Universitat Autònoma de Barcelona

How does seminal plasma protect mammalian spermatozoa? Species-linked variations within the genus Equus

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Species-linked variations within the genus *Equus*

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"La science sans conscience n'est que ruine de l'âme", Rabelais

Universitat Autònoma de Barcelona

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CERTIFICAN

Que la Tesis titulada **"***How does seminal plasma protect mammalian spermatozoa? Species-linked variations within the genus* Equus" presentada por Marion Papas para optar al grado de Doctor en Medicina y Sanidad Animales por la Universidad Autónoma de Barcelona se ha realizado bajo su dirección y, considerándola terminada y cumpliendo los requisitos para poder optar a la Mención Internacional, autorizan su presentación para ser juzgada por la Comisión correspondiente.

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ABBREVIATIONS

AI	Artificial insemination
ALH	Amplitude of lateral head displacement
ANOVA	Analysis of variance
ART	Artificial reproduction techniques
BCF	Beat cross frequency
CASA	Computer assisted sperm analysis
CAT	Catalase
CPA	Cryoprotective agents
CRISP	Cysteine-rich secretory protein
DGC	Density gradient centrifugation
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EV	Electronic volume
GFE	Good freezability ejaculate
GOT	Glutamic oxaloacetic transaminase
GPT	Glutamate pyruvate transaminase
GPX	Glutathione peroxidase
GSR	Glutathione reductase
H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
HE	Hydroethidine
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'tetraethyl-benzimidazolylcarbocyanine iodide
$JC\text{-}1_{agg}$	JC-1 aggregate

LIN	Percentage of linearity
M540	Merocyanine 540
Min	Minutes
MMP	Mitochondrial membrane potential
$O2^{-\bullet}$	Superoxide
PBS	Phosphate buffered saline solution
PFE	Poor freezability ejaculate
PI	Propidium iodide
PMN	Polymorphonuclear neutrophil
PMOT	Percentage of progressive motile spermatozoa
PUFA	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SEM	Standard error of the mean
SLC	Single layer centrifugation
SOD	Superoxide dismutase
SP	Seminal plasma
SP1-4	Sperm subpopulation 1-4
STR	Straightness of sperm trajectory (%)
SYBER®	Commercial name of nucleic acids synthetic dyes ($C_{32}H_{37}N_4S$)
TMOT	Percentage of total motile spermatozoa
VAP	Average path velocity
VCL	Curvilinear velocity (μm/s)
VSL	Straight-linear velocity
WOB	Percentage of wobble coefficient
YO-PRO®-1	Commercial name of a non-permeant nuclear dye ($C_{24}H_{29}I_2N_3O$)

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ABSTRACT

More than a simple vehicle for sperm, seminal plasma represents an important and active contributor to mammalian fertility. Specific components of seminal plasma are adsorbed onto the surface of ejaculated spermatozoa while they pass through the male reproductive tract and provide sperm cells with the ability to reach and fertilize the oocyte. Among the components of seminal plasma, there are enzymatic and non-enzymatic antioxidants that modulate the production of reactive oxygen species (ROS), which are by-products generated during mitochondrial electron transport. Oxidative stress, which is induced by an incorrect balance between antioxidants and ROS production, impairs sperm function. In this context, seminal plasma represents the most important defense system able to prevent the damages that these species exert upon sperm cells. In spite of this, removal of seminal plasma is usually recommended prior to sperm storage, cooled or cryopreserved, as the presence of this fluid may be detrimental for sperm longevity. Against this background, the main aim of this Dissertation was to determine the relevance of the antioxidant enzymes present in seminal plasma for equine reproduction strategies.

On the one hand, and as a first objective of this work, we observed much higher activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GSR) in jackass seminal plasma than in that of stallions. Since equids are seasonal breeders, this study also investigated season influence on the antioxidant composition of seminal plasma. Interestingly, activities of SOD and GPX in seminal plasma showed seasonal variations in jackasses but not in stallions. On the other hand, because the demand of cryopreserved semen in the equine industry is increasing and no study had previously addressed whether the activities of these four enzymes are related to sperm cryotolerance, the second and third study evaluated the relationship between SOD, CAT, GPX and GSR activities in fresh seminal plasma and the sperm ability to withstand cryopreservation in these two species. It was found that the specific SOD activity in seminal plasma is related to sperm cryotolerance in both stallions and jackasses. Based on these results, one can

also conclude that a short contact between seminal plasma components and spermatozoa is enough to provide a cryoprotective effect.

Since seminal plasma has been reported to modulate ROS balance in several mammalian species, this Dissertation also investigated how the presence of seminal plasma modulates ROS generation by jackass spermatozoa under oxidative stress induced exogenously by hydrogen peroxide. With regard to these results, however, prolonged contact of seminal plasma was found to have limited effect against oxidative stress induced exogenously to jackass spermatozoa.

Finally, sperm selection is getting prominent in equine assisted reproduction technologies and also leads to the removal of seminal plasma components. The last objective of this study aimed to investigate the effects of single layer centrifugation (SLC) with Equicoll on jackass sperm parameters and its interactions with the female reproductive tract. While SLC-Equicoll was found to increase the percentages of viable spermatozoa after selection, the impact on sperm motility parameters was marginal. In addition, removal of seminal plasma components through SLC-Equicoll increased sperm phagocytosis by polymorphonuclear neutrophils (PMNs), thereby confirming that seminal plasma modulates the interaction between sperm and PMNs.

In conclusion, this Dissertation indicates that although the activities of seminal plasma antioxidants differ between stallions and jackasses, SOD appears to be a good predictor of sperm cryotolerance in these two species. In addition, this Dissertation has also demonstrated, for the first time, that the modulating role of seminal plasma with regard to the interaction between jackass spermatozoa and PMNs is more apparent than that observed when oxidative stress is induced by hydrogen peroxide.

Keywords: Donkey; Stallion; Seminal plasma; Antioxidants; Oxidative Stress; Cryopreservation

RESUMEN

Más que un simple medio de transporte de los espermatozoides, el plasma seminal representa un importante y activo contribuyente para la fertilidad de los mamíferos. Los componentes específicos del plasma seminal se adsorben a la superficie de los espermatozoides eyaculados mientras pasan a través del tracto reproductor masculino y proporcionan a los gametos la capacidad de alcanzar y fecundar el oocito. Entre los componentes del plasma seminal se encuentran los antioxidantes enzimáticos y no enzimáticos que modulan la producción de especies reactivas de oxígeno (ROS), que son subproductos generados por la cadena de transporte de electrones. El estrés oxidativo, inducido por un equilibrio incorrecto entre los antioxidantes y la producción de ROS, perjudica las funciones del espermatozoide. De esta manera, el plasma seminal representa el sistema de defensa más importante capaz de prevenir los daños que dichas especies reactivas de oxígeno pueden provocar en los espermatozoides. A pesar de ello, se suele recomendar la eliminación del plasma seminal como paso previo a la conservación de los espermatozoides (refrigeración o congelación), ya que la presencia de este fluido puede ser perjudicial para la longevidad de los gametos. En este contexto, el objetivo principal de esta Tesis Doctoral fue determinar la importancia de los enzimas antioxidantes del plasma seminal en la estrategia reproductiva de los equinos.

Por un lado, y como primer objetivo de este trabajo, se observó que las actividades de la superóxido dismutasa (SOD), la catalasa (CAT), la glutatión peroxidasa (GPX) y la glutatión reductasa (GSR) en el plasma seminal de burros eran mucho más altas que en el de caballos. Además, dado que los équidos son reproductores estacionales, este estudio también investigó la influencia de la estación en la composición de antioxidantes del plasma seminal. Curiosamente, las actividades de SOD y GPX en el plasma seminal mostraron variaciones estacionales en los burros, pero no en los caballos. Por otro lado, debido a que la demanda de semen congelado en la industria equina está aumentando, y que ningún estudio previo abordó la relación entre las actividades de estos cuatro enzimas y la capacidad de los espermatozoides de resistir a la congelación, los estudios segundo y tercero evaluaron la relación entre las actividades de SOD,

CAT, GPX y GSR en el plasma seminal fresco y la criotolerancia de los espermatozoides de burro y caballo. Se encontró que la actividad específica del SOD en el plasma seminal está relacionada con una mejor tolerancia de los espermatozoides a la congelación tanto en caballos como en burros. Basándose en estos resultados, también se puede concluir que un periodo corto de contacto entre los componentes del plasma seminal y los espermatozoides es suficiente para proporcionar este efecto crioprotector.

Dado que otros estudios previos han observado que el plasma seminal modula la producción de ROS por parte de los espermatozoides en distintas especies de mamíferos, esta Tesis Doctoral también estudió si la presencia de plasma seminal es capaz de controlar la generación espermática de ROS en condiciones de estrés oxidativo inducido por la adición de peróxido de hidrógeno. Sorprendentemente, se observó que el contacto prolongado de los espermatozoides de burro con el plasma seminal tiene un efecto compensatorio limitado en cuanto a los respuesta de aquéllos al estrés oxidativo inducido exógenamente.

Por último, la selección de los espermatozoides, que comporta la eliminación de los componentes del plasma seminal, se está convirtiendo en una tecnología prominente en el ámbito de la reproducción asistida equina. Por consiguiente, el último objetivo de esta Tesis Doctoral fue investigar los efectos de la centrifugación con una sola fase/capa (SLC) mediante Equicoll sobre los parámetros de calidad de los espermatozoides de burro y sus interacciones con el tracto reproductivo femenino. Aunque la selección con Equicoll mejoró los porcentajes de viabilidad espermática después de la selección, el impacto sobre los parámetros de motilidad fue mínimo. Además, la extracción de los componentes del plasma seminal mediante Equicoll aumentó la fagocitosis de los espermatozoides por los neutrófilos, lo que confirma que el plasma seminal modula la interacción entre los gametos masculinos y estas células inmunitarias.

En conclusión, esta Tesis Doctoral indica que, aunque las actividades de los antioxidantes seminales del plasma difieren entre caballos y burros, la SOD parece ser, en ambas especies, un buen predictor de la capacidad de los espermatozoides en resistir a la congelación. Además, esta Tesis Doctoral

también demostró, por primera vez, que el papel modulador del plasma seminal es más evidente respecto a la interacción entre los espermatozoides de burro y los neutrófilos, que en cuanto a la respuesta de aquéllos al estrés oxidativo inducido por el peróxido de hidrógeno.

RESUM

Més que un simple mitjà de transport dels espermatozoides, el plasma seminal representa un important i actiu contribuent a la fertilitat dels mamífers. Els components específics del plasma seminal s'adsorbeixen a la superfície dels espermatozoides ejaculats mentre passen a través del tracte reproductor masculí i proporcionen als gàmetes la capacitat d'arribar a l'oviducte i fecundar l'oòcit. Entre els components del plasma seminal s'hi troben els antioxidants enzimàtics i no enzimàtics que modulen la producció d'espècies reactives d'oxigen (ROS), que són subproductes obtinguts de l'activitat de la cadena de transport d'electrons. L'estrès oxidatiu, induït per un equilibri incorrecte entre els antioxidants i la producció de ROS, perjudica la funcionalitat espermàtica. D'aquesta manera, el plasma seminal representa el sistema de defensa més important quant a la prevenció dels danys que aquestes espècies d'oxigen provoquen en els espermatozoides. Tot i això, es sol recomanar l'eliminació del plasma seminal com a pas previ a la conservació (refrigeració o criopreservación) dels espermatozoides, atès que la presència d'aquest fluid pot ser perjudicial per la longevitat dels gàmetes. En aquest context, l'objectiu principal d'aquesta Tesi Doctoral va ser determinar la importància dels enzims antioxidants del plasma seminal per l'estratègia reproductiva dels èquids.

D'una banda, i com a primer objectiu d'aquesta Tesi Doctoral, es va observar que les activitats de la superòxid dismutasa (SOD), la catalasa (CAT), la glutatió peroxidasa (GPX) i la glutatió reductasa (GSR) en el plasma seminal dels rucs eren molt més altes que en el dels cavalls. A més, atès que els èquids són reproductors estacionals, aquest estudi també va investigar la influència de les estacions en la composició d'enzims antioxidants del plasma seminal. Curiosament, les activitats de SOD i GPX del plasma seminal van mostrar variacions estacionals en els rucs, però no en els cavalls. D'altra banda, i atès que s'està produint un augment de la demanda de semen congelat dels èquids i que cap estudi previ ha abordat la relació entre les activitats d'aquests quatre enzims i la capacitat dels espermatozoides de resistir a la criopreservació, els estudis segon i tercer van avaluar la relació entre les activitats dels enzims SOD, CAT, GPX i GSR, presents en el plasma seminal, i la criotolerància dels

espermatozoides de ruc i cavall. Es va observar que l'activitat específica de la SOD en el plasma seminal està relacionada amb la criotolerància dels espermatozoides de rucs i cavall. Els resultats d'aquests dos estudis també permeten concloure que, per tal que s'observi aquest efecte crioprotector, n'hi ha prou amb un breu contacte entre els components del plasma seminal i els espermatozoides.

Atès que estudis previs han demostrat que el plasma seminal modula la producció espermàtica de ROS en diferents espècies de mamífers, aquesta Tesi Doctoral també va estudiar si la presència del plasma seminal és capaç de controlar la generació espermàtica de ROS en condicions d'estrès oxidatiu induït pel peròxid d'hidrogen. Sorprenentment, es va observar que el contacte perllongat del plasma seminal amb els espermatozoides de ruc té un efecte limitat a l'hora de contrarestar l'estrès oxidatiu induït exògenament en aquests gàmetes.

Finalment, la selecció d'espermatozoides, que comporta l'eliminació dels components del plasma seminal, s'està convertint en una tecnologia important dins l'àmbit de la reproducció assistida equina. Per tant, l'últim objectiu d'aquesta Tesi Doctoral va ser investigar els efectes de la centrifugació amb una sola fase/capa (SLC) mitjançant Equicoll sobre els paràmetres de qualitat del espermatozoides de ruc i les seves interaccions amb el tracte reproductor femení. Tot i que la selecció amb Equicoll va millorar els percentatges de viabilitat espermàtica després de la selecció, l'impacte sobre els paràmetres de motilitat va ser molt limitat. A més, l'extracció dels components del plasma seminal mitjançant Equicoll va augmentar la fagocitosi dels espermatozoides per part dels neutròfils, la qual cosa confirma que el plasma seminal modula la interacció entre els gàmetes masculins i aquestes cèl·lules immunitàries.

En conclusió, aquesta Tesi Doctoral indica que, encara que les activitats dels antioxidants del plasma seminal difereixen entre cavalls i rucs, la SOD és, en ambdues espècies, un bon predictor de la capacitat dels espermatozoides de resistir a la criopreservació. A més, aquesta Tesi Doctoral també ha demostrat, per primera vegada, que el paper modulador del plasma seminal és més evident quant a la interacció entre els espermatozoides de burro i els neutròfils,

que respecte a l'estrès oxidatiu induït en els gàmetes masculins d'aquesta espècie pel peròxid d'hidrogen.

RÉSUMÉ

Plus qu'un simple moyen de transport des spermatozoïdes, le plasma séminal contribue activement à la fertilité des mammifères. Certains de ses composants, adsorbés par la surface des spermatozoïdes dès lors qu'ils traversent l'appareil reproducteur masculin, offrent en effet à ces cellules la capacité d'atteindre et de féconder l'ovocyte. Parmi ces composants, antioxydants enzymatiques et non enzymatiques modulent la génération d'espèces réactives d'oxygène (ROS), sous-produits obtenus à partir de la chaine de transport des électrons. Le plasma séminal représente ainsi un important système de défense capable de prévenir les dommages cellulaires provoqués par un stress oxydatif. Ce dernier, résultat d'un mauvais équilibre entre les antioxydants et la production de ROS, peut effectivement nuire gravement aux fonctions des spermatozoïdes. Cependant, le plasma séminal est usuellement enlevé avant la conservation, réfrigérée ou congelée, de la semence. La présence de ce fluide pouvant en effet diminuer la longévité des spermatozoïdes. Le sujet de cette Thèse était donc de déterminer, au regard des stratégies actuelles de reproduction équine, le rôle exact que peuvent être amenés à jouer ces enzymes antioxydantes présentes dans le plasma séminal.

Les premières expériences ont permis d'observer que les activités de la superoxyde dismutase (SOD), la catalase (CAT), la glutathion peroxydase (GPX) et la glutathion réductase (GSR) étaient beaucoup plus élevées dans le plasma séminal de l'âne que dans celui des chevaux. Etendues à la possible influence des saisons sur la composition antioxydante séminale, elles ont aussi permis de démontrer que les activités de SOD et GPX dans le plasma séminal ne présentaient des variations saisonnières que chez l'âne. Les expériences suivantes, motivées par l'augmentation de la demande de congélation du sperme dans l'industrie équine, ont quant à elles permis d'évaluer la relation entre les activités SOD, CAT, GPX et GSR du plasma séminal et la capacité du sperme à être congelé chez ces deux espèces. Chez l'une et l'autre, elles ont démontré que l'activité spécifique de la SOD dans le plasma séminal est liée à la cryotolérance des spermatozoïdes. L'idée qu'un bref contact entre les

composants séminaux et les spermatozoïdes suffit à fournir l'effet protecteur recherché est ainsi validé.

Puisqu'il a été démontré que le plasma séminal modulait la production de ROS chez plusieurs espèces de mammifères, cette Thèse a également étudié comment la présence de plasma séminal permettait de contrôler l'excès de ROS lorsque les spermatozoïdes d'âne sont soumis à un stress oxydatif induit par le peroxyde d'hydrogène. Au regard de ces résultats, un contact prolongé du plasma séminal s'est avéré avoir un effet limité.

Enfin, puisque la sélection des spermatozoïdes prend de plus en plus d'importance dans les technologies de procréation assistée équine et mène également à l'élimination des composants du plasma séminal, le dernier objectif de cette Thèse visait à déterminer les effets de la centrifugation monocouche (CSL) avec Equicoll sur les paramètres du sperme de l'âne et ses interactions avec l'appareil reproducteur féminin. Bien que la sélection via Equicoll augmente la viabilité des spermatozoïdes, l'impact sur la motilité reste marginal. De plus, l'élimination des composants du fluide séminal via Equicoll a augmenté la phagocytose des spermatozoïdes par les neutrophiles, confirmant ainsi que le plasma séminal module l'interaction entre les spermatozoïdes et les cellules inflammatoires.

Cette Thèse a permis ainsi de conclure que même si les activités antioxydantes du plasma séminal diffèrent entre les chevaux et les ânes, la SOD se révèle être un bon prédicteur de la cryotolérance du sperme chez les uns et les autres. Elle permet aussi d'affirmer, et ceci pour la première fois dans la sphère de la reproduction équine, que le rôle modulateur du plasma séminal est, chez l'âne, plus probant, au niveau de l'interaction entre les spermatozoïdes et les neutrophiles que dans la protection contre le stress oxydatif induit par le peroxyde d'hydrogène.

ASTRATTO

Il plasma seminale è più di un semplice veicolo dello sperma, essendo un elemento fondamentale per la fertilità dei mammiferi. Gli spermatozoi infatti, durante il loro tragitto all'interno del tratto riproduttivo maschile assorbono specifici componenti del plasma seminale che aiutano a raggiungere e fecondare l'oocita. Fra i componenti del liquido seminale ritroviamo antiossidanti enzimatici e non che regolano la produzione di radicali liberi dell'ossigeno (ROS). Lo stress ossidativo è quindi indotto da un disequilibrio fra gli antiossidanti e i ROS prodotti compromettendo la funzionalità dello sperma. Il plasma seminale è il più importante sistema di difesa in grado di prevenire il danno ossidativo indotto dai ROS agli spermatozoi. Contrariamente a ciò, è consigliato rimuovere il plasma seminale prima della congelazione o crioconservazione dello sperma perché la presenza di fluidi può essere dannosa per la longevità dello sperma. Premesso ciò, il principale obiettivo di questa Tesi è determinare il valore degli enzimi antiossidanti nel plasma seminale di equidi usati per la riproduzione.

Nella prima parte di questo lavoro, abbiamo osservato più alte attività di superossido dismutasi (SOD), catalasi (CAT), glutathione perossidasi (GPX) e glutathione reduttasi (GSR) nel plasma seminale degli asini rispetto che nei cavalli. Inoltre, considerando che gli equini sono riproduttori stagionali, questo studio ha investigato l'influenza della stagione sulla tipologia di antiossidanti del liquido seminale. Curiosamente, l'attività di SOD e GPX nel plasma seminale varia negli asini ma non nel cavallo. Inoltre, siccome la domandi di seme crioconservato è in aumento, e ad oggi nessuno studio ha valutato se l'attività di questi quattro enzimi influisce sulla criotolleranza dello sperma, nel secondo e terzo studio abbiamo valutato l'attività di SOD, CAT, GPX e GSR nel plasma seminale fresco e la capacità dello sperma di resistere alla crioconservazione nelle due specie. I risultati di questi studi dimostrano che l'attività specifica dell'enzima SOD nel plasma seminale è relazionato alla criotolleranza dello sperma in entrambe le specie. Detto ciò, possiamo concludere dicendo che anche un breve contatto fra i componenti del plasma seminale e gli spermatozoi è sufficiente a garantire gli effetti protettivi ad essi. Dal momento

che in alcuni studi hanno riportato che il plasma seminale modula l'attività dei ROS, questa Tesi valuta inoltre il meccanismo per cui il plasma seminale previene la formazione dei ROS nello sperma di asini sottoposti a stress ossidativo indotto da esogene idrogeno perossidasi. I risultati di questo studio dimostra che un prolungato contatto degli spermatozoi con il plasma seminale ha un effetto limitato contro i danni ossidativi indotti dall'esterno agli spermatozoi di asino.

