation. Thus, it is used to detect the presence of intracellular ROS. It is excited with a blue laser, emitting green fluorescence.

- **PNA Alexa Fluor 647** (PNA-647): PNA is an agglutinin that can not penetrate an intact acrosomal membrane. It used to determine acrosomal status since it binds to β-galactose residues on the inner side of the acrosomal outer membrane. Therefore, only damaged or reacted acrosomes spermatozoa are stained. It is excited with a red laser, emitting red fluorescence.

Flow cytometry analyses of the functional parameters of bull sperm were carried out using a CyAn ADP flow cytometer (Beckman Coulter, Inc., Brea, USA) fitted with three diode lasers (violet at 405 nm, blue at 488 nm, and red at 635 nm). The staining solutions were prepared on the same day of analysis in Falcon-type tubes. The sperm samples were incubated in 300 µL of PBS 0.5% BSA with different fluorescent probes, at a concentration of 1.7×10^6/ml, 38 °C for 15 min in the dark.

The combinations of fluorescent followed the sets indicated above: H342/PI/YO-PRO-1/M540/MT (viability, apoptosis, capacitation, mitochondrial activity) and H258/CM-H_2DCFDA/MitoSOX/PNA-647 (viability, acrosomal status, cytoplasmic ROS, and mitochondrial superoxide). Fluorescence was collected in the cytometer by the different photodetectors provided with filters: In the violet line, 450/50 nm (blue fluorescence: H342, H258); in the blue line, 530/40 nm (green fluorescence: YO-PRO-1, CM-H_2DCFDA), 575/25 nm for orange fluorescence (orange: M540), 613/20 nm for red fluorescence (red: PI, MitoSOX); and in the red line, 665/20 nm for red fluorescence (red PNA-647, MT). Sample acquisition was controlled by using the Summit V4.3.02 software.

In order to discard non-sperm residues (small debris and other particles), a cytogram for forward and side scatter (FSC/SSC) was used in all analyses in the first group of stains for gating out debris. In the set of stains including H342, a cytogram for SSC/Hoechst 33342 enabled to gate out debris in a more specific manner. In the second set of stains, we also used a SSC/Hoechst 33258 cytogram. However, in this case, it was less specific since viable spermatozoa are not stained by H258. The acquisition was stopped after reaching 5,000 cells in each sample. The data obtained by cytometry were processed by using Weasel v. 3.2 (Frank Battye, Melbourne, Australia). Figures 4.1 and 4.2 show examples of cytograms.

The parameters obtained from the flow cytometry analyses were:

- **Viability**

The YO-PRO-1/PI provided the proportions of viable and "apoptotic" spermatozoa (Figure 4.1A). The sperm subpopulations were categorized as:

- PI-, viable spermatozoa.
- YO-PRO-1+/PI-, apoptotic spermatozoa.
- PI+, dead spermatozoa.

Table 4.2. List of the fluorochromes used for flow cytometry.

Fluorochrome	Company	Final concentration	Emission (nm)
Hoescht-33342	Sigma-Aldrich	4.5 µM	486
Propidium iodide	Sigma-Aldrich	3 µm	617
YO-PRO-1	Thermo Fisher	100 nM	510
Merocyanine 540	Thermo Fisher	2 µM	578
Mitotracker deep red	Thermo Fisher	100 nM	665
Hoechst 33258	Thermo Fisher	4.5 µM	486
MitoSOX	Thermo Fisher	1 µM	580
CM-H_2DCFDA	Thermo Fisher	5 µM	523
PNA Alexa Fluor 647	Thermo Fisher	1 µg/mL	670

We extracted the variables for viable spermatozoa (PI-), apoptotic (YO-PRO-1+/PI-) and the ratio for apoptotic within viable (apoptotic/viable).

- **Plasma membrane disorder (capacitation)**

The YO-PRO-1/M540 combination provided an estimation of capacitated spermatozoa (Figure 4.1B). The sperm subpopulations were categorized as:

- YO-PRO-1-/M540-, viable spermatozoa (non-apoptotic) with intact plasmalemma.
- YO-PRO-1-/M540+, viable spermatozoa (non-apoptotic) with capacitated plasmalemma.

We obtained the ratio of capacitated-viable spermatozoa (YO-PRO-1-/M540+) to the total viable spermatozoa (YO-PRO-1-) for further analysis.

- Mitochondrial membrane potential ($\Delta\psi_m$)

We used the combination YO-PRO-1/MT (Figure 4.1C). The selected subpopulations were:

- YO-PRO-1-/MT+, viable spermatozoa with high $\Delta\psi_m$.
- YO-PRO-1-/MT-, viable spermatozoa with low $\Delta\psi_m$.

We used the proportion of viable spermatozoa with high $\Delta\psi_m$ for analysis.

- **Reactive oxygen species (ROS) in the cytoplasm**

We used the combination H258/CM-H_2DCFDA (Figure 4.2B). As an estimation of cytoplasmic ROS, we obtained the median fluorescence intensity (MFI) of the intracellular concentration of reactive oxygen species in viable sperm (H258-), which was expressed as the median fluorescence intensity of the CM-H_2DCFDA probe.

- **Mitochondrial superoxide ($O_2^{\bullet-}$) assay**

We used the combination H258/MitoSOX (Figure 4.2C). The selected subpopulations were:

- H258-/MitoSOX-, viable spermatozoa with low mitochondrial $O_2^{\bullet-}$ production.
- H258-/MitoSOX+, viable spermatozoa with high mitochondrial $O_2^{\bullet-}$ production.

We used the ratio of viable spermatozoa presenting high mitochondrial $O_2^{\bullet-}$ production (H258-/MitoSOX+) respect to the total viable spermatozoa (H258-).

- **Acrosome integrity**

We used a combination of H258/PNA-Alexa 647 (Figure 4.2D). The selected subpopulations were:

- Non-viable with damaged acrosomes (H258+/PNA+).
- Viable with intact acrosome (H258-/PNA-).
- Viable with damaged acrosome (H258-/PNA+).

We used both the total proportion of spermatozoa with damaged or reacted acrosomes (H258+/PNA+ plus H258-/PNA+), and the ratio of viable spermatozoa presenting damaged or reacted acrosomes (H258-/PNA+) respect to the total viable spermatozoa (H258-).

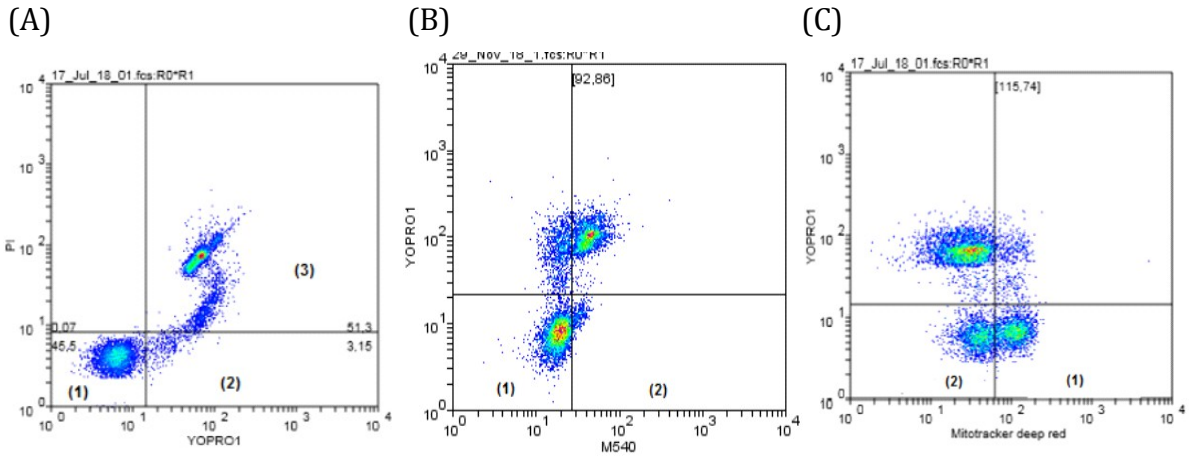

Figure 4.1. Cytograms representing sperm populations obtained with the H342/PI/YO-PRO-1/M540/MT probe combination; quadrants allow the identification of different sperm subpopulations. (A) Cytogram of a YO-PRO-1/PI stain. PI does not stain spermatozoa with the intact plasma membrane (viable) (1 and 2), thus enabling the identification of dead (membrane-damaged) spermatozoa (3). YO-PRO-1 labels apoptotic and dead spermatozoa (2 and 3), allowing to identify live but apoptotic spermatozoa together with PI (2). (B) Cytogram of M540/YO-PRO-1. The M540 probe indicates the degree of lipid disorder of the cell membrane; thus YO-PRO-1/M540-, events correspond to viable (non-apoptotic) spermatozoa (1), while YO-PRO-1/M540+ spermatozoa are considered viable (non-apoptotic) with higher membrane disorder (capacitated) (2). (C) Cytogram for the YO-PRO-1/MT combination, for identifying viable (non-apoptotic) spermatozoa $_{wi}$th high $\Delta\psi m$ (1), and with low $\Delta\psi m$ (2).

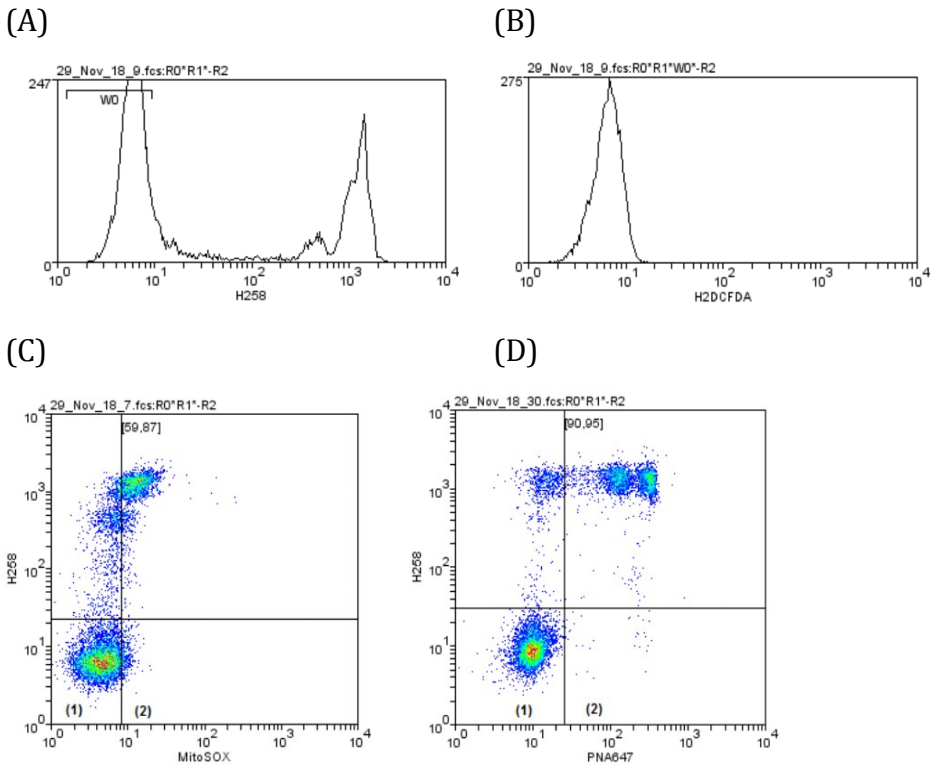

Figure 4.2. Cytograms representing sperm populations obtained with the H258/CM-H_2DCFDA/MitoSOX/PNA-647 probe combination; regions and quadrants allow the identification of different sperm subpopulations. (A) Histogram for the H258 probe fluorescence intensity, allowing identifying viable spermatozoa (H258-), as the peak within the W0 region. (B) Histogram for the CM-H_2DCFDA fluorescence intensity (only viable spermatozoa, gated in (A) through the W0 region); this histogram provides the median fluorescence intensity of (MFI) for this probe, as an estimate of intracellular ROS levels. (C) Cytogram of H258/MitoSOX, allowing to identify viable spermatozoa with low (1) and high (2) mitochondrial superoxide anion production. (D) Cytogram of H258/PNA-Fluor 647, allowing to identify viable spermatozoa with intact acrosome (1) and with damaged or reacted acrosomes (2).

4.7 Sperm chromatin integrity

We used the SCSA (Sperm DNA Structure Assay) to assess sperm DNA integrity and chromatin compaction. We diluted the sperm sample (directly post-thawing and after incubation) in TNE buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4), to a final concentration around 2×10^6/ml, and stored the samples at -80 °C. The samples were thawed on crushed ice prior to analysis. A volume of 200 µl was pipetted in a flow cytometry tube and immediately mixed with 400 µl of acid-detergent solution (0.1% Triton X-100, 150 mM NaCl, and 80 mM HCl, pH 1.2) to induce denaturation of DNA *in situ*. Exactly thirty seconds later, the sample was stained by adding 1.2 ml of acridine orange (AO) solution (0.1 M citric acid, 0.2 M

Na_2HPO_4, 1 mM disodium EDTA, and 0.15 M NaCl, pH 6.0; 6 μg/ml AO). The tube was kept in crushed ice and run in the cytometer within three to five minutes. We used the FACSCalibur flow cytometer (Becton Dickinson; Franklin Lakes, NJ) to analyze the stained samples. The cytometer was set to acquire at least 5,000. The AO emits red fluorescence if it binds to single-strand DNA (susceptible to denaturation, with breaks) and green fluorescence if it binds to double-strand DNA (resistant to denaturation, no breaks). Flow cytometry files (FSC v.2) were analyzed in the R statistical environment (Team; 2019) by using the flowCore (Ellis et al., 2019; Team; 2019) library for access and processing of cytometry data. The DNA fragmentation index (DFI) was calculated for each spermatozoon as the ratio of red to total (red+green) fluorescence, times 1000. We obtained the standard deviation of the DFI (SD-DFI) and the proportion of spermatozoa with a DFI higher than 250 (%DFI). Chromatin compaction was estimated from the green fluorescence intensity, considering the spermatozoa with a fluorescence intensity higher than 650 (%HDS).

4.8 Statistical analysis

Statistical analyses were carried out in the R statistical environment (Team, 2019). The data was analyzed using linear-mixed effects models.

In the first experiment, for the experiment carried out at SERIDA, the equilibration time and antioxidant treatment were used as fixed factors, whilst bull, breed, and replicate were included in the random part of the models. For the part carried out at Xenética Fontao, the equilibration time was used as a fixed factor, and bull and replicate were used as random effects. In the second experiment, the supplement type was used as a fixed factor, whilst bull, breed, and replicate were included in the random part of the models. In the third experiment, the antioxidant treatment was the fixed factor, whilst bull was the grouping factor in the random part. In the fourth experiment, the colloid treatment was the fixed factor, whilst bull and replicate were included in the random part of the model.

RESULTS

5.1 Impact of the Extension of the Equilibration Time on Bull Semen Cryopreservation

5.1.1 BIOXcell extender and GSH supplementation

The extension of the equilibration time before freezing using BIOXcell extender (the experiment in SERIDA) resulted in small, however significant, changes in post-thawing sperm quality. The supplementation with GSH 2 mM had no effect in general, saving a few exceptions.

Figures 5.1a and 5.1b show the results for the motility variables obtained by CASA post-thawing and after the 5-h incubation. The total motility (Fig. 5.1a) did not change post-thawing between equilibration times. In contrast, progressive motility and motility parameters were significantly higher in the samples frozen after the 4-h equilibration (Figures 5.1a and 5.1b). These differences increased after incubation, with lower quality for the 24-h equilibration, including total motility. The GSH treatment slightly affect WOB post-thawing and after incubation, and LIN after incubation (Fig. 5.1b).

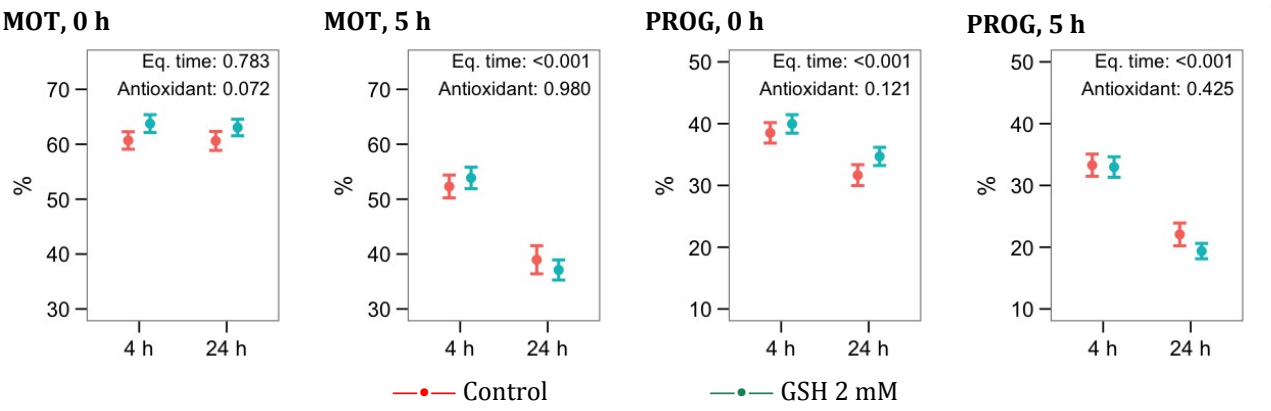

Figure 5.1a. Effect of the extension of equilibration time from 4 h to 24 h and GSH 2 mM, using the BIOXcell extender on CASA variables. The plots show mean ± SEM for total motility (MOT) and progressive motility (PROG) (continue to Fig. 5.1b), directly post-thawing (0 h), and after 5-h incubation (5 h). The results also show the impact of GSH (2 mM) on these parameters (control as red and GSH as blue). Insets show *P* values for the effects of equilibration time and GSH. The overall effect of the incubation was $P<0.001$.

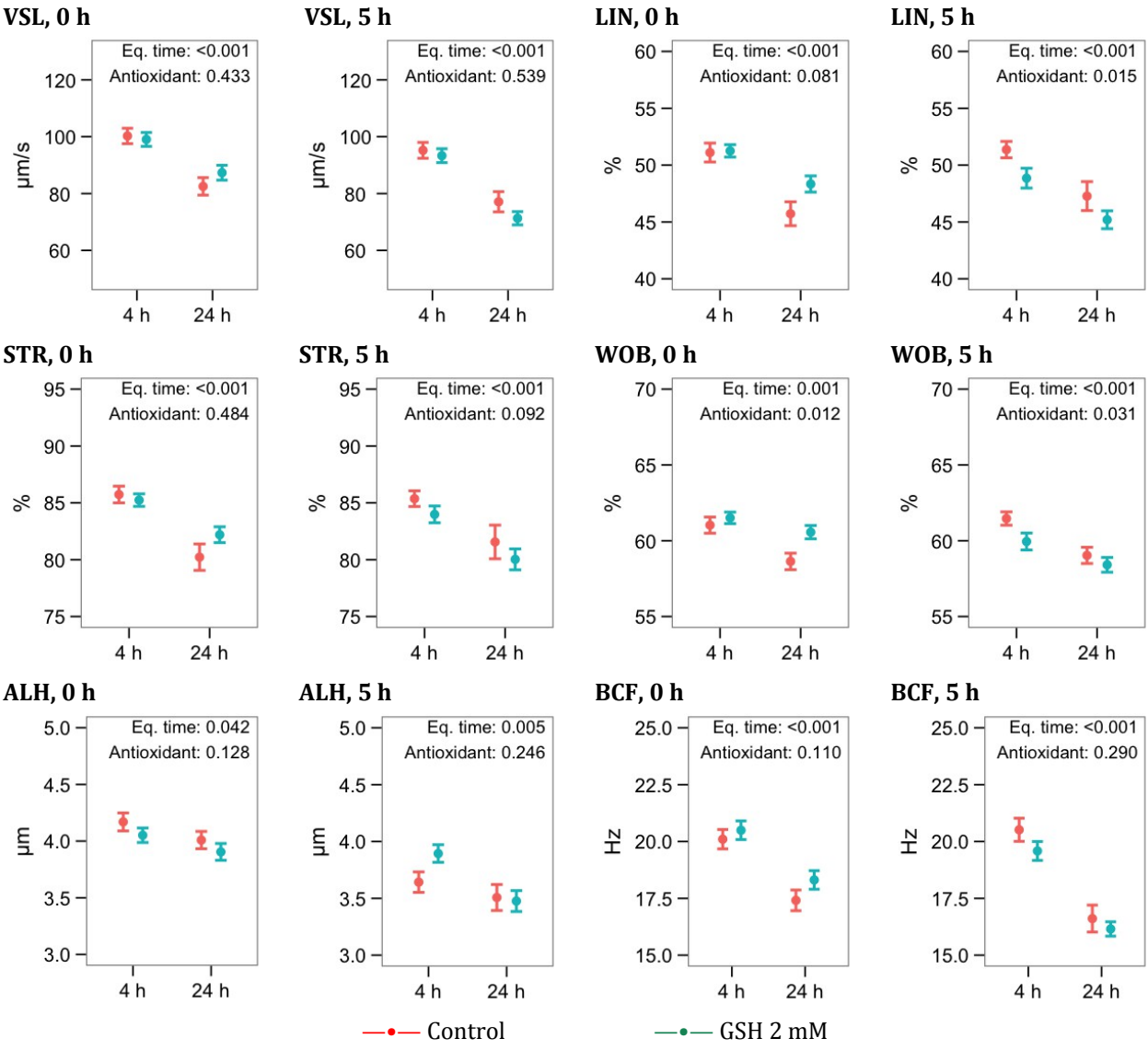

Figure 5.1b. Effect of the extension of equilibration time from 4 h to 24 h and GSH 2 mM, using the BIOXcell extender, on CASA variables (continued from Fig. 5.1a). The plots show mean ± SEM for the kinetic parameters, directly post-thawing (0 h), and after 5-h incubation (5 h). The results also show the impact of GSH (2 mM) on these parameters (control as red and GSH as blue). Insets show *P* values for the effects of equilibration time and GSH. The overall effects of the incubation were $P<0.001$, except for STR ($P>0.05$), ALH ($P=0.048$) and BCF ($P<0.004$).

The subpopulation analysis of the CASA data yielded two groups, Rapid and Slow (Table 5.1). There were no differences in post-thawing. After the incubation, the 24-h equilibration showed a significantly lower proportion of Rapid and higher of Slow spermatozoa (Figure 5.2).

Table 5.1. Descriptive statistics (median ± MAD) for the two motility subpopulations found in the data for the extension equilibration time experiment using the BIOXcell extender.