In ultimo, il processo di selezione dello sperma sta diventando prominente nell' industria della riproduzione assistita equina e di conseguenza anche la rimozione del plasma seminale dallo sperma. L'ultimo obiettivo di questa Tesi è di valutare gli effetti della "single layer centrifugation" (SLC) con Equicoll sui parametri dello sperma di asino e la sua interazione con l'apparato riproduttore femminile. Da un lato, SLC-Equicoll dimostra di migliorare la vitalità degli spermatozoi, dall'altro l'impatto sulla motilità degli spermatozoi è marginale. Inoltre, la rimozione del plasma seminale con il sistema SLC-Equicoll aumenta la fagocitosi da parte dei neutrofili degli spermatozoi, confermando quindi che il plasma seminale modula il meccanismo di interazione fra I neutrofili e lo sperma.

In conclusione, questa Tesi dimostra che l'attività degli antiossidanti del plasma seminale è differente fra cavalli e asini, che l'enzima SOD è un buon indicatore della criotolleranza dello sperma nelle due specie. Inoltre, è stato dimostrato per la prima volta, il ruolo modulatore del plasma seminale nell'interazione fra gli spermatozoi e i neutrofili, che è invece meno chiaro di quello osservato durante danno ossidativo con idrogeno perossidasi.

INTRODUCTION

1. Genus *Equus*

1.1. Evolution and domestication

Equidae is a taxonomic family of mammals composed of different species of genus *Equus*, which belongs to the order of Perissodactyla. The individuals of this order present hoofed toes (ungulates) and an odd number of fingers, such as the family of tapirs (e.g. rhinoceros; Houghton Brown et al., 2003). According to mitochondrial DNA cleavage maps and fossil records, the genus *Equus* includes three zebras (*E. grevyi*, *E. zebra*, *E. burchelli*), two donkeys (*E. asinus*, *E. hemionus*), and the true horse (*E. caballus*; which includes the domestic and Przewalski's horse) (Fig. 1; Forstén, 1992). These species differ both morphologically and chromosomically. For instance, the domestic horse has a diploid (2n) chromosome number of 64, whereas donkeys possess 62 (2n) chromosomes. Despite these differences, almost all individuals of these species are able to reproduce among themselves giving interspecific hybrids (Wilborn and Pugh, 2011).

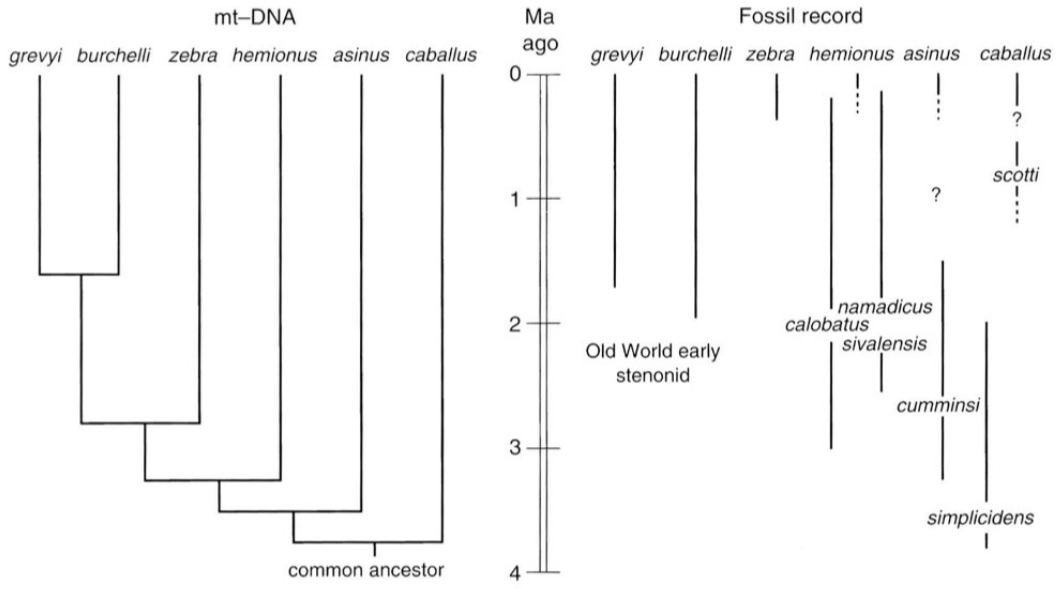

Figure 1. Species of genus Equus (Forstén, 1992)

Equines have evolved over a period of approximately 60 million years, from the forest-dwelling, fox-like *Eohippus* (Fig. 2; also known as *Hyratotherium* or dawn horse) to Merychippus (20 to 25 million year ago) and to Pliohippus (6 to 12 million years ago), which presented a structure very similar to the modern horse. About 6,000 years ago, the nomadic tribes from Eurasia, especially from around the Caspian Sea and the Black Sea, began to keep and breed these animals in captivity (Fig. 3; Houghton Brown et al., 2003). The first use was for meat and milk production. Then, equines were used for transport, drawing wagons and chariots, and they started to be ridden for herding purposes and war.

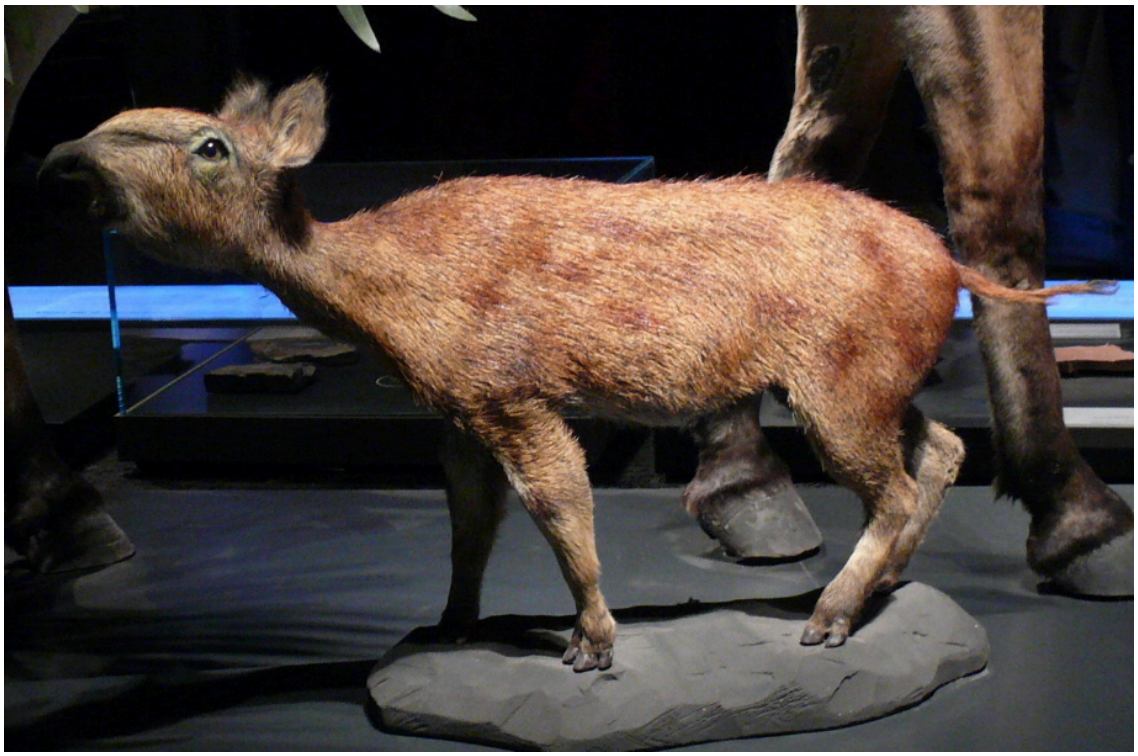

Figure 2. Eohippus, reconstructed in the Natural History Museum, Berlin

The process of domestication exercised a substantial influence on the development of equines and was concurrent with their spread and diversification (Goodwin, 2003). In that way, the modern horse is the product of artificial selection and as a result, there are variations in size, appearance, color and behavior. Nowadays, horses may be divided in four categories: hot-blood, warm-blood, cold-blood and ponies (Houghton Brown et al., 2003). They are

essentially used for sports and hobbies. While domestic donkeys represent a valuable aid in the fields of agriculture and transport for populations without access to modern technologies, they have been gradually replaced in industrialized countries (Goodwin, 2003). Nevertheless, breeding programs for the production of donkey's milk are getting developed and they are also used as guards for protecting small ruminants, or as companion animals (Wilborn and Pugh, 2011). In addition, the use of jackasses to breed mules is also on demand (Canisso et al., 2011).

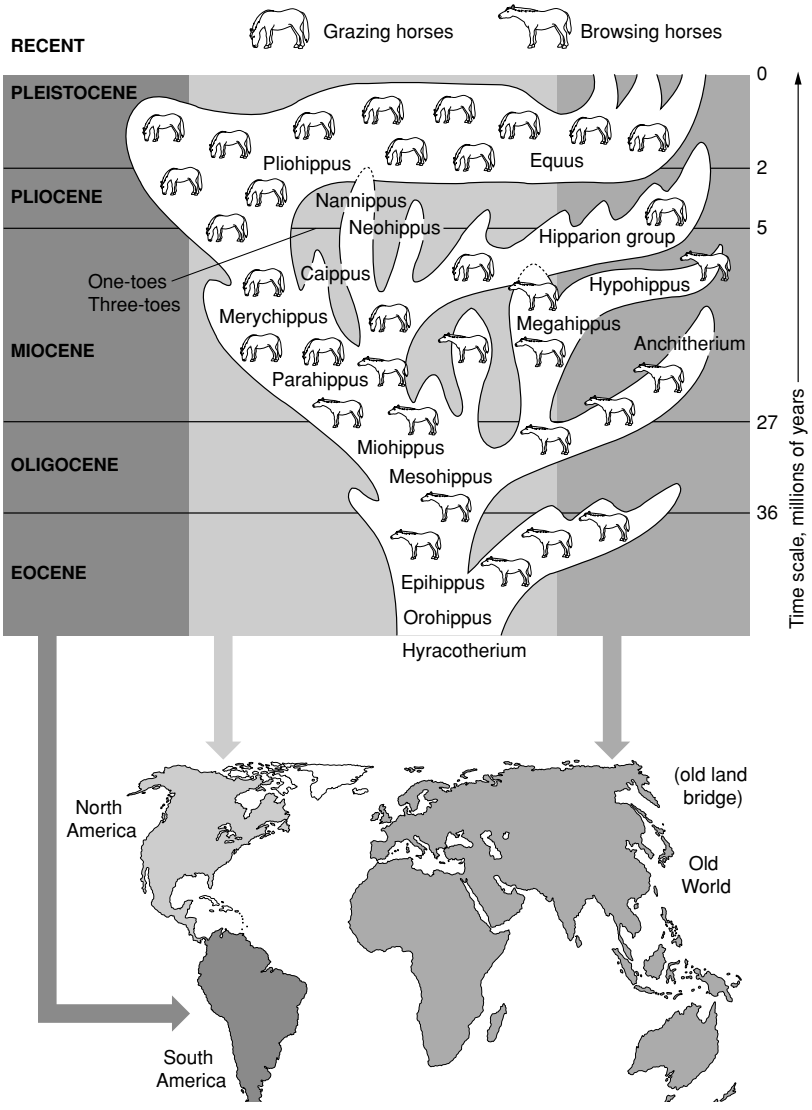

Figure 3. Evolutionary pathway of the equids. (Houghton Brown et al., 2003)

1.2. Reproduction and seasonality

Evolution of equine species has been profoundly impacted by the seasonal changes in the daily pattern of light/dark exposure (Dardente et al., 2016). In effect, the evolution of these species has taken into consideration the biological rhythm of their reproductive competence and they have also had to develop an appropriate physiological or behavioral response. As other seasonally breeding mammals, equids display a circannual cycle governed by photoperiod, which coordinates their reproductive activity throughout the year (Senger, 2012). For this reason, mild climatic conditions and optimum nutrition are provided for the lactating mare and offspring are born at a time of year that is advantageous to their survival.

Seasonal reproduction system is up- and down-regulated by environmental and endogenous signals which all converge upon a neuroendocrine pathway, the brain-pituitary-gonadal-axis (Lehman et al., 1997). Briefly, sufficient secretion of the gonadotropin-releasing hormone (GnRH) stimulates the hypothalamus to release gonadotropins which promote gametogenesis, steroidogenesis and development of reproductive tissues (Gerlach and Aurich, 2000). The pineal gland lies above and behind the hypothalamus and secretes melatonin, an antigonadotrophic; its function is inhibited with the increase of day-length (Roser, 2009). Environmental conditions, such as social interaction, nutrition and stress, may also influence the onset of the breeding season (Senger, 2012). In that way, it is worth mentioning that domestic horses and donkeys are submitted to different social organizations. Indeed, horses are characterized by being a non-territorial family band of one stallion and up to six mares; in the case of donkeys, jackasses are territorial, no lasting bonds are formed, and jennies may range over several male territories (Goodwin, 2003).

Mares and jennies are both polyestrous breeders; at the end of a variable follicular phase, spontaneous ovulation occurs. Their estrus cycle is associated with day-length. Therefore, in the temperate zone of the northern hemisphere, the natural breeding season extends, approximately, from May to October (Fig. 4; England, 2005). While the reproductive cyclicity between mares and jennies is

similar, it is worth noting, in this context, that shorter estrous cycle and interovulatory period have been described in jennies (Ginther et al., 1987).

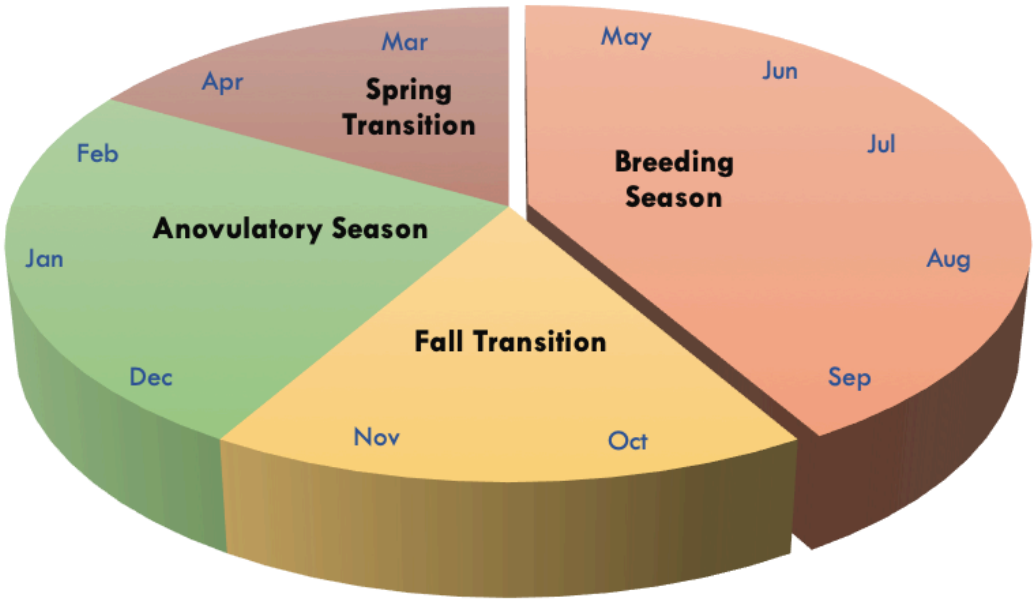

Figure 4. Annual stages of the mare's estrous cycle

Seasonality is often less marked in the male than in the female (Hoffmann and Landeck, 1999). However, since endocrine secretions and the subsequent concentration of sex hormones are influenced by seasonal fluctuations (Fig. 5; Senger, 2012), variations in male sexual behavior and germinative testicular function should also be observed. In stallions, seasonal variations have been found in the number of spermatogonia, spermatocytes and daily production of sperm (Meyers, 2009). In addition, fatty acid composition of sperm plasma membrane in Shetland pony stallions also undergoes seasonal changes (Aurich et al., 2018). In effect, from January to March, the content of polyunsaturated fatty acids (PUFAs) increases, whereas that of saturated fatty acids decreases throughout the rest of the year. Influence of seasons has also been reported regarding sperm quality parameters, although there are inconsistencies across the literature (Hoffmann and Landeck, 1999; Gamboa et al., 2010). Furthermore, Gebauer et al. found that the influence of the photoperiod on the composition of seminal plasma is larger than on sperm characteristics (Gebauer et al., 1976).

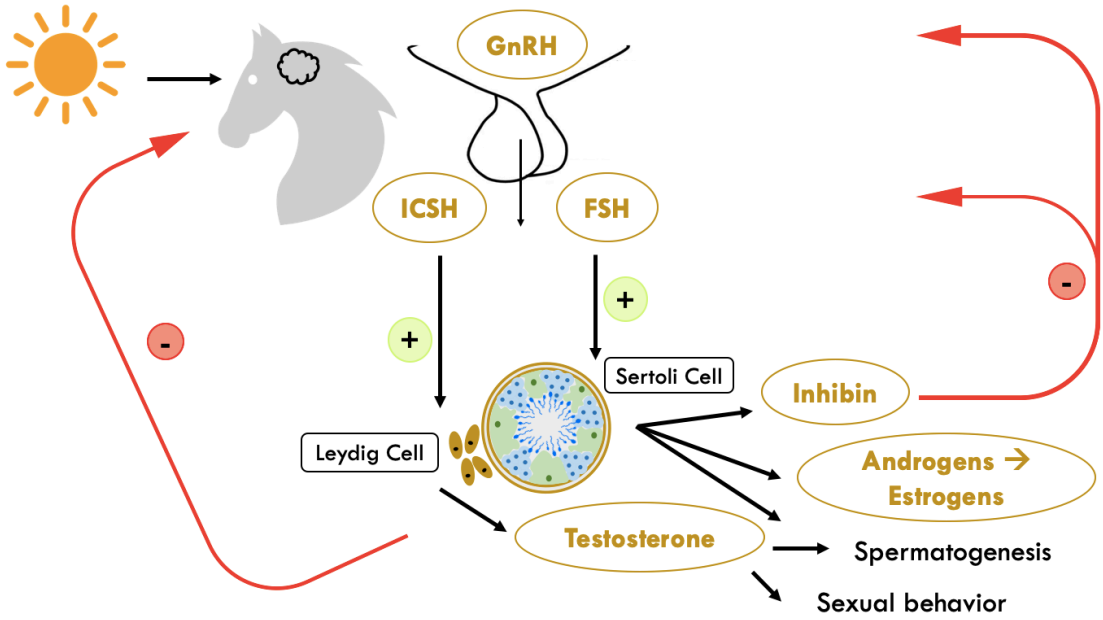

Figure 5. Reproductive endocrinology in the stallion.

Regarding sperm quality parameters in jackasses, conflictive results have been observed about the influence of season. Thus, while no variations have been observed in jackasses from Martina Franca breed (Carluccio et al., 2013), those from the Andalusian breed appear to exhibit lower sperm progressive motility during the summer and autumn than over the other seasons (Dorado et al., 2014). Moreover, semen pH and secretory activity of the prostate gland in male donkeys has been related to seasonal variations, this activity being the highest during spring (Gastal et al., 1997; Abou-Elhamd et al., 2013).

2. Male reproductive system

2.1. Anatomy

2.1.1. General overview

The male reproductive system is comparable to a "manufacturing complex" whose product consists of fertile spermatozoa (Senger, 2012). The male reproductive tract consists of a pair of scrotal testes, suspended by a spermatic cord and external cremaster muscle, the epididymes, the deferens ducts, the accessory sex glands and the penis (Fig. 6; McCue, 2014).

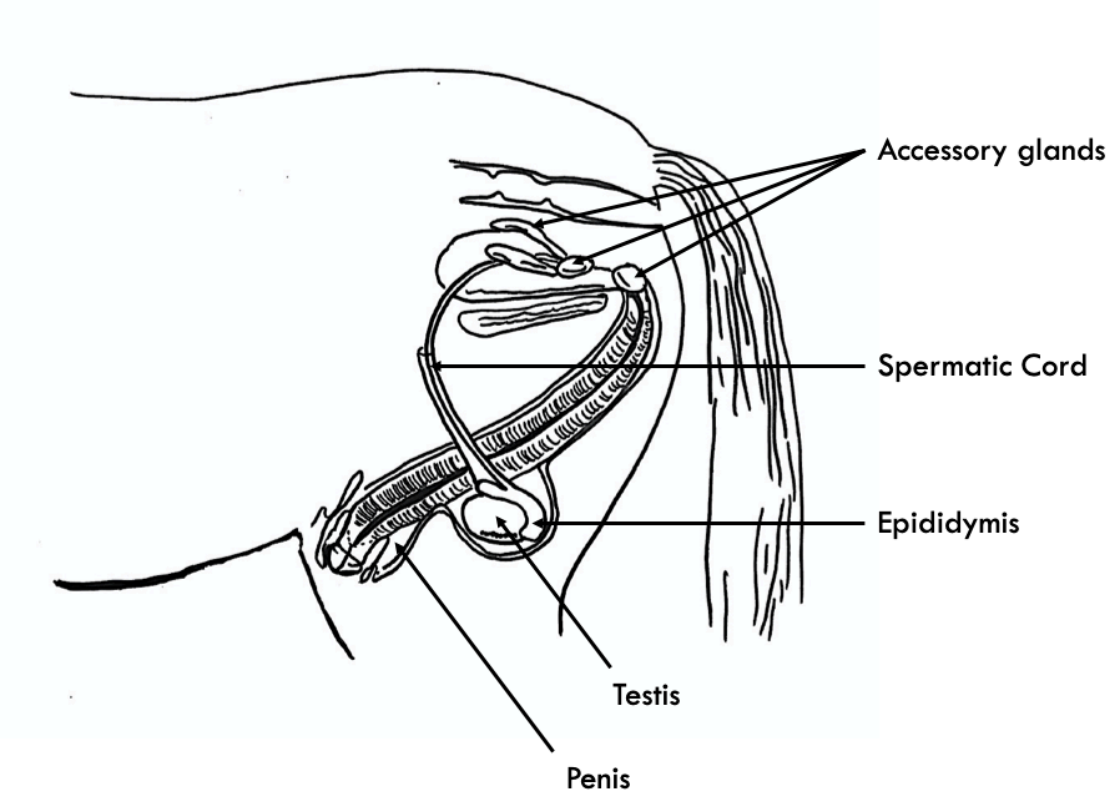

Figure 6. Reproductive tract of the stallion

2.1.2. Penis

The penis is the copulatory organ consisting of three parts, the root, the body and the glans penis. The root is attached to the ischial arch. The body, or the shaft, has the major erectile tissues: corpora cavernosa, which are two columns that lie on the dorsal part of the penis, and the corpus spongiosum, which lies in the ventral groove between the two corpora cavernosa and surrounds the urethra. The glans penis is normally retracted in the prepuce and represents an area richly innervated and sensitive. The penis is rounded by specific muscles and nerves responsible for erection, protrusion an ejaculation of semen. (Senger, 2012).

2.1.3. Spermatic cord

The spermatic cord extends from the inguinal ring to the dorsal pole of the testis where it is attached (Fig. 7). It provides the testicular vasculature, and the lymphatic and neural connection to the body. It also encloses a specialized network of capillaries, the pampiniform plexus, which acts as a thermoregulatory mechanism since the heat in the warmer arterial supply is lost to the cooler venous return system. The spermatic cord also harbors the ductus deferens and the cremaster muscle (Senger, 2012).

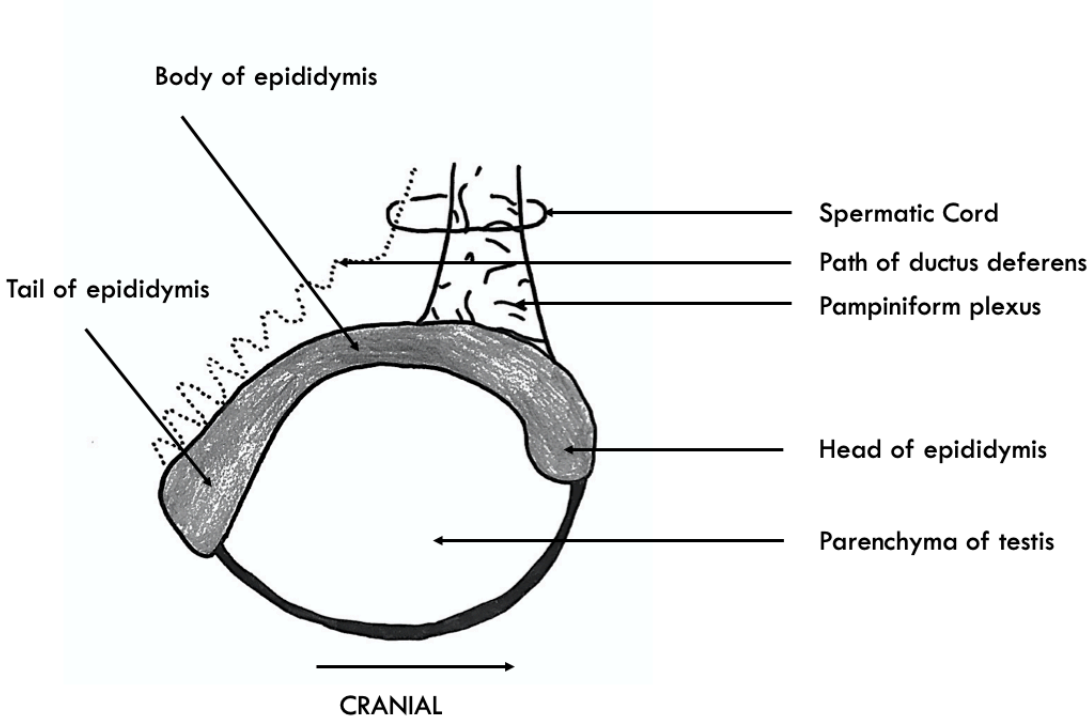

Figure 7. Lateral view of the testis

2.1.4. Testis

Testicles are the site where spermatozoa and androgenic hormones are produced. In stallions and jackasses, they are located within the pendulous scrotum, which consists of a two lobed sac with four major layers, the skin, the tunica dartos, the scrotal fascia and the parietal vaginal tunica (Chenier, 2007). The scrotum acts as a thermosensor, a swamp cooler and a protective sac (Senger, 2012). Testicles have to stay 4-6ºC cooler than the body for the optimal process of spermatogenesis. This different temperature is maintained thanks to

the cremaster muscle, which allows the testes to be let down away from the abdomen, and the pampiniform plexus.

2.1.5. Epididymis

The epididymis consists of a single tubule connecting the efferent ducts to the vas deferens. They play a key role for sperm transit, concentration, protection, maturation and storage. Three regions are distinguishable: the caput, which is attached to the cranial pole of the testis; the corpus, which lies to the dorsal part of the testis; and the cauda, which is linked to the caudal part of the testicle via the proper ligament of the testis (Fig. 7; Burns, 2007).

2.1.6. Accessory glands

The accessory sex glands are located along the male genital tract, between the end of the vas deferens and the root of the penis (Fig. 8). Both stallions and jackasses have the full set of accessory glands, which includes ampullae, prostate, seminal vesicle and bulbourethral glands. Morphology and secretory ability of the male accessory apparatus rely on species-linked and individual attributes (Mann, 1981). These differences are reflected on the total ejaculate volume, ranging from 50-150 mL in stallions and jackasses (Mann, 1981). Ampullae are an enlargement of the distal part of the vas deferens. Since they are not strictly discrete structures separated from their site of secretion by ducts, they are sometimes not classified as accessory glands (Davies Morel, 1999). They contain a variable amount of yellowish and creamy fluid. In stallions, an outstanding characteristic is the presence of very large ampulla (16 to 20 mm in diameter), even larger in jackasses (Fig. 9).

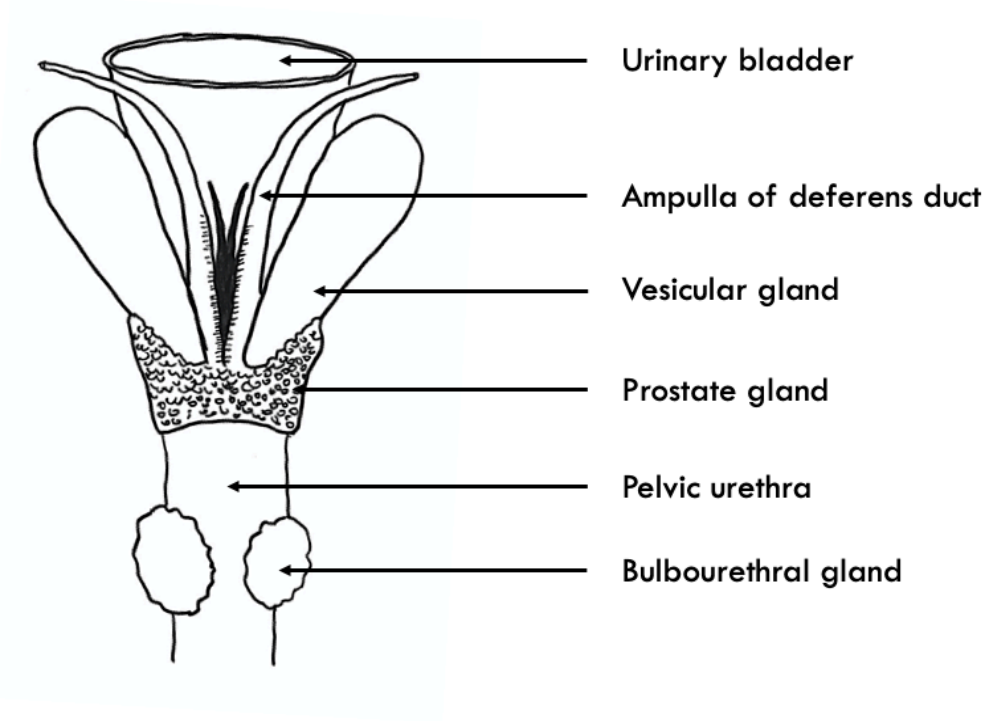

Figure 8. Accessory sex glands in the stallion

Seminal vesicles are, in both stallions and jackasses, shaped like ovoid sacs with muscular walls lined with a thick mucous membrane. The fluid produced by the seminal vesicles is transparent or slightly opaque and viscous. They measure approximately 160-200 mm in length, 25 mm in width and 13 mm in height (Davies Morel, 1999). Seasonal changes have been observed regarding the gelatinous aspect of seminal vesicle secretions. The prostate gland is composed of two nodular lobes and produces a thin and watery fluid. An isthmus provides a connection between the two lobes over the mid-line, and measures approximately 70×40×20 mm (Davies Morel, 1999). An increase of the lobes and isthmus thickness of the prostate can be observed after sexual arousal After ejaculation, it significantly declines. As seminal vesicles, prostate is mostly formed by connective tissue, making their transrectal palpation difficult. The bulbourethral glands, also known as Cowper's glands, are formed by two club-shaped lobes situated at the pelvic outlet, dorsolateral to the urethra. They measure, on average, 19×32 mm (Davies Morel, 1999). In terms of volume, fluid secretion from the bulbourethral glands represent the minor portion.

Normal growth, metabolism and secretory activity of all accessory sex glands depends on the availability of testosterone in the peripheral blood.

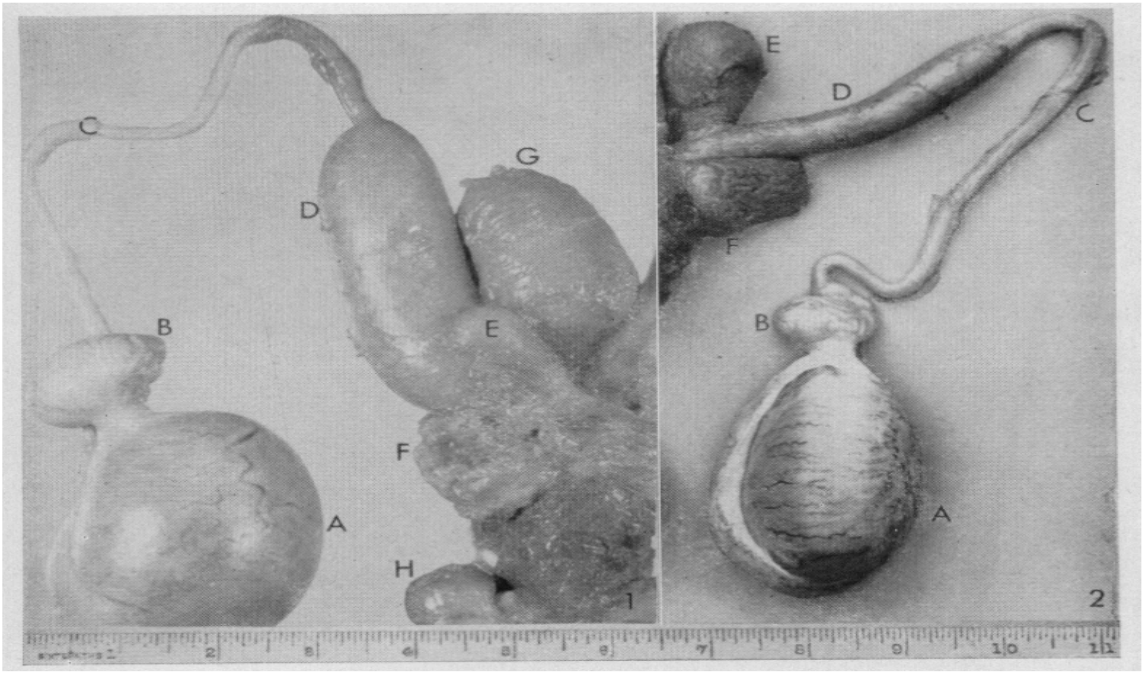

Figure 9. 1. Jackass accessory sex glands 2. Stallion accessory sex glands (Mann, 1963)

2.2. Semen

2.2.1. Ejaculate

Mammalian semen reflects a heterogenous cell suspension that differs between species and individuals within the same species. Amongst others, the variations observed concern sperm number, volume, pH, and total protein content (Mann, 1981). Semen is made up of two parts. The first one consists of the total number of spermatozoa, reflecting sperm production by the testes. The second one is the seminal, plasma, which is the fluid produced by the secretory activity of the accessory sex organs (Meyers, 2009). Within the male tract and during the ejaculatory process, spermatozoa are mixed with secretory fluids produced by accessory sex glands.

Ejaculation involves three sequential processes: erection, emission and ejaculation. Mammalian ejaculates vary markedly between species, individuals, age, breeding season, size of the testes, and environmental conditions, including nutrition, drug intake and social stress (Mann, 1981). The various

components of the ejaculate originate from different parts of the male reproductive tract and are released in a concrete order. In equids, emission and ejaculation follow a series of strong and pulsatile contractions of the urethralis and bulbospongiosus muscles. As a consequence, ejaculates are the result of six to nine jets of semen (Katila and Kareskoski, 2006). Split ejaculation begins with the watery pre-sperm faction, derived from the urethral glands. The milky, but not gelatinous, sperm-rich fraction follows. This portion comes from the epididymis and the ampulla and possesses a high content of glycerylphosphorylcholine (epididymal), ergothioneine (ampulla) and some traces of citric acid. The third fraction is the viscous and gelatinous post-sperm fraction, mainly coming from vesicular secretions (Mann, 1981).

2.2.2. Spermatozoa

a. Structure

The spermatozoon is the mature male gamete that contains the genetic information and fertilizes the oocyte. They are highly polarized cells characterized by an oval head with a long tail. While differences in shape and size may be observed between mammalian species, the structure and function of the male gamete are common (Mann, 1981). Three sections, enclosed by a differentiated plasma membrane, are distinguished: the head, the connecting piece and the tail (Fig. 10).

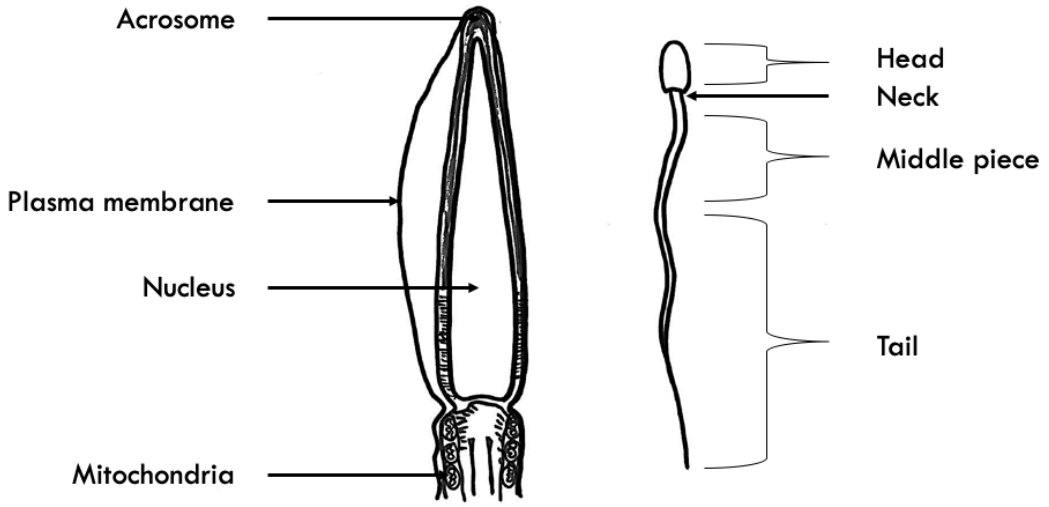

Figure 10. Stallion spermatozoon

The head comprises the acrosome, located in the anterior head region, the nucleus, and a very small volume of cytoplasm. The sperm haploid nucleus contains the male genetic material made up of deoxyribonucleic acid conjugated mainly with protamines and, to a lesser extent (~10%), histones (Meyer, 2009). The acrosome is a membrane-limited vesicle, rich in proteolytic enzymes, such as acrosin, trypsin, hyaluronidase and proteases. Upon interaction of the apical sperm membrane and oocyte vestments, and following sperm capacitation, acrosome reaction occurs releasing these proteolytic enzymes, which allows spermatozoa to pass through those vestments (including cumulus cells and zona pellucida; Yeste, 2013). The neck is the short link between the base of the head and the tail and contains the proximal centriole. In the case of the tail, three regions are distinguished: the mid piece, also known as mitochondrial or intermediate; the principal (main) piece, and the terminal (end) piece. The mid-piece contains the mitochondria, which play a crucial role in providing energy (ATP) through oxidative phosphorylation (Davies Morel, 1999). Although mammalian sperm are able to obtain energy from glycolysis, the activity of the electron transport chain in stallion spermatozoa is known to be higher than in other mammalian species (Rodriguez-Gil, 2006).

Prior to acrosome reaction, the whole surface of the sperm head and tail is covered by the plasma membrane, which, in a similar fashion to other cells, has a typical, complex mosaic structure. It consists of a lipid bilayer membrane with sterols, integral and peripheral proteins, most of which are glycosylated. (Ladha, 1998). Hydrophobic fatty acid chains of membrane phospholipids occupy the interior of the lipid bilayer and confers impermeability to water-soluble, polar and charged molecules. These fatty acids may be saturated or unsaturated, and palmitic acid is one of the predominant saturated fatty acids in the membrane phospholipids of horse spermatozoa (Miller et al., 2004; Aurich et al., 2018). The other phospholipids contain unsaturated fatty acids, which have one or more double carbon-to-carbon bonds in their chain. Since the kinks of unsaturated fatty acid tails prevent tight packing, the high amounts of PUFAs in sperm plasma membrane contribute to membrane fluidity and flexibility (Flesh and Gadella, 2000). Docosapentaenoic acid is one of the most predominant PUFA in the stallion sperm membrane, with significant variations between stallions (Miller et al., 2004; Aurich et al., 2018). Finally, cholesterol is able to interact with fatty acid chains of phospholipids leading to a decrease of the membrane mobility and providing rigidity and stability (Fig. 11).

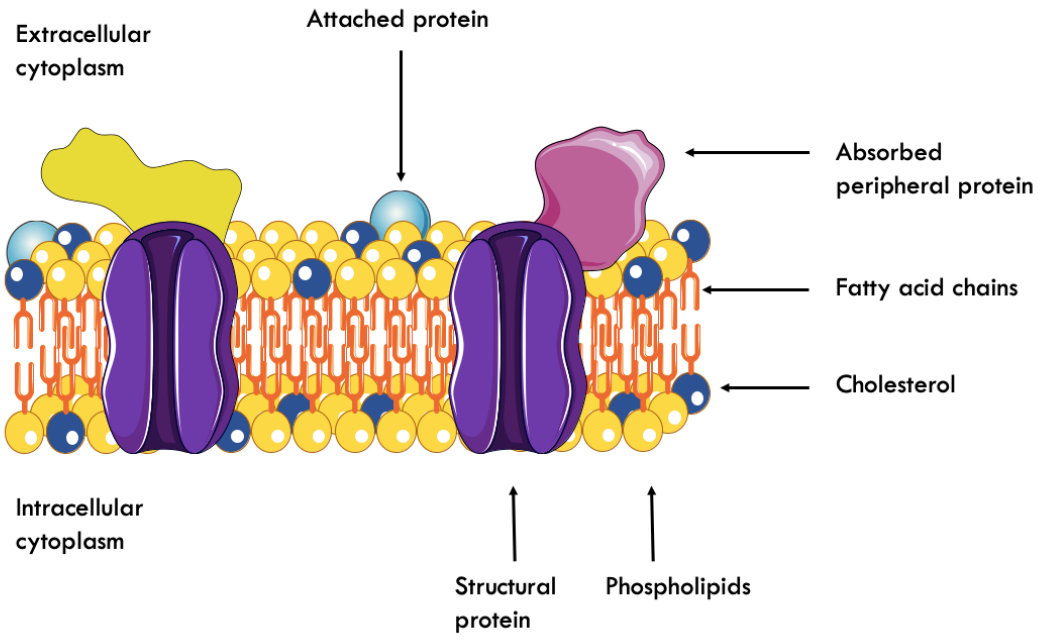

Figure 11. Simplified structure of the plasma membrane of the equine spermatozoon

b. Physiology

As aforementioned, mammalian spermatozoa have a complex structure which undergoes sequential changes from the beginning of the spermatogenesis to fertilization. Spermatogenesis consists of the production of sperm by the seminiferous epithelium during which spermatogonial stem cells generate spermatocytes, the latter differentiating into spermatids and spermatozoa (Meyers, 2009). In this germinal epithelium, three developmental phases are distinguished: a) spermatocytogenesis, which are the mitotic divisions of spermatogonia; b) meiosis, which goes from primary spermatocytes to spermatids; and c) spermiogenesis, which is the differentiation of spermatids into spermatozoa.