Cluster	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF	%
Rapid	194.1 ±54.9	91.1 ±42	113.6 ±34	50.9 ±14.2	86.6 ±11	60.9 ±9.4	4.2 ±1.5	20.4 ±7.6	69.8
Slow	62.6 ±34.8	13.3 ±11.1	32.3 ±18.8	24.4 ±15.8	49.1 ±26.2	52.5 ±12.2	1.7 ±0.7	7 ±4.4	30.2

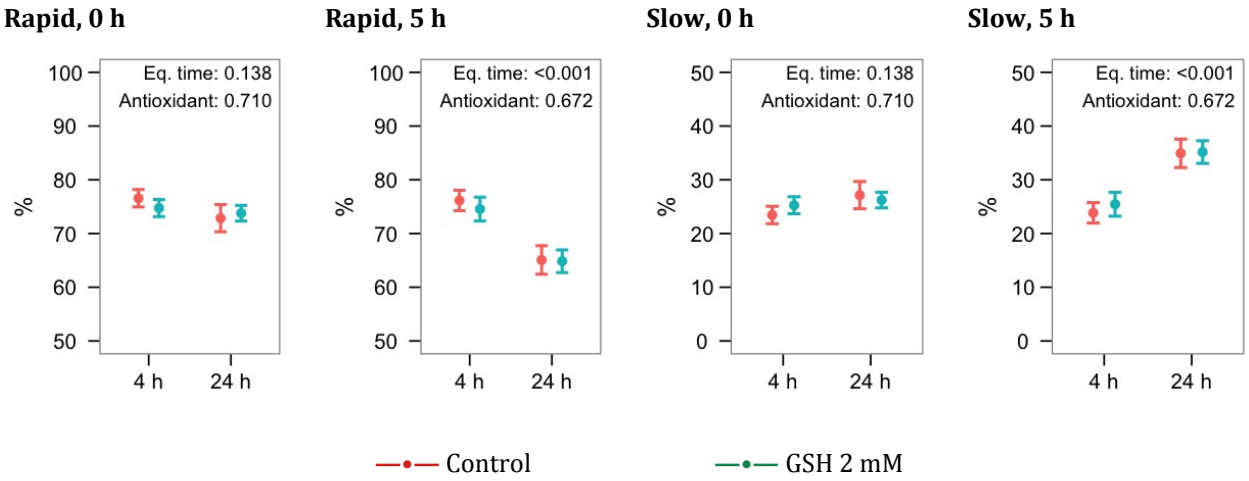

Figure 5.2. Effect of the extension of equilibration time from 4 h to 24 h and GSH 2 mM, using the BIOXcell extender, on CASA subpopulations. The plots show mean ± SEM for the Rapid and Slow subpopulations, directly post-thawing (0 h), and after 5-h incubation (5 h). The results also show the impact of GSH (2 mM) on these parameters (control as red and GSH as blue). Insets show *P* values for the effects of equilibration time and GSH. The overall effect of the incubation was *P*=0.003 for both variables.

Considering the flow cytometry variables (Figures 5.3a and 5.3b), the 24-h equilibration showed higher viability and mitochondrial activity, and lower capacitation ratio (respect to viable spermatozoa) and %HDS, when assessing the samples post-thawing. We noticed a slight increase in the cytoplasmic ROS production and in the proportion of viable spermatozoa with damaged acrosomes. However, when assessing the samples after the post-thawing incubation, the viability was lower in the longer equilibration and there were no differences for mitochondrial activity and sperm capacitation. Cytoplasmic ROS significantly increased in this analysis, together with the proportion of spermatozoa with damaged acrosomes. However, the ratio of viable spermatozoa with damaged acrosomes dropped. The production of the mitochondrial superoxide anion (ratio of viable spermatozoa) or DNA fragmentation

(%DFI) were not affected at any of the analysis points. Interestingly the %DFI parameter was very low and not affected by the post-thawing incubation.

The only effect of supplementing BIOXcell with 2 mM glutathione was a small modification of the LIN and WOB kinematic parameters (Figure 5.1b) and decreasing the production of superoxide anion in the mitochondria, detected at the two analysis points (Figure 5.3b).

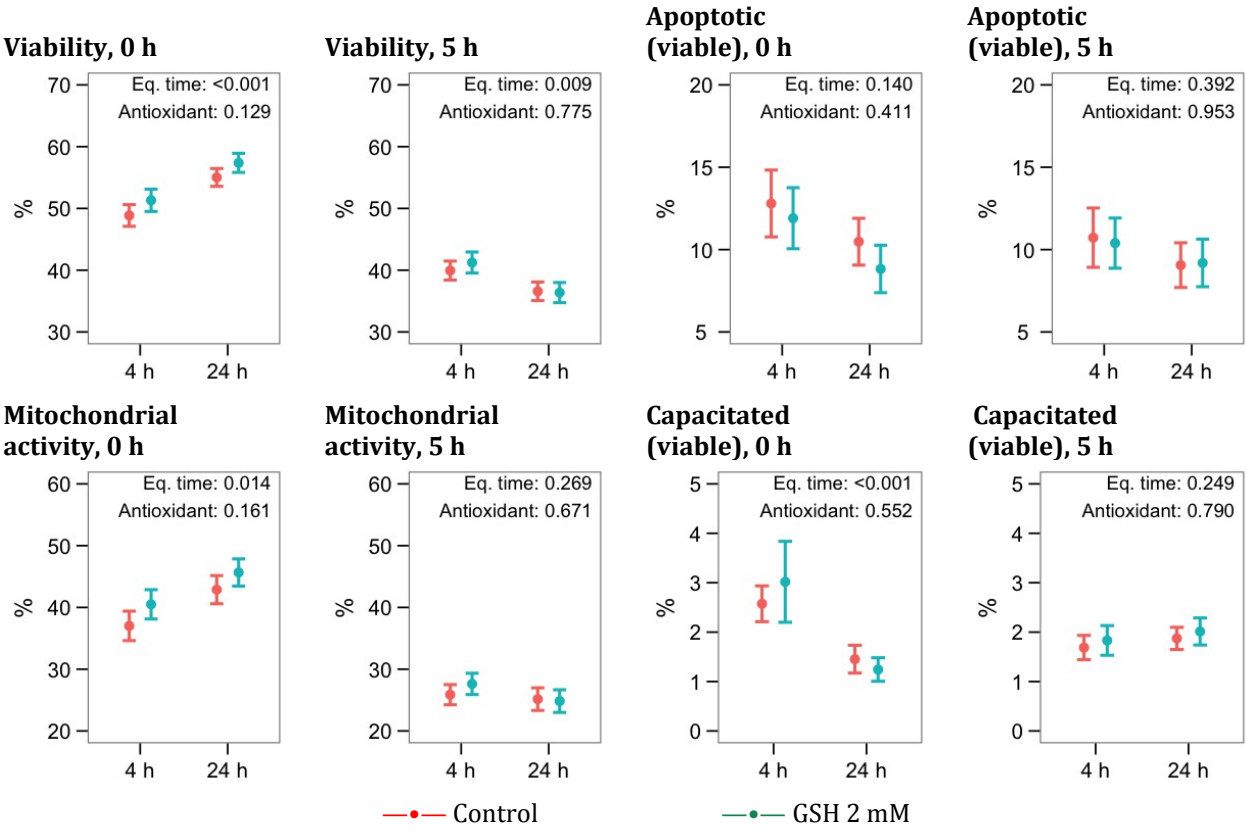

Figure 5.3a. Effect of the extension of equilibration time from 4 h to 24 h and GSH 2 mM, using the BIOXcell extender, on flow cytometry variables (continue to Fig. 5.3b). The plots show mean ± SEM for sperm viability (YO-PRO-1$^-$), apoptotic spermatozoa (YO-PRO-1$^+$ within PI$^-$), mitochondrial activity (MitoTracker$^+$), capacitated spermatozoa (M540$^+$ within YO-PRO-1$^-$), cytoplasmic ROS (H_2DCFDA within PI$^-$), and production of the superoxide anion $O_2^{\bullet-}$ in mitochondria (MitoSOX$^+$ within YO-PRO-1$^-$), directly post-thawing (0 h), and after incubation for five hours (5 h). The results also show the impact of GSH (2 mM) on these parameters (control as red and GSH as blue). Insets show *P* values for the effects of equilibration time and GSH.

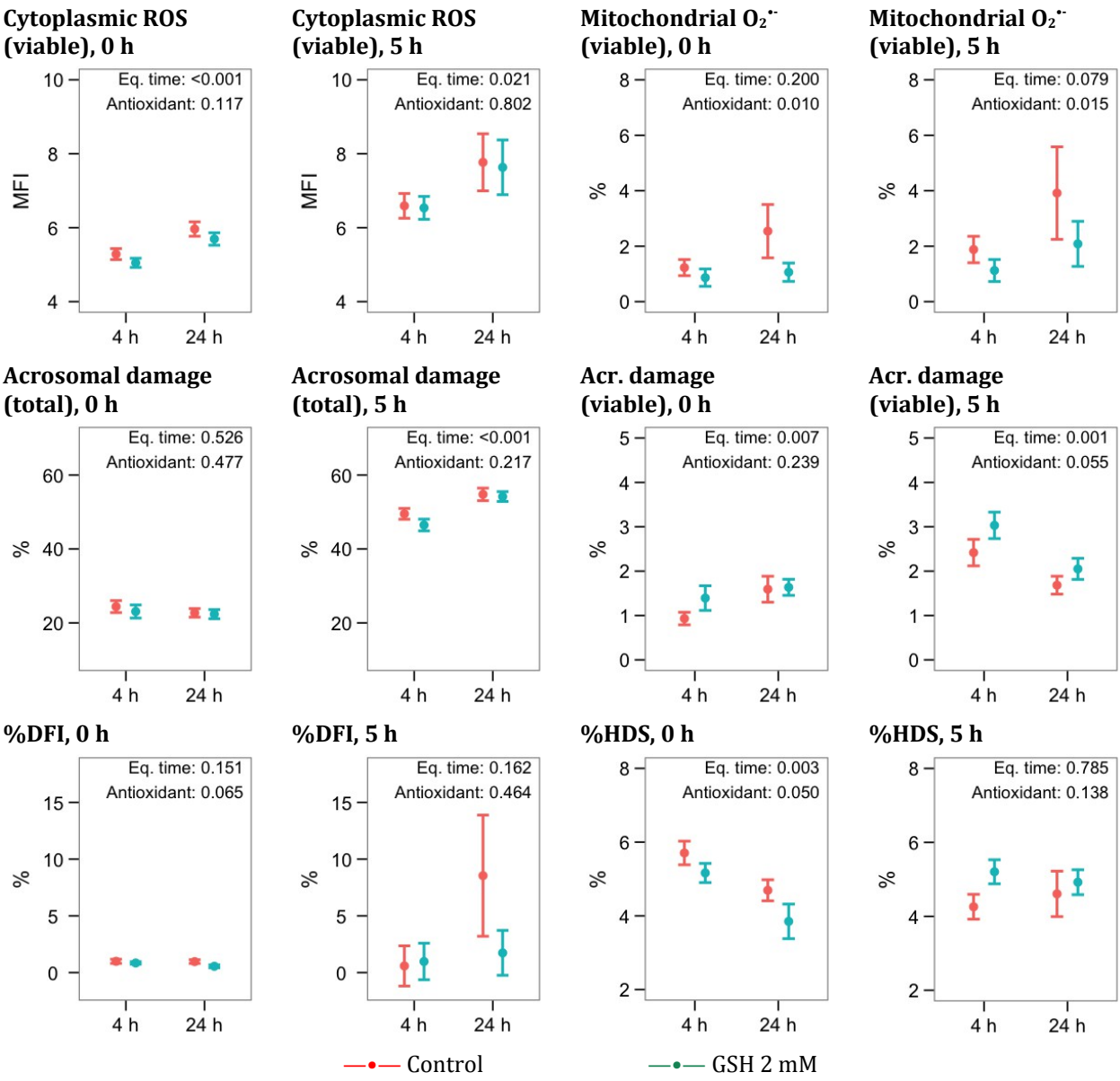

Figure 5.3b. Effect of the extension of equilibration time from 4 h to 24 h and GSH 2 mM, using the BIOXcell extender, on flow cytometry variables (continue from Fig. 5.3a). The plots show mean ± SEM for sperm acrosomal damage (PNA^+ for total and viable —YO-PRO-1^-— spermatozoa), DNA fragmentation (%DFI), and chromatin immaturity (%HDS), directly post-thawing (0 h), and after incubation for five hours (5 h). The results also show the impact of GSH (2 mM) on these parameters (control as red and GSH as blue). Insets show *P* values for the effects of equilibration time and GSH.

5.1.2 OPTIXcell extender

The results using the OPTIXcell extender (experiment at Xenética Fontao) were similar to those obtained with the BIOXcell extender, with a few differences. The CASA parameters assessed post-thawing (except VCL and ALH) were higher in the samples frozen after the 4.h equilibration treatment. After the post-thawing incubation, most parameters decreased (again, VCL and ALH showed a contrary trend) and the differences between both equilibration times were not significant in any case (Figures 5.4a and 5.4b).

The subpopulation analysis yielded three groups in this case, Rapid, Slow, and Hyper (because of a certain resemblance to hyperactive motility), described in Table 5.2. Differently than for the BIOXcell extender, we found that the 4-h equilibration time showed an effect on the subpopulation pattern after thawing, with a higher proportion of Rapid and a concomitant reduction in Hyper (Figure 5.5). In this case, we did not find any significant effect after the 4-h incubation, which again caused a significant decrease for most motility parameters, except VCL and ALH.

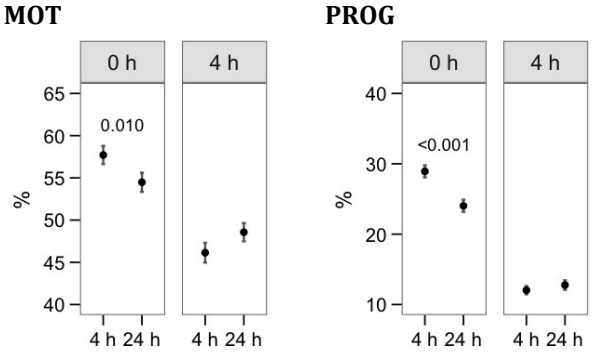

Figure 5.4a. Effect of the extension of equilibration time from 4 h to 24 h using the OPTIXcell extender on CASA variables. The plots show mean ± SEM for total motility (MOT) and progressive motility (PROG) (continue to Fig. 5.4b), directly post-thawing (0 h), and after the 4-h incubation (4 h). Insets show *P* values for the effects of equilibration time. The overall effect of the incubation was *P*<0.001.

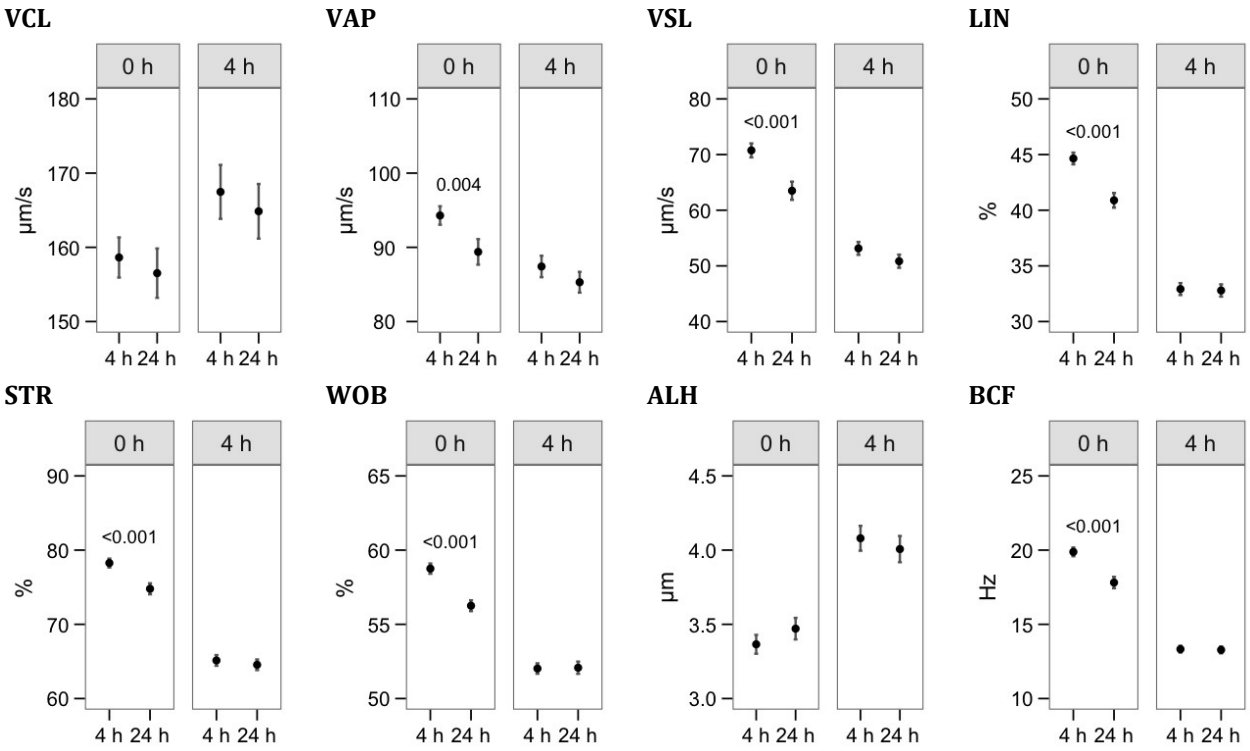

Figure 5.4b. Effect of the extension of equilibration time from 4 h to 24 h using the OPTIXcell extender on the CASA kinematic variables. The plots show mean ± SEM for the kinematic variables (continued from Fig. 5.4a), directly post-thawing (0 h), and after the 4-h incubation (4 h). Insets show *P* values for the effects of equilibration time. The overall effect of the incubation was $P<0.001$ except for VCL.

Table 5.2. Descriptive statistics (median ± MAD) for the three motility subpopulations found in the data for the extension equilibration time experiment using the OPTIXcell extender.

Cluster	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF	%
Rapid	144.6 ±47.9	80.8 ±27.8	94.4 ±27	56.3 ±12	88.3 ±8.3	65.3 ±8.6	3 ±1.1	24 ±8.4	32.4
Slow	56.9 ±23.5	9.2 ±5.6	27.3 ±11.7	16.8 ±11.3	37.6 ±23.4	47.5 ±11.8	1.8 ±0.6	6 ±3	19.0
Hyper	196.2 ±93.2	50.3 ±36.1	95.5 ±39.2	30.1 ±13.2	62.6 ±23.8	50 ±8.5	4.7 ±2.3	14 ±5.9	32.4

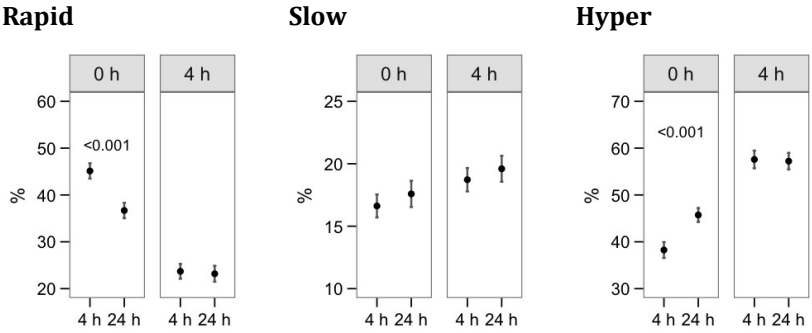

Figure 5.5. Effect of the extension of equilibration time from 4 h to 24 h, using the OPTIXcell extender, on the motility subpopulations (Table 5.2). The plots show mean ± SEM for the Rapid, Slow, and Hyper (hyperactive-like) subpopulations, directly post-thawing (0 h), and after the 4-h incubation (4 h). Insets show *P* values for the effects of equilibration time. The overall effect of the incubation was $P<0.001$ for Rapid and Hyper, and $P=0.013$ for Slow.

Flow cytometry analyses (Figures 5.6a and 5.6b) showed higher viability and lower capacitated spermatozoa (as ratio within viable), total acrosomal damage, and chromatin immaturity (%HDS) for the 24-h equilibration time in the post-thawing analysis.

After the incubation, we observed a decrease in sperm viability, mitochondrial activity, acrosomal damage as ratio within viable, and %HDS. In parallel, apoptosis (ratio within viable), mitochondrial superoxide production, and total acrosomal damage increased. In this analysis point, we observed a lower capacitation ratio, total acrosomal damage, and %HDS for the 24-h equilibration time, as in the post-thawing analysis. Additionally, the mitochondrial superoxide production was lower in the extended equilibration time.

The DNA fragmentation as %DFI was very low in all the cases, as observed in the BIOXcell experiment. This parameter was not affected by any treatment or even the post-thawing incubation.

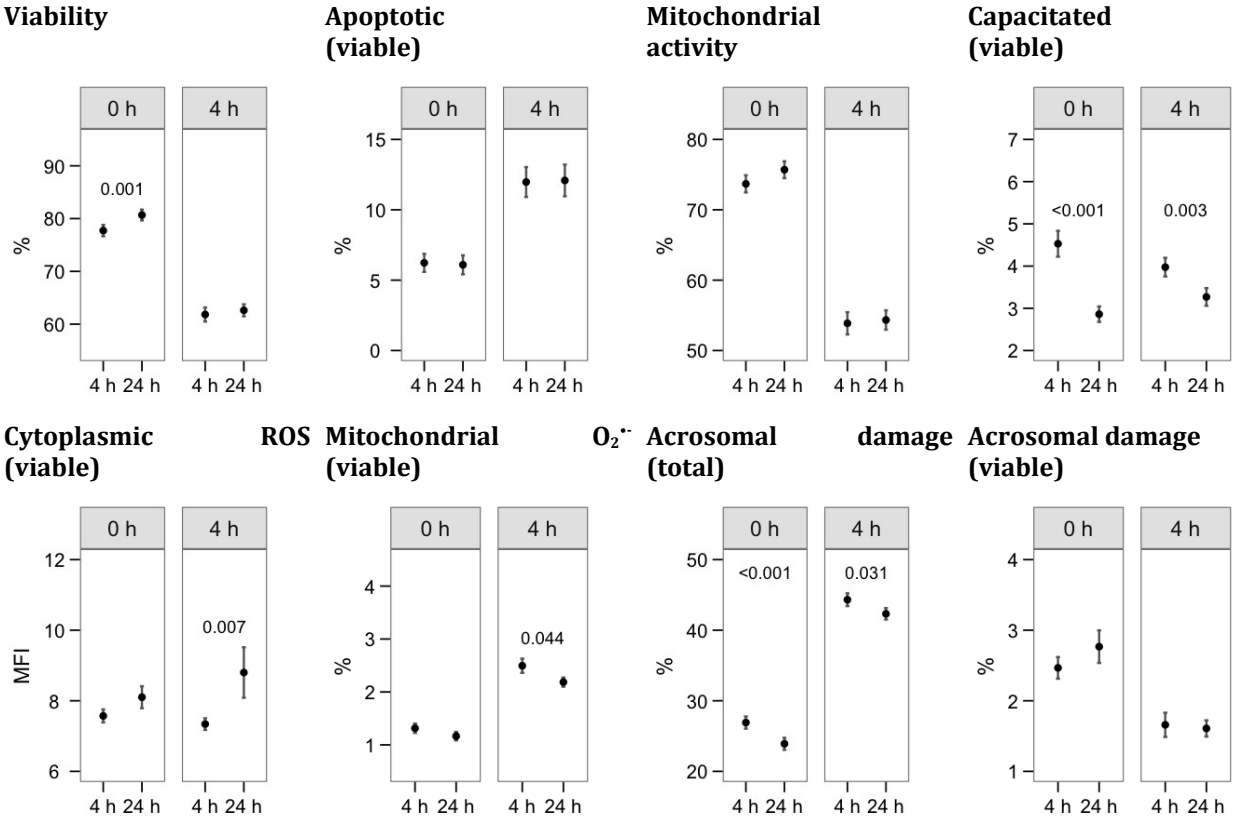

Figure 5.6a. Effect of the extension of the equilibration time from 4 h to 24 h using OPTIXcell on flow cytometry variables (continue to Fig. 5.6b). The plots show mean ± SEM for sperm viability (YO-PRO-1-), apoptotic spermatozoa (YO-PRO-1$^+$ within PI$^-$), mitochondrial activity (MitoTracker$^+$), capacitated spermatozoa (M540$^+$ within YO-PRO-1$^-$), cytoplasmic ROS (H_2DCFDA within PI$^-$), and production of the superoxide anion $O_2^{\bullet-}$ in mitochondria (MitoSOX$^+$ within YO-PRO-1$^-$) and acrosomal damage (PNA$^+$ for total and viable —YO-PRO-1$^-$— spermatozoa), directly post-thawing (0 h) and after incubation for four hours (4 h). The *P* values are indicated for the equilibration time effect. The overall effect of the incubation was $P<0.001$ except for capacitated and cytoplasmic ROS.

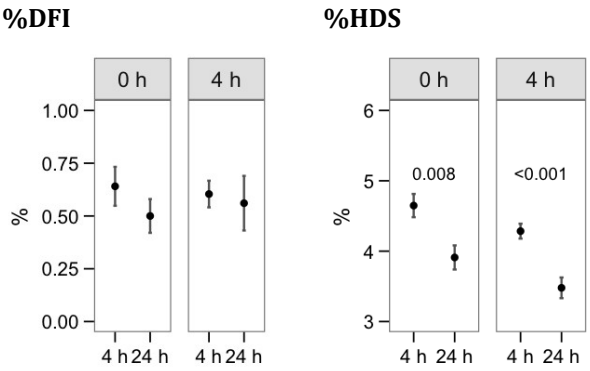

Figure 5.6b. Effect of the extension of the equilibration time from 4 h to 24 h using OPTIXcell on flow cytometry variables (continued from Fig. 5.6a), on sperm DNA fragmentation (%DFI) and chromatin immaturity (%HDS), directly post-thawing (0 h) and after incubation for four hours (4 h). The *P* values are indicated for the equilibration time effect. The overall effect of the incubation was *P*=0.024 for %HDS.

5.2 Quality of Bull Semen Cryopreserved with BIOXcell Supplemented with Trehalose and Glutathione (GSH)

In this experiment, we tested the ability of trehalose and GSH as supplements for the BIOXcell extender to improving the post-thawing quality of bull semen. In general, GSH yielded no advantage, and we found trehalose to be detrimental for that purpose.

Figures 5.7a and 5.7b show the results for the motility analyses. Trehalose 50 mM yielded lower total and progressive motility comparing to the control, both post-thawing and post-incubation (P<0.001). Kinematic variables followed the same trend, also significantly in most cases. When we tested trehalose at 100 mM, average values plummeted, almost abolishing motility. GSH affected some kinematic variables, with 5 mM significantly decreasing LIN (Fig. 5.7a), STR, WOB, and BCF (Fig. 5.7b), after incubation (reflecting in lower progressivity too), and ALH post-thawing. Interestingly, both GSH concentrations increased WOB post-thawing (Fig. 5.7b).

The subpopulation analysis yielded two groups, termed Rapid and Slow, as described in Table 5.3. The study of the proportions of these two groups showed that the application of GSH 5 mM and trehalose 50 mM slightly decreased the presence of Rapid while increasing Slow both post-thawing and after the incubation. Trehalose 100 mM caused a dramatic drop in Rapid and a rise of Slow, whereas GSH 2 mM did not show any significant change for these subpopulations (Figure 5.8).

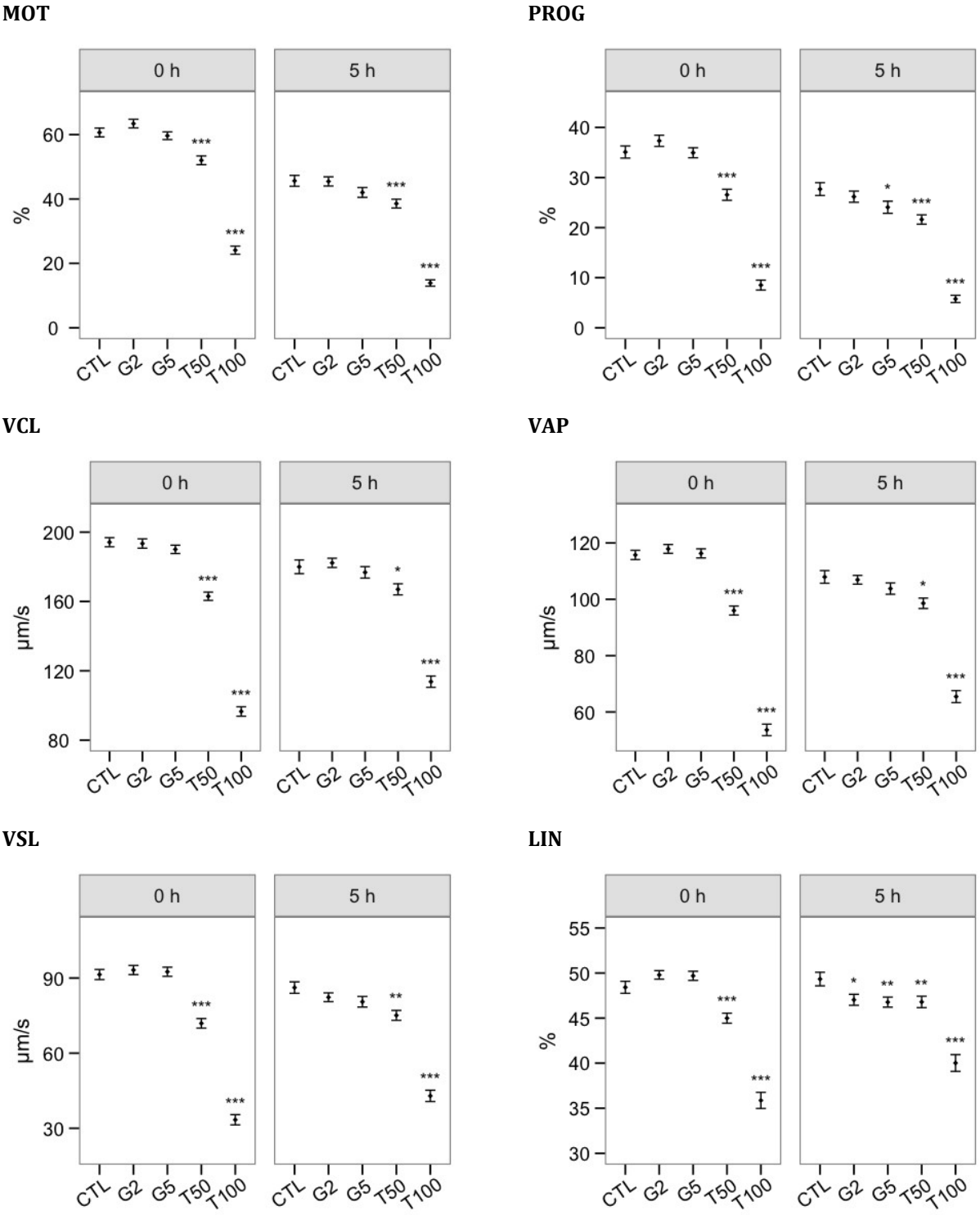

Figure 5.7a. Effect of the antioxidant treatments (CTL: Control; G2, G5: GSH 2 and 5 mM; T50, 100: Trehalose 50 and 100 mM) on CASA variables. The plots show mean ± SEM for total motility (MOT), progressive motility (PROG), and kinematic variables (continued to Fig. 5.7b), directly post-thawing (0 h), and after the 5-h incubation (5 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.001$ for MOT and PROG, $P>0.05$ for the remainder.

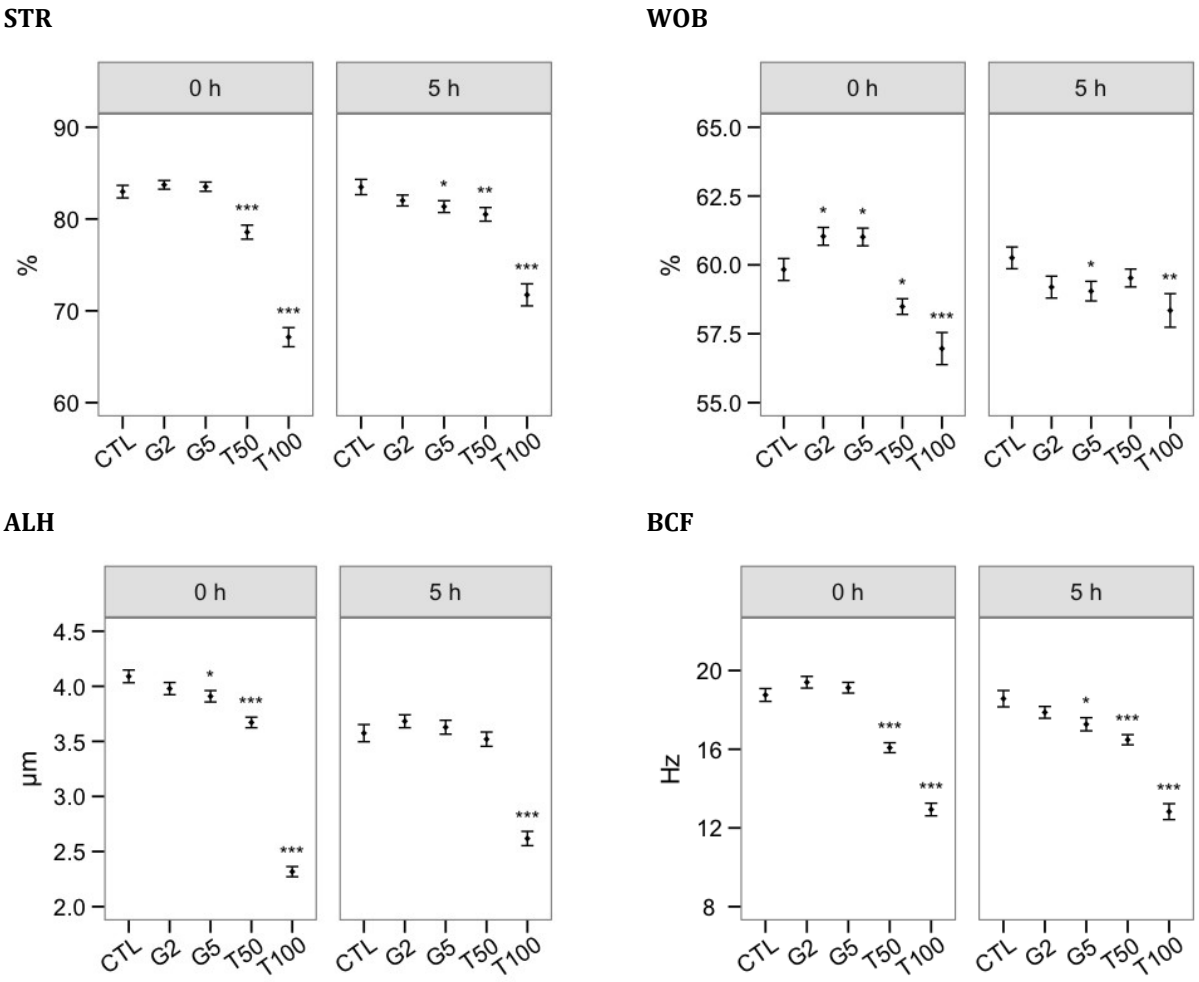

Figure 5.7b. Effect of the antioxidant treatments (CTL: Control; G2, G5: GSH 2 and 5 mM; T50, 100: Trehalose 50 and 100 mM) on CASA variables. The plots show mean ± SEM for kinematic variables and subpopulations (continued from Fig. 5.7a), directly post-thawing (0 h), and after the 5-h incubation (5 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.001$ for ALH and BCF, $P>0.05$ for the remainder.

Table 5.3. Descriptive statistics (median ± MAD) for the two motility subpopulations found in the data for the experiment on the effect of trehalose and GSH for freezing bull semen.

Cluster	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF	%
Rapid	194.1 ±54.9	91.1 ±42	113.6 ±34	50.9 ±14.2	86.6 ±11	60.9 ±9.4	4.2 ±1.5	20.4 ±7.6	69,8
Slow	62.6 ±34.8	13.3 ±11.1	32.3 ±18.8	24.4 ±15.8	49.1 ±26.2	52.5 ±12.2	1.7 ±0.7	7 ±4.4	30,2

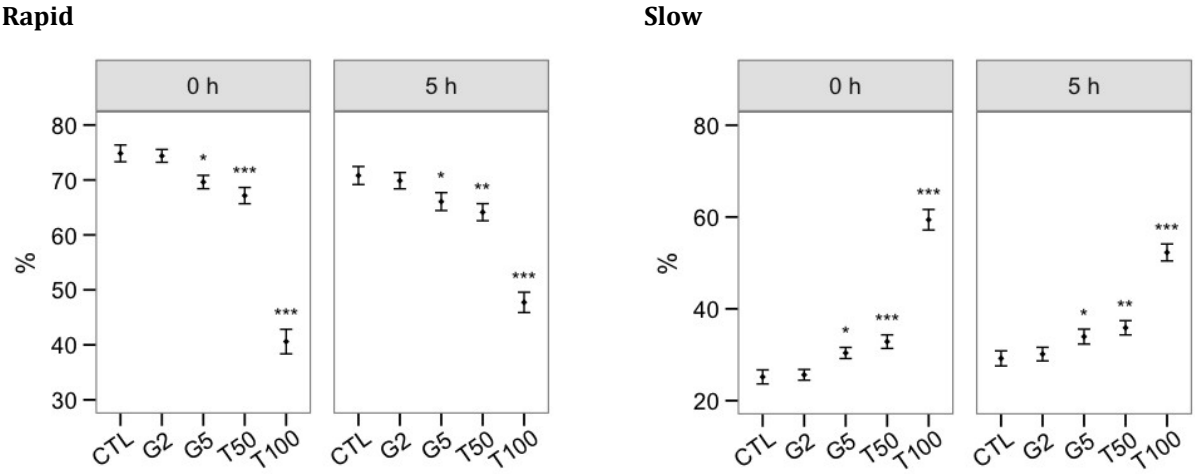

Figure 5.8. Effect of the antioxidant treatments (CTL: Control; G2, G5: GSH 2 and 5 mM; T50, 100: Trehalose 50 and 100 mM) on CASA variables. The plots show mean ± SEM for the proportions of the motility subpopulations (Table 5.3), directly post-thawing (0 h), and after the 5-h incubation (5 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P>0.05$.

Considering the flow cytometry variables (Figures 5.9a and 5.9b), we found a less dramatic effect of the treatments. T100 showed lower viability overall, and lower mitochondrial activity after the incubation (Fig. 5.9a). This treatment increased the proportion of acrosomal damage at both analysis points (Fig. 5.9b) and of viable spermatozoa with apoptotic features after the incubation (Fig. 5.9a). Interestingly, it also significantly decreased the levels of cytoplasmic ROS post-thawing (Fig. 5.9a) and acrosomal damage in viable spermatozoa after the incubation (Fig. 5.9b). G2 showed a small significant increase in viable sperm acrosomal damage.

The proportion of viable spermatozoa producing mitochondrial superoxide dropped in all the treatments (Fig. 5.9a), both post-thawing and after the incubation (except for T100 after the incubation). These changes in ROS production did not reflect in the chromatin analysis. Whereas both GSH treatments showed lower %HDS after thawing, this trend changed after the incubation, with the effect of the 5 mM treatment being significant (Fig. 5.9b).

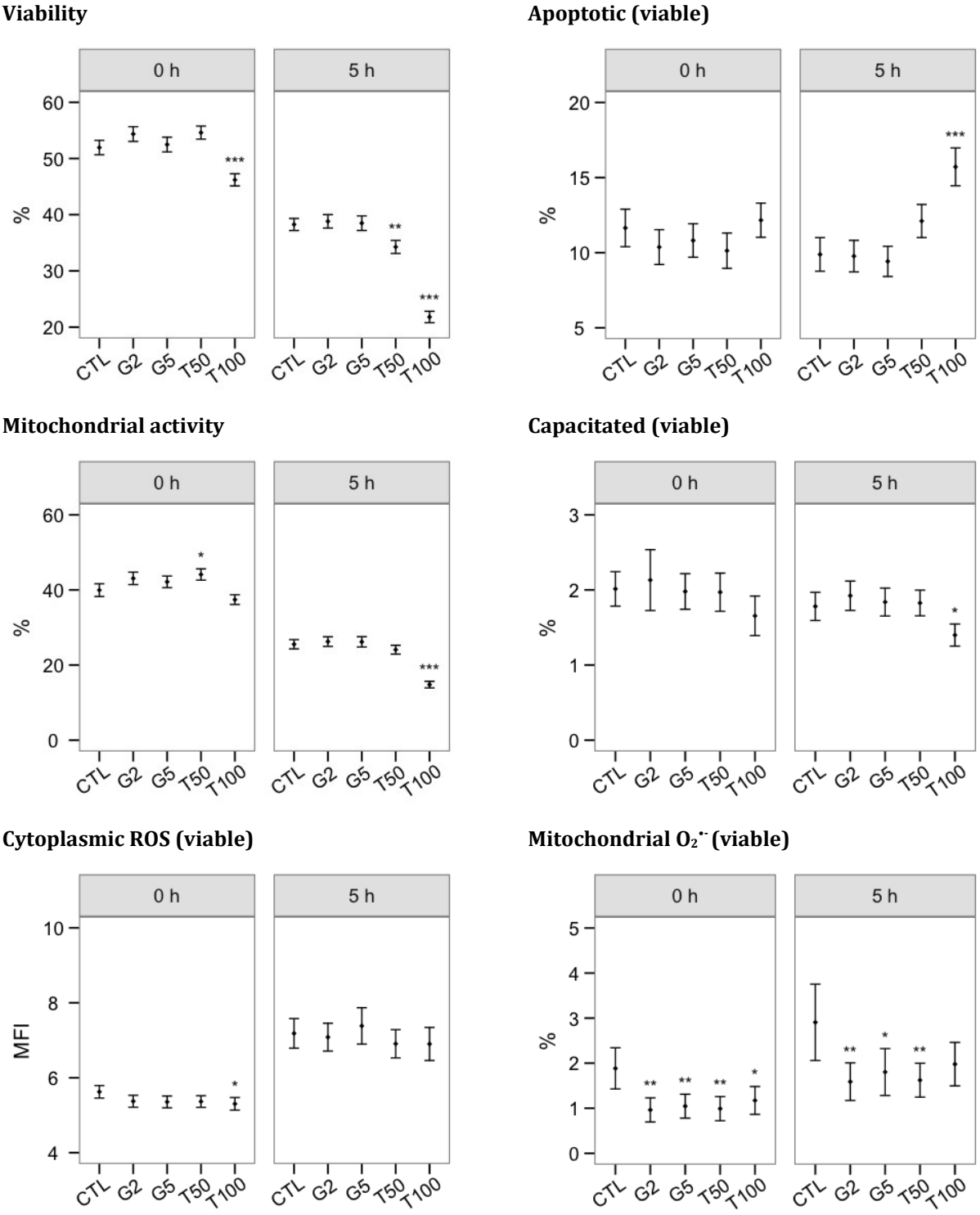

Figure 5.9a. Effect of the antioxidant treatments (CTL: Control; G2, G5: GSH 2 and 5 mM; T50, 100: Trehalose 50 and 100 mM) on flow cytometry variables. The plots show mean ± SEM (continued to Fig. 5.9b), directly post-thawing (0 h), and after the 5-h incubation (5 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.001$ for viability, mitochondrial activity, cytoplasmic ROS, and mitochondrial superoxide.

As in previous experiments, DNA fragmentation as %DFI (Fig. 5.9b) was very low in all cases and not affected by the treatments. Nevertheless, we observed an increase in the dispersion of the control samples after the incubation, possibly reflecting the between-male variability in the resistance to the DNA damage. In this case, the treatments seem to homogenize the results, maybe by protecting the most vulnerable samples.

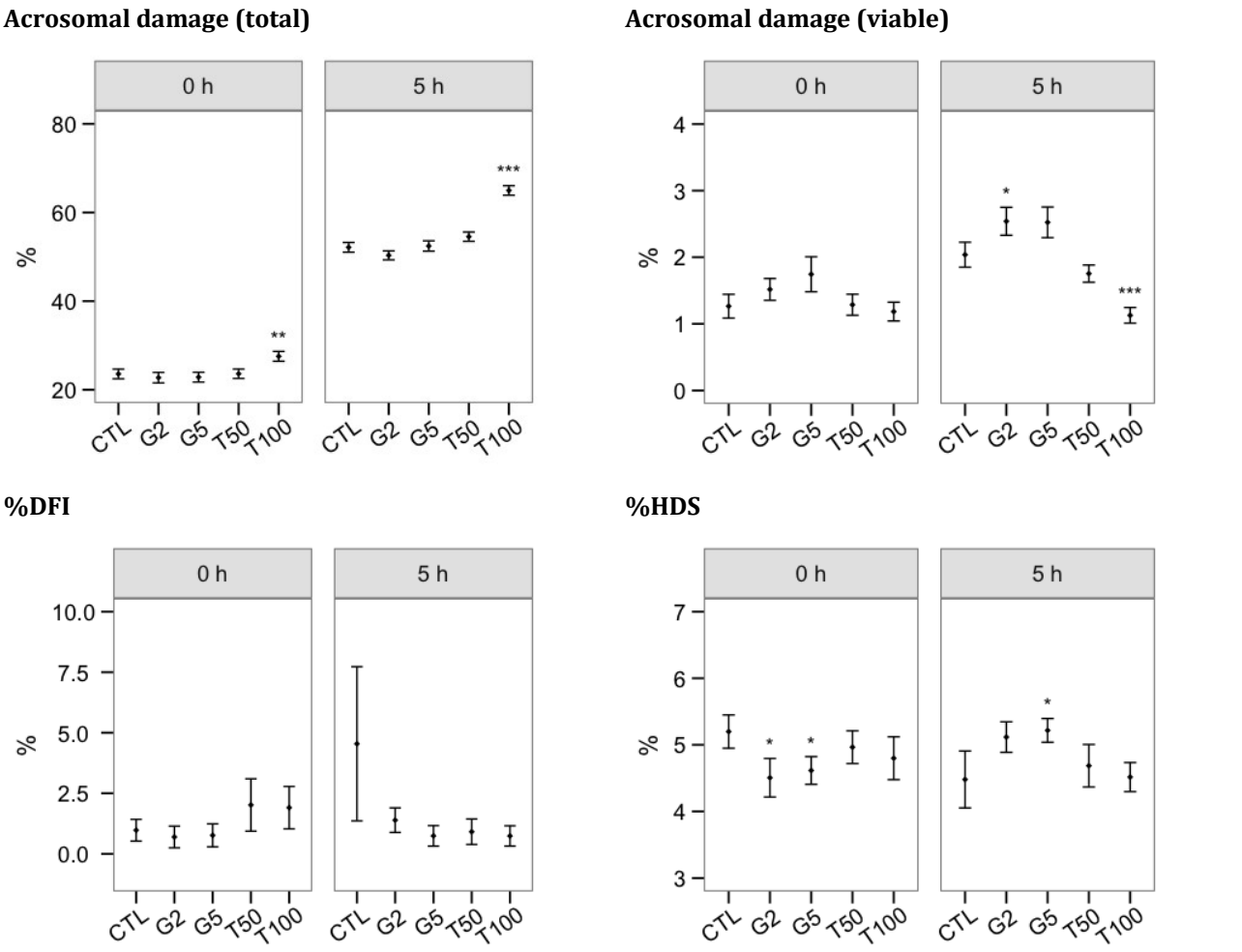

Figure 5.9b. Effect of the antioxidant treatments (CTL: Control; G2, G5: GSH 2 and 5 mM; T50, 100: Trehalose 50 and 100 mM) on flow cytometry variables. The plots show mean ± SEM (continued from Fig. 5.9a), directly post-thawing (0 h), and after the 5-h incubation (5 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.001$ for acrosomal status variables.