Mammalian spermatozoa originate from the testis with neither progressive motility nor fertilizing ability. They acquire gradually these two properties through their transit throughout the epididymis. The spermatogenic wave comprises all spermatogenesis steps and epididymal maturation. In stallions and jackasses, a spermatogenic wave lasts about 65-70 days, since two months are required between the initial and terminal stages of spermatogenesis and an additional week is needed for spermatozoa to pass throughout the epididymis (Mann, 1981).

Spermatozoa undergo further changes within the female reproductive tract, where sperm capacitation occurs. Sperm capacitation takes places within the mare oviduct, lasts for 6 hours, and involves a long and complex process that leads sperm cells to acquire the full capacity to undergo the acrosome reaction, and bind and fertilize the oocyte (Meyers, 2009). Upon ejaculation, spermatozoa are bound by spermadhesins, which are glycoproteins that coat the sperm membrane and provide protection during the passage through the mare's reproductive tract, delaying premature capacitation (Yeste, 2013). Removal of this protective layer and subsequent exposure of receptor sites is an essential step for sperm capacitation. At this point, there is an increase in the intracellular levels of bicarbonate and calcium, which activate the soluble adenylyl cyclase and change the motility pattern (hyperactivation) of the

flagellum (Meyers, 2009). During that process, there is an efflux of cholesterol from the plasma membrane, which makes this membrane to fuse with the outer acrosome membrane. As a consequence, the acrosome reaction occurs upon interaction of the spermatozoon with cumulus cells, and acrosome enzymes are subsequently released (Yeste et al., 2017). After this process, the spermatozoon penetrates through cumulus cells and zona pellucida, and binds and fuses with the oocyte membrane.

As aforementioned, both the development and function of the male gamete are supported by the production of ATP, which is derived from two metabolic pathways: a) glycolysis, which takes place in the principal piece of the flagellum; and b) oxidative phosphorylation, which occurs in the mitochondria and is very important during sperm capacitation (Du Plessis et al., 2015).

2.2.3. Seminal plasma

Seminal plasma is the confluent fluid, issuing from the testis, the epididymis and accessory glands situated along the male reproductive tract. During ejaculation, spermatozoa are mixed with the seminal complex mixture, which becomes essential for sperm physiology and metabolism (Fig. 12). The secretory activity of accessory glands relies on an apocrine mechanism, which means that a portion of the cytoplasm is discharged by the secretory cells together with the secretory products.

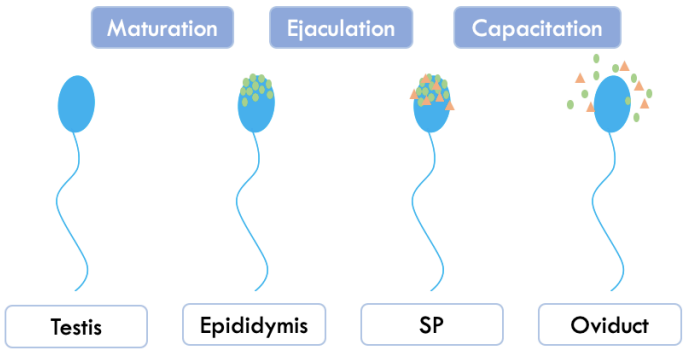

Figure 12. Physiological sequence of sperm surface changes (adapted from Leahy and Gadella, 2011)

As far as the constituents of seminal plasma are concerned, proteins represent an important part. They include spermadhesins, proteins containing fibronectin type II domains, and proteins exhibiting enzymatic, inhibitory, and immunomodulation activities (Jonakova et al., 2010). In addition to proteins, there are other compounds, such as hormones, sugars, amino acids, and growth factors help sperm cells to optimize their chance to fertilize the oocyte (Alghamdi et al., 2009). With regard to sugars and while stallion and jackass seminal plasma is almost free of fructose, the spermatozoa of these species are able to metabolize, both aerobically and anaerobically, the fructose added to the medium (Mann et al., 1963). Finally, and unlike other body fluids, seminal plasma is well endowed with free amino acids, notably glutamic acid, as well as small molecular peptides (Mann, 1981).

More than a natural diluent and transport vehicle, seminal plasma plays an important role for mammalian fertility, both with regard to sperm-related processes and within the female reproductive tract. On the one hand, it acts as a nutritive and buffering medium of pH 7.35-7.50, providing protection from the acidic environment of the vagina. On the other hand, seminal plasma is also involved in sperm function. In effect, the increased levels of bicarbonate present in seminal fluid activates, as aforementioned, sperm motility through the activity of soluble adenylyl cycles residing within the cell (Suarez and Wolfner, 2017). Furthermore, semenogelin (SEMG), secreted by the vesicular glands, induces semen coagulation in primates and mice (Prins and Lindgren, 2015), and participates in the subsequent formation of the copulatory plug, which controls the backflow of semen from the vagina. Semenogelin may also delay sperm capacitation until sperm reach the oviduct, when SEMG is degraded by proteases present in the seminal plasma, such as the prostate-specific antigen (Suarez and Wolfner, 2017). Following removal of SEMG, spermatozoa may establish a reservoir at the lower portion of the oviductal isthmus and at the uterotubal junction. This oviductal reservoir allows a given population of viable spermatozoa to be maintained for a short period of time until the oocyte is released from the ovary (Jonakova et al., 2010). This reservoir is also established thanks to binder sperm proteins (BSP), which are also seminal plasma proteins

that attach to the apical regions of sperm plasmalemma and mediate the interaction with oviductal epithelial cells (Suarez and Wolfner, 2017). This family of proteins is also involved in sperm capacitation, as BSP proteins interact with physiological inducers of sperm capacitation such as high density lipoproteins and glycosaminoglycans (Jonakova et al., 2010). Seminal plasma can also inhibit sperm-oocyte fusion and suppress sperm capacitation through the action of cysteine-rich secretory protein-1 (CRISP-1), secreted by the epididymal epithelium (Roberts et al., 2007). It is worth highlighting that the sequential shedding and uptake of proteins from the seminal plasma modulate capacitation and decapacitation status. Since decapacitated spermatozoa are unable to undergo the acrosome reaction, coating material spread over the plasma membrane prevents or inhibits sperm-oocyte interaction.

To conclude with this section, one should note that seminal plasma also elicits smooth muscle contractions in both male and female reproductive tracts, notably through prostaglandin activity. Its components also include signaling molecules that stimulate female tissues and increase the chances of conception and progression to pregnancy. Indeed, seminal plasma is related to the activation of regulatory T cells which stimulate the female immune response to tolerate pregnancy, preventing rejection of the genetically different fetus (Schjenken, 2014). Furthermore, seminal plasma has been shown to reduce the physiologic influx of neutrophils after semen deposition (Alghamdi et al., 2009). Specifically, cysteine-rich secretory protein-3 (CRISP-3) isolated from seminal plasma and seminal DNase have been described to protect spermatozoa from binding to neutrophils (Doty et al., 2011), and free sperm cells from neutrophil extracellular traps (Alghamdi and Foster, 2005), respectively. Finally, seminal plasma also protects sperm from oxidative damage (Schjenken, 2014). Given that the relationship between this function and the topic of this Dissertation, this issue will be taken up again in S*ection 4.3.*

3. Semen storage

As aforementioned, seasonality represents an important factor constraining reproduction in some domestic animals, such as equids. Moreover, in most species, the survival of mammalian spermatozoa after deposition into the female reproductive tract does not excess several days (Mann, 1981). Under this scenario, artificial insemination and assisted reproductive techniques appear to be an essential tool for equine reproduction management and have become a routine practice in the equine breeding industry. Advantages of artificial insemination are many: facilitation and acceleration of genetic improvement of breeds; routine evaluation of semen; improvement of the reproductive performance of subfertile stallions and mares; removal of geographical restrictions, allowing long-term storage of semen; assistance in the preservation of rare breeds; and control of venereal diseases.

3.1. Artificial insemination and semen storage

Artificial insemination involves collection and transfer of an ejaculate from a male into a sexually receptive female, at the proper time, i.e. close to ovulation. Several semen collection methods exist. An exhaustive list includes the use of an artificial vagina, with a phantom, a teaser female or directly on the ground, pharmacological collection, and epididymal sperm harvest (Samper, 2007). The most widely way to collect stallion and jackass semen is via the use of a pre-warmed artificial vagina. Various models of artificial vagina are available commercially such as the Missouri and the Colorado models, which are the most common models seen in North America (Brinsko et al., 2011). In Europe, the most commonly used is the Hannover model (Fig. 13; Sitters, 2014). Despite the differences, all models consist of a tubular inner liner, which is surrounded by a plastic case that can be filled with water (McCue, 2014).

Figure 13. Hannover artificial vaginal model (Minitüb Ibérica, S.L.; Tarragona, Spain)

Raw semen is very sensitive to changes of the environment and should be transported to the laboratory, immediately after collection, with no agitation, no exposure to light and avoiding cold-shock. Gel fraction or debris have to be removed using an in-line filter during or after collection. Then, semen volume and sperm concentration are recorded and diluted with pre-warmed (37ºC) extender (Samper, 2007). Extenders protect spermatozoa from cold shock and provide buffering and nutrients for sperm. The equine seminal extenders commonly used are milk-based with glucose and antibiotics, and are based on the original Kenney extender, described in 1975. The antibiotics mostly used are a combination of potassium penicillin, amikacin sulfate and/or gentamicin (Samper, 2007).

In a typical artificial insemination program, mares or jennies are inseminated with 250 to 500 millions progressively motile spermatozoa (Brinsko, 2011). Since a final sperm concentration of $25x10^6$ spz/mL is recommended to achieve an adequate sperm longevity (Varner et al., 1987), the volume dose for insemination ranges between 10 and 20 mL of raw semen plus

extender (McCue, 2014). The standard insemination technique consists of introducing the insemination catheter through the swollen cervix, placing the semen in the body of the uterus (Samper, 2007). In the case of doses with low sperm concentration, deep uterine insemination close to the oviductal papilla is recommended, as this increases the chances of fertilization success (Samper, 2007).

3.2. Cooled semen

Processing techniques for semen preservation are determined by the expected longevity of a given ejaculate. Fresh extended semen cannot be preserved for more than 12 hours at 15ºC-20ºC. Cooled semen can be stored for up to 72 hours at 4-6ºC. It is important to highlight that stallion spermatozoa requires a slow cooling rate of -0.5º to -0.1ºC per minute between 20º and 5ºC to maximize sperm viability (Samper, 2007).

After semen processing, packaging is performed under anaerobic conditions (Samper, 2007). It is worth mentioning that differences do exist between stallion and jackass semen regarding the rate of aerobic metabolism. In effect, motility and respiratory sperm activities in jackass semen diluted with an egg-yolk medium at 5ºC are maintained much longer than in stallion semen (Mann et al., 1963).

The presence of seminal plasma during storage at 5ºC for 24-48 hours has been described to be detrimental for sperm motility (Jasko et al., 1991). In addition, different studies have demonstrated that removal of 90% of seminal plasma contributes to the stabilization of sperm plasma membrane (Brinsko et al., 2000; Barrier-Battut et al., 2013). Furthermore, removal of seminal plasma has been correlated with better maintenance of sperm viability after 48 hours of storage at 4-5ºC in Zamorano-Leonés (Serres et al., 2002) and Catalonian jackasses (Miró et al., 2009), without beneficial effects being observed in the Amiata breed (Rota et al., 2008). In spite of the aforementioned, the inclusion of a low proportion of seminal plasma, from 1 to 20%, has been reported to optimize sperm motility (Jasko et al., 1992). Reduction of the amount of seminal

plasma in cooled semen can be accomplished through dilution of semen with extender. While centrifugation of the ejaculate and subsequent removal of the supernatant can be an alternative, differences between species have been observed with regard to the sperm sensitivity to centrifugation. Gravitation forces of 600 g and below do not significantly damage mammalian spermatozoa (Mann, 1981).

Finally, differences between individuals in terms of their response to cooled storage have also been observed (Brinsko et al., 2000; Morrell et al., 2016). For this reason, stallions and jackasses may be classified as good or poor coolers depending on how their sperm progressive motility and viability are maintained throughout liquid-storage at 4ºC-5ºC.

3.3. Cryopreserved semen

When stallion or jackass sperm have to be preserved for more than 48-72 hours, they must be cryopreserved. Cryopreservation is the process that preserves structurally intact living cells by cooling to subzero temperatures (Pegg, 2007). At present, cryopreservation is the best method for long-term sperm storage and allows the establishment of germplasm banks. However, freeze-thawing involves a reduction in sperm function and survival, decreasing their fertilizing ability compared to fresh or cooled semen. The success of cryopreservation relies upon many factors such as cryoprotectants, extenders, the composition of plasma membrane, and cooling and warming rates. In addition, other factors, such as the intrinsic ejaculate freezability and the impact of the season, also influence sperm cryopreservation outcomes. After collection and initial evaluation, the ejaculate is diluted in an isotonic medium which is usually an extender for cooled semen. Then, seminal plasma is removed through centrifugation and the pellet is diluted in a cryopreservation medium containing permeable and non-permeable cryoprotecting agents (Samper, 2007).

Cryoprotectants protect the cell from ice crystallization by decreasing the freezing point of intracellular components, as it reduces the amount of salts and solutes present in the liquid phase. According to their capacity to cross cell

membranes, they are classified as permeating and non-permeating cryoprotectants. Since they are not able to diffuse passively across the plasma membrane, non-permeating cryoprotectants act extracellularly. They include milk and egg yolk proteins, sugars, and other compounds of high molecular weight (i.e. polyethylene glycol; Yeste, 2016). Permeating cryoprotectants are able to diffuse through plasma membrane, replacing water in the sperm cell and, consequently, altering their properties. They include glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, and propylene glycol (Hezavehei et al., 2018). Glycerol is the most used permeable cryoprotectant for cryopreserving stallion spermatozoa. It is a non-electrolyte, i.e. does not ionize when dissolved, and, thus, reduces the electrolyte concentration in the residual unfrozen solution (Jang et al., 2017). Permeable cryoprotectant agents may be toxic at high concentrations (Hezavehei et al., 2018). Furthermore, high sensitivity of jackass ejaculates to glycerol should be considered since a half dose of glycerol (2.2%) used for stallion sperm cryopreservation seems to be cytotoxic (Vidament et al., 2009). Therefore, setting a cryopreservation protocol should contemplate different factors, such as the composition of the extender (egg-yolk, milk, buffers, cryoprotective agents) and the rates of cooling/freezing and warming.

Species-linked differences are related to the sensitivity of sperm cells to withstand freezing and thawing procedures. Differences are also observed between males belonging to the same species (Hoffmann et al., 2011; Al-Essawe et al., 2018), and even between ejaculates from the same animal (Yeste et al., 2015). For this reason, stallions and jackasses, or their ejaculates, may be classified as of "good" or "poor" freezability, which is based on sperm motility, both total and progressive, and survival. Such influence of the donor on the sperm resilience to withstand cryopreservation has been related, amongst others, to the composition of seminal plasma. Related with this, the addition of seminal plasma from the sperm-rich fraction has been found to be positively correlated with post-thaw sperm motility (Kareskoski et al., 2006). Moreover, addition of seminal plasma from ejaculates with good freezability increases post-thaw sperm quality parameters from poor freezability ejaculates (Aurich et al., 1996).

Freezing and thawing procedures can be detrimental for sperm plasma membrane and consequently affect the function of the cell (Watson, 2000). Cryodamage results, among others, from thermal shock, formation of intracellular ice crystals, cellular dehydration and osmotic shock. The phase change of intracellular and extracellular water represents the main challenge for cell cryosurvival (Yeste, 2016). Indeed, between -5ºC and -15ºC, while ice is present in the extracellular compartment, the intracellular compounds remain unfrozen. The potential created induces the water flowing out the cell in order to freeze externally. Consequently, a very high cooling rate does not allow intracellular water to flow out completely and ice crystals are formed into the cytoplasm resulting in cell cryoinjury. In the case of very low cooling rates, most of the water flows out giving rise to dehydration and shrinkage of the cell (Yeste, 2016).

3.3.1. Cold shock

As aforementioned, sperm plasma membrane is rich in PUFAs, which confer fluidity to the membrane, but display a high sensitivity to cold shock. Such cold shock refers to sperm damage, generally irreversible, induced by a rapid temperature drop from 20 to 0ºC. When temperature drops, lipid phases in the sperm membrane are transformed into a crystalline state. Following this, the lipid bilayer undergoes a transition from fluid to gel state (Sieme et al., 2015). Re-ordering of phases and fusion of PUFAs change the normal configuration of the lipid bilayer and the subsequent distribution of components, and make the plasma membrane more fragile (Fig. 14; Rasit Ugur et al., 2019). As a consequence of this lipid restructuration, integral membrane proteins and some cholesterol molecules also undergo structural alterations (Yeste, 2016). Disruption in the interaction between components of the plasma membrane affects the integrity of plasma and acrosome membranes. This leads to a premature depolymerization of actin filaments from the cytoskeleton, and changes plasma membrane permeability to some crucial ions, such as calcium and bicarbonate (Yeste, 2016). As a result, both cell structure and function are affected, metabolism rates and sperm motility are decreased and the

percentage of non-viable spermatozoa is increased (Watson, 2000).

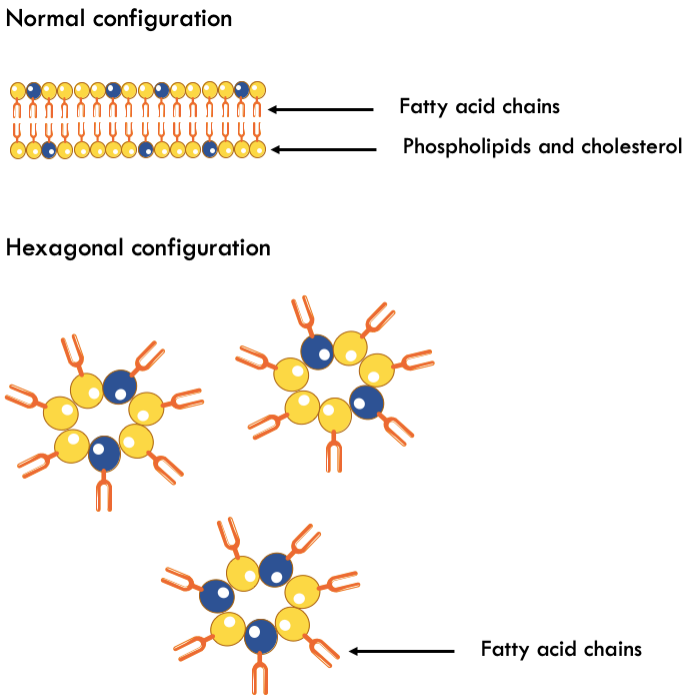

Figure 14. Alteration of function state lipid membrane (Adapted from Davies Morel, 1999)

3.3.2. Osmotic stress

As temperature drops, ice crystals are preferentially formed in the extracellular solution, which is hypertonic. Consequently, intracellular water flows out from the cell and the cell shrinks until intra and extracellular solute concentration is equilibrated (Peña et al., 2011). Furthermore, through hypertonic stress, electrolyte balance may be disrupted and this can affect the mechanisms that regulate cell volume (Fig. 15; Yeste, 2016). Under this scenario, the most challenging point is to find the best cooling rate, since ice crystallization occurs when cooling rates are too fast or too slow, and the optimal cooling rate is the one that generates the lowest amount of intracellular ice crystals (Jang et al., 2017). Success of cryopreservation procedure also implies cell survival along thawing. When ice crystals melt during thawing, sperm organelles can once again be damaged, due to the changes in the osmolality of both intra and extracellular environments. Therefore, osmotic stress may be the main

detrimental effect produced by freezing and thawing procedures (Peña et al., 2011). In the case of stallion sperm, the most appropriate protocol for 0.5 mL straws is thawing at 37ºC for 30-60 seconds (Alvarenga et al., 2016).