5.3 Quality of Bull Semen Cryopreserved with BIOXcell Supplemented with the Antioxidants Curcumin, Crocin, and GSH

The use of the antioxidants curcumin and crocin for bull semen cryopreservation, with glutathione as a reference, yielded mixed results. There were no effects of the antioxidants on the CASA parameters post-thawing (Figures 5.10, 5.11a, and 5.11b). Only after the incubation, MOT, PROG, and LIN declined with both crocin concentrations, LIN was also lowered by GSH, and WOB decreased with all the treatments.

As in the previous experiments, we found two motility subpopulations, Rapid and Slow (Table 5.4). However, there were no significant effects on the proportions of these subpopulations (Figure 5.11b).

Table 5.4. Descriptive statistics (median ± MAD) for the two motility subpopulations found in the data for curcumin, crocin and GSH effects for freezing bull semen.

Cluster	VCL	VSL	VAP	LIN	STR	WOB	ALH	BCF	%
Rapid	198.6 ±55.5	97.3 ±44.7	119.8 ±33.9	50.9 ±14.2	87.0 ±10.9	61.0 ±8.6	4.2 ±1.5	21.5 ±8.3	82,2
Slow	49.6 ±27.4	8.5 ±4.8	22.8 ±12.5	18.6 ±11.2	41.4 ±22.1	47.4 ±11.4	1.5 ±0.6	6.0 ±4.4	17,8

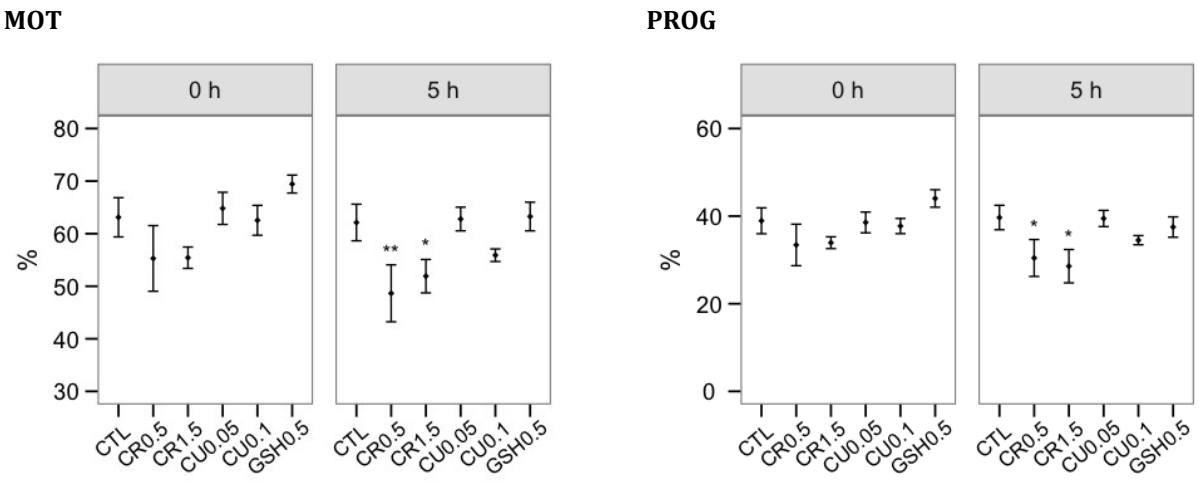

Figure 5.10. Effect of the antioxidant treatments (CTL: Control; CR0.5, CR1.5: Crocin 0.5 and 1.5 mM; CU0.05, CU0.1: Curcumin 0.05 and 0.1 mM; GSH0.5: GSH 0.5 mM) on CASA variables. The plots show mean ± SEM for total motility (MOT), progressive motility (PROG), directly post-thawing (0 h), and after the 5-h incubation (5 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.05$ for MOT.

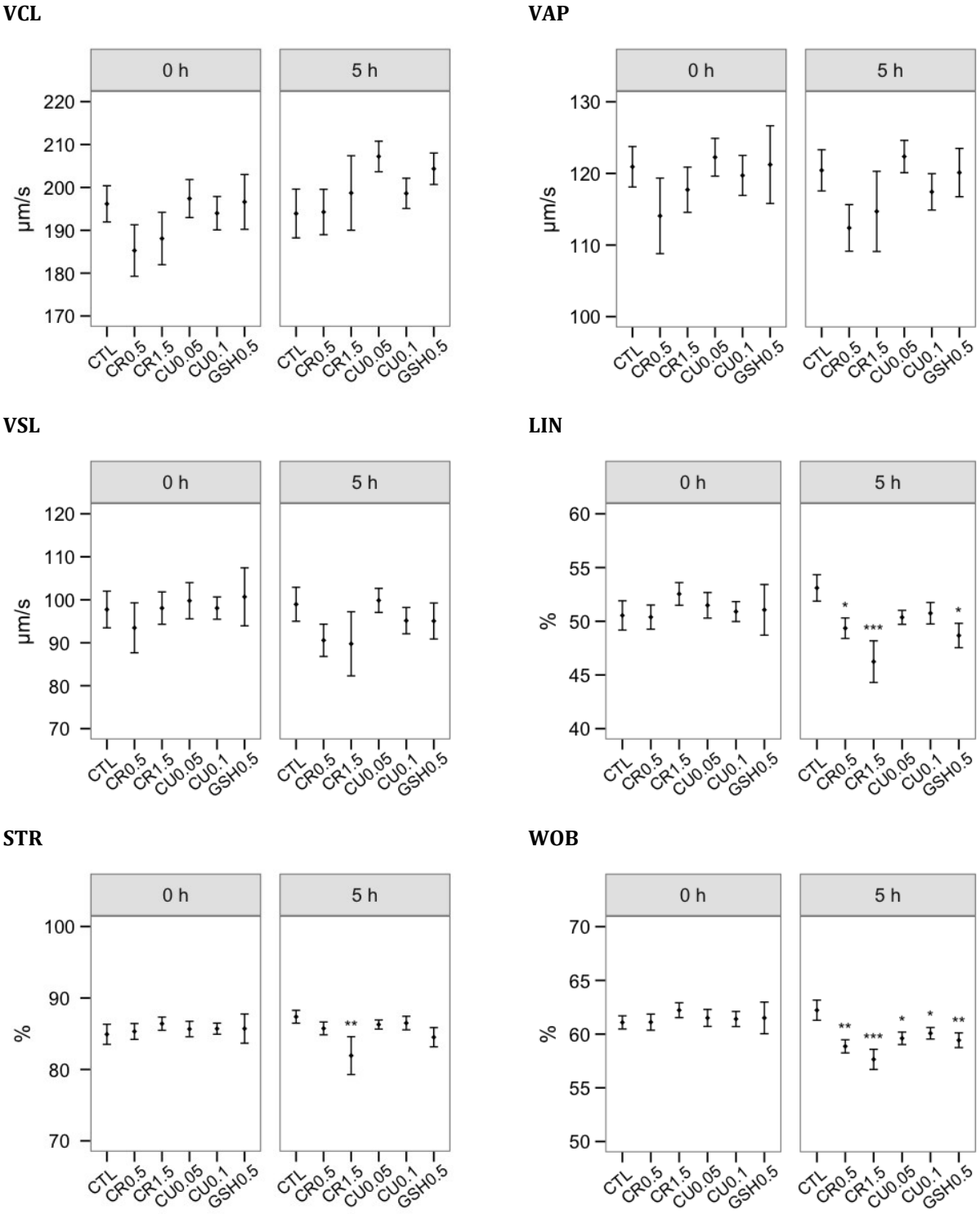

Figure 5.11a. Effect of the antioxidant treatments (CTL: Control; CR0.5, CR1.5: Crocin 0.5 and 1.5 mM; CU0.05, CU0.1: Curcumin 0.05 and 0.1 mM; GSH0.5: GSH 0.5 mM) on CASA variables. The plots show mean ± SEM for kinematic variables (continued to Fig. 5.11b), directly post-thawing (0 h), and after the 5-h incubation (5 h). Insets show *P* values for the effects of the treatments respect to the Control (* *P*<0.05; ** *P*<0.01; *** *P*<0.001). The overall effect of the incubation was *P*<0.05 for VCL.

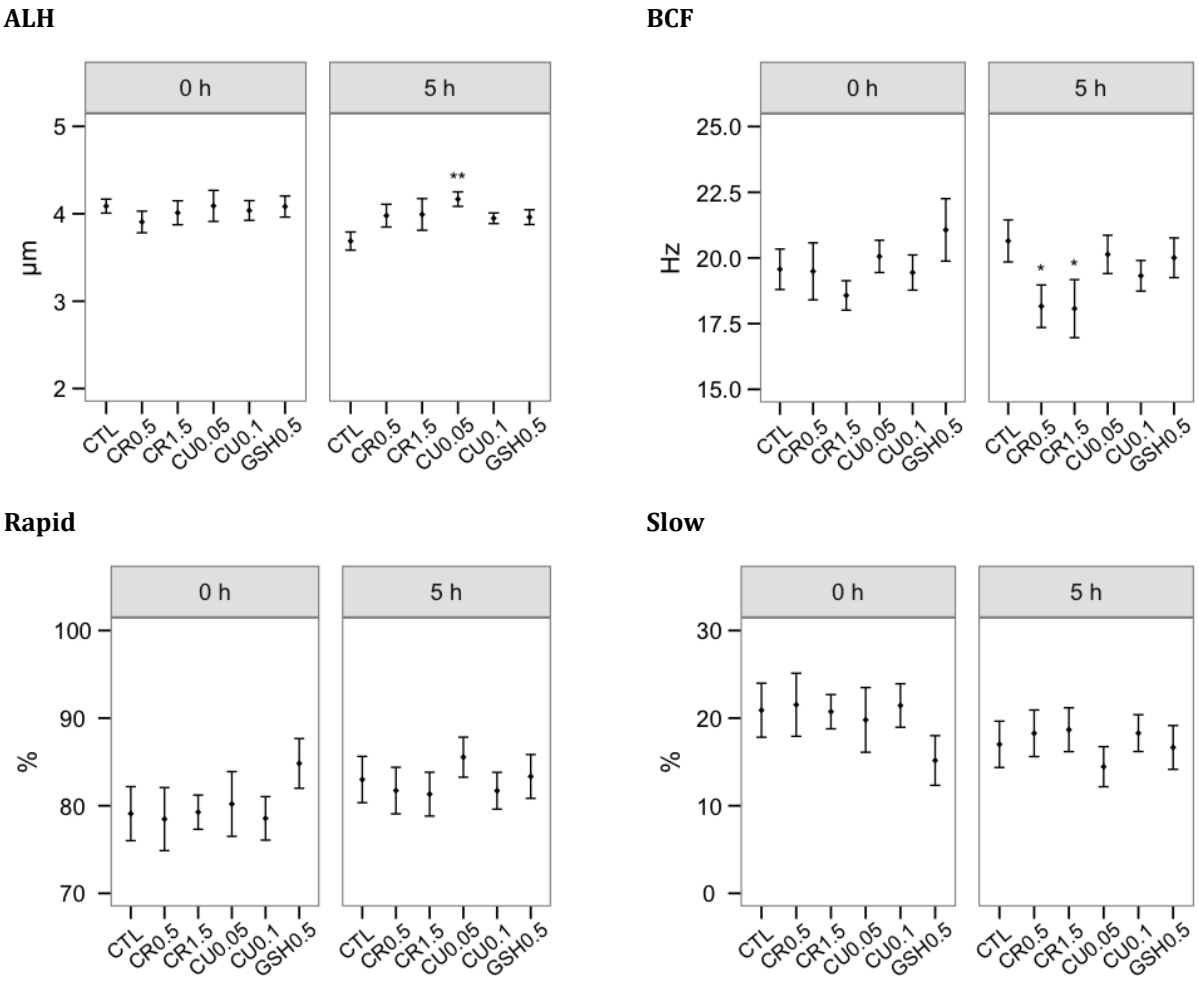

Figure 5.11b. Effect of the antioxidant treatments (CTL: Control; CR0.5, CR1.5: Crocin 0.5 and 1.5 mM; CU0.05, CU0.1: Curcumin 0.05 and 0.1 mM; GSH0.5: GSH 0.5 mM) on CASA variables. The plots show mean ± SEM for kinematic variables and subpopulations (continued from Fig. 5.11a), directly post-thawing (0 h), and after the 5-h incubation (5 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.001$ for WOB, $P>0.05$ for the remainder.

Flow cytometry parameters followed a similar pattern than the motility data (Figures 5.12a and 5.12b). However, these analyses were more sensitive. Crocin caused a clear quality decrease post-thawing. Viability and the mitochondrial activity significantly dropped with the two crocin concentrations, which also induced a surge in the apoptotic ratio, and decreased the capacitation ratio (Figure 5.12a). Interestingly, both concentrations affected these parameters similarly, except for the production of mitochondrial superoxide (Figure 5.12b), which significantly rose with 1.5 mM. Part of these effects remained after the incubation, except for the apoptotic ratio and the mitochondrial superoxide production. In this analysis point, crocin increased the total acrosomal damage (Figure 5.12b). GSH significantly decreased the mitochondrial superoxide production after the incubation.

The cytoplasmic ROS (Figure 5.12b) were not affected by neither crocin nor GSH, but they went up with both curcumin concentrations. As we found with curcumin in most cases, CU0.05 and CU0.1 did not differ in this effect.

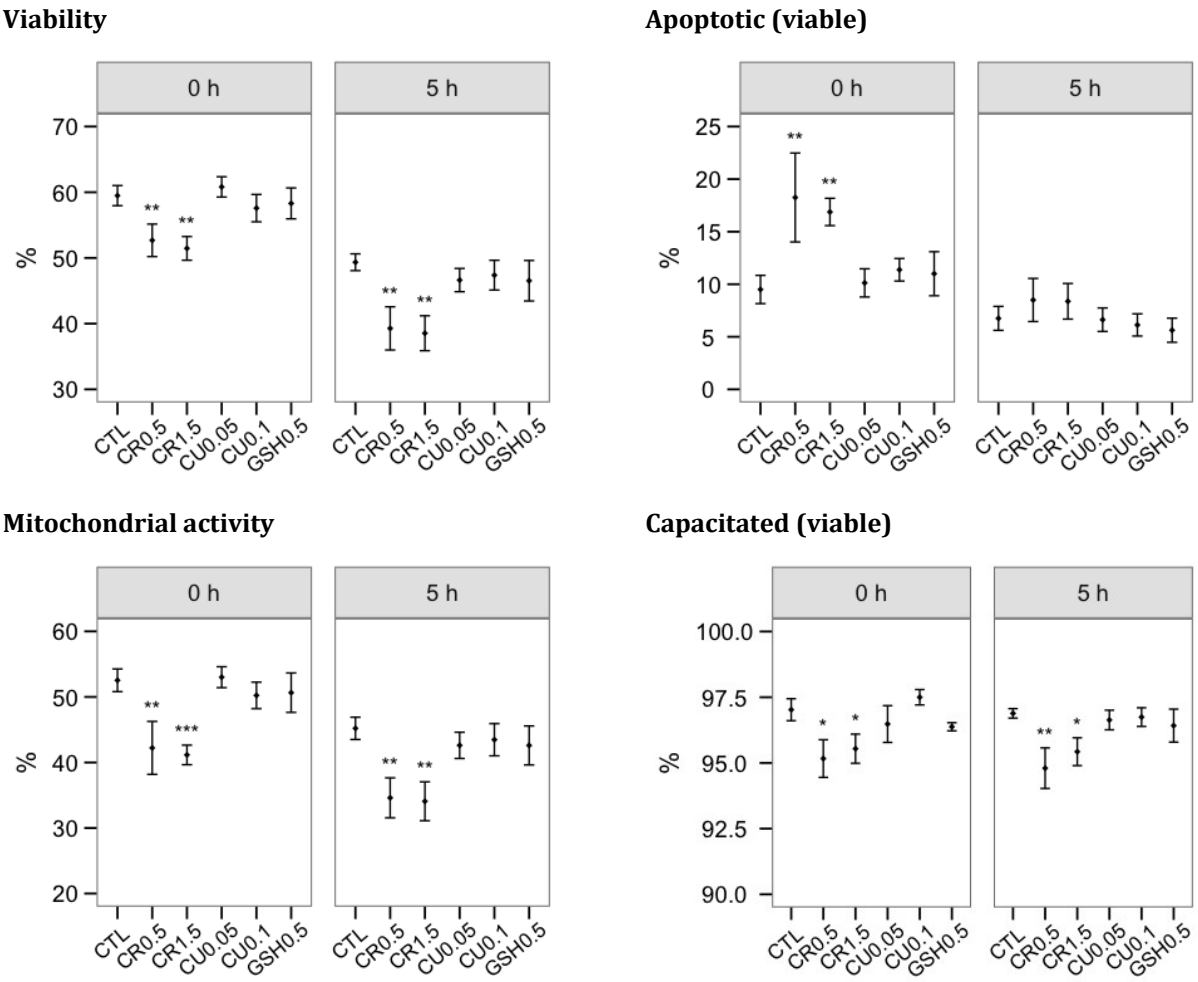

Figure 5.12a. Effect of the antioxidant treatments (CTL: Control; CR0.5, CR1.5: Crocin 0.5 and 1.5 mM; CU0.05, CU0.1: Curcumin 0.05 and 0.1 mM; GSH0.5: GSH 0.5 mM) on flow cytometry variables. The plots show mean ± SEM (continued to Fig. 5.12b), directly post-thawing (0 h), and after the 5-h incubation (5 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.001$ except for capacitation ratio.

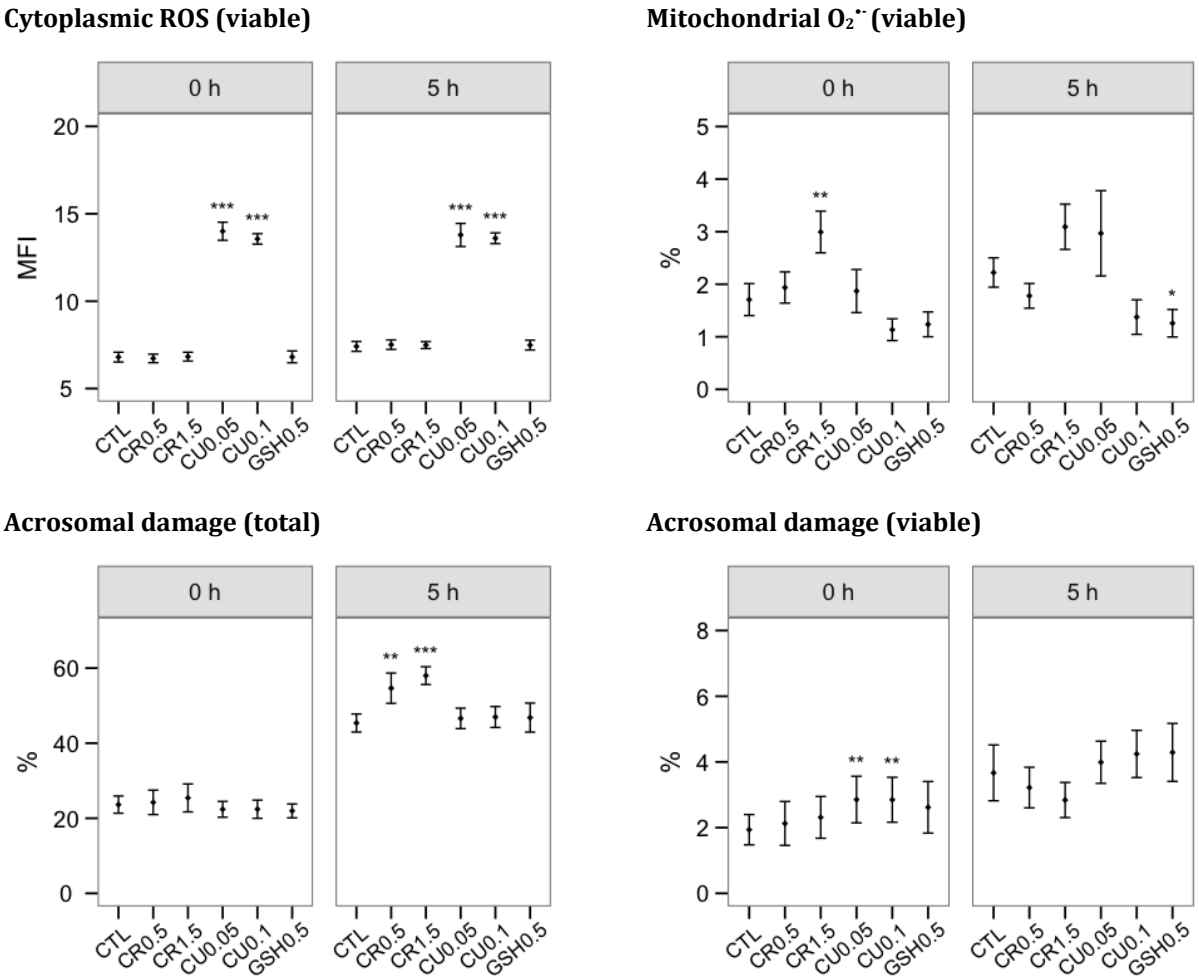

Figure 5.12b. Effect of the antioxidant treatments (CTL: Control; CR0.5, CR1.5: Crocin 0.5 and 1.5 mM; CU0.05, CU0.1: Curcumin 0.05 and 0.1 mM; GSH0.5: GSH 0.5 mM) on flow cytometry variables. The plots show mean ± SEM (continued from Fig. 5.12a), directly post-thawing (0 h), and after the 5-h incubation (5 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.001$, except $P<0.01$ for cytoplasmic ROS.

Figure 5.13 shows the chromatin assessment results. DNA fragmentation as %DFI was very low as in previous experiments and not affected by the treatments. However, crocin 1.5 mM post-thawing and both concentrations after the incubation had a decreasing effect on %HDS. In this case, the effect size was larger for the highest concentration.

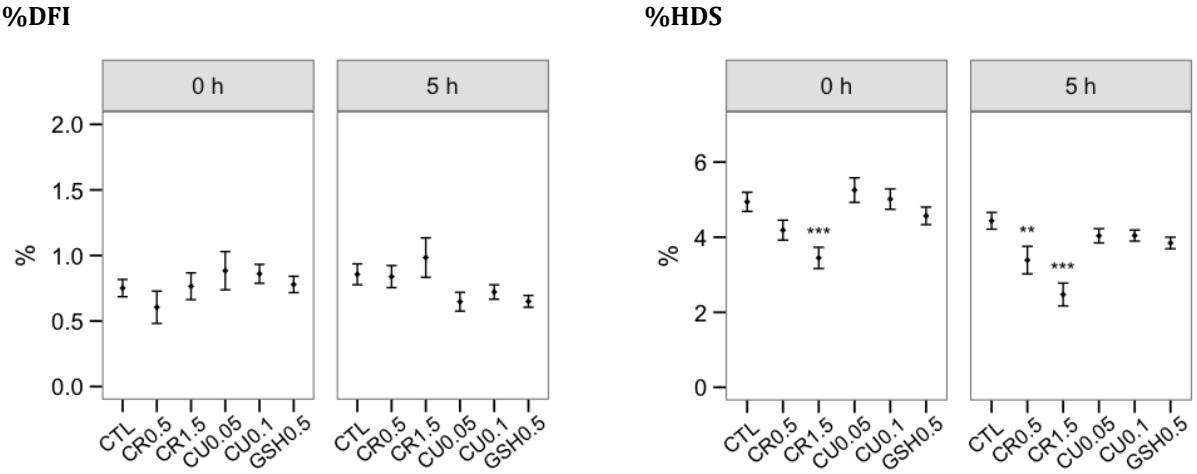

Figure 5.13. Effect of the antioxidant treatments (CTL: Control; CR0.5, CR1.5: Crocin 0.5 and 1.5 mM; CU0.05, CU0.1: Curcumin 0.05 and 0.1 mM; GSH0.5: GSH 0.5 mM) on the chromatin assessment variables. The plots show mean ± SEM, directly post-thawing (0 h), and after the 5-h incubation (5 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.001$ except for %DFI with $P>0.05$.

5.4 Improvement of Bull Sperm Post-thawing Quality by Using Single Layer (SLC) and Double Layer (DLC) Colloid Centrifugation Before Freezing

The use of colloid centrifugation prior to processing the samples for freezing improved the post-thawing sperm quality. Total and progressive motility in both SLC and DLC were significantly higher compared to the control (Figure 5.14) post-thawing. Interestingly, after incubation SLC lowered the total motility, while progressive motility was best preserved in both SLC and DLC (Figure 5.14). Both colloids decreased sperm velocity (Figure 5.15), especially VCL, at both analysis points (except SLC for VAP post-thawing). However, VSL increased significantly for both colloids post-thawing and for SLC after the incubation, reflecting in a considerable increase at both analysis points in the linearity parameters LIN, STR, and WOB. The colloids also reduced ALH while increasing BCF at both points (Figure 5.15).

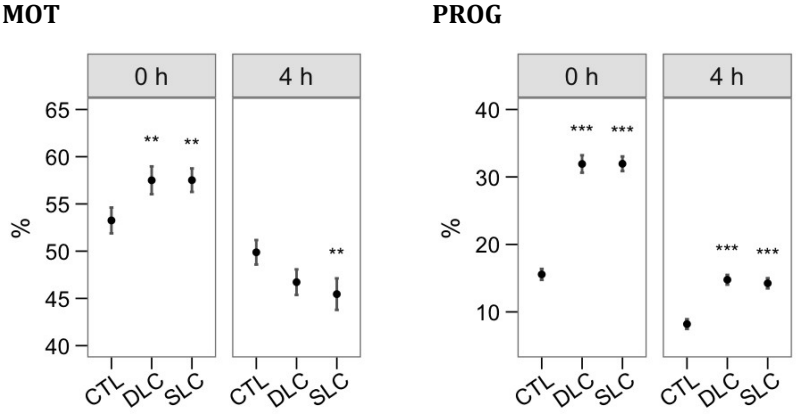

Figure 5.14. Effect of the colloid centrifugation (CTL: Control; DLC: Double Layer Centrifugation; SLC: Single Layer Centrifugation) on CASA variables. The plots show mean ± SEM for total motility (MOT) and progressive motility (PROG), directly post-thawing (0 h), and after the 4-h incubation (4 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.001$.

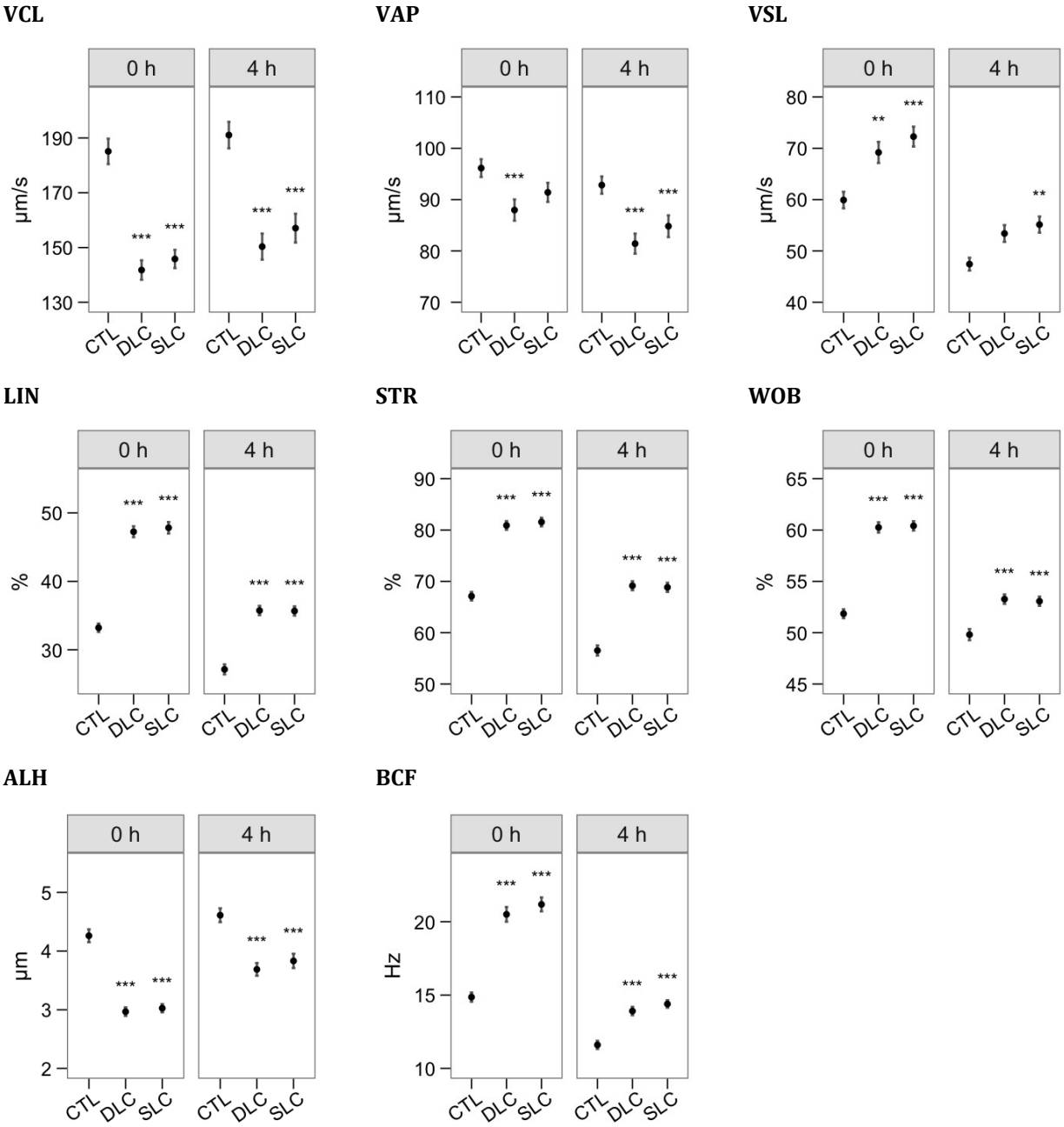

Figure 5.15. Effect of the colloid centrifugation (CTL: Control; DLC: Double Layer Centrifugation; SLC: Single Layer Centrifugation) on CASA variables. The plots show mean ± SEM for the kinematic variables directly post-thawing (0 h), and after the 4-h incubation (4 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.001$ except for VCL.

The motility subpopulations were obtained from the same experiment on the extension of the equilibration time with OPTIXcell, thus using the same groups described in Table 5.2. Figure 5.16 shows the effects of the colloid treatments on the subpopulations. Both Rapid and Slow subpopulations significantly increased in SLC and DLC both post-thawing and after the incubation, whereas the Hyper subpopulation followed an inverse and significant change.

The colloids positively affected the flow cytometry variables, with small differences between both treatments (Figure 5.17). The viability and mitochondrial activity were higher post-thawing for DLC and SLC, with lower apoptotic ratio, mitochondrial superoxide production, and acrosomal damage. However, the capacitation ratio and acrosomal damage ratio were higher for DLC and even higher for SLC (P<0.001 SLC vs. DLC), and DLC-processed samples showed a higher post-thawing cytoplasmic ROS production.

After the incubation, the positive effect on sperm viability and mitochondrial activity maintained, but the effects on apoptotic ratio and acrosomal damage dissipated while keeping the increase in the acrosomal damage ratio. Moreover, the production of mitochondrial superoxide was higher for the samples submitted to the colloid centrifugation (Figure 5.17).

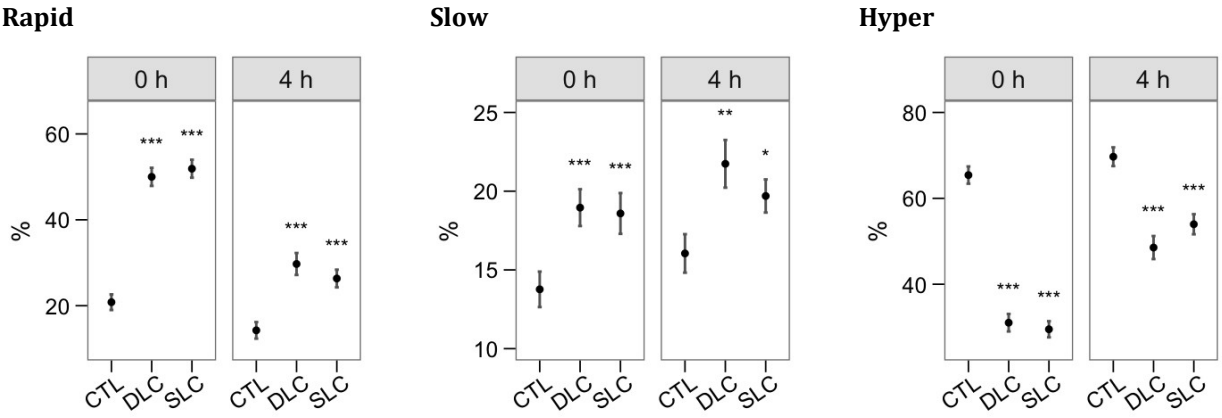

Figure 5.16. Effect of the colloid centrifugation treatments (CTL: Control; DLC: Double Layer Centrifugation; SLC: Single Layer Centrifugation) on CASA variables. The plots show mean ± SEM for the proportions of the motility subpopulations, directly post-thawing (0 h), and after the 4-h incubation (4 h). Insets show P values for the effects of the treatments respect to the Control (* P<0.05; ** P<0.01; *** P<0.001). The overall effect of the incubation was P<0.001 for Rapid and Hyper, and P<0.05 for Slow.

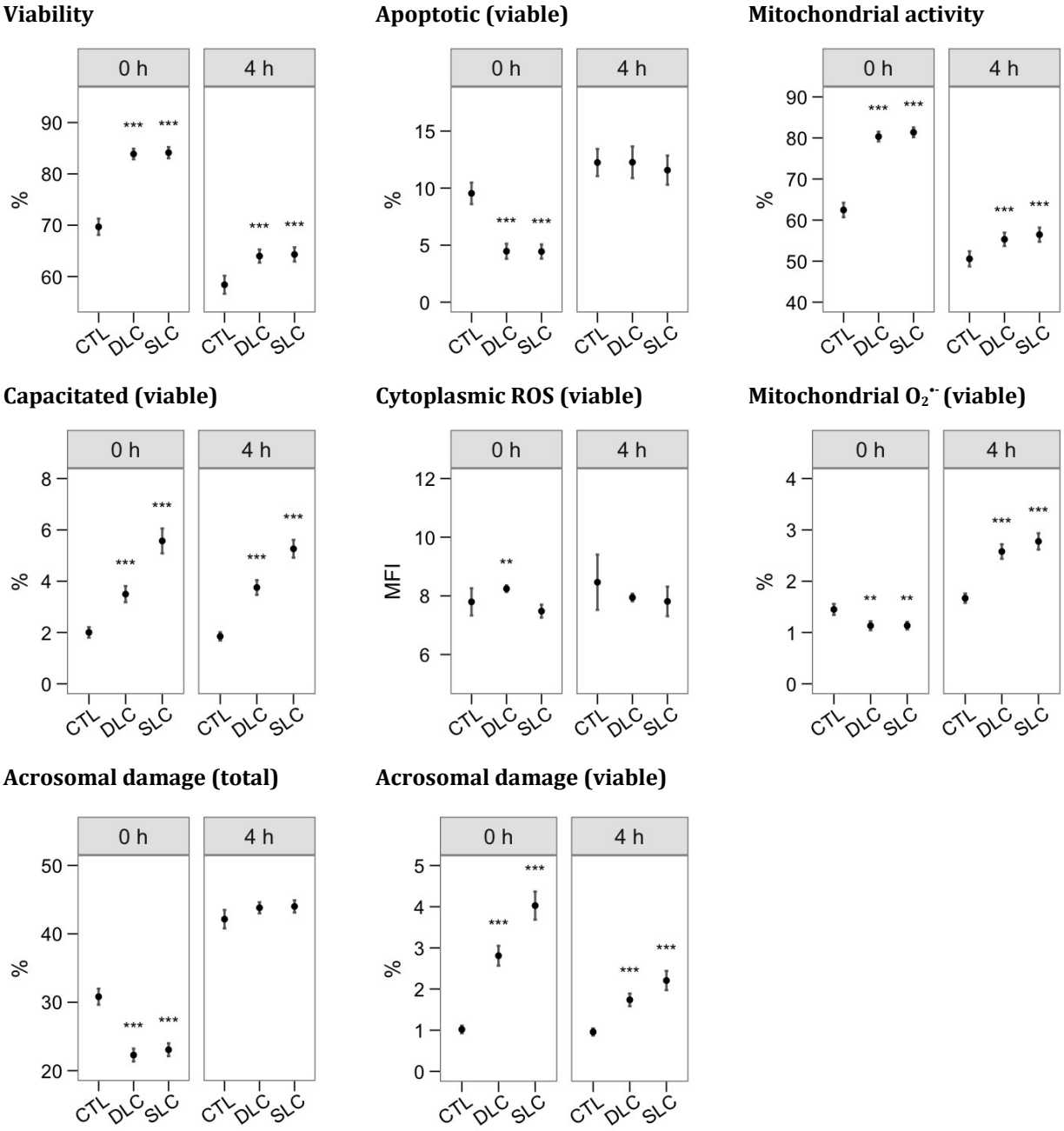

Figure 5.17. Effect of the colloid centrifugation treatments (CTL: Control; DLC: Double Layer Centrifugation; SLC: Single Layer Centrifugation) on flow cytometry variables. The plots show mean ± SEM, directly post-thawing (0 h), and after the 4-h incubation (4 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.001$, except for the capacitation ratio and cytoplasmic ROS.

Figure 5.18 shows the effects of the treatments on chromatin status. Whereas the DNA fragmentation (%DFI) was very low in all cases, the pre-freezing application of both treatments almost totally abolished the presence of DNA-damaged spermatozoa ($P<0.001$). Chromatin immaturity was not affected in any case.

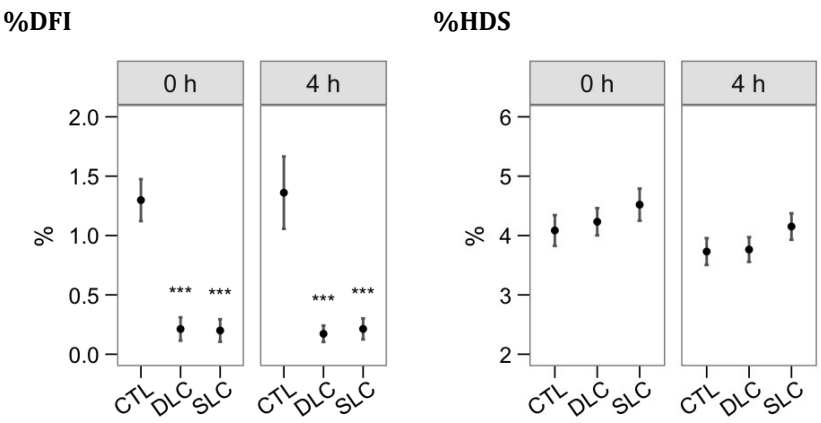

Figure 5.18. Effect of the colloid centrifugation treatments (CTL: Control; DLC: Double Layer Centrifugation; SLC: Single Layer Centrifugation) on flow cytometry variables. The plots show mean ± SEM, directly post-thawing (0 h), and after the 5-h incubation (5 h). Insets show *P* values for the effects of the treatments respect to the Control (* $P<0.05$; ** $P<0.01$; *** $P<0.001$). The overall effect of the incubation was $P<0.05$ for %HDS.

DISCUSSION

6.1 Impact of Extending the Equilibration Time on Post-thawing Bull Semen Quality

In this thesis, we extended the equilibration time from four to twenty-four hours in two different experiments using BIOXcell extender in SERIDA and OPTIXcell extender in the Xenética Fontao Artificial Insemination Center. The aim was to improve the post-thawing bull semen quality and also to test the possibility of different AI centers that use these extenders to apply the extension of the equilibration times to the day after semen collection. This may be appropriate with the work schedule of some of these centers. We conducted these experiments based on the many previous studies (Foote and Kaproth, 2002; Anzar et al., 2011; Shahverdi et al., 2014; Fleisch et al., 2017) which confirmed the enhancing effect of the prolongation of the equilibration time on post-thawing bull and buffalo sperm quality using different extenders and equilibration times.

Many authors (Mazur, 1984; Salamon and Maxwell, 2000; Bailey et al., 2000; Meryman, 2007; Leite et al., 2010) have shown that equilibration time is essential for the adaptation of the sperm plasma membrane to the freezing step, and thus maintaining the integrity of sperm membranes during cryopreservation process. However, there is no agreement on which equilibration time is more appropriate for the survival and fertility of bull sperm (Foote and Kaproth, 2002).

Our results, in contrast to many studies (Foote and Kaproth, 2002; Anzar et al., 2011; Shahverdi et al., 2014; Fleisch et al., 2017), showed that the post-thaw sperm motility in the two experiments was lower (except the total motility with BIOXcell and VCL and ALH with OPTIXcell) in prolonged equilibration time semen. These differences between our findings and those obtained through these studies may be related to the different extenders and procedures used.

The type of extender affects the impact of the equilibration time on the post-thawing semen quality, as confirmed by many studies on bull and buffalo semen. For instance, Muiño et al. (2007) reported that Tris-egg yolk based extender Biladyl resulted in higher post-thaw bull sperm quality, comparison to the soybean-derived extenders AndroMed and Biociphos Plus when the equilibration time extended to 18 h. Fleisch et al. (2017) showed that both Triladyl egg yolk-based extender and OPTIXcell liposome-based extender yielded higher post-

thaw bull sperm quality compared to AndroMed when the equilibration time was prolonged from 4 h to 24, and 48 h. Shahverdi et al. (2014), in a study on buffalo sperm, confirmed that BIOXcell extender gave higher post-thaw progressive motility and motility parameters than Tris-egg yolk extender with extended the equilibration times from 2 h to 4, 8, and 16 h. However, the extender type did not affect the total motility and sperm physiology parameters.

From these previous studies, we can infer that there is an interaction between the type of extender in semen cryopreservation and the extension of the equilibration time, with a preference for the egg yolk-based extenders over soy bean-derived extenders. Several authors (Bergeron et al., 2004; Bergeron et al., 2006; Muiño et al., 2007) connected these effects with the protective influences of egg yolk low-density lipoproteins (LDL) in egg yolk-based extenders. LDLs are composed of 84–89% lipids, which are comprised of about 26% phospholipids and 5% cholesterol (Eser et al., 2014). Phospholipids and cholesterol in the sperm plasma membrane are essential to maintain the fluidity and functionality of the membrane at physiological temperature (Elmoazzen et al., 2009; Lemma, 2011; Grötter et al., 2019). The lipid transition phase, which may happen during the cryopreservation process, leads to disordered fatty acids of the cell membrane phospholipids (Lemma, 2011) and a decrease in the proportion of cholesterol in the membrane (Holt, 2000). These together cause a reduction in the stability of the membrane during the cooling phase (Holt, 2000). The LDL can compensate for the lack of phospholipids and cholesterol in the sperm plasma membrane during cryopreservation. Another potential cryoprotective mechanism of egg yolk is by sequestration of bovine seminal plasma (BSP) proteins by LDL (Bergeron et al., 2006). BSP proteins stimulate cholesterol and phospholipids efflux from the sperm membrane. Thus LDL reduces this efflux and, consequently, maintains the resistance of the plasma membrane to the cooling and freezing temperatures (Bergeron et al., 2004; Bergeron et al., 2006).

Soybean lecithin can also replace lost phospholipids from sperm plasma membranes, or it may form a protective layer on plasma membranes (Ondřej et al., 2019). The adding of the LDL extracted from egg yolk to the soybean lecithin-based extenders AndroMed and BIOXcell led to enhance the post-thawing bull sperm quality (Ondřej et al., 2019). Thus, this is evidence of that Tris-egg yolk extenders due to their content of LDL provide high protection to spermatozoa when the cooling phase is extended before freezing. In contrast, soy bean-derived extenders might have a limited effect on that, due to their lack of phospholipids compared to egg yolk based extenders.

Another substitute for egg yolk is artificially prepared liposomes (generally from lecithin), for instance in the commercially available OPTIXcell extender (Ondřej et al., 2019). Liposome-based extenders may stabilize spermatozoa during cooling and freezing temperatures by modifying sperm membranes by liposomes, which consist of one or more phospholipid bilayers, thus exchanging lipids and cholesterol in the membranes (Akbarzadeh et al., 2013; Fleisch et al., 2017). Nevertheless, these mechanisms are still poorly understood (Fleisch et al., 2017). However, OPTIXcell provided lower protective efficiency for bull semen stored for up to three days post-collection at 18°C, comparison to different commercial semen extenders (Murphy et al., 2017).

While BIOXcell, a soybean-based semen extender, is a widely-used commercial extender, no reports were found in the literature about using this extender while at the same time prolonging the equilibration time of bull semen to twenty-four hours. This made us test this extender in the first experiment, while we used OPTIXcell, a liposome-based extender, in the second experiment.

Regarding the sperm physiology parameters, our results agree with many reports (Foote and Kaproth, 2002; Anzar et al., 2011; Shahverdi et al., 2014; Fleisch et al., 2017), as the extension of the equilibration time enhanced sperm viability post-thawing in both our experiments. The decrease in sperm motility, which is accompanied by an enhancement in the sperm viability in the extended equilibration time, maybe be due to viable spermatozoa maintaining membrane integrity while becoming immotile.

Both extenders, with extending the equilibration time, did not affect the %DFI, and this is in line with Shahverdi et al. (2014) as they did not find an effect of the equilibration time on the %DFI in their study on buffalo semen. Both extenders resulted in a decrease in the %HDS post-thawing in the twenty-four hours equilibration time. The similarity in different semen quality parameters between the two extenders used in our study at post-thawing semen quality agrees with the findings of Amal et al. (2019) as they found no differences between BIOXcell and OPTIXcell extenders when they compared them regarding the post-thawing bull semen quality equilibrated at four hours.