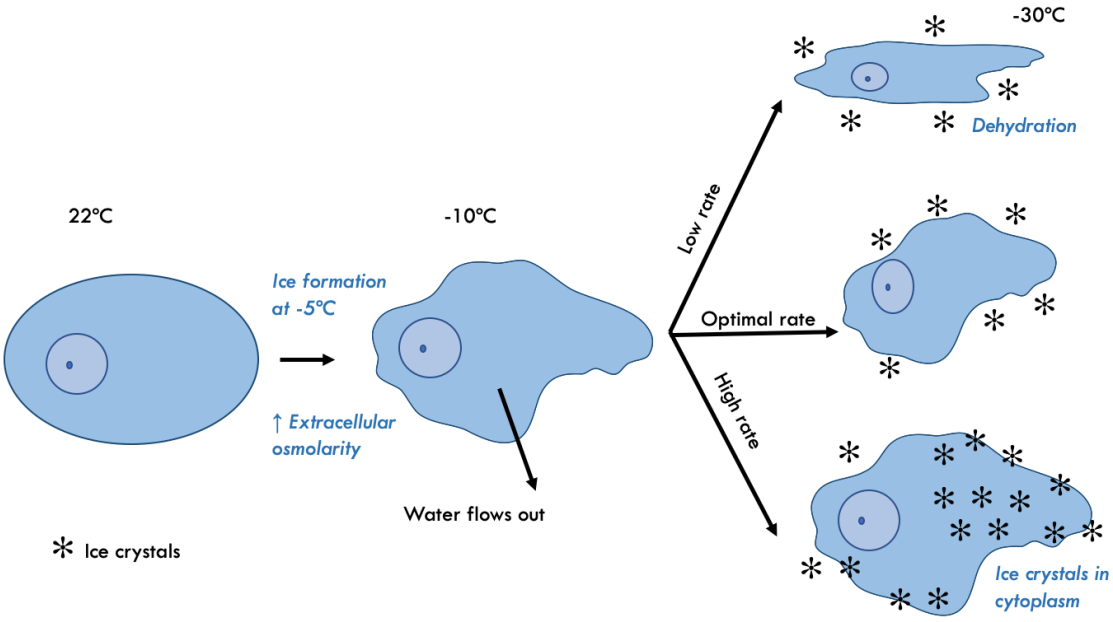

Figure 15. Cryoinjuries of cells during freezing and thawing (Adapted from Yeste, 2016)

It is worth mentioning that cryoinjuries are not restricted to sperm membrane since other compartments are also affected, such as sperm chromatin and mitochondria. In addition, cryopreservation induces apoptotic-like changes in spermatozoa as it may activate caspases (Peña et al., 2011).

4. Oxidative stress

During sperm handling for preservation, artificial insemination or assisted reproduction techniques, semen is exposed to many non-physiological changes in temperature, pH, partial pressure of oxygen and carbon dioxide, light, and absence of female genital secretions. Consequently, sperm can exacerbate oxidative stress, which may impair their survival and fertilizing ability (Aitken and Baker, 2006).

4.1. Reactive oxygen species

Reactive oxygen species (ROS) cover a wide range of by-products derived from reduction of molecular oxygen (O_2), which includes free radicals such as the superoxide anion ($O_2^{-\bullet}$) and nitric oxide ($NO^{\bullet}$), and oxidants, such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl), peroxyl and alkoxyl radicals ($ROO^{\bullet}$, $RO^{\bullet}$). Due to the unpaired number of electrons in the outer atomic shell, free radicals are very energetic molecules, so they try to find out other electrons from other atoms/molecules with which to pair. Consequently, these metabolites are very reactive and are able to modify a wide range of other biomolecule structures including proteins, lipids and nucleic acids (Agarwal et al., 2014).

Low levels of ROS are naturally produced by spermatozoa as a by-product of the electron transport chain during oxidative phosphorylation (Aitken and De Iuliis, 2011). In fact, low ROS generation is vital for crucial physiologic pathways. For instance, their interaction with cholesterol from the sperm surface is essential for capacitation and the subsequent acrosome reaction (Baumber et al., 2003; Leahy and Gadella, 2011). However, the high reactive capacity and the large number of possible targets lead ROS to generate oxidative damage (Aitken and De Iuliis, 2011). Therefore, oxidative stress is a condition associated with the unbalance between ROS generation and the antioxidant defense system, which aims to scavenge ROS and prevents further damage on sperm cells (Agarwal et al., 2014).

4.2. ROS-induced damage

As aforementioned, ROS can be spontaneously produced in mammalian spermatozoa by mitochondria through oxidative phosphorylation (Ball, 2008) or through apoptotic-like changes (Fig. 16; Gavriliouk and Aitken, 2015). Immature, damaged or dead spermatozoa can also produce ROS (Morrell and Rodriguez-Martinez, 2009). In the male and female genital tracts, infiltrating phagocytic leukocytes induced by an inflammatory process may be another potential source of ROS (Baumber et al., 2002; Gavriliouk and Aitken, 2015).

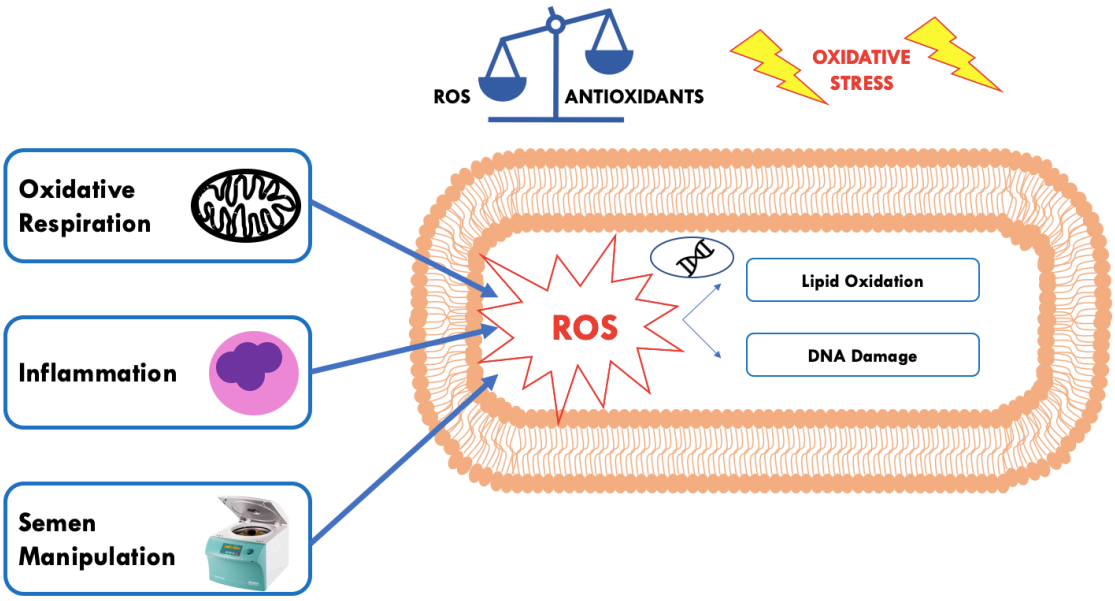

Figure 16. Source of ROS generation in mammalian spermatozoa

In addition, sperm preparation techniques, such as storage, centrifugation or cryopreservation, are associated with an increase of ROS generation (Watson, 2000). As a result, mitochondrial damage, reduction in the membrane fluidity and loss of integrity of acrosome and plasma membranes are observed, in association with decreased motility, viability and ability to fertilize the oocyte (Ball, 2008). One should also note that, in addition to produce cell injuries, sperm cryopreservation leads to reduction in the antioxidant intracellular contents (Agarwal et al., 2014).

4.2.1. Lipid peroxidation

Sperm membranes are abundant in PUFA, which provides the fluidity required for events associated to membrane remodeling, sperm capacitation, and fertilization (Wathes et al., 2007). PUFA, and particularly docosahexanoic acid, are less stable than saturated fatty acids, which makes them a major target for ROS and are more vulnerable to their oxidation (Aitken and De Iuliis, 2011). In effect, PUFA in plasma membranes react with oxygen to produce peroxyl radicals, the primary free radical intermediate of lipid peroxidation producing an autocatalytic self-propagating reaction (Agarwal et al., 2014). The generated

lipid peroxides and aldehydes have a direct inhibitory action on sperm motility. The disruptive effect of lipid peroxidation leads to changes in sperm membrane fluidity, alters signaling pathways essential for sperm function, and affects the sperm ability to fuse with the oocyte membrane during fertilization (Aitken et al., 2016).

4.2.2. DNA fragmentation

Since most histones are replaced by protamines during spermiogenesis, sperm DNA has a highly condensed structure and chromatin is minimally exposed to ROS-related insult (Agarwal et al., 2014). However, incomplete protamination of sperm chromatin makes DNA more vulnerable to oxidative damage and premature decondensation, allowing the possibility of chromosomal rearrangements to occur (Agarwal et al., 2014). Since stable DNA packaging is crucial for proper decondensation upon fertilization, delivery of paternal genome and fertilizing ability are impaired when DNA is fragmented or chromatin condensation is altered in response to oxidative damage (Agarwal and Allamaneni, 2005; Rasit Ugur et al., 2019).

4.3. Antioxidant protection

Both spermatozoa and seminal plasma contain antioxidant systems able to scavenge ROS and thus prevent cell damage. Indeed, in addition to the fact that spermatozoa are protected by intracellular antioxidants provided by mitochondria and cytosol, highly specialized extracellular antioxidant enzymes are secreted by the male reproductive tract during epididymal maturation (Wathes et al., 2007). Since spermatozoa lose most of their cytoplasm during spermiogenesis and epididymal maturation, and consequently lack endogenous enzymatic defenses, mature ejaculates are mainly protected from oxidative damage by seminal plasma (Yu et al., 2011). Seminal plasma is endowed with low-molecular enzymatic and non-enzymatic antioxidant components able to neutralize ROS and preserve sperm function (Agarwal et al., 2014). As a consequence, removal of seminal plasma may make sperm more vulnerable to

oxidative damage in front of ROS generation. Actually, in human sperm, removal of antioxidant protection from seminal plasma through centrifugation has been reported to increase DNA fragmentation (Yu et al., 2011).

The antioxidant components of seminal plasma are so important that the total antioxidant capacity of seminal plasma has been described as a biochemical predictor for men fertility (Adeel, 2011). Non-enzymatic oxidants include vitamins B, C and E, carotenoids, carnitine, cysteine, pentoxifylline, taurine, melatonin, albumin and metals (Hezavehei et al., 2018). Enzymatic compounds include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GSR) (Fig. 17).

With regard to the reactions that antioxidant enzymes catalyze, SOD allows the dismutation of $O_2^{-\bullet}$ to produce H_2O_2. SOD should only be considered as an antioxidant if coupled with additional enzymes able to metabolize the H_2O_2 generated (Aitken and De Iuliis, 2011). In this regard, SOD activities are completed by those of CAT and those of GPX. Indeed, CAT reduces hydrogen peroxide into water and molecular oxygen (Ighodaro and Akinloye, 2017). Then, due to the detrimental nature of H_2O_2, lipid peroxidation or DNA damage occur; the glutathione peroxidase – glutathione reductase system is responsible for peroxide metabolism. The function of GPX is to reduce hydrogen peroxide to water (Ighodaro and Akinloye, 2017). Glutathione reductase is also associated with ROS balance, as catalyzes the reduction of glutathione disulfide (GSSG) to the reduced form of glutathione (GSH) and hydrogen peroxide (Zura Zaja et al., 2016).

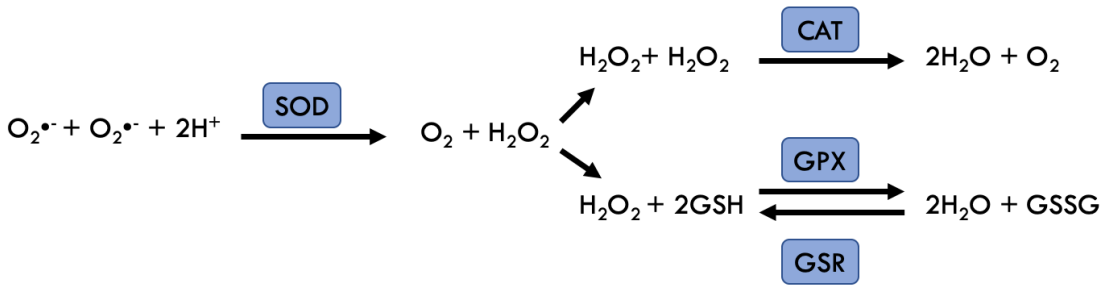

Figure 17. Enzymes reactions

Finally, it is worth noting that, in previous studies, seasonal variation in the antioxidant system of seminal plasma has been observed in both seasonal (ram) and non-seasonal (boar) species (Muiño-Blanco et al., 2008). Given the lack of evidence in stallions and jackasses, this Dissertation aimed to address whether seasonal variations in the activities of these four antioxidant enzymes also exist in those species (See Objectives).

5. Sperm selection

Sperm selection techniques in the equine breeding industry aim at the improvement of "problematic" ejaculates for artificial insemination, removal of pathogens, increase of the resilience to cryopreservation, and selection of spermatozoa for intracytoplasmic sperm injection (Morrell, 2012). Since sperm are submitted to a selection mechanism within the female reproductive tract, especially in the cervix (in this case, depending on the species), the uterotubal junction and in the oviduct (Morrell and Rodriguez-Martinez, 2009), these techniques try to mimic that selection, thereby removing seminal plasma and keeping these spermatozoa with better quality parameters, including normal morphology, and membrane and chromatin integrity (Morrell and Rodriguez-Martinez, 2009).

The techniques for improving semen quality can be classified into "separation" and "selection" techniques. Separation techniques consist of sperm washing by simple centrifugation immediately after collection. The resulting pellet is resuspended in an extender and spermatozoa are thus separated from most of the seminal plasma components. However, since the pellet is a concentrate from the original ejaculate, the sample obtained contains viable, moribund dead, and abnormal spermatozoa (Morrell and Rodriguez-Martinez, 2009). Sperm selection methods include sperm migration, sperm filtration and sperm centrifugation through a colloid. In the case of equine species, sperm migration-based techniques, based on "swim-up", and centrifugation through colloid are the most used (Morrell et al., 2016).

5.1. Migration

The "swim up" method is the oldest one. It is based on the ability of motile spermatozoa to swim into the culture medium of a different composition. The culture medium is layered over the pellet recovered from previously centrifuged semen (Fig. 18; Beydola et al., 2014). The movement of sperm from the pellet to the upper part of the medium may allow the separation of motile spermatozoa from the immotile ones (Volpes et al., 2016) and from the seminal plasma (Morrell and Rodriguez-Martinez, 2009). However, no selection on head morphology and chromatin integrity is achieved with this method. In addition, the recovery rate is low (10-20%) (Morrell and Rodriguez-Martinez, 2009). The advantage of this technique is that it does not generate significant amounts of ROS (Beydola et al., 2014)

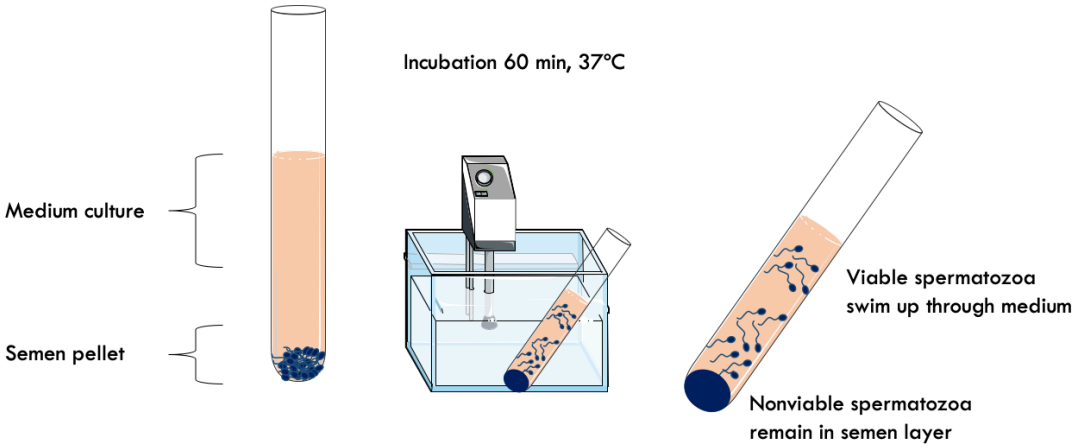

Figure 18. The "swim up" technique.

5.2. Filtration

Filtration methods rely on the ability of viable and motile spermatozoa to move throughout the solid phase while non-viable cells tend to adhere to the matrix. The recovery rate is around 63%; however, it has been reported that not all of seminal plasma and cellular debris are removed (Morrell and Rodriguez-Martinez, 2009).

5.3. Colloid centrifugation

Colloids are coated silica particles in a buffered salt solution of two different densities (Morrell et al., 2009). During centrifugation through colloids, spermatozoa with good viability, motility and chromatin integrity are separated from seminal plasma and from other cells sub-populations (Morrell and Rodriguez-Martinez, 2009). Two techniques of colloid centrifugation are used in equine, density gradient centrifugation (DGC) and single layer centrifugation (SLC) (Morrell et al., 2016).

5.3.1. Density gradient centrifugation

During density gradient centrifugation, heterogeneous cell populations move to the point of the gradient that matches with their own density (isopycnic point) (Morrell et al., 2016). Density gradually increases from the upper layer to the bottom. Since morphologically normal and abnormal spermatozoa have different densities (Beydola et al., 2014), morphologically normal, motile spermatozoa with an intact DNA pass through the colloid, whereas the others are retained in the upper phase (Fig. 19; Morrell et al., 2009). Furthermore, the seminal plasma mixed with the extender is retained above the upper layer and, consequently, spermatozoa in the pellet obtained after centrifugation are free of seminal plasma. In addition, density gradient centrifugation through colloids prolongs survival of stallion spermatozoa (Morrell et al., 2009). It has also been described that this technique is able to isolate from the low-density interphase, and that equine spermatozoa display apoptotic-like changes (Brum et al., 2008).

Figure 19. Density gradient centrifugation

5.3.2. Single layer centrifugation

Single layer centrifugation (SLC) is a variation of colloid centrifugation using one layer of colloid (Fig. 20; Morrell et al., 2016). During centrifugation through a species-specific formulation, seminal plasma is retained on the top of the colloid while motile spermatozoa move down and form the pellet. Since only one layer of colloid is employed, this technique seems to offer a more practical method than DCG for selection of high quality spermatozoa. Moreover, whereas voluminous samples can be processed by SLC, the maximum volume that can be prepared through DGC is 1.5 mL (Morrell and Rodriguez-Martinez, 2009). Another advantage of SLC is the reduction of pathogens contained in semen samples, such as *Trypanosoma equiperdum* in equine semen (Yasine et al., 2019) or circovirus in boar semen (Morrell and Rodriguez-Martinez, 2009). In stallion ejaculates, SLC selects spermatozoa with good motility and normal morphology, not only from fresh/raw semen but also after 24 hours of liquid-storage at 4ºC (Morrell et al., 2011). Furthermore, SLC-selected spermatozoa produce less hydrogen peroxide (Morrell et al., 2016) and display better ability to withstand cryopreservation (Al-Essawe et al., 2018). Regarding jackasses, spermatozoa selected through SLC after 24 hours of cooled-storage (Ortiz et al., 2014) and after freezing-thawing procedures (Ortiz et al., 2015) display better sperm quality than unselected cells. Finally, in pigs, SLC is able to remove

seminal plasma proteins PSP-I and –II, as well as, part of the cholesterol from the sperm membrane (Kruse et al., 2011).

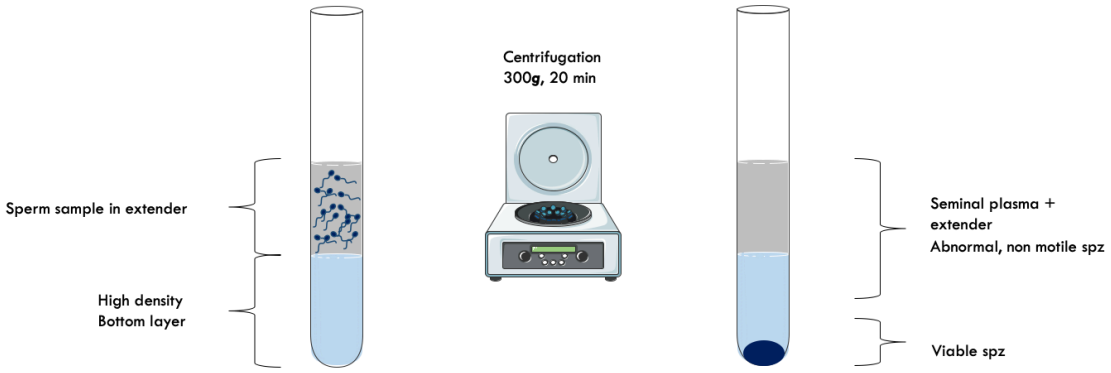

Figure 20. Single Layer Centrifugation

OBJECTIVES

The objectives of this Dissertation are:

1. To compare the activities of seminal plasma antioxidant enzymes (SOD, CAT, GPX and GSR) in stallion and jackasses.
2. To investigate whether the activities of these enzymes differ between seasons.
3. To determine the relationship between the sperm quality parameters in fresh and frozen-thawed semen and the activity of these four seminal plasma antioxidant enzymes.
4. To assess whether the activities of SOD, CAT, GPX, and GSR in seminal plasma are related with the sperm ability to withstand cryopreservation, in both stallions and jackasses.
5. To evaluate whether the presence of seminal plasma is able to modulate the sperm response to oxidative stress exogenously induced in jackasses.
6. To investigate the effects of selecting jackass spermatozoa with single layer centrifugation (SLC).