The post-thawing sperm incubation at 38 °C for 4 h or 5 h was conducted *in vitro* to mimic the female body temperature, wherein sperm should remain viable and motile for some time. The decline in semen quality after incubation post-thawing in our experiments, which agrees with other studies (Rahoo et al., 2011; Rastegarnia et al., 2013), may reveal the

potential cryoinjuries which result from the freezing-thawing process which then appear after semen incubation.

In the experiment in which we used the BIOXcell extender, we supplemented the extender with 2 mM GSH to test the possible compensate impact of this antioxidant on the decrease in semen quality. This antioxidant had a very small effect, and we discuss it in the next chapter. In the same experiment, we used two cattle breeds Asturiana de los Valles and Holstein-Friesian. The interaction between the equilibration time and the breed was mostly insignificant, and this could be associated with the small number of bulls used. However, we believe this was a relevant observation, due to a lack of such studies on the Asturiana de los Valles local breed.

Many authors (Rodriguez-Martinez, 2003; Christensen et al., 2011) have pointed out that different sperm quality variables that can be used to predict the fertility of the bull semen sample; however, there is a lack of consensus on which variables to use. Moce and Graham (2008) showed that a specific sperm quality variable isolated from the rest of the variables are not useful to predict the fertility of a semen sample. Similarly, Holt (2000) showed that a combination of different laboratory assays of sperm quality provides a more precise indicator of the potential fertility of an ejaculate. On the other hand, Fleisch et al. (2017) showed that although the extension of the equilibration period from 4 h to 72 h affected the post-thawing bull semen quality; however, there was no difference in the field fertility capacity of those samples.

The differences in the post-thawing semen variables between the two equilibration times in our experiments were small. Thus according to these findings and the previous studies, we can conclude that the application of extension the equilibration time to the day after the day of semen collection is available with the two extenders tested in our experiments and according to the AI center's requirement. Further studies are required to evaluate the fertilizing capacity of those samples, to create a more comprehensive picture of these treatments.

6.2 Supplementation of Trehalose and GSH to the Semen Freezing Extender

The sperm cryopreservation process increases the levels of ROS production in spermatozoa and seminal plasma (Chatterjee and Gagnon, 2001). The ROS leads to the initiation of lipid peroxidation (LPO) in the sperm plasma membrane and therefore gives rise to damage of the membrane (Agarwal et al., 2014; Harchegani et al., 2019). This may reduce sperm fertility post-thawing (Mazzilli et al., 1995; Chatterjee and Gagnon, 2001). Spermatozoa contain low levels of endogenous antioxidants (Peris et al., 2007; Agarwal et al., 2014), while ROS may cause the inactivation of enzymes and the depletion of endogenous antioxidants in the sperm (Baumber et al., 2000; Agarwal et al., 2014; Tvrda et al., 2016a; Harchegani et al., 2019; Tvrda et al., 2019). This makes them more vulnerable to oxidative stress (Peris et al., 2007; Agarwal et al., 2014).

From these studies, it seems clear that the antioxidant system in sperm is weak, and the cryopreservation process may aggravate the problem. Therefore, different reports (Eidan, 2016; Shah et al., 2017a) confirmed that the addition of exogenous antioxidants to the semen cryopreservation extenders has a beneficial effect and protects sperm against oxidative damage.

Trehalose, as a cryoprotectant, modulates cell dehydration and increases membrane fluidity during cryopreservation (Aboagla and Terada, 2003; Hu et al., 2010). Several reports have confirmed the beneficial effects on post-thawing sperm quality of trehalose supplemented to freezing extenders in bull (Hu et al., 2010; Chhillar et al., 2012; Oh et al., 2012; Kumar et al., 2013; Ozturk et al., 2017), buffalo (Kumar et al., 2013; Iqbal et al., 2018), and ram (Matsuoka et al., 2006) semen.

In contrast to these reports, we could not find an enhancement in post-thawing sperm quality due to the supplementation of the freezing extender with different concentrations of trehalose (we estimated these concentrations from these studies). On the contrary, we found that trehalose was detrimental to sperm. Our results show a reduction in post-thawing sperm motility due to the supplementation of 50 and 100 mM of trehalose (the decrease in T100 was more) to the BIOXcell freezing extender. They also show that there is a reduction in sperm viability and an increase in the acrosomal damage in T100. The previous studies were done

using egg yolk or milk-based extenders, while ours is the first study done using soybean-derived freezing extender BIOXcell supplemented with trehalose in a bull.

Trehalose cannot penetrate the cell membrane when it is present in the freezing extender. It also causes the drying of spermatozoa to prevent inside ice formation (Sieme et al., 2016; Grötter et al., 2019). Several reports confirmed the correlation among the concentrations of different ingredients in semen extenders like glycerol and trehalose, and also other components in the extender, like egg yolk, to affect the results in terms of post-thawing sperm quality and *in vivo* fertility (Büyükleblebici et al., 2014; Iqbal et al., 2018).

Glycerol behaves in a different manner as a cryoprotectant. It decreases the ice crystal formation in the extracellular matrix, and also, by entering inside the cell, it prevents the formation of osmotic pressure differences (Öztürk et al., 2020). While glycerol may also be toxic and lead to an initial osmotic disturbance, thus shrinkage and damage of the spermatozoa, when the extender is added to the semen (Holt, 2000; Sieme et al., 2016), especially when glycerol added in high concentrations (Öztürk et al., 2020).

Many authors (Najafi et al., 2013; Iqbal et al., 2018; Öztürk et al., 2020), showed that trehalose has a synergic effect with glycerol when they are found at specific concentrations in the freezing extender, and this may affect the post-thawing sperm quality and may depend on the extender type. Furthermore, others have confirmed that glycerol could be replaced by trehalose in the semen extender to give equivalent post-thawing semen quality (Athurupana et al., 2015). From that, we suspect that the high concentrations of trehalose added to the extender, which may not be compatible with the glycerol concentration, expose the spermatozoa to high dehydration and osmotic stress during the cryopreservation process.

The decrease in sperm quality in our study that results from the supplementing of trehalose (especially with the higher concentration) to the BIOXcell may be due to a disturbance in the synergic effect of this sugar with glycerol, which might expose spermatozoa to hyper-dehydration and hyperosmotic conditions during the cooling and thawing phases. Therefore, membrane injuries could happen, and with this, a decrease in sperm motility and viability. The harmful effects of osmotic stress due to the high concentrations of trehalose supplemented to the freezing extender on post-thawing semen quality has also been reported in ram (Aisen et al., 2002; Öztürk et al., 2020), boar (Hu et al., 2009), and chicken (Thananurak et al., 2016) semen.

Trehalose at concentrations used in our study slightly reduced the production of superoxide anions ($O_2^{\bullet-}$) post-thawing and after incubation. This might be due to the antioxidant properties of trehalose, which were also mentioned in other reports (Kumar et al., 2013; Iqbal et al., 2018). While the decrease of the ROS did not compensate for the overall reduction in sperm quality.

We can conclude that the supplementation of trehalose to BIOXcell extender leads to reduce the post-thawing sperm quality due to osmotic damage of spermatozoa. It is possible that the glycerol content in BIOXcell (40.2 g/L; Penitente-Filho et al., 2014), or other components, are not compatible with the trehalose concentrations tested. We have to point out that this was the first attempt of supplementing BIOXcell with trehalose, and the addition of trehalose may be useful with freezing extenders containing a concentration of glycerol lower than BIOXcell.

Reduced glutathione (GSH) is one of the essential antioxidants in the cells (Meister, 1994; Bilodeau et al., 2001). The high levels of ROS produced during the cryopreservation process cause a reduction in the endogenous GSH content in semen and are consequently associated with a decrease in sperm quality. This has been reported in bovine (Bilodeau et al., 2000), boar (Gadea et al., 2004), and human (Gadea et al., 2011) studies. Therefore, the supplementation of GSH to the freezing semen extenders to reduce oxidative stress and compensate for the lack of GSH, thus enhancing post-thawing sperm quality and fertility has also been reported in bull (Sariözkan et al., 2009; Shah et al., 2017a), goat (Razliqi et al., 2015), boar (Yeste et al., 2014), red deer (Wang and Dong, 2017), buffalo (Ansari et al., 2012), and human (Gadea et al., 2011) studies.

There is only one report (Sariözkan et al., 2009) regarding the supplementation of BIOXcell extender with GSH in bull semen cryopreservation. In that study, researchers tested the effect of just one concentration of GSH (2 mM). This resulted in a decrease in the acrosomal damage and the total abnormalities of post-thawing sperm. The lack of available information on the nature of such molecules in BIOXcell, prompted Stradaioli et al. (2007) to analyze this extender. They showed that it contains 450 μM of GSH, in comparison with 40 μM in egg yolk-Tris-glycerol extender (EYTG). In our diverse experiments, we tested different concentrations of GSH (0.5, 2, and 5 mM) supplemented to the BIOXcell extender to discover the correct concentration which may lead to an improvement in the post-thawing bull semen quality.

We found no improvement in the overall post-thawing bull semen quality. However, there was a slight reduction in the proportion of viable spermatozoa producing mitochondrial superoxide with all concentrations of GSH used. There was also a slight decrease in the post-thawing %HDS in G2 (in both experiments where we used this concentration) and G5. At the same time, there was a slight decrease in sperm motility in G5. These findings disagree with those of Ansari et al. (2012), where they found an increase in the post-thawing sperm quality using egg yolk-based extender with different concentrations of GSH in buffalo semen. While our results are consistent with those of Tuncer et al. (2010), where they confirmed that the supplementation of milk-based extender, Laiciphose, with GSH (0.5 and 2 mM) did not improve the post-thawing bull sperm quality and fertility. Similarly, the fertility of bull semen cryopreserved in a milk-based extender containing different concentrations of GSH did not result in any improvement (Foote et al., 2002). Gadea et al. (2008), in a study on the supplementation of GSH to the bull semen thawing medium, found that it affected neither motility parameters nor acrosomal damage.

Our results of the experiments where we used BIOXcell showed that there was low ROS production in the control semen samples (with no antioxidants treatment), both post-thawing and even after incubation. This might indicate the presence of sufficient levels of antioxidants in this extender. GSH, which is one of the components of BIOXcell, led to no improvements in semen quality in our experiments with extra supplementation of this antioxidant. Foote et al. (2002) also concluded similarly, posing that the presence of natural antioxidants (casein) in a milk-based extender decreased the requirement of more antioxidant supplementation.

Thus, we conclude that when using BIOXcell extender in bull semen cryopreservation, extra supplementation of GSH might not result in improved post-thawing semen quality.

6.3 Supplementation of Curcumin and Crocin to the Semen Freezing Extender

Curcumin (CUR) and crocin (CR) are natural antioxidants that have been used in several studies as supplements to semen freezing or thawing extenders to reduce ROS in semen and thus improve semen quality. Curcumin has excellent ROS scavenging properties and helps preserve the antioxidant characteristics of the endogenous semen antioxidants (Tvrda et al., 2016a; Tvrda et al., 2016b). It thus improves the post-thawing sperm quality if it is supplemented to the semen extender (Bucak et al., 2012; Tvrda et al., 2016b; Shah et al., 2017b). There have been few studies conducted on adding curcumin to the bull semen freezing extender. These studies confirmed the improvement effects of it in terms of post-thawing sperm motility (Tvrda et al., 2016b), a decrease of sperm abnormalities, and enhancement of the sperm viability in post-thawing semen (Bucak et al., 2012). Improvement effects of supplementing the freezing extender with curcumin on post-thawing sperm quality were also reported in buffalo (Shah et al., 2017b), goat (Bucak et al., 2010), and boar (Chanapiwat and Kaeoket, 2015) semen.

Our results show that the supplementation of curcumin 0.05 and 0.1 mM to the freezing extender did not improve the post-thawing semen quality. These findings disagree with other studies that reported on bull (Bucak et al., 2012; Tvrda et al., 2016b) and buffalo (Shah et al., 2017b) semen. Bucak et al. (2012) showed an improvement in the post-thawing bull semen quality using 0.5 mM of curcumin supplemented to the freezing extender, while they did not find such increases with the concentration of 2.0 mM. Similarly, Shah et al. (2017b) showed that 1.5 mM of curcumin in comparison to 0.5, 1.0, and 2.0 mM resulted in the best improvement on the post-thawing buffalo semen quality. While Tvrda et al. (2016b) confirmed that 0.05 mM of curcumin supplemented to the freezing extender improved the post-thawing semen quality. Moreover, Naz (2011) showed that the incubation of human and murine sperm with curcumin at a concentration of 0.12 mM led to a decrease in sperm quality while increasing the curcumin concentration to higher than 0.25 mM led to the complete inhibition of sperm motility.

Our results also show a surprising increase in cytoplasmic ROS both post-thawing and after incubation due to supplementing the freezing extender with the two concentrations of curcumin. Some authors reported that curcumin, despite to its antioxidant properties, may

also increase the generation of ROS in the cells directly, or due to its an impact to reduce the endogenous GSH in the cells (Atsumi et al., 2007; Sanchez et al., 2010). For instance, Atsumi et al. (2007) showed that curcumin could be used as a cytotoxic therapy to the cancer cells by causing an increase in the generation of ROS. In that study, they confirmed that the incubation of the cells for one hour with curcumin at 0.01 mM resulted in the same levels of GSH as in the control (without curcumin addition). However, increasing the dose of curcumin to 0.03 mM, the GSH level was reduced to 37% than that of the control. On the other hand, Bucak et al. (2012) pointed out that curcumin at 0.5 mM led to the maintenance of the total glutathione levels in post-thawed bull sperm.

Thus we suggest that the increase in the cytoplasmic ROS levels in our study (in curcumin treatment samples) could be due to the effect of curcumin, while this may explain the slight increase in the ratio of viable sperm with acrosomal damage post-thawing in curcumin treatment samples. However, curcumin did not negatively affect sperm motility and other sperm physiology parameters in our study. The cause of that may be due to the amount of GSH found in BIOXcell, as we discussed before. Thus these amounts of GSH could compensate for the high levels of ROS and prevent the oxidative stress to sperm due to curcumin addition.

Crocin, as an antioxidant, has a high ability to scavenge free radicals (Galano et al., 2010; Sapanidou et al., 2016; Esposito et al., 2019). Our results show that the supplementation of the freezing extender with the two concentrations of crocin led to a slight decline in sperm motility after incubation. At the same time, there was a reduction in the sperm physiology parameters post-thawing and after incubation.

Our study is the first on the supplementing of semen freezing extender with crocin in bull, while there are two other studies about the supplementation of the thawing medium with crocin in bull (Sapanidou et al., 2015; Tsantarliotou et al., 2016). These studies obtained good results in terms of sperm motility, viability, and acrosomal integrity. Sapanidou et al. (2015) showed that incubation of frozen-thawed bull sperm with crocin (1 mM) for 120 and 240 minutes led to enhance the semen quality and *in vitro* fertility of sperm. The same previous research group (Tsantarliotou et al., 2016) did another experiment by incubation of the frozen-thawed bull sperm with crocin (1 mM) for 30 min and 60 minutes in a media contain H_2O_2 to induce oxidative stress to sperm. They found an improvement in the sperm motility and physiology parameters after the incubation, comparison to the control group. Our

results show, in contrast, that both concentrations (0.5 and 1.5 mM) of crocin used in our study did not improve the post-thawing sperm motility. At the same time, there was a slight reduction in sperm physiology parameters, except for a reduction in the post-thawing %HDS in crocin 1.5 mM. The small number of bulls used in the previous studies (4 bulls), and the utterly different protocol between these studies and ours, maybe the cause for getting different results.

As we discussed before, the GSH levels in the BIOXcell extender may be led to no improvement in semen quality with the further addition of exogenous antioxidants to this extender. This is similar to the previous study using 2 mM and the same extender when the ROS production in the control group in this study was minimal even after incubation.

We can conclude that curcumin and crocin supplemented to the BIOXcell freezing extender are not useful to improve the post-thawing bull semen quality. Overall, from the different studies in this thesis, there would be no gain in adding non-enzymatic antioxidants to BIOXcell, possibly due to its optimized composition. However, we cannot discard that specific antioxidants (targeted to specific organelles or reactive molecules) could be useful.

6.4 Semen Selection by Single Layer (SLC) and Double Layer (DLC) Colloid Centrifugation

Colloid centrifugation is a mechanism for selecting high-quality spermatozoa from the ejaculate (Morell and Rodriguez-Martinez, 2009). Semen cryopreservation causes an increase in dead and abnormal spermatozoa, which is harmful to the entire batch of semen because they produce toxic substances or free radicals (Aitken and Clarkson, 1987; Agarwal et al., 2006). The elimination of these cells by colloid centrifugation may cause a direct enhancement in semen quality by increasing the ratio of robust sperm to damaged ones (Martinez-Alborcia et al., 2013; Valeanu et al., 2015), while it may also decrease the harmful impact of these sperm on the healthy sperm, overall increasing the post-thawing semen quality (Morell and Rodriguez-Martinez, 2009).

BoviPure is one of the colloids which can be used as SLC or DLC and is formulated explicitly for bull semen (Samardzija et al., 2006b; Valeanu et al., 2015). Many reports (Samardzija et al., 2006b; Valeanu et al., 2015; Arias et al., 2017) had confirmed the efficiency of this colloid when it used as SLC on post-thawing bull semen to increase the quality and

fertility of spermatozoa which have been isolated. For instance, Samardzija et al. (2006a) showed that the post-thawed bull sperm separated by BoviPure showed a higher *in vitro* fertility rate than those separated by Percoll colloid. However, there are no reports in the literature about using this colloid with pre-freezing bull semen to improve freezability and post-thawing semen quality. In our study, we used BoviPure as SLC and DLC for processing bull semen pre-freezing, and as the first study in this field.

Our results proved that using SLC and DLC with bull semen pre-freezing led to a dramatic improvement in the post-thawing sperm motility and sperm physiology parameters. These findings are consistent with those of Nongbua et al. (2017). They showed that the pre-freezing application of Bovicoll as SLC with bull semen increased the mitochondrial membrane potential post-thawing. However, in contrast to our results, they did not find an effect of SLC on sperm viability and chromatin integrity, whereas they found an increase in superoxide production. They attributed the increase in the mitochondrial membrane potential and superoxide production to the selection of the most metabolically active spermatozoa. Indeed, we found a small rise in the cytoplasmic ROS production ratio in DLC post-thawing, but a slight reduction in the post-thawing superoxide production in both colloid treatments, concomitant to an increase in the mitochondrial membrane potential.

The varying results between the study by Nongbua et al. (2017) and ours could be explained by the methodology. The colloid (Bovicoll) and the procedure differed. Whereas they extended the semen with Tris buffer containing egg yolk before centrifugation, we used BoviWash, also after centrifugation to wash the pellets. BoviWash (by Nidacon) is a specialist medium, which is optimized for protecting the spermatozoa in these steps. Another difference, maybe minor, was in the centrifugation time thirty minutes in our study versus twenty minutes. These differences may be the reason we get better results regarding post-thawing semen quality than that study.

Our findings agree with those by Valeanu et al. (2015), who confirmed that using the BoviPure gradient as SLC on bull sperm post-thawing led to improved sperm motility and physiology. They used the same colloid and procedure as ours, only post-thawing instead of pre-freezing. Similar results were also obtained by Arias et al. (2017) using Percoll and BoviPure colloids as SLC on bull semen post-thawing. They found that both colloids enhanced the sperm motility and mitochondrial membrane potential comparison to control. We should remark that the successful use of DGC pre-freezing enables the application of new

cryopreservation and AI protocols, which could lead to the optimization of procedures for the AI centers.

The SLC and DLC treatments in our study caused a high reduction in the %DFI of post-thawed semen, even after incubation. Nevertheless, the chromatin damage was low even in the control group (%DFI was 1.0%-1.5%), indeed as found in most samples examined in this thesis. The reduction of the chromatin damage is an advantage for using SLC and DLC, and it offers a bright prospect considering that some valuable bulls could produce chromatin-damaged spermatozoa eventually. Our results are consistent with the findings of Valeanu et al. (2015) on bull, and García et al. (2009) on stallion by using SLC on post-thawed semen. Similarly, Brahem et al. (2011) reported that the DGC caused a reduction in the sperm DNA fragmentation in fresh human semen. However, Nongbua et al. (2017) and Arias et al. (2017) could not find such results, possibly due to the low DNA fragmentation values found in bull semen. Many authors (Hu et al., 2008; Lymberopoulos and Khalifa, 2010) confirmed the effect of extender used on sperm chromatin integrity.

The decrease in the %DFI by using colloid centrifugation could be explained by the removal of immature, morphologically abnormal, and dead sperm containing a high proportion of DNA fragmentation. SLC and DLC may reduce the presence of these sperm in the semen sample, as reported previously (Gloria et al., 2016). Moreover, the sperm maturation leads to an increase in their density and motility (Oshio, 1988), which may raise their chances of passing through the gradients during colloid centrifugation.

Until recently there was a lack of animal-specific colloid formulations, being one of the causes for the widespread application of the DGC in processing animal spermatozoa (Martinez-Alborcia et al., 2013; Thys et al., 2009), being limited to embryo production and similar techniques. The developing of species-specific colloid formulations and the modification of the DGC as SLC as a simplified method could help its practical application as a routine step in animal breeding (Morell and Rodriguez-Martinez, 2009; Thys et al., 2009), for instance, when males of high genetics value present low semen freezability. On the other hand, the disadvantage of these techniques is that the recovery rate of spermatozoa can be low, decreasing the number of doses obtained (Stuhtmann et al., 2012). In a recent study, Gloria et al. (2016) showed that both SLC and DLC caused a noticeable reduction in sperm recovery, with no differences between SLC and DLC. However, they pointed out that both

techniques could be performed in practice when the advantages (higher sperm quality) balanced the loss of spermatozoa.

Our results showed little difference in SLC and DLC regarding post-thawing semen quality. These findings are consistent with those of Thys et al. (2009), who found that both methods using two colloids (glycerolpropylsilane-coated silica beads, later called Bovicoll, and Percoll) offered equivalent results regarding post-thawed bull semen quality and *in vitro* fertility. Similarly, Gloria et al. (2016) showed that post-thawing bull semen quality did not differ between SLC and DLC when using iodixanol colloid, while both colloids increased the semen quality comparison to the control group. In favor of SLC, Thys et al. (2009) showed that the duration of the preparation of this technique is shorter than the preparation of DLC, and the process is less complicated and more convenient. Indeed, our experience at Xenética Fontao showed us that SLC could be more practical and economical.

Therefore, we can propose that colloid centrifugation on pre-freezing bull semen both as SLC and DLC is suitable for enhancing post-thawing semen quality, and it could be performed in practice in AI centers. Future studies could focus on studying individual variations among bulls after DGC, regarding the ability of this technique to enhance sperm freezability. Moreover, since both techniques showed equivalent results, we recommend using the SLC rather than DLC, being simple, more practical, and more economical due to time and material savings.

CONCLUSIONS

Considering the results obtained in the present thesis, we can draw the following conclusions:

1. Extending the equilibration time from four to twenty-four hours using BIOXcell or OPTIXcell showed mixed results, reducing the post-thawing sperm motility while improving sperm viability. However, differences were small and opened the possibility of using longer processing times when the center's logistics require it.

2. The supplementation of the BIOXcell extender with trehalose at both 50 and 100 mM decreased the post-thawing sperm quality, although we detected a reduction of reactive oxygen species. Trehalose, at least at these concentrations added to BIOXcell, does not seem suitable as a supplement for bull semen.

3. The supplementation of BIOXcell with GSH at 0.5, 2, and 5 mM in the diverse experiments did not improve the post-thawing sperm quality. However, we observed some positive effects, such as reducing the production of mitochondrial superoxide anion. While not being useful for the overall improvement of bull semen cryopreservation, GSH could be considered in future formulations.

4. The supplementation of BIOXcell with curcumin at 0.05 and 0.1 mM or crocin at 0.5 and 1.5 mM did not improve the post-thawing sperm quality while raising the production of cytoplasmic ROS (curcumin) or decreasing some sperm quality parameters (crocin). Curcumin and crocin do not appear to be suitable for cryopreserving bull semen at the conditions tested in this study.

5. Both pre-freezing SLC and DLC colloid centrifugation using BoviPure improved the post-thawing sperm motility and sperm physiology parameters. They also reduced the sperm DNA fragmentation. These procedures could be of practical use for enhancing the efficiency of bull semen cryopreservation, especially with samples of low freezability.

6. There were no differences in the results of using SLC and DLC concerning the post-thawing sperm quality. Therefore, SLC could be suitable for practical use in breeding centers, being cheaper and easier to perform.

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SUMMARY OF THE THESIS IN SPANISH

UNIVERSIDAD DE LEÓN
FACULTAD DE CIENCIAS BIOLÓGICAS Y AMBIENTALES

Programa de Doctorado en Biología Molecular y Biotecnología

UNIVERSITY OF LEON
FACULTY OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

PhD Program in Molecular Biology and Biotechnology

Bases celulares y optimización de la supervivencia de la criopreservación de semen de toro

Directores:

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A report presented by Amer Qasim Salman as a part of the requirements to apply for the title of Doctor

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OBJETIVOS

El objetivo principal del presente estudio es contribuir a mejorar los protocolos de criopreservación para el semen de toro. Con base en los antecedentes bibliográficos y en nuestra hipótesis, los objetivos específicos definidos para esta tesis fueron:

1. Probar el efecto de extender el tiempo de equilibrado en la calidad del semen de toro después de la descongelación, para mejorar la criopreservación de semen y el manejo de los centros de inseminación.
2. Mejorar la criopreservación del semen de toro suplementar el diluyente de congelación con trehalosa y GSH.
3. Mejorar la criopreservación del semen de toro suplementar el diluyente de congelación con los antioxidantes curcumina y crocina.
4. Probar la centrifugación coloidal pre-congelación de una y dos capas para seleccionar una subpoblación espermáticas optimizada para conseguir una mayor calidad después de la descongelación.

MATERIALES Y MÉTODOS

4.1 Diseño experimental

4.1.1 Experimento 1. Extensión del tiempo de equilibrado y suplementación del diluyente de congelación de semen con GSH

Probamos el efecto de extender el tiempo de equilibrado y agregar GSH al diluyente de congelación en la calidad del semen de toro después de la descongelación. En el experimento, que se realizó en SERIDA, utilizamos doce toros (8 Holstein y 4 AV). El semen extendido (BIOXcell) se equilibró a 5 °C (después de enfriar desde temperatura ambiente a 5 °C en noventa minutos) durante cuatro y veinticuatro horas. Cada dosis también se dividió para evaluar el efecto de la suplementación de GSH 2 mM al diluyente de congelación en el semen post-descongelación, y su interacción con el tiempo de equilibrado.

Este experimento continuó en colaboración con Xenética Fontao (Lugo), probando los mismos tiempos de equilibrado con doce toros Holstein y el diluyente OPTIXcell.

Las muestras se analizaron después de descongelar y después de cuatro horas (dosis de Xenética Fontao) o cinco horas (experimento SERIDA) de incubación a 38 °C. En ambos experimentos, la motilidad y varios parámetros de fisiología espermática se estimaron utilizando CASA y citometría de flujo inmediatamente después de la descongelación.

4.1.2 Experimento 2. Suplementación de trehalosa y GSH al diluyente de congelación de semen

Realizamos este experimento para evaluar el posible efecto de mejora de los suplementos (trehalosa y GSH) en el diluyente de congelación en la calidad del semen post-descongelado. Este experimento se realizó en SERIDA-Cenero. Utilizamos doce toros (8 Holstein y 4 AV). Los eyaculados (en BIOXcell) se dividieron en cinco partes: control (sin suplementación), trehalosa a 50 y 100 mM, y a GSH 2 y 5 mM, y luego el semen se congeló. El semen descongelado fue evaluado por CASA y citometría de flujo, directamente después de descongelar y después de cinco horas de incubación a 38 °C.

4.1.3 Experimento 3. Suplementación de curcumina, crocina y GSH al diluyente de congelación de semen

Probamos los antioxidantes curcumina, crocina y GSH como suplementos del diluyente de congelación para mejorar la calidad del semen de toro después de la descongelación. Utilizamos ocho toros Holstein en este experimento. Los toros se encontraban en Centro de Inseminación Artificial (Centro de Selección y Reproducción Bovina de Cenero, SERIDA), donde se realizó el experimento. Dividimos los eyaculados en el control (sin suplementación) y complementamos el diluyente con curcumina 0,05 y 0,1 mM, crocina 0,5 y 1,5 mM y GSH 0,5 mM, y luego el semen se congeló. Tras la descongelación, el semen se evaluó por CASA y citometría de flujo inmediatamente después de la descongelación y después de cinco horas de incubación a 38 °C.

4.1.4 Experimento 4. Selección de semen por centrifugación coloidal de una sola capa (SLC) y doble capa (DLC)

Probamos la idoneidad del coloide BoviPure para llevar a cabo SLC y DLC antes de la congelación para mejorar la calidad del semen de toro después de la descongelación, y para mejorar la congelabilidad de machos seleccionados. Se utilizaron muestras de semen de doce toros Holstein. Cada eyaculado se dividió en tres partes alícuotas: control (sin procesamiento), SLC y DLC. Los pellets resultantes de SLC y DLC se resuspendieron y los tres tipos de muestras se criopreservaron en OPTIXcell. La calidad del semen post-descongelación fue evaluada por CASA y citometría de flujo, inmediatamente después de descongelar y después de cuatro horas de incubación a 38 °C.

4.2 Reactivos y soluciones

Los reactivos generales, cuando no se indica en otro sentido, se compraron a Sigma-Aldrich (Merck KGaA, Darmstadt, Alemania). Los diluyentes de congelación BIOXcell y OPTIXcell se obtuvieron de IMV (L'Aigle, Francia). La solución coloidal BoviPure y su medio de lavado complementario BoviWash se compraron a Nidacon (Gotemburgo, Suecia).

Utilizamos PBS (solución salina tamponada con fosfato) para diluir el semen de toro antes de estudiar la motilidad y para preparar las soluciones de tinción para los análisis de citometría de flujo. Preparamos un stock de PBS 10X como se muestra en la Tabla 4.1, diluyéndolo diez veces con agua milli-Q antes de su uso. El pH final se ajustó a 7,4.

Los consumibles y las soluciones para citometría de flujo se adquirieron de Beckman Coulter (Brea, CA, EE. UU.) Y Thermo Fisher (Waltham, MA, EE. UU.). Las sondas fluorescentes para citometría de flujo se compraron de Sigma-Aldrich o Thermo Fisher, y se describen en la sección correspondiente.

4.3 Animales y colección de muestras

Los investigadores involucrados en los experimentos no tuvieron contacto con ningún animal en ningún caso. El semen se obtuvo mediante vagina artificial (42 °C a 46 °C, lubricante no espermicidal) de animales entrenados sometidos a recolección de rutina para congelación de semen e IA (Bhattacharyya et al., 2009). La recolección fue realizada por personal capacitado de SERIDA o Xenética Fontao, y se entregaron alícuotas a los investigadores. Todos estos centros están calificados y certificados para su actividad como centros de cría, siguiendo la normativa española y europea (Reales Decretos 2256/1994, 2129/2008, 841/2011).

En el primer experimento, utilizamos toros criados en los centros de cría SERIDA y Xenética Fontao. En la parte realizada en el SERIDA, utilizamos doce toros (cuatro Asturiana del los Valles y ocho Holstein-Friesian). En la parte realizada en Xenética Fontao, utilizamos doce toros Holstein-Friesian. Todos los animales estaban en servicio regular de IA y estaban alojados en establos medio abiertos (un toro en cada establo) en entornos natural. Gozaban de buena salud y cumplían los requisitos de la UE para toros utilizados en centros de inseminación artificial. Se recolectó semen de estos toros (un eyaculado por día) en días

diferentes (al menos, dos días entre colecciones), totalizando tres réplicas por cada toro realizado dentro de un período de un mes.

En el segundo experimento, utilizamos los mismos doce toros en SERIDA, como en el primer experimento, totalizando tres réplicas para cada toro realizado dentro de un período de un mes. Las condiciones de los animales y la metodología de recolección fueron las mismas.

En el tercer experimento, utilizamos ocho toros Holstein-Friesian que se mantuvieron en SERIDA. La recolección de semen se realizó como se describe para los experimentos 1 y 2, con uno réplica por toro.

En el cuarto experimento, utilizamos los mismos toros en Xenética Fontao, como en el primer experimento, con la misma cantidad de réplicas y eyaculaciones por día.

4.4 Procesamiento de semen

Después de la recolección de semen, el volumen de eyaculación se estimó directamente de los tubos graduados, mientras que la concentración se determinó por absorbancia con un fotómetro adaptado calibrado para semen de toro (Accucell, IMV Technologies, L'Aigle, Francia).

En todos los experimentos, las dosis de semen (pajuelas de 0,25 ml) se congelaron en vapores de nitrógeno líquido utilizando un congelador programable (Digit-cool; IMV Technologies). La congelación siguió una curva estándar para el semen bovino, según lo descrito por Muiño et al. (2008): -5 °C/min desde +4 °C, hasta -10 °C; -40 °C/min de -10 °C a -100 °C y -20 °C/min de -100 °C a -140 °C, y luego se almacenaron en nitrógeno líquido. La descongelación se realizó en un baño de agua a 37 °C durante treinta segundos. Se descongelaron dos pajuelas en cada caso, y el semen se reunió en tubos de 1,5 ml precalentados (a 25 °C). La calidad de las muestras criopreservadas fue verificada por CASA y la citometría de flujo como se describe en las secciones correspondientes, directamente después de descongelar y después de la incubación.

4.4.1 Experimento 1

En el primer experimento, se aplicaron dos tiempos de equilibrado antes de congelar el semen. La mitad de cada muestra de semen diluida (con diluyente BIOXcell en SERIDA y con diluyente OPTIXcell en Xenética Fontao) se equilibró a 5 °C durante cuatro horas; para la otra mitad, el tiempo de equilibrado se extendió a veinticuatro horas. Después de cada tiempo de equilibrado, el semen fue empaquetado en pajuelas de 0,25 ml y fue criopreservado.

En la parte realizada en SERIDA para el experimento 3, se preparó GSH 4 mM a temperatura ambiente en BIOXcell. El semen prediluido con BIOXcell (184×10^6/ml) se dividió en dos partes alícuotas en tubos de 15 ml. El tubo de control recibió otro volumen de BIOXcell, mientras que el tubo GSH recibió otro volumen de BIOXcell con 4 mM GSH. Por lo tanto, la concentración final de GSH fue 2 mM en el tubo suplementado, mientras que la concentración de semen en cada tubo fue 92×10^6/ml. Después de este paso, las alícuotas se trataron como se indicó anteriormente para lograr un diseño factorial de 2 tiempos de equilibrado × 2

tratamientos de suplementación. Después de cada tiempo de equilibrado, el semen fue empaquetado en pajuelas de 0,25 ml y criopreservado.

4.4.2 Experimento 2

En el segundo experimento, se prepararon dos concentraciones de trehalosa (100 mM, 200 mM) y dos concentraciones de GSH (4 mM, 10 mM) a temperatura ambiente usando BIOXcell. El semen prediluido con BIOXcell (184×10^6/ml) se dividió en cinco partes alícuotas en tubos de 15 ml. Agregamos otro volumen de BIOXcell (Control) o BIOXcell suplementado a los tubos correspondientes, logrando una concentración de semen de 92×10^6/my trehalosa 50 mM y 100 mM, y GSH, 2 mM y 5 mM (tubos T50, T100, G2 , G5). El semen se dejó enfriar a 5 °C durante noventa minutos y se equilibró en un refrigerador a 5 °C durante cuatro horas. Posteriormente, fue empaquetado en pajuelas de 0,25 ml y criopreservado.

4.4.3 Experimento 3

En el tercer experimento, se prepararon dos concentraciones de curcumina (100 μM y 200 μM), dos concentraciones de crocina (1 mM y 3 mM) y una concentración de GSH (1 mM) usando BIOXcell a temperatura ambiente. La extensión y la suplementación de semen se llevaron a cabo como en los experimentos tres y cuatro, con una concentración final de semen de 92×10^6/ml y seis tubos de 15 ml: Control, 0.05 mM (CU0.05) y 0.1 mM (CU0.1) curcumina y 0.5 mM (CR0.5), 1.5 mM (CR1.5) crocina y 0.5 mM (GSH0.5) GSH. Estos tubos se dejaron enfriar a 5 °C durante noventa minutos, luego se equilibraron a 5 °C durante cuatro horas. Después de este paso, el semen fue empaquetado en pajuelas de 0,25 ml y criopreservado.

4.4.4 Experimento 4

En el cuarto experimento, cada eyaculado se dividió a temperatura ambiente en tres partes alícuotas: control (sin procesamiento coloidal), SLC y DLC. SLC y DLC se prepararon en Tubos de 15 ml a temperatura ambiente:

SLC: 10 ml de coloide BoviPure™ al 80%.

DLC: 5 ml de BoviPure™ 40% y 5 ml de BoviPure™ 80%.

Se preparó pipeteando la capa de mayor densidad en el fondo del tubo, luego se colocaron cuidadosamente 5 ml de la capa de menor densidad en la parte superior.

El procedimiento para la centrifugación coloidal SLC y DLC fue el siguiente: se pipeteó 1 ml de semen en cada uno de los dos tubos (15 ml cada uno), luego se diluyó lentamente con BoviWash™ a temperatura ambiente. Se agregaron dos mililitros de BoviWash a un mililitro de semen, de modo que la concentración final de semen fue inferior a 250×10^6/ml. Luego, el semen diluido se pipeteó lentamente sobre la capa superior de cada columna de BoviPure (para SLC y DLC). Los tubos coloides se centrifugaron durante treinta minutos a 300×g, sin freno. El sobrenadante se eliminó cuidadosamente y se añadió 1 ml de BoviWash™ a los pellets. Luego, el semen se extendió con OPTIXcell a la concentración final (alrededor de

100×10^6/ml), se enfrió a 5 °C durante noventa minutos, se mantuvo en equilibrado durante cuatro horas a 5 °C y finalmente, se envasó en pajuelas de 0.25 ml para la criopreservación.

4.5 Determinación de la motilidad de los espermatozoides por CASA (análisis de semen asistido por computadora)

CASA (Computer Assisted Sperm Analysis) permite analizar la motilidad de los espermatozoides con precisión y repetibilidad (Amann y Waberski, 2014). El sistema CASA además permite el análisis de la cinética espermática en un campo microscópico, al capturar y digitalizar las imágenes de espermatozoides, que se identifican de acuerdo con el tamaño, la forma y el brillo de sus cabezas.

Para analizar la motilidad y los parámetros de motilidad en nuestros estudios, colocamos 5 µl de cada muestra diluida en PBS-0,5% BSA (preparada a partir de PBS 10X diluido 10 veces con ddH_2O y agregando 5 mg/ml de albúmina de suero bovino) con una concentración de semen de alrededor de 30 a 50×10^6/ml, en una cámara de conteo de semen Makler® de 10 µm de profundidad (Sefi-Medical Instruments, Haifa, Israel) que fue previamente atemperada a 37 °C. Se examinó de inmediato bajo un microscopio (Nikon E400, Tokio, Japón), con un objetivo de contraste de fase negativo ×10 y usando una cámara de video (Basler A312fc, Basler Vision Components, Ahrensburg, Alemania). Se capturaron al menos tres campos, con alrededor de doscientos espermatozoides/campo. Los parámetros de motilidad se determinaron utilizando el sistema ISAS® V.1.019 (Proiser I + D, Paterna, Valencia, España), estableciendo el área total de la cabeza entre 25 y 80 μm^2 y la velocidad de adquisición de la imagen a 53 s^{-1}. Los parámetros estimados utilizando el sistema computarizado ISAS se muestran a continuación:

- **Motilidad total de los espermatozoides (MOT)**: proporción de espermatozoides con VCL> 10 µM/s.

- **Motilidad progresiva (PROG)**: proporción de espermatozoides con desplazamiento progresivo, definida como VCL> 10 µm/s y STR> 80%.

- **Velocidad de trayectoria curvilínea (VCL)**: Velocidad con respecto a la trayectoria real, expresada en µm/s.

- **Velocidad de trayectoria recta (VSL)**: velocidad con respecto a la trayectoria recta o lineal, expresado en µm/s.

- **Velocidad media (VAP)**: velocidad media de la ruta según la trayectoria suavizada o promedio, expresada en µm/s.

- **Índice de linealidad (LIN)**: relación entre VSL y VCL, expresada como porcentaje.

- **Índice de rectitud (STR)**: relación entre VSL y VAP, expresada como porcentaje.

- **Índice de oscilación (WOB)**: relación entre VAP y VCL, expresada como un porcentaje. Proporciona una estimación de la sinuosidad de la ruta real.

- **Amplitud del movimiento lateral de la cabeza (ALH)**: amplitud de las variaciones de la trayectoria real de la cabeza del semen a lo largo de su trayectoria suavizada (ALH media de la trayectoria), expresada en μm.

- **Frecuencia del cruce del batido (BCF)**: la tasa de tiempo promedio a la que la trayectoria real de los espermatozoides cruza su trayectoria promedio, expresada en Hz.

Los datos obtenidos por el sistema CASA a partir del movimiento de cada espermatozoide se resumieron extrayendo los valores medios para cada muestra. Se realizó un análisis de subpoblación de espermatozoides aplicando el procedimiento de algoritmo de agrupación de AGNES de dos pasos (Ledesma et al., 2017). Este análisis permite una agrupación de los datos de motilidad para clasificar los espermatozoides en grupos caracterizados por patrones de motilidad distintivos.

4.6 Análisis de citometría de flujo

Utilizamos varias sondas fluorescentes en la misma solución para teñir los espermatozoides. Combinamos las sondas de acuerdo con sus espectros de emisión de fluorescencia. En la tabla 4.2 se muestra un resumen de las sondas utilizadas y sus máximos de emisión. Preparamos dos combinaciones de sondas fluorescentes para permitir la evaluación de diferentes variables fisiológicas, como se detalla a continuación:

El primer grupo de tinción contenía:

- **Hoechst 33342 (H342)**: se utiliza para descartar residuos en la muestra. Tiñe el ADN de todas las células con un núcleo, ya que puede atravesar membranas intactas. Se excita con un láser ultravioleta o violeta, emitiendo fluorescencia azul.

- **Yoduro de propidio (PI)**: esta sonda sólo ingresa a las células cuando se compromete la membrana plasmática, uniéndose al ADN; por lo tanto, los espermatozoides teñidos con PI (núcleo rojo fluorescente) se consideran no viables o muertos. Se excita con un láser ultravioleta o azul a verde, emitiendo fluorescencia roja.

- **YO-PRO-1**: sonda de unión al ADN impermeable a la membrana. Solo puede ingresar a las células que han comenzado el proceso de apoptosis, alterándose la permeabilidad de la membrana plasmática a través de la apertura de los canales de pannexina. Se excita con un láser azul y emite fluorescencia verde.

- **Merocianina 540 (M540)**: esta sonda indica el grado de desorden lipídico de la membrana celular. Se une a membranas con altos niveles de desorden lipídico. Se utiliza para evaluar el grado de fluidez de la membrana plasmática, que se asocia con la capacitación espermática. Se excita con un láser azul y emite fluorescencia naranja.

- **MitoTracker deep red (MT)**: esta sonda se acumula dentro de las mitocondrias y emite fluorescencia roja si tienen un alto potencial de membrana (mitocondrias activas). Se excita con un láser rojo y emite fluorescencia roja.

El segundo grupo de tinción contenía:

- **Hoechst 33258 (H258)**: esta sonda impermeable a la membrana solo tiñe el ADN de las células con membranas dañadas. Los espermatozoides H258 + se consideran no viables o muertos. Se excita con un láser ultravioleta o violeta, emitiendo fluorescencia azul.

- **MitoSOX**: esta sonda se dirige a las mitocondrias y detecta selectivamente el radical anión superóxido ($O_2^{\bullet -}$) producido en las mitocondrias. Emite fluorescencia roja cuando se oxida con el anión superóxido.

- **CM-H_2DCFDA**: esta sonda penetra en la membrana plasmática, se hidroliza a una forma impermeabilizante de membrana mediante citoplasmas esterasas y emite fluorescencia verde después de la oxidación. Por lo tanto, se utiliza para detectar la presencia de ROS intracelulares. Se excita con un láser azul y emite fluorescencia verde.

- **PNA Alexa Fluor 647 (PNA-647)**: PNA es una aglutinina que no puede penetrar en una membrana acrosómica intacta. Determina el estado acrosómico, ya que se une a los residuos de β-galactosa en el lado interno de la membrana externa acrosomal. Por lo tanto, solo los espermatozoides con acrosomas dañados o reaccionados se tiñen. Se excita con un láser rojo y emite fluorescencia roja.

Los análisis de citometría de flujo de los parámetros funcionales de semen de toro se llevaron a cabo utilizando un citómetro de flujo CyAn ADP (Beckman Coulter, Inc., Brea, EE. UU.) Equipado con tres láseres de diodo (violeta a 405 nm, azul a 488 nm y rojo a 635 nm). Las soluciones de tinción se prepararon el mismo día de análisis en tubos de tipo Falcon. Las muestras de semen se incubaron en 300 µL de PBS 0.5% BSA con diferentes sondas fluorescentes, a una concentración de 1.7×10^6/ml, 38 °C durante 15 minutos en la oscuridad.

Las combinaciones de fluorocromos se hicieron según los grupos indicados anteriormente: H342/PI/YO-PRO-1/M540/MT (viabilidad, apoptosis, capacitación, actividad mitocondrial) y H258/CM-H_2DCFDA/MitoSOX/PNA-647 (viabilidad, estado acrosómico , ROS citoplasmático y superóxido mitocondrial). La fluorescencia se analizó en el citómetro mediante los diferentes fotodetectores provistos de filtros: en la línea violeta, 450/50 nm (fluorescencia azul: H342, H258); en la línea azul, 530/40 nm (fluorescencia verde: YO-PRO-1, CM-H_2DCFDA), 575/25 nm para fluorescencia naranja (naranja: M540), 613/20 nm para fluorescencia roja (rojo: PI, MitoSOX); y, en la línea roja, 665/20 nm para fluorescencia roja (PNA-647 rojo, MT). La adquisición de muestras se controló utilizando el software Summit v4.3.02.