PAPER COMPENDIUM

1. Activities of antioxidant seminal plasma enzymes (SOD, CAT, GPX and GSR) are higher in jackasses than in stallions and are correlated with sperm motility in jackasses

2. Specific activity of superoxide dismutase in stallion seminal plasma is related to sperm cryotolerance

3. Total and specific activity of superoxide dismutase in donkey seminal plasma is related to sperm cryotolerance

4. Seminal plasma has limited counteracting effects following induction of oxidative stress in donkey spermatozoa

5. Effects of single layer centrifugation through EquicollTM on donkey fresh spermatozoa and on its interaction with polymorphonuclear cells

Activities of antioxidant seminal plasma enzymes (SOD, CAT, GPX and GSR) are higher in jackasses than in stallions and are correlated with sperm motility in jackasses

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Article

Specific Activity of Superoxide Dismutase in Stallion Seminal Plasma Is Related to Sperm Cryotolerance

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Abstract: While the removal of seminal plasma is a routine practice prior to equine sperm cryopreservation, this fluid contains the main source of antioxidant enzymes able to scavenge these reactive oxygen species. Therefore, stallion seminal plasma components may have an impact on ejaculate freezability. Against this background, this study was designed to investigate whether the activities of the main stallion seminal plasma antioxidant enzymes are related to sperm cryotolerance. With this purpose, 16 ejaculates were collected from 14 healthy stallions, and each ejaculate was split into two aliquots. The first one was used to evaluate the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GSR) in seminal plasma. The second aliquot was extended and then processed for cryopreservation. Sperm motility and viability were evaluated before and after cryopreservation, and ejaculates were classified as of good (GFE) or poor freezability (PFE) based on total motile and viable spermatozoa at post-thaw. We observed that, while the specific activities of CAT, GPX, and GSR were similar between GFE and PFE, that of SOD was significantly ($p < 0.05$) higher in GFE than in PFE. We can thus conclude that, in stallions, the specific activity of SOD in the seminal plasma of a given ejaculate might be related to its freezability.

Keywords: seminal plasma; sperm; equine; cryopreservation; antioxidant enzymes

1. Introduction

Cryopreserved stallion semen is widely used in the equine breeding industry. Indeed, not only does cryopreservation of stallion sperm facilitate their availability and transport, but it also preserves the genetic material for an unlimited period [1]. However, the plasma membrane of stallion sperm contains an elevated content of polyunsaturated fatty acids [2], which makes this cell highly sensitive to oxidative stress and subsequent lipid peroxidation. Since the process of freezing and thawing exposes sperm to severe cold shock and osmotic stress, their survival and fertilizing ability can be greatly compromised [3]. For this reason, efforts have been made to improve stallion sperm cryopreservation over the last years, testing the use of different cryoprotectants, including glycerol [1]. However, optimizing cryopreservation protocols is still needed, as there is room to increase the fertilizing ability of frozen–thawed stallion sperm.

While in cattle, selection of sires is based on the assessment of reproductive parameters, including sperm freezability, stallions are selected according to a specific phenotype mainly related to sport performance, which is not usually in line with sperm cryotolerance [1,4]. Moreover, in horses, as in other mammalian species, there is a high individual variability in the ability of sperm to withstand freezing and thawing procedures [4–8]. These differences, which appear to be partially related to the lipid composition of sperm plasma membrane, lead stallions to be classified as of "good" or "poor" freezers [4,6].

Seminal plasma, which is the fluid containing the sperm at ejaculation, is produced by the epididymis and accessory sex glands, and is made up of proteins, ions, and organic substances, such as amino acids, lipids, monosaccharides, and hormones [7,9]. In mammals, this fluid is known to play a vital role for sperm function, both in the male and female reproductive tract [10]; however, conflicting results have been reported with regard to their beneficial or detrimental effects during storage of cooled and cryopreserved equine semen [6,8,9,11]. For this reason, most of the seminal plasma is usually discarded before cryopreservation, as this minimizes the negative impact on sperm motility and viability during sperm storage. However, the inclusion of a low proportion of seminal plasma, from 1% to 20%, has been reported to have a positive effect on sperm motion characteristics [7,8,12].

On the other hand, seminal plasma plays an important antioxidant role against the oxidative damage of spermatozoa. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GSR) are the major antioxidant enzymes present in mammalian seminal plasma, and are involved in the scavenging of reactive oxygen species (ROS) [13,14]. As stated above, at the time of ejaculation, sperm and seminal plasma mix. Thus, even in those cases in which seminal plasma is completely removed before cryopreservation, sperm are in contact with seminal plasma for a short period of time. While variations in the composition of seminal plasma have been reported to exist between individual stallions [15,16], no previous study has determined whether the activity of antioxidant enzymes in seminal plasma is related to sperm cryotolerance.

Against this background, the aim of the present work was to determine whether the activities of SOD, CAT, GPX, and GSR in seminal plasma (fresh semen) are related to the sperm ability to withstand the freezing and thawing procedures, as not only could this shed light on how sperm cryopreservation works in equines, but it could also be a useful tool to predict the suitability of a given stallion ejaculate for sperm cryopreservation.

2. Materials and Methods

2.1. Experimental Design

Each ejaculate was split into two fractions. One fraction was centrifuged to recover the seminal plasma and to evaluate the activity of each enzyme, whereas the other was used to evaluate sperm concentration, motility, and viability and then cryopreserved. Upon thawing, sperm motility and viability were also assessed. Based on sperm post-thaw motility and viability, ejaculates were classified as of good (GFE) or poor (PFE) freezability through cluster analyses (see Section 2.9).

2.2. Semen Collection

Throughout the year, semen samples were collected from 14 warmblood and Arabian stallions, aged from 5 to 21 years old. All animals were housed at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain). This is an EU-approved semen collection center (Authorization code: ES09RS01E) that operates under strict protocols of animal welfare and health control. All stallions used in this study were semen donors housed at that center and were collected under CEE health conditions (free of Equine Arteritis, Infectious Anemia, and Contagious Metritis). Since this service already runs under the approval of the Regional Government of Catalonia (Spain) and because no manipulation to the animals other than semen collection was carried out, the ethics committee of our institution indicated that no further ethical approval was

required. A total of 16 ejaculates (2 from 2 stallions, and 1 from each of the other 12 stallions) was acquired in a regular schedule using a phantom and an artificial vagina Hannover model (Minitüb Ibérica, S.L.; Tarragona, Spain) filled with warm water to obtain a temperature range inside the lumen between 48 °C and 50 °C. An inline nylon filter was used to remove contaminants and the gel fraction. Two equal volume fractions of gel-free semen were obtained from each ejaculate. The first aliquot was used to recover the seminal plasma, whereas the other was diluted 1:4 (*v:v*) with a preheated (37 °C) skim milk extender (4.9% glucose, 2.4% skim milk, 100 mL double-distilled water). This latter aliquot was used to evaluate sperm concentration, viability, and motility in fresh semen, and the remaining volume was intended for cryopreservation.

2.3. Seminal Plasma Collection

Immediately after ejaculate collection, raw semen was placed in 50 mL conical tubes and loaded in a centrifuge at 3000× g and 4 °C for 10 min (JP Selecta S.A., Barcelona, Spain). The supernatant was examined for the presence of sperm cells and centrifuged again and again at the same conditions until seminal plasma was free of spermatozoa. The absence of cells was assessed by a phase-contrast microscope (Olympus Europe, Hamburg, Germany) at 200×. The number of centrifugations depended on the ejaculate, and centrifugation was performed as many times as needed until samples were sperm-free. Usually, around five centrifugations were required. Thereafter, samples were stored in 5 mL tubes at −80 °C until enzyme activities were measured. Before analysis, seminal plasma samples were thawed on ice (4 °C).

2.4. Determination of Enzyme Activities

Tubes containing seminal plasma were thawed prior to evaluating their total protein content and their total and specific activities of the following four antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GSR).

Total protein content was determined with a biuret-based total protein reagent, which consisted of copper ions in an alkaline reagent that reacted with peptide protein bonds. This reaction resulted in a purple color with a maximum absorbance of 540 nm, directly proportional to the concentration of total protein in the sample. In parallel, a standard curve with bovine serum albumin was prepared.

Total enzyme activities of SOD, GPX, and GSR in seminal plasma were evaluated using a commercial kit (Randox Laboratories Ltd, Crumlin, UK) following the instructions of the manufacturer. In brief, the assessment of GPX activity (Ransel kit) was performed following the oxidation of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) with cumene hydroperoxide and glutathione reductase. Activity of GSR (Glut Red kit) was measured based on the oxidation of NADPH. Finally, the evaluation of SOD activity (Ransod kit) was based on the formation of red formazan dye generated by the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) with the superoxide radical produced by xanthine and xanthine oxidase. An Olympus AU400 analyzer (Beckman Coulter, Hamburg, Germany) was used to perform the analytical assessment.

CAT activity was measured through a spectrophotometric assay that monitors the change in absorbance at 240 nm for 30 s, while H_2O_2 is reduced to H_2O and O_2 [17]. Briefly, 20 µL seminal plasma was diluted in 830 µL phosphate buffer (62.5 mM, pH = 7.2) in a spectrophotometric cuvette. The reaction started following the addition of 150 µl H_2O_2 in order to reach a final concentration of 30 mM. Enzyme activity (U/mL) was calculated using the following formula [18]:

$$CAT\ activity\left(\frac{U}{L}\right) = \frac{\Delta A}{\varepsilon \times \ell} \times\ dilution\ factor$$

where: *A: absorbance*, *ε: extinction molar coefficient (H_2O_2)*, *ℓ: light path, dilution factor: 1000*

To evaluate the purity of the enzyme in seminal plasma samples, specific activities of the four enzymes were determined by normalizing the activity of each enzyme with the amount of total protein in seminal plasma samples [19].

2.5. Sperm Cryopreservation

Samples intended for cryopreservation were centrifuged in a Medifriger BL-S programmable refrigerated centrifuge (JP Selecta S.A., Barcelona, Spain) at 600× g and 20 °C for 15 min. Immediately after centrifugation, the supernatant was discarded using a vacuum pump and the sperm pellet was resuspended in a commercial extender containing cryoprotectants (methylformamide and glycerol; BotuCrio, Botupharma, Sweden). Sperm concentration, motility, and viability were again evaluated, and the same commercial extender was added until a final concentration of 200×10^6 viable spermatozoa per mL was reached. Samples were then loaded into 0.5 mL plastic straws and then sealed. Thereafter, straws were cooled and frozen using an Ice-Cube 14S programmable freezer (Minitüb Ibérica, S.L.; Tarragona, Spain) in three steps. First, semen was cooled from 20 °C to 5 °C for 60 min at a rate of −0.25 °C/min. In the second step, the temperature was reduced from 5 °C to −90 °C for 20 min, at a rate of −4.75 °C/min. Finally, the temperature decreased from −90 °C to −120 °C for 2.7 min, at a rate of −11.11 °C/min. Straws were immediately plunged into liquid nitrogen and stored in tanks until thawing and analysis. The thawing protocol consisted of incubating the straws at 37 °C for 30 s in a water bath followed by dilution with three volumes of prewarmed Kenney extender at 37 °C (final concentration: 50×10^6 spermatozoa/mL). After thawing, sperm motility and viability were evaluated at 10 min post-thaw.

2.6. Evaluation of Sperm Concentration

In order to determine sperm concentration, 10 μL of semen was placed in 990 μL buffered saline containing 2% formaldehyde. Three independent counts under a phase-contrast microscope (Olympus 200×; Europe, Hamburg, Germany), using a Neubauer chamber (Paul Marienfeld GmbH & Co. KG; Lauda-Königshofen, Germany), were made.

2.7. Evaluation of Sperm Motility

Sperm motility was evaluated, before and after cryopreservation, using a computer-assisted sperm motility analysis (CASA) system (ISAS 1.0, Proiser; Valencia, Spain) in a prewarmed Neubauer chamber under an Olympus BX41 phase-contrast microscope (Olympus 20× 0.30 PLAN objective; Olympus Europe, Hamburg, Germany) with a heated stage at 37 °C. At least 1000 spermatozoa were counted and the following motility parameters were evaluated: total (TMOT) and progressive sperm motility (PMOT), curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), amplitude of lateral head displacement (ALH, μm), beat cross frequency (BCF, Hz), linearity (LIN, %), straightness (STR, %), motility parameter wobble (WOB, %). Settings of the CASA system were: frame frequency = 25 Hz; cell size range = 4–75 μm^2; connectivity = 12. A spermatozoon was classified as motile when VAP >10 μm/s, and progressively motile when STR ≥75%.

2.8. Evaluation of Sperm Viability

Sperm viability was evaluated before and after cryopreservation through SYBR14 and propidium iodide (PI) using the Live/Dead Sperm Viability kit (Invitrogen Molecular Probes, Thermofisher; Waltham, Massachusetts, USA) and a flow cytometer (Cell Laboratory QuantaSC cytometer, Beckman Coulter; Fullerton, CA, USA). Samples, previously adjusted to 1×10^6 sperm/mL, were stained with SYBR14 (final concentration: 100 nM) at 38.5 °C for 10 min, and with PI (final concentration: 12 μM) at the same temperature for 5 min. Following this, spermatozoa were excited with an argon ion laser emitting at 488 nm and set at a power of 22 mW. The sheath flow rate was set at 4.17 μL/min. Sperm were selected on the basis of electronic volume (EV) and side scatter (SS), and the non-sperm specific events were gated out. The EV channel was periodically calibrated using 10 μm Flow-Check fluorospheres (Beckman Coulter, Brea, California, United States) by positioning this size bead in channel 200 on the EV scale. A total of 10,000 events were evaluated and two optical filters were used: FL1 (green fluorescence): Dichroic/Splitter, DRLP: 550 nm, BP filter: 525 nm; and FL3 (red fluorescence): LP filter: 670/730 nm. Signals were logarithmically amplified and photomultiplier settings were adjusted to

each staining method. FL1 was used to detect SYBR14-fluorescence and FL3 was used to detect PI-red fluorescence. Three sperm populations were identified: (i) viable spermatozoa ($SYBR14^+/PI^-$); (ii) non-viable spermatozoa showing red fluorescence ($SYBR14^-/PI^+$); and (iii) non-viable spermatozoa stained both green and red ($SYBR14^+/PI^+$). Non-sperm, debris particles were identified as non-stained either for SYBR14 or PI ($SYBR14^-/PI^-$). SYBR14 spillover into the PI channel was compensated (2.45%).

2.9. Statistical Analyses.

Data were analyzed with a statistical package (IBM SPSS for Windows Ver. 25.0; IBM Corp., Armonk, NY, USA) and are shown as mean ± standard error of the mean (SEM). First, data were checked for normality (Shapiro–Wilk test) and homoscedasticity (Levene test). Following this, ejaculates were classified into two freezability groups (GFE or PFE) based on their post-thaw sperm viability and total motility, using the likelihood distance and the Schwarz's Bayesian criterion (cluster analysis). Total and specific enzyme activities between these two groups were compared with a *t*-test for independent samples. Sperm motility and viability before and after freeze–thawing in GFE and PFE were compared through a linear mixed model (intrasubjects factor: before and after cryopreservation; intersubjects factor: GFE v.s. PFE) followed by post hoc Sidak's test. Pearson coefficient was used to calculate correlations between sperm parameters, and total and specific enzyme activities. The level of significance was set at $p \leq 0.05$.

3. Results

3.1. Classification of Ejaculates into Good (GFE) or Poor Freezability (PFE)

According to their post-thaw sperm viability and total motility, stallion ejaculates were classified as of good (GFE) or poor freezability (PFE). From the 16 ejaculates included in this study, 8 were classified as GFE and the other 8 were classified as PFE. Cut-off values for an ejaculate to be considered as GFE were found to be: %TMOT: 52.1%; % viable spermatozoa: 58.6%. Table 1 shows sperm parameters (as mean ± SEM) in fresh and frozen–thawed semen from GFE and PFE. Before cryopreservation, semen quality parameters including viability and progressive and total motilities did not differ between both groups. After thawing, viability, progressive and total motilities, and other kinetic parameters, such as VSL, VAP, LIN, WOB, and ALH, were lower ($p < 0.05$) in PFE than in GFE.

Table 1. Quality parameters (mean ± SEM) of fresh and frozen–thawed semen from good and poor freezability ejaculates ($n = 16$).

	Fresh		Frozen-Thawed	
	GFE	**PFE**	**GFE**	**PFE**
PMOT	45.2 ± 2.9	52.3 ± 1.2	35.1 ± 1.9 **	18.4 ± 1.0 **
TMOT	81.9 ± 3.8	85.6 ± 3.5	58.6 ± 2.1 **	42.0 ± 1.4 **
VCL	102.0 ± 4.1	96.2 ± 4.5	66.3 ± 1.5	63.8 ± 2.4
VSL	53.5 ± 1.7	52.9 ± 2.7	34.8 ± 1.2 *	28.0 ± 1.0 *
VAP	75.1 ± 4.6	67.5 ± 4.5	43.9 ± 2.0 **	35.2 ± 1.3 **
LIN	53.3 ± 3.0	55.0 ± 1.4	52.6 ± 0.7 **	44.0 ± 0.4 **
STR	73.5 ± 4.5	79.2 ± 1.0	79.7 ± 1.1	79.6 ± 0.7
WOB	73.4 ± 2.6	69.5 ± 1.9	66.0 ± 1.5 **	55.2 ± 0.1 **
ALH	2.8 ± 0.2 **	3.6 ± 0.1 **	2.7 ± 0.0 **	3.3 ± 0.1 **
BCF	10.0 ± 0.7 **	12.1 ± 0.5 **	11.7 ± 0.5	12.3 ± 0.3
Viability	79.0 ± 1.3	77.9 ± 1.8	60.0 ± 0.5**	51.35 ± 2.1 **

Abbreviations: GFE: good freezability ejaculates; PFE: poor freezability ejaculates; PMOT: progressive motility; TMOT: total motility; VCL: curvilinear velocity (μm/s); VSL: straight-line velocity (μm/s); VAP: average path velocity (μm/s); LIN: linearity coefficient (%); STR: straightness coefficient (%); WOB: wobble coefficient (%); ALH: lateral head displacement (μm); BCF: frequency of head displacement (Hz). *$p < 0.05$; **$p < 0.01$.

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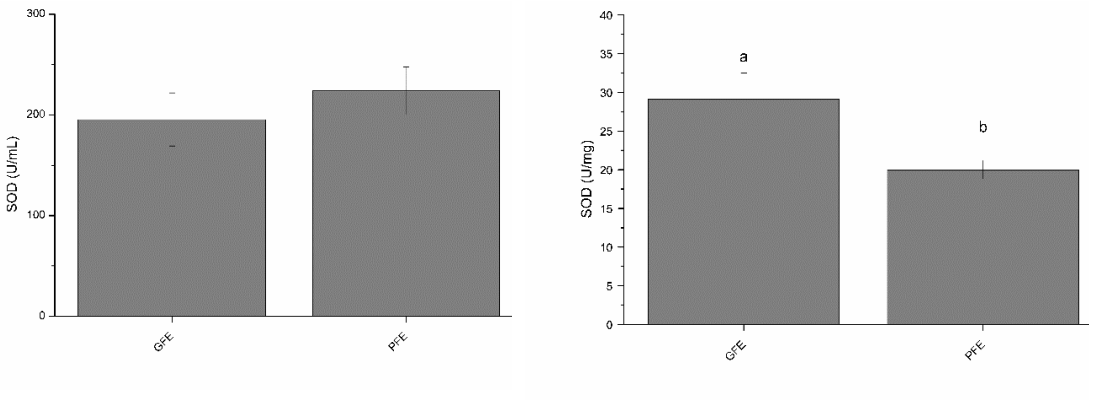
3.2. Activity of Superoxide Dismutase in Seminal Plasma of Good and Poor Freezability Ejaculates

Total SOD activity in the seminal plasma of GFE and PFE are shown in Figure 1a (as mean ± SEM). No significant differences were observed between GFE and PFE. However, as shown in Table 2, total SOD activity in seminal plasma negatively correlated ($p < 0.05$) with percentages of progressively motile spermatozoa after thawing. Figure 1b shows the specific SOD activities in GFE and PFE. GFE exhibited higher ($p < 0.05$) specific activity of SOD than PFE. Moreover, SOD specific activities were positively correlated ($p < 0.05$; Table 3) with sperm viability, total sperm motility, VSL, and LIN.



(**a**) (**b**)

Figure 1. Superoxide dismutase activities in stallion seminal plasma (mean ± SEM): (**a**) total activity; (**b**) specific activity. Different letters (a, b) mean significant ($p < 0.05$) differences between good (GFE) and poor freezability ejaculates (PFE).