Para descartar eventos no espermáticos (desechos pequeños y otras partículas), se utilizó un citograma para dispersión frontal y lateral (FSC/SSC) en todos los análisis. Cuando se incluyó H342, se utilizó un citograma SSC/Hoechst 33342 para eliminar los residuos de una manera más específica. En el segundo conjunto de tinciones también utilizamos un citograma SSC/Hoechst 33258. Sin embargo, en este caso fue menos específico, ya que los espermatozoides viables no se tiñen con H258. La adquisición se detuvo después de llegar a 5000 células en cada muestra. Los datos obtenidos por citometría se procesaron utilizando Weasel v. 3.2 (Frank Battye, Melbourne, Australia). Las Figuras 4 y 5 muestran ejemplos de citogramas.

Los parámetros obtenidos de los análisis de citometría de flujo fueron:

- **Viabilidad**

El YO-PRO-1/PI proporcionó las proporciones de espermatozoides viables y "apoptóticos" (Figura 4.1A). Las subpoblaciones de semen se clasificaron como:

PI-, espermatozoides viables.

YO-PRO-1 +/PI-, espermatozoides apoptóticos.

PI +, espermatozoides muertos.

Se extrajeron las variables para espermatozoides viables (PI-), apoptóticos (YO-PRO-1+/PI-) y la proporción de apoptóticos dentro de viables (apoptóticos/viables).

- **Trastorno de la membrana plasmática (capacitación)**

La combinación YO-PRO-1/M540 proporcionó una estimación de espermatozoides capacitados (Figura 4.1B). Las subpoblaciones de semen se clasificaron como:

YO-PRO-1-/M540-, espermatozoides viables (no apoptóticos) con plasmalema intacto.

YO-PRO-1-/M540+, espermatozoides viables (no apoptóticos) con plasmalema capacitado.

Obtuvimos la proporción de espermatozoides capacitados viables (YO-PRO-1-/M540+) con respecto al total de espermatozoides viables (YO-PRO-1-) para su posterior análisis.

- **Potencial de membrana mitocondrial (Δψm)**

Utilizamos la combinación YO-PRO-1/MT (Figura 4.1c). Las subpoblaciones seleccionadas fueron:

YO-PRO-1-/MT+, espermatozoides viables con Δψm alto.

YO-PRO-1-/MT-, espermatozoides viables con Δψm bajo.

Utilizamos la proporción de espermatozoides viables con Δψm alto para el análisis.

- **Especies reactivas de oxígeno (ROS) en el citoplasma**

Utilizamos la combinación H258/CM-H_2DCFDA (Figura 4.2B). Como una estimación de ROS citoplasmático, obtuvimos la mediana de la intensidad de fluorescencia (MFI) de La concentración intracelular de especies reactivas de oxígeno en semen viable (H258-), se expresó como la mediana de la intensidad de fluorescencia de la sonda CM-H_2DCFDA.

- **Ensayo de superóxido mitocondrial ($O_2^{\bullet -}$)**

Utilizamos la combinación H258/MitoSOX (Figura 4.2c). Las subpoblaciones seleccionadas fueron:

H258-/MitoSOX-, espermatozoides viables con baja producción de $O_2^{\bullet -}$ mitocondrial.

H258-/MitoSOX+, espermatozoides viables con alta producción mitocondrial de $O_2^{\bullet -}$.

Utilizamos la proporción de espermatozoides viables que presentan una alta producción mitocondrial de $O_2^{\bullet -}$ (H258-/MitoSOX+) con respecto a los espermatozoides viables totales (H258-).

- **Integridad del acrosoma**

Utilizamos la combinación H258/PNA-Alexa 647 (Figura 4.2D). Las subpoblaciones seleccionadas fueron:

No viable con acrosomas dañados (H258+/PNA+).

Viable con acrosoma intacto (H258-/PNA-).

Viable con acrosoma dañado (H258-/PNA+).

Utilizamos tanto la proporción total de espermatozoides con acrosomas dañados o reaccionados (H258+/PNA + más H258-/PNA+) como la proporción de espermatozoides viables que presentan acrosomas dañados o reaccionados (H258-/PNA+) con respecto a los espermatozoides viables totales (H258-).

4.7 Integridad de la cromatina espermática

Utilizamos el SCSA (Sperm DNA Structure Assay) para evaluar la integridad del ADN espermático y la compactación de la cromatina. Diluimos la muestra de semen (directamente después de descongelar y después de la incubación) en tampón TNE (Tris-HCl 10 mM, NaCl 150 mM, EDTA 1 mM, pH 7,4), a una concentración final de alrededor de 2×10^6/ml, y almacenamos el muestras a -80 °C. Las muestras se descongelaron en hielo picado antes del análisis. Se pipeteó un volumen de 200 µl en un tubo de citometría de flujo, y se mezcló inmediatamente con 400 µl de solución de detergente ácido (Triton X-100 al 0,1%, NaCl 150 mM y HCl 80 mM, pH 1,2) para inducir la desnaturalización del ADN in situ Exactamente treinta segundos después, la muestra se tiñó agregando 1,2 ml de solución de naranja de acridina (AO) (ácido cítrico 0,1 M, Na_2HPO_4 0,2 M, EDTA disódico 1 mM y NaCl 0,15 M, pH 6,0; 6 µg/ml de AO). El tubo se mantuvo en hielo picado y se analizó en el citómetro de tres a cinco minutos más tarde. Utilizamos el citómetro de flujo FACSCalibur (Becton Dickinson; Franklin Lakes, NJ) para analizar las muestras teñidas. El citómetro estaba configurado para adquirir al menos 5000 eventos. El AO emite fluorescencia roja si se une al ADN monocatenario (susceptible a la desnaturalización, con roturas) y fluorescencia verde si se une al ADN de doble cadena (resistente a la desnaturalización, sin roturas). Los archivos de citometría de flujo (FSC v.2) se analizaron en el entorno estadístico R (Team; 2019) utilizando la biblioteca flowCore (Ellis et al., 2019; Team; 2019) para acceder y procesar los datos de citometría. El índice de fragmentación del ADN (DFI) se calculó para cada espermatozoide como la relación de fluorescencia roja a total (rojo + verde), multiplicada por 1000. Obtuvimos la desviación estándar del DFI (SD-DFI) y la proporción de espermatozoides con un DFI superior a 250 (%DFI). La compactación de la cromatina se estimó a partir de la intensidad de fluorescencia verde, considerando los espermatozoides con una intensidad de fluorescencia superior a 650 (%HDS).

4.8 Análisis estadístico

Los análisis estadísticos se llevaron a cabo en el entorno estadístico R (R team, 2019). Los datos se analizaron utilizando modelos de efectos lineales mixtos.

En el primer experimento, para el experimento realizado en SERIDA, el tiempo de equilibrado y el tratamiento antioxidante se usaron como factores fijos, mientras que el toro, la raza y la réplica se incluyeron en la parte aleatoria de los modelos. Para la parte realizada en Xenética Fontao, el tiempo de equilibrado se utilizó como factor fijo, y el toro y la réplica se utilizaron como efectos aleatorios. En el segundo experimento, el tipo de suplemento se utilizó como factor fijo, mientras que el toro, la raza y la réplica se incluyeron en la parte aleatoria de los modelos. En el tercer experimento, el tratamiento antioxidante fue el factor fijo, mientras que el toro fue el factor de agrupación en la parte aleatoria. En el cuarto experimento, el tratamiento coloidal fue el factor fijo, mientras que el toro y la réplica se incluyeron en la parte aleatoria del modelo.

RESULTADOS

5.1 Impacto de la extensión del tiempo de equilibrado en la criopreservación del semen de toro

5.1.1 Diluyente BIOXcell y suplementación con GSH

La extensión del tiempo de equilibrado antes de la congelación utilizando el diluyente BIOXcell (experimento en el SERIDA) dio como resultado pequeños, aunque significativos, cambios en la calidad del semen después de la descongelación. La suplementación con GSH 2 mM no tuvo efecto en general, salvo algunas excepciones.

Las Figuras 5.1a y 5.1b muestran los resultados para las variables de motilidad obtenidas por CASA después de la descongelación y después de la incubación de 5 h. La motilidad total (Fig. 5.1a) no cambió después de la descongelación entre los tiempos de equilibrado. En contraste, la motilidad progresiva y los parámetros de motilidad fueron significativamente mayores en las muestras congeladas después del equilibrado de 4 h (Figuras 5.1a y 5.1b). Estas diferencias aumentaron después de la incubación, con menor calidad para el equilibrado de 24 h, incluida la motilidad total. El tratamiento con GSH afecta ligeramente a WOB después de la descongelación y después de la incubación, y LIN después de la incubación (Fig. 5.1b).

El análisis de subpoblación de los datos de CASA arrojó dos grupos, Rápido y Lento (Tabla 5.1). No hubo diferencias después de la descongelación. Después de la incubación, el equilibrado de 24 h mostró una proporción significativamente menor de espermatozoides rápidos y más alta de lentos (Figura 5.2).

Teniendo en cuenta las variables de citometría de flujo (Figuras 5.3a y 5.3b), el equilibrado de 24 h mostró una mayor viabilidad y actividad mitocondrial, y una tasa de capacitación más baja (con respecto a los espermatozoides viables) y %HDS, al evaluar las muestras después de la descongelación. Notamos un ligero aumento en la producción de ROS citoplasmáticos y en la proporción de espermatozoides viables con acrosomas dañados. Sin embargo, al evaluar las muestras después de la incubación posterior a la descongelación, la viabilidad fue menor en el equilibrado más prolongado y no hubo diferencias en la actividad mitocondrial y la capacitación de los espermatozoides. Las ROS citoplasmáticas aumentaron significativamente en este análisis, junto con la proporción de espermatozoides con acrosomas dañados. Sin embargo, la proporción de espermatozoides viables con acrosomas dañados disminuyó. La producción del anión superóxido mitocondrial (proporción de espermatozoides viables) o fragmentación de ADN (%DFI) no se vieron afectados en ninguno de los puntos de análisis. Curiosamente, el parámetro %DFI fue muy bajo y no se vio afectado por la incubación posterior a la descongelación.

El único efecto de suplementar BIOXcell con glutatión 2 mM fue una pequeña modificación de los parámetros cinemáticos LIN y WOB (Figura 5.1b) y la disminución de la

producción de anión superóxido en las mitocondrias, detectado en los dos puntos de análisis (Figura 5.3b).

5.1.2 Diluyente OPTIXcell

Los resultados con el diluyente OPTIXcell (experimento en Xenética Fontao) fueron similares a los obtenidos con el diluyente BIOXcell, con algunas diferencias. Los parámetros de CASA evaluados después de la descongelación (excepto VCL y ALH) fueron mayores en las muestras congeladas después del tratamiento de equilibrado 4 h. Después de la incubación posterior a la descongelación, la mayoría de los parámetros disminuyeron (nuevamente, VCL y ALH mostraron una tendencia contraria) y las diferencias entre ambos tiempos de equilibrado no fueron significativas en ningún caso (Figuras 5.4a y 5.4b).

El análisis de subpoblación arrojó tres grupos en este caso, Rapid, Slow y Hyper (debido a cierto parecido con la motilidad hiperactiva), descritos en la Tabla 5.2. De manera diferente que para el diluyente BIOXcell, encontramos que el tiempo de equilibrado de 4 h mostró un efecto en el patrón de subpoblación después de la descongelación, con una mayor proporción de Rapid y una reducción simultánea en Hyper (Figura 5.5). En este caso, no encontramos ningún efecto significativo después de la incubación de 4 h, lo que nuevamente causó una disminución significativa para la mayoría de los parámetros de motilidad, excepto VCL y ALH.

Los análisis de citometría de flujo (Figuras 5.6a y 5.6b) mostraron una viabilidad más alta y espermatozoides capacitados más bajos (como proporción dentro de los viables), daño acrosómico total e inmadurez de cromatina (%HDS) para el tiempo de equilibrado de 24 h en el análisis posterior a la descongelación.

Después de la incubación, observamos una disminución en la viabilidad de los espermatozoides, la actividad mitocondrial, el daño acrosómico como proporción dentro de viable y el% de HDS. Paralelamente, la apoptosis (proporción viable), la producción de superóxido mitocondrial y el daño acrosómico total aumentaron. En este punto de análisis, observamos una tasa de capacitación más baja, daño acrosómico total y% de HDS para el tiempo de equilibrado de 24 h, como en el análisis posterior a la descongelación. Además, la producción de superóxido mitocondrial fue menor en el tiempo de equilibrado extendido.

La fragmentación del ADN como %DFI fue muy baja en todos los casos, como se observó en el experimento BIOXcell. Este parámetro no se vio afectado por ningún tratamiento o incluso la incubación posterior a la descongelación.

5.2 Calidad del semen de toro criopreservado con BIOXcell suplementado con trehalosa y glutatión (GSH)

En este experimento, probamos la capacidad de la trehalosa y el GSH como suplementos para el diluyente BIOXcell para mejorar la calidad post-descongelación del semen de toro. En general, GSH no produjo ninguna ventaja, y encontramos que la trehalosa es perjudicial para ese propósito.

Las Figuras 5.7a y 5.7b muestran los resultados de los análisis de motilidad. La trehalosa 50 mM produjo una motilidad total y progresiva más baja en comparación con el control, tanto después de la descongelación como después de la incubación (P <0,001). Las variables cinemáticas siguieron la misma tendencia, también significativamente en la mayoría de los casos. Cuando probamos la trehalosa a 100 mM, los valores promedio cayeron en picado, casi aboliendo la motilidad. GSH afectó algunas variables cinemáticas, con 5 mM disminuyendo significativamente LIN (Fig. 5.7a), STR, WOB y BCF (Fig. 5.7b), después de la incubación (reflejando también una progresividad más baja), y ALH después del descongelación. Curiosamente, ambas concentraciones de GSH aumentaron la WOB después de la descongelación (Fig. 5.7b).

El análisis de subpoblaciones arrojó dos grupos, denominados Rapid y Slow, como se describe en la Tabla 5.3. El estudio de las proporciones de estos dos grupos mostró que la aplicación de GSH 5 mM y trehalosa 50 mM disminuyó ligeramente la presencia de Rapid al tiempo que aumentó Slow tanto después de la descongelación como después de la incubación. Trehalose 100 mM causó una caída dramática en Rapid y un aumento de Slow, mientras que GSH 2 mM no mostró ningún cambio significativo para estas subpoblaciones (Figura 5.8).

Considerando las variables de citometría de flujo (Figuras 5.9a y 5.9b), encontramos un efecto menos dramático de los tratamientos. T100 mostró una viabilidad general más baja y una actividad mitocondrial más baja después de la incubación (Fig. 5.9a). Este tratamiento aumentó la proporción de daño acrosómico en ambos puntos de análisis (Fig. 5.9b) y de espermatozoides viables con características apoptóticas después de la incubación (Fig. 5.9a). Curiosamente, también disminuyó significativamente los niveles de ROS citoplasmáticos después de la descongelación (Fig. 5.9a) y el daño acrosómico en espermatozoides viables después de la incubación (Fig. 5.9b). G2 mostró un pequeño aumento significativo en el daño acrosómico de semen viable.

La proporción de superóxido mitocondrial productor de espermatozoides viables disminuyó en todos los tratamientos (Fig. 5.9a), tanto después de la descongelación como después de la incubación (excepto T100 después de la incubación). Estos cambios en la producción de ROS no se reflejaron en el análisis de cromatina. Mientras que ambos tratamientos con GSH mostraron un menor porcentaje de %HDS después de la descongelación, esta tendencia cambió después de la incubación, siendo significativo el efecto del tratamiento de 5 mM (Fig. 5.9b).

Como en experimentos anteriores, la fragmentación del ADN como %DFI (Fig. 5.9b) fue muy baja en todos los casos y no se vio afectada por los tratamientos. Sin embargo, observamos un aumento en la dispersión de las muestras de control después de la incubación, posiblemente reflejando la variabilidad entre machos en la resistencia al daño del ADN. En este caso, los tratamientos parecen homogeneizar los resultados, quizás protegiendo las muestras más vulnerables.

5.3 Calidad del semen de toro criopreservado con BIOXcell suplementado con los antioxidantes curcumina, crocina y GSH

El uso de los antioxidantes curcumina y crocina para la criopreservación del semen de toro, con glutatión como referencia, arrojó resultados mixtos. No hubo efectos de los antioxidantes en los parámetros de CASA después de la descongelación (Figuras 5.10, 5.11a y 5.11b). Solo después de la incubación, MOT, PROG y LIN disminuyeron con ambas concentraciones de crocina, GSH también redujo LIN y WOB disminuyó con todos los tratamientos.

Como en los experimentos anteriores, encontramos dos subpoblaciones de motilidad, rápida y lenta (Tabla 5.4). Sin embargo, no hubo efectos significativos sobre las proporciones de estas subpoblaciones (Figura 5.11b).

Los parámetros de citometría de flujo siguieron un patrón similar al de los datos de motilidad (Figuras 5.12a y 5.12b). Sin embargo, estos análisis fueron más sensibles. La crocina causó una clara disminución de la calidad después de la descongelación. La viabilidad y la actividad mitocondrial disminuyeron significativamente con las dos concentraciones de crocina, lo que también indujo un aumento en la tasa apoptótica y disminuyó la tasa de capacitación (Figura 5.12a). Curiosamente, ambas concentraciones afectaron estos parámetros de manera similar, a excepción de la producción de superóxido mitocondrial (Figura 5.12b), que aumentó significativamente con 1,5 mM. Parte de estos efectos permanecieron después de la incubación, a excepción de la tasa apoptótica y la producción de superóxido mitocondrial. En este analisis punto, la crocina aumentó el daño acrosómico total (Figura 5.12b). GSH disminuyó significativamente la producción de superóxido mitocondrial después de la incubación.

Las ROS citoplasmáticas (Figura 5.12b) no se vieron afectadas por la crocina ni el GSH, pero aumentaron con ambas concentraciones de curcumina. Como encontramos con la curcumina en la mayoría de los casos, CU0.05 y CU0.1 no difirieron en este efecto.

La Figura 5.13 muestra los resultados del ensamblaje de la cromatina. La fragmentación del ADN como %DFI fue muy baja como en experimentos anteriores y no se vio afectada por los tratamientos. Sin embargo, la crocina 1,5 mM después de la descongelación y ambas concentraciones después de la incubación tuvieron un efecto decreciente sobre el% de HDS. En este caso, el tamaño del efecto fue mayor para la concentración más alta.

5.4 Mejora de la calidad posterior a la descongelación del semen de toro mediante el uso de la centrifugación coloidal de capa única (SLC) y doble capa (DLC) antes de la congelación

El uso de centrifugación coloidal antes de procesar las muestras para congelar mejoró la calidad del semen después de la descongelación. La motilidad total y progresiva tanto en SLC como en DLC fue significativamente mayor en comparación con el control (Figura 5.14) después de la descongelación. Curiosamente, después de la incubación, la SLC redujo la motilidad total, mientras que la motilidad progresiva se conservó mejor tanto en la SLC como en la DLC (Figura 5.14). Ambos coloides disminuyeron la velocidad de los espermatozoides (Figura 5.15), especialmente VCL, en ambos puntos de análisis (excepto SLC para VAP después de la descongelación). Sin embargo, VSL aumentó significativamente tanto para los coloides después de la descongelación como para SLC después de la incubación, lo que refleja un aumento considerable en ambos puntos de análisis en los parámetros de linealidad LIN, STR y WOB. Los coloides también redujeron la ALH al tiempo que aumentaban el FBC en ambos puntos (Figura 5.15).

Las subpoblaciones de motilidad se obtuvieron del mismo experimento sobre la extensión del tiempo de equilibrado con OPTIXcell, utilizando así los mismos grupos descritos en la Tabla 5.2. La Figura 5.16 muestra los efectos de los tratamientos coloides en las subpoblaciones. Las subpoblaciones rápidas y lentas aumentaron significativamente en SLC y DLC tanto después de la descongelación como después de la incubación, mientras que la subpoblación de Hyper siguió un cambio inverso y significativo.

Los coloides afectaron positivamente las variables de citometría de flujo, con pequeñas diferencias entre ambos tratamientos (Figura 5.17). La viabilidad y la actividad mitocondrial fueron mayores después de la descongelación para DLC y SLC, con una tasa apoptótica más baja, producción de superóxido mitocondrial y daño acrosómico. Sin embargo, la tasa de capacitación y la tasa de daño acrosómico fueron más altas para DLC e incluso más altas para SLC (P <0,001 SLC vs. DLC), y las muestras procesadas con DLC mostraron una mayor producción de ROS citoplasmático post-descongelación.

Después de la incubación, el efecto positivo sobre la viabilidad de los espermatozoides y la actividad mitocondrial se mantuvo, pero los efectos sobre la tasa apoptótica y el daño acrosómico se disiparon mientras se mantenía el aumento en la tasa de daño acrosómico. Además, la producción de superóxido mitocondrial fue mayor para las muestras sometidas a la centrifugación coloidal (Figura 5.17).

La Figura 5.18 muestra los efectos de los tratamientos sobre el estado de la cromatina. Mientras que la fragmentación del ADN (%DFI) fue muy baja en todos los casos, la aplicación previa a la congelación de ambos tratamientos abolió casi por completo la presencia de espermatozoides dañados por el ADN (P <0,001). La inmadurez de la cromatina no se vio afectada en ningún caso.

CONCLUSIONES

Considerando los resultados obtenidos en la presente tesis, podemos proponer las siguientes conclusiones:

1. Extender el tiempo de equilibrado de cuatro a veinticuatro horas usando BIOXcell u OPTIXcell mostró resultados mixtos, reduciendo la motilidad de los espermatozoides después de la descongelación y mejorando la viabilidad de los espermatozoides. Sin embargo, las diferencias fueron pequeñas y abren la posibilidad de utilizar tiempos de procesamiento más largos cuando la logística del centro lo requiere.

2. La suplementación del diluyente BIOXcell con trehalosa a 50 y 100 mM disminuyó la calidad del semen después de la descongelación, aunque detectamos una reducción de las especies reactivas de oxígeno. La trehalosa, al menos a estas concentraciones y agregadas a BIOXcell, no parece adecuada como suplemento para el semen de toro.

3. La suplementación de BIOXcell con GSH a 0,5, 2 y 5 mM en los diversos experimentos no mejoró la calidad del semen después de la descongelación. Sin embargo, observamos algunos efectos positivos, como la reducción de la producción de anión superóxido mitocondrial. Si bien no es útil para la mejora general de la criopreservación de semen de toro, GSH podría considerarse en futuras formulaciones.

4. La suplementación de BIOXcell con curcumina a 0.05 y 0.1 mM o crocina a 0.5 y 1.5 mM no mejoró la calidad del semen después de la descongelación, al tiempo que aumentó la producción de ROS citoplasmáticos (curcumina) o disminuyó algunos parámetros de calidad del semen (crocina). La curcumina y la crocina no parecen ser adecuadas para cropreservar semen de toro en las condiciones probadas en este estudio.

5. Tanto la centrifugación coloidal SLC previa a la congelación como la centrifugación coloidal DLC utilizando BoviPure mejoraron la motilidad y los parámetros fisiológicos de los espermatozoides después de la descongelación. También redujeron la fragmentación del ADN espermático. Estos procedimientos podrían ser de uso práctico para mejorar la eficiencia de la criopreservación de semen de toro, especialmente con muestras de baja congelabilidad.

6. No hubo diferencias en los resultados del uso de SLC y DLC con respecto a la calidad del semen después de la descongelación. Por lo tanto, SLC podría ser adecuado para el uso práctico en centros de cría, siendo más barato y más fácil de realizar.