Table 2. Pearson correlation coefficients between total enzyme activities and post-thawed sperm quality parameters ($n = 16$).

	SOD (U/mL)	GPX (U/L)	GSR (U/L)	CAT (U/L)
PMOT	−0.44 *	−0.04	−0.32	−0.17
TMOT	−0.22	−0.38	−0.09	−0.11
VCL	−0.32	−0.50 *	0.24	−0.11
VSL	−0.33	−0.44	0.02	−0.35
VAP	−0.37	−0.41	0.19	−0.23
LIN	−0.23	−0.22	−0.22	−0.48 *
STR	0.25	0.16	−0.81 **	−0.49 *
WOB	−0.31	−0.25	0.07	−0.29
ALH	0.18	−0.13	0.29	0.44
BCF	0.29	−0.15	−0.62 **	−0.32
Viability	−0.02	−0.30	−0.47 *	0.06

Abbreviations: GFE: good freezability ejaculates; PFE: poor freezability ejaculates; PMOT: progressive motility; TMOT: total motility; VCL: curvilinear velocity (μm/s); VSL: straight-line velocity (μm/s); VAP: average path velocity (μm/s); LIN: linearity coefficient (%); STR: straightness coefficient (%); WOB: wobble coefficient (%); ALH: lateral head displacement (μm); BCF: frequency of head displacement (Hz). *$p < 0.05$; **$p < 0.01$.

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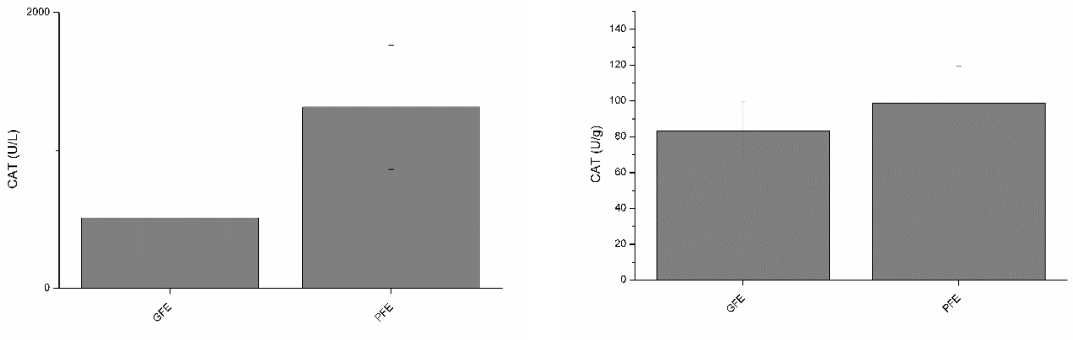
Table 3. Pearson correlation coefficients between specific enzyme activities and post-thawed sperm quality parameters ($n = 16$).

	SOD (U/mg)	GPX (U/g)	GSR (U/g)	CAT (U/g)
PMOT	0.05	0.33	0.38	0.13
TMOT	0.50*	0.08	0.49 *	0.29
VCL	0.18	−0.20	0.60 **	0.27
VSL	0.50 *	0.06	0.66 **	0.11
VAP	0.37	0.03	0.73 **	0.24
LIN	0.62 **	0.26	0.45	−0.12
STR	0.33	0.18	-0.67 **	−0.75 **
WOB	0.45	0.19	0.64 **	0.13
ALH	−0.40	−0.48 *	−0.19	0.26
BCF	0.36	−0.16	−0.50 *	−0.50 *
Viability	0.46 *	0.04	−0.02	0.23

Abbreviations: GFE: good freezability ejaculates; PFE: poor freezability ejaculates; PMOT: progressive motility; TMOT: total motility; VCL: curvilinear velocity (μm/s); VSL: straight-line velocity (μm/s); VAP: average path velocity (μm/s); LIN: linearity coefficient (%); STR: straightness coefficient (%); WOB: wobble coefficient (%); ALH: lateral head displacement (μm); BCF: frequency of head displacement (Hz). *$p < 0.05$; **$p < 0.01$.

3.3. Activity of Catalase in Seminal Plasma of Good and Poor Freezability Ejaculates

Neither total nor specific activities of CAT were found to differ between GFE and PFE (Figure 2a and b). However, total CAT activity was negatively ($p < 0.05$) correlated with percentages of LIN and STR at post-thaw (Table 2). Moreover, specific CAT activity was negatively correlated (Table 3) with percentages of STR ($p < 0.01$) and BCF ($p < 0.05$) at post-thaw.

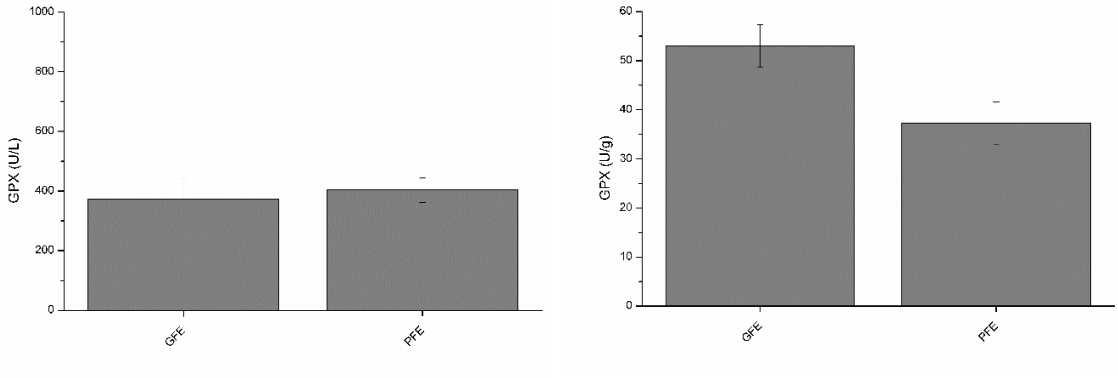


(**a**) (**b**)

Figure 2. Catalase activities in stallion seminal plasma (mean ± SEM): (**a**) total activity; (**b**) specific activity. No significant ($p > 0.05$) differences between good (GFE) and poor freezability ejaculates (PFE) were observed.

3.4. Activity of Glutathione Peroxidase in Seminal Plasma of Good and Poor Freezability Ejaculates

Total and specific activities of GPX are shown in Figure 3a and b, respectively. Although no significant differences between GFE and PFE were observed, total GPX activity was negatively ($p < 0.05$) correlated with post-thaw VCL (Table 2). Moreover, specific GPX activity was negatively ($p < 0.05$) correlated with post-thaw ALH (Table 3).

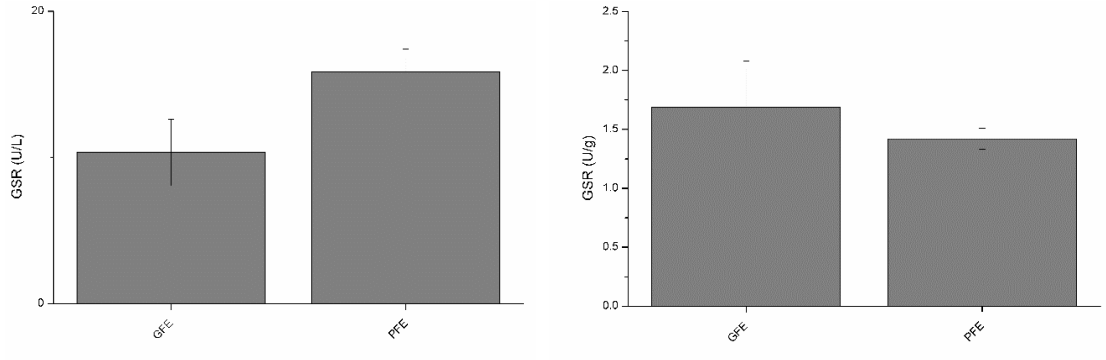


(**a**) (**b**)

Figure 3. Glutathione peroxidase activities in stallion seminal plasma (mean ± SEM): (**a**) total activity; (**b**) specific activity. No significant ($p > 0.05$) differences between good (GFE) and poor freezability ejaculates (PFE) were observed.

3.5. Activity of Glutathione Reductase in Seminal Plasma of Good and Poor Freezability Ejaculates

Total and specific activities of GSR are shown in Figure 4a,b, respectively. No significant differences were observed between GFE and PFE. However, total GSR activity was negatively ($p < 0.01$) correlated with percentages of viable spermatozoa, STR, and BCF at post-thaw (Table 2). Moreover, specific GSR activity (Table 3) was negatively correlated with post-thaw STR ($p < 0.01$) and BCF ($p < 0.05$). Specific GSR activity was also positively correlated with percentages of total motile spermatozoa ($p < 0.05$), VCL ($p < 0.01$), VSL ($p < 0.01$), VAP ($p < 0.01$), and WOB ($p < 0.01$) at post-thaw.



(**a**) (**b**)

Figure 4. Glutathione reductase activities in stallion seminal plasma (mean ± SEM): (**a**) total activity; (**b**) specific activity. No significant ($p > 0.05$) differences between good (GFE) and poor freezability ejaculates (PFE) were observed.

4. Discussion

This work was conducted to investigate whether total and specific activities of SOD, CAT, GPX, and GSR in seminal plasma were related to the ability of stallion sperm to withstand cryopreservation. We found that the specific activity of SOD was higher in GFE than in PFE.

Seminal plasma is the fluid, produced by the testis, the epididymis, and the male accessory glands, that accompanies sperm at ejaculation [20]. It provides a suitable environment for sperm, and also represents the most important source of antioxidants, enzymatic and non-enzymatic, able to remove the excess of reactive oxygen species inducing oxidative stress in semen. In that way, removal of seminal plasma prior to cryopreservation may be one of the reasons for the lower fertility of frozen–thawed

equine semen [21]. Differences in seminal plasma composition have been observed between and within individuals, and within ejaculates [21]. Equine seminal plasma contains a plethora of enzymes able to scavenge reactive oxygen species, in particular, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase/glutathione reductase (GPX/GSR), which are able to catabolize superoxide anion, hydrogen peroxide, and lipid peroxide. The main finding from this study is that, in stallions, the specific SOD activity of seminal plasma is higher in GFE than in PFE. In addition, the specific activity of that enzyme is positively and significantly correlated with post-thaw sperm viability. SOD plays a key role in cell protection against oxidative stress, as it catalyzes the dismutation of superoxide anion into hydrogen peroxide and molecular oxygen [22]. While, in stallion spermatozoa, hydrogen peroxide is usually considered to be the most detrimental ROS, there is, at present, some debate as to whether superoxides are also detrimental [23], since these species do not appear to be converted into hydrogen peroxide [24]. The relationship between SOD activity and sperm function and survival has been already investigated in human and livestock species. Barranco et al. observed that total activity of SOD in boar seminal plasma is positively related with total and progressive sperm motilities and hydrogen peroxide generation by viable spermatozoa, following 72 h of liquid storage at 17 °C [25]. In humans, the reduction of SOD activity in spermatozoa during cryopreservation has been related to lipid peroxidation and subsequent loss of motility after thawing [26]. Moreover, positive correlations have been found between SOD content and the ability of human sperm to withstand freezing and thawing [27]. Based on other studies [28], Buffone et al. assumed a close association between SOD content and SOD enzymatic activity and observed that post-thaw motility recovery was positively correlated with SOD content in mature spermatozoa [27]. Remarkably, in the same study, no significant correlation was found between SOD content in men seminal plasma and motility recovery of spermatozoa after thawing [27], which appears to not match with our results. Under this scenario, the antioxidant power of semen appears to differ between species. Therefore, we suggest that SOD antioxidant protection in human semen is mainly provided by mature spermatozoa rather than by seminal plasma as it occurs in stallions [21]. Moreover, and according to a previous study in stallions, high levels of SOD activity have been found in the ampulla and the prostate, although some activity has also been observed in the other accessory reproductive tissues [21]. Based on our findings, we can hypothesize that stallion semen with higher SOD content in seminal plasma better withstands oxidative stress during cryopreservation. Furthermore, since the activity of this enzyme was evaluated right after ejaculation and seminal plasma was removed prior to cryopreservation, we suggest that a short contact between sperm and seminal plasma proteins is enough to produce a beneficial effect on sperm cryosurvival. Finally, a previous work demonstrated that adding epididymal stallion spermatozoa with seminal plasma may increase the fertility of frozen–thawed semen [29]. Thus, we hypothesize that a quick interaction between sperm cells and seminal fluid is required to increase the sperm resilience to cryopreservation, but a prolonged contact may be detrimental for sperm storage [6].

Scavenging activity of SOD is completed by the activity of CAT, which reduces hydrogen peroxide into water and molecular oxygen [22]. In stallions, total and specific activities of CAT in seminal plasma have been shown to be high [14,30], although no correlation has been observed between total activity of CAT in stallion seminal plasma and kinematic sperm parameters [14]. Interestingly, several studies have demonstrated that the addition of CAT to semen prevents the adverse effects of oxidative stress on human [31], mouse [32], boar [33], and stallion [34] spermatozoa. These previous studies support the importance of CAT activity for ROS scavenging and the subsequent modulation of oxidative stress. However, it is worth mentioning that conflicting results have been reported regarding fertility. Indeed, while low total CAT activity has been associated with male infertility in humans [35], the total activity of this antioxidant enzyme has been negatively correlated with the fertility of Arabian horses [36]. Moreover, although previous research supports that total CAT activity in equine semen decreases after freeze–thawing [37], our study has found a negative correlation between total and specific CAT activities and post-thaw kinematic sperm parameters (LIN, STR, and BCF). These differences

warrant further research aimed at understanding the importance of the antioxidant system in different mammalian species.

Protection against ROS-induced damage and maintenance of sperm function and survival are also carried out by the glutathione system (GPX/GSR) [38]. The function of GPX is to reduce hydrogen peroxide to water [22]. Following freeze–thawing, a reduction in the total GPX activity in equine semen has been observed [37]. Furthermore, in Arabian horses, positive correlations of total GPX activity with quality and fertility of fresh equine ejaculate have been observed [36]. In our study, however, total GPX activity was negatively correlated with post-thaw VCL. While these data indicate that the functional role of GPX differs between fresh and frozen–thawed equine spermatozoa, it is worth mentioning that differences between species also exist. Indeed, the relative content of GPX1 and GPX5, which are enzymes that belong to the GPX family, has been found to be positively correlated with sperm cryotolerance, both in humans [38] and in boars [39].

GSR is also associated with antioxidant protection [40] and catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form of glutathione (GSH). In the present study, we have not observed a clear relationship between total and specific GSR activities and post-thaw sperm quality parameters, which makes the use of this enzyme as a freezability marker difficult. These results match with previous works in humans, in which the relative content of GSR does not appear to be affected during cryopreservation [38]. Although the main finding of this study is that the specific SOD activity in seminal plasma is related to sperm cryotolerance, the other three enzymes investigated (i.e., CAT, GPX, and GSR) were also correlated to some extent negatively or positively to post-thaw sperm motility. In this context, it is worth mentioning that a previous study from our group observed no correlation between SOD, CAT, GPX, and GSR activities in seminal plasma and fresh sperm parameters [38]. In spite of this, the relevance of these four enzymes matches with a previous study from Aurich et al. in which the addition of seminal plasma from GFE was found to improve the sperm resilience to cryopreservation, as it better maintained post-thaw sperm viability and progressive motility [6]. Consequently, the composition of seminal plasma, which appears to differ between GFE and PFE, influences the freezability of stallion spermatozoa. In this context, it is worth remembering that during freezing and thawing, spermatozoa are exposed to changes in osmotic pressure and variations in temperature, which induce oxidative stress and subsequent cryoinjuries [1]. Overproduction of ROS leads to a decrease in survival, acrosome integrity, motility, and fertilizing ability of equine sperm cells [41,42]. In addition, Yeste et al. determined that ROS levels in frozen–thawed stallion spermatozoa differ between GFE and PFE [43]. All these data support the great importance of antioxidant enzymes such as CAT, GPX, GSR, and especially SOD, as its specific activity differs between GFE and PFE, in the correct balance between the radical-generating and radical-scavenging potential. Our results also suggest that the relationship between seminal plasma and the way that sperm cells handle oxidative stress is due, at least in part, to the activity of these four enzymes.

Finally, this study supports the use of specific SOD activity in seminal plasma as a sperm freezability marker in stallions. In this context, it is worth keeping in mind that looking for markers that predict the ability of the ejaculate to be cryopreserved represents a challenge not only in equine but also in other species. In boar seminal plasma, proteins such as non-heparin-binding spermadhesin (PSP-II) or lipocalin enzyme (L-PGDS) have been identified as potential modulators of sperm freezability [44]. In the horse, Bucci et al. observed that the activity of alkaline phosphatase in seminal plasma is positively correlated with post-thaw sperm viability [45]. Herein, we have identified that the specific SOD activity in seminal plasma may also be used as a freezability marker, as it differs between GFE and PFE.

5. Conclusions

In summary, the results obtained in this study support the hypothesis that the interaction of seminal plasma components with stallion sperm impacts their freezability. The present study also evidences that the specific activity of SOD in stallion seminal plasma is positively correlated with sperm

ability to withstand cryopreservation. Further research to determine whether specific SOD activity varies between ejaculates of the same individual is warranted, since this could address if specific SOD activity in seminal plasma could be useful to classify a given stallion as a 'good' or 'bad' freezer.

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Total and specific activity of superoxide dismutase in seminal plasma is related to the cryotolerance of donkey spermatozoa

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Cryobiology
Under review

Abstract

This study investigated the activity of antioxidant enzyme systems in seminal plasma (SP), as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase plus reductase system (GPX/GSR), are related to the ability of donkey sperm to withstand cryopreservation. Eighteen ejaculates from 16 healthy donkeys were collected and split into two aliquots. The first one was centrifuged (3,000×g, 4ºC for 10 min) and used to determine the activities of these four enzymes in SP, whereas the other was diluted in a skim-milk extender and then cryopreserved. Assessment of sperm motility and viability was performed before and after cryopreservation. Based on the percentages of total motile and viable spermatozoa at post-thaw, samples were classified as good (GFE) or poor (PFE) freezability ejaculates through cluster analyses. Total and specific activities of SOD in SP were higher ($P<0.05$) in GFE than in PFE, whereas no significant differences between GFE and PFE were observed regarding total and specific activities of CAT, GPX and GSR. However, post-thaw sperm parameters were positively correlated with total and specific activities of CAT and negatively correlated with those of GSR. In conclusion, determination of SOD activity in donkey SP may predict the ability of a given jackass ejaculate to withstand cryopreservation.

Keywords: donkey; seminal plasma; antioxidant enzymes; cryopreservation

1. Introduction

In the last few decades, donkeys (*Equus asinus*) have been rediscovered in an attempt to protect biodiversity and develop marginal agricultural areas [14]. In addition, the production of donkey milk intended for human consumption has increased in the last years, as it represents an interesting alternative for children suffering from specific diseases, such as allergy to cow milk [28]. Together with this, the anti-ageing properties of donkey milk make it an ideal constituent for cosmetic products [14]. Therefore, improving breeding management in donkeys, which includes the use of assisted reproductive technologies, such as artificial insemination (AI) and semen preservation, is crucial for genetic selection and conservation.

In modern livestock breeding programs, conducting AI with cryopreserved semen is growing, as it allows for maximizing the use of genetically valuable animals [42]. However, AI with frozen-thawed sperm is known to result in a decreased reproductive performance when compared to fresh semen [47], especially in the case of jackasses [43,17]. Indeed, frozen-thawed spermatozoa exhibit lower viability and sublethal dysfunction as a consequence of cold shock, osmotic stress, and oxidative damage that occur during cryopreservation [50]. In addition, in both the mare and the jenny, the inflammatory response of the endometrium to frozen-thawed sperm is more severe than that observed with the raw/fresh ejaculate [22,44,30]. Related with this, it has been observed that while previous addition of jackass frozen-thawed sperm with seminal plasma has no impact upon sperm quality parameters, such as motility, plasma membrane and DNA integrity [41], it does improve conception rates in jennies [40].

Seminal plasma is the heterogeneous fluid to which spermatozoa are mixed upon ejaculation. Secretion, volume and composition of this complex mixture depend on the accessory glands situated along the male genital tract [26]. Although removal of seminal plasma by centrifugation is a common step before cryopreservation [4], seminal plasma represents the most important source of antioxidant systems in mammalian semen [32,9]. Indeed, seminal plasma is endowed with many enzymes scavengers that remove the excess of

reactive oxygen species (ROS). An excess of ROS generation leads to lipid peroxidation of sperm plasma membrane and DNA damage [2]. Since cryopreservation procedure leads to dysfunction of mitochondria and subsequent overproduction of ROS [35], a correct balance between ROS and antioxidants is crucial to avoid internal cell disturbances, and therefore removal of seminal plasma during semen processing may increase the sperm susceptibility to ROS-induced damages.

In stallions and jackasses, enzymatic scavengers are mostly provided by seminal plasma and include catalase (CAT), superoxide dismutase (SOD), and the system glutathione peroxidase – glutathione reductase (GPX/GSR) [8,34]. However, wide disparities in the composition of seminal plasma as well as in the sperm resilience to withstand freezing and thawing exist between species and individuals, and even between ejaculates from the same animal [26,31]. Freezability or cryotolerance is defined as the sperm capacity to withstand cryopreservation procedures [49], which makes ejaculates to be classified as 'good freezability ejaculates' (GFE) or 'poor freezability ejaculates' (PFE) [50,15]. Related with this, while specific seminal plasma proteins have been found to be related with ejaculate freezability in stallions and boars [20,37], it is yet to be determined whether total and specific activities of the most important antioxidant enzymes in seminal plasma (CAT, SOD, GPX and GSR) are related to sperm cryotolerance in the donkey.

Against this background, the aims of this work were: 1) to determine the putative relationship between activities of antioxidant enzymes in jackass seminal plasma and frozen-thawed sperm quality parameters; and 2) to investigate whether total and specific activities of antioxidant enzymes in seminal plasma may predict sperm cryotolerance in jackasses.

2. Materials and Methods

2.1. Collection of semen samples

All animals involved in the study were housed at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain), an EU-approved equine semen collection center (authorization number: ES09RS01E). This center operates under strict protocols of animal welfare and health control. All jackasses were semen donors housed at that center and were collected under CEE health conditions (free of Equine Arteritis, Infectious Anemia and Contagious Metritis). Since this Service already runs under the approval of the Regional Government of Catalonia (Spain) and because no manipulation to the animals other than semen collection was carried out, the Ethics committee of our institution indicated that no further ethical approval was required.

A total of 16 healthy Catalan and Catalan crossbreed jackasses, aged from 3 to 11 years old and with proven fertility, were involved in this study. All animals received a standard diet (with mixed hay and basic concentrate) and were provided with water ad libitum. They underwent regular semen collection, every other day during the week, under the same conditions. Samples were collected throughout the year. A total of 18 ejaculates (four ejaculates came from two jackasses, and the other 14 came from one jackass each) were collected using a pre-warmed artificial vagina Hannover model (Minitüb Ibérica, S.L.; Tarragona, Spain). Gelatinous fraction was eliminated through an in-line nylon filter. After removing 5.0 mL sample of each raw ejaculate to obtain seminal plasma (see section 2.2), semen was diluted 1:5 (v:v) in a pre-warmed (37ºC) skim-milk extender (4.9% glucose, 2.4% skim milk, 100 mL double distilled water). Sperm quality of each sample was evaluated immediately after dilution. The parameters assessed included sperm concentration, viability, and motility.

2.2. Collection of seminal plasma

Seminal plasma was harvested immediately after semen collection by several centrifugations of the ejaculate at 3,000×g and 4ºC for 10 min (JP Selecta S.A., Barcelona, Spain). The supernatant was examined under a phase-contrast microscope at 200× (Olympus Europe; Hamburg, Germany) to ensure that samples were sperm-free. The number of cycles of centrifugation ranged between five and seven, depending on the ejaculate. Then, 2-mL aliquots of seminal plasma were stored in at -80ºC. Previous to determination of enzyme activities, seminal plasma samples were places into ice to be thawed at 4ºC.

2.3. Enzyme activities assessment

Total protein amount and the activity of four antioxidant enzymes (superoxide dismutase, SOD; glutathione peroxidase, GPX; glutathione reductase, GSR; and catalase, CAT) were assessed in each seminal plasma sample.

Total protein was determined with Total Protein Reagent for Olympus System (Beckman Coulter, Hamburg, Germany), which results from the reaction of cupric ions in an alkaline solution. This solution reacts with peptide protein bonds and acquires a violet color. A calibration curve was built in parallel with bovine serum albumin. The absorbance of the complex at 540 nm is directly proportional to the concentration of total protein in the sample.

Total activities of SOD, GPX and GSR in seminal plasma were determined using commercial available kits (Randox Laboratories Ltd, Crumlin, UK) following the instructions of the manufacturer and adapted to Olympus AU400 system (Beckman Coulter, Hamburg, Germany). The activities of GPX was measured by a Ransel kit, which is linked to the oxidation of NADPH in the presence of Cumene Hydroperoxide and. GSR (Glut Red kit) was determined by the oxidation of NADPH. SOD (Ransod kit) was measured based on the xanthine oxidase method.

Activity of CAT was estimated by the method of Maehly and Chance [24]. The enzyme catabolizes hydrogen peroxide, which can be measured directly

using spectrophotometry (Biochrom Ltd, Holliston, MA, USA), through the decrease of the absorbance at 240 nm for 30 seconds. With this purpose, a 20-µL aliquot of seminal plasma was diluted in 830 µL of phosphate buffer (pH=7.2). Based on the following formula, enzyme activity was calculated [1]:

CATactivity (U/mL) = (initial absorbance-final absorbance)/0.0394 L/ cm*µmol (molar extinction coefficient of H2O2) x dilution factor.

The activity of each enzyme was normalized with the amount of total protein in seminal plasma samples in order to determine the specific activity, and indirectly, the purity of the enzymes [36].

2.4. Sperm cryopreservation

The method used for freezing and thawing was that described by Yeste et al. [50]. Briefly, after assessing sperm concentration, motility and viability, semen samples were centrifuged at 600×g and 20ºC for 15 min using a Medifriger BL-S programmable refrigerated centrifuge (JP Selecta S.A., Barcelona, Spain) and the supernatant was removed using a vacuum pump. The pellet was extended with a cryoprotectant including a combination of methylformamide and glycerol (BotuCrio, Botupharma, Sweden). Sperm concentration, motility and viability of the resuspended pellet were re-evaluated. Samples were diluted to a final concentration of 200×106 viable spermatozoa per mL, and then packaged into 0.5 mL plastic straws using an automatic filling and sealing machine. Once the semen was loaded, the cooling/freezing curve was performed in three stages, using an Ice-Cube 14S programmable freezer (Minitüb). The first stage consisted of cooling from 20ºC to 5ºC for 60 min at a rate of -0.25°C/min. During the second stage, straws were frozen from 5ºC to -90°C for 20 min, at a rate of -4.75°C/min. Finally, the last stage consisted of freezing from -90°C to -120 °C for 2.7 min, at a rate of -11.11°C/min. Straws were placed directly into liquid nitrogen and stored in tanks until thawing and analysis. Straws were thawed in a water bath at 37ºC for 30 seconds and diluted with three volumes of pre-warmed Kenney extender at 37ºC (final concentration: 50x106 spz/mL). After 10 minutes of incubation, sperm parameters were evaluated.

2.5. Evaluation of sperm concentration

Ten µL of non-extended semen, from each ejaculate, were placed in a 990 µL buffered saline, 2% formaldehyde solution. Sperm concentration count was performed in triplicate in a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany), using a phase-contrast microscope at 200× magnification (Olympus).

2.6. Evaluation of sperm motility

Total and progressive sperm motility and kinematic parameters were evaluated before (diluted semen) and after cryopreservation. In all cases, spermatozoa were incubated at 37ºC for 15 min prior to assessment. A computer-assisted sperm motility analysis (CASA) system (ISAS: Proiser, Valencia, Spain) was used, and settings were those described by Miró et al. [29] for Catalonian jackass semen. Briefly, 5 µL of diluted semen was placed into a pre-warmed (37ºC) Neubauer chamber under a phase-contrast microscope (Olympus 20× 0.30 PLAN objective). For each evaluation, 1,000 cells spermatozoa were, at least, counted. Evaluation of sperm motility included the following parameters, total motility (TMOT; %), progressive motility (PMOT; %), curvilinear velocity (VCL; µm/s), average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), amplitude of lateral head displacement (ALH; µm), beat cross frequency (BCF; Hz), linearity index (LIN=VSL/VCL; %), straightness index (STR=VSL/VAP; %), and oscillation index (WOB=VAP/VCL; %).The CASA setting was the following: frame acquired 25 Hz; particles size: 4 to 75 µm2; connectivity: 12. Spermatozoa with a mean average path velocity (VAP) < 15 µm/s were considered as immotile. Progressive motile spermatozoa were those that exhibited STR>75%.

2.7. Evaluation of sperm viability

Before and after cryopreservation, sperm viability was determined through SYBR14 and propidium iodide (PI) using the Live/Dead Sperm Viability kit

(Invitrogen Molecular Probes, Thermofisher; Waltham, Massachusetts, USA) and a flow cytometer (QuantaSC cytometer, Beckman Coulter; Fullerton, CA, USA). First, samples were adjusted to 1×106 spermatozoa/mL. Following this, sperm sample were incubated with SYBR14 (final concentration: 100 nM) at 38.5ºC for 10 min, and with PI (final concentration: 12 µM) at the same temperature for 5 min. Whereupon, samples were excited with an argon ion laser emitting at 488 nm and set at a power of 22 mW. The sheath flow rate was set at 4.17 µL/min. Selection of spermatozoa was based on electronic volume (EV) and side scatter (SS), and the non-sperm specific events were gated out. Periodically, the EV channel was calibrated using 10-µm Flow-Check fluorospheres (Beckman Coulter) by positioning this size bead in channel 200 of the EV scale. Evaluation of 10,000 events, in total, was performed and two optical filters were used: FL1 with green fluorescence: Dichroic/Splitter, DRLP: 550 nm, BP filter: 525 nm; and FL3 with red fluorescence: LP filter: 670/730 nm. Signals were logarithmically amplified, and photomultiplier settings were adjusted to each staining method. SYBR14-fluorescence was detected by FL1 and PI-red fluorescence by FL3. SYBR14-spill over into the PI-channel was compensated (2.45%). Three sperm populations were identified in dot-plots: (i) viable spermatozoa (SYBR14+/PI-); (ii) non-viable spermatozoa displaying red fluorescence (SYBR14-/PI+); and (iii) non-viable spermatozoa shown both green and red fluorescence (SYBR14+/PI+). Non-stained events for either SYBR14 or PI (SYBR14-/PI-) were identified as non-sperm/debris particles.

2.8. Experimental procedures

For each ejaculate, sperm motility and viability were evaluated before and after cryopreservation. Determination of total and specific activities of SOD, CAT, GPX and GSR in seminal plasma of each ejaculate was conducted separately. Based on post-thaw total sperm motility and viability, ejaculates were classified into two groups of freezability (GFE and PFE) through cluster analyses.

2.9. Statistical analyses.

Results were analyzed with a statistical package (IBM SPSS for Windows Ver. 25.0; IBM Corp., Armonk, NY, USA) and are shown as mean ± standard error of the mean (SEM). Data were checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene test). Ejaculates were then classified into two freezability groups (GFE or PFE) based on their post-thaw sperm viability and total motility, using the likelihood distance and the Schwarz's Bayesian criterion. Total and specific activities of SOD, CAT, GPX and GSR in seminal plasma were compared between GFE and PFE with a t-test for independent samples. Total and progressive sperm motilities, kinematic parameters (VCL, VAP, VSL, LIN, STR, WOB, BCF and ALH) and sperm viability before and after freeze-thawing in GFE and PFE were compared through a linear mixed model (intra-subjects factor: before and after cryopreservation; inter-subjects factor: GFE vs. PFE). Post-hoc Sidak's test was used for pair-wise comparisons. Pearson coefficient was used to calculate correlations between post-thaw sperm parameters and total and specific enzyme activities in seminal plasma. The level of significance was set at $P \leq 0.05$.

3. Results

3.1. Classification of ejaculates into good (GFE) or poor freezability (PFE)

Jackass ejaculates were divided into two freezability groups according to their post-thaw total sperm motility and viability. Following cluster analysis, GFE resulted to be those that had total motile spermatozoa > 56.3% and sperm viability > 58.9 %. From the total of 18 ejaculates involved in this study, 10 and 8 ejaculates were classified as GFE and PFE, respectively. Sperm quality parameters of fresh and frozen-thawed semen from these two groups are shown in Table 1 (as mean ± SEM). Before cryopreservation, no sperm parameter differed between GFE and PFE. After thawing, and as expected, total sperm motility, specific kinetic parameters, such as LIN and ALH, and sperm viability were significantly ($P<0.05$) lower in PFE than in GFE.

Table 1. Quality parameters (mean ± SEM) of fresh and frozen-thawed jackass semen from good and poor freezability ejaculates (*n*=18).

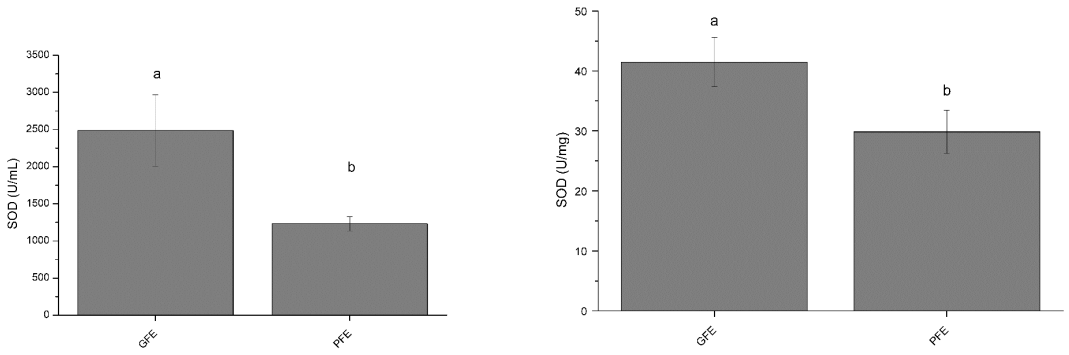
	Fresh		**Frozen-Thawed**	
	GFE	PFE	GFE	PFE
PMOT	43.1±5.0	34.0±4.3	22.5±3.3	15.7±2.0
TMOT	92.4±2.7	89.6±2.6	58.1±0.8**	36.8±2.6**
VCL	102.0±6.6	117.6±11.3	54.9±2.7	53.8±4.7
VSL	60.8±5.6	62.6±4.3	29.9±1.8	34.6±4.2
VAP	81.6±6.7	90.6±7.4	37.4±1.9	41.2±5.1
LIN	59.0±3.0	55.3±3.9	54.3±0.8**	63.1±3.5**
STR	74.0±2.1	70.4±3.3	79.9±2.1	84.0±1.5
WOB	79.3±2.2	77.9±1.9	68.3±1.7	74.9±3.4
ALH	3.3±0.2	4.2±0.4	2.7±0.1**	2.2±0.1**
BCF	7.1±0.9	7.6±0.8	11.4±0.8	10.7±0.6
Viability	93.0±2.3	92.7±2.4	60.4±2.1**	40.2±1.7**

Abbreviations: GFE: good freezability ejaculate; PFE: poor freezability ejaculate; PMOT: progressive motility; TMOT: total motility; VCL: sperm curvilinear velocity; VSL: sperm linear velocity; VAP: mean velocity; LIN: linear coefficient; STR: straightness coefficient; WOB: wobble coefficient; ALH: mean lateral head displacement; BCF: frequency of head displacement. *P < 0.05; **P < 0.01.

3.2. Activity of superoxide dismutase in seminal plasma of good and poor freezability ejaculates

Total activities of SOD in the seminal plasma of GFE and PFE are shown in Figure 1.a (as mean ± SEM). Total SOD activity in seminal plasma was significantly higher (P<0.05) in GFE than in PFE. Moreover, as shown in Table 2, total activity of SOD in seminal plasma was positively correlated (P<0.01) with post-thaw BCF and negatively correlated (P<0.05) with post-thaw motility parameter WOB.

Figure 1.b (mean ± SEM) shows the specific activities of SOD in GFE and PFE. GFE showed significantly (P<0.05) higher specific activity of SOD in their seminal plasma than PFE. Moreover, SOD specific activity in seminal plasma was positively correlated (P<0.05) (Table 3) with the percentages of viable spermatozoa.



(a) (**b**)

Figure 1. Superoxide dismutase activities in jackass seminal plasma (mean ± SEM): (**a**) Total activity; (**b**) Specific activity. Different letters (a, b) mean significant ($P < 0.05$) differences between GFE and PFE.

Table 2. Correlation coefficients between enzyme activities and post-thawed jackass sperm quality parameters (n=18).

	SOD (U/mL)	**GPX (U/L)**	**GSR (U/L)**	**CAT (U/L)**
PMOT	0.30	0.20	-0.13	0.10
TMOT	0.36	0.20	-0.33	0.01
VCL	-0.03	-0.02	-0.47*	-0.11
VSL	-0.15	-0.04	-0.39	0.04
VAP	-0.25	-0.15	-0.51*	-0.12
LIN	-0.23	-0.04	-0.10	0.32
STR	0.38	0.40	0.34	0.58*
WOB	-0.53*	-0.32	-0.37	-0.30
ALH	0.13	-0.13	-0.29	-0.39
BCF	0.67**	0.46	0.45	0.63**
Viability	0.42	0.16	-0.38	0.13

Abbreviations: SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase; GSR: glutathione reductase; PMOT: progressive motility; TMOT: total motility; VCL: sperm curvilinear velocity; VSL: sperm linear velocity; VAP: mean velocity; LIN: linear coefficient; STR: straightness coefficient; WOB: wobble coefficient; ALH: mean lateral head displacement; BCF: frequency of head displacement. *P < 0.05; **P < 0.01.

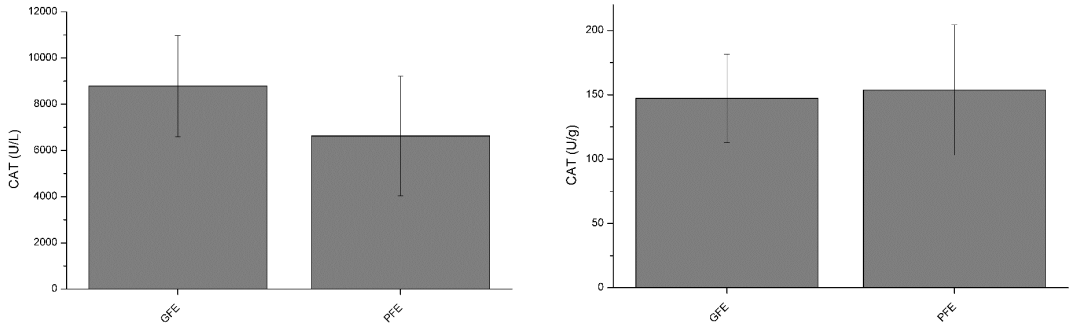
Table 3. Correlation coefficients between specific enzyme activities and post-thawed jackass sperm quality parameters (*n*=18).

	SOD (U/mg)	GPX (U/g)	GRS (U/g)	CAT (U/g)
PMOT	0.35	-0.47	-0.34	0.06
TMOT	0.46	0.26	0.05	-0.01
VCL	0.36	0.29	-0.14	-0.08
VSL	0.18	0.28	-0.16	0.05
VAP	0.16	0.36	0.01	-0.08
LIN	-0.10	0.13	-0.11	0.32
STR	0.18	-0.24	-0.67**	0.47
WOB	-0.22	0.33	0.33	0.05
ALH	0.22	-0.01	0.16	-0.38
BCF	0.43	-0.29	-0.50*	0.56*
Viability	0.50*	0.22	-0.02	0.05

Abbreviations: SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase; GSR: glutathione reductase; PMOT: progressive motility; TMOT: total motility; VCL: sperm curvilinear velocity; VSL: sperm linear velocity; VAP: mean velocity; LIN: linear coefficient; STR: straightness coefficient; WOB: wobble coefficient; ALH: mean lateral head displacement; BCF: frequency of head displacement. *P < 0.05; **P < 0.01.

3.3. Activity of catalase in seminal plasma of good and poor freezability ejaculates

Total and specific activities of CAT in the seminal plasma of GFE and PFE are shown in Figures 1 and 2 (as mean ± SEM). No significant differences (P>0.05) between GFE and PFE were observed. However, total CAT activity in seminal plasma was positively correlated with percentages of STR (P<0.01) and BCF (P<0.05) after thawing (Table 2). Similarly, specific CAT activity in seminal plasma was positively correlated with percentages of beat cross frequency (BCF) (P<0.05) at post-thaw (Table 3).

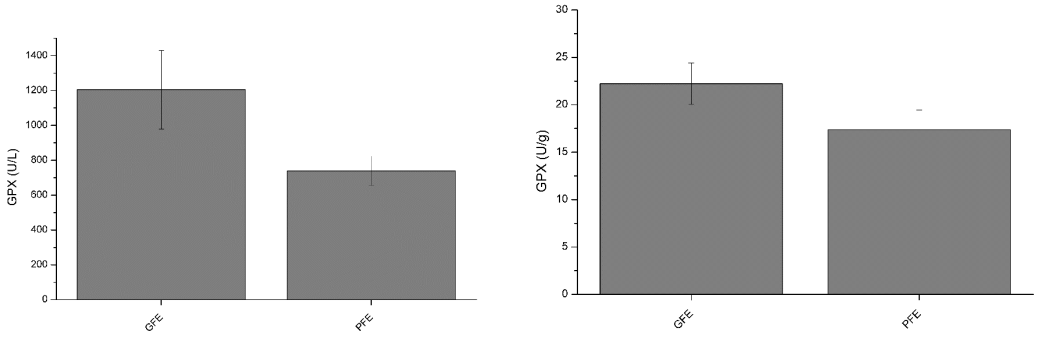


(a) (**b**)

Figure 2. Catalase activities in jackass seminal plasma (mean ± SEM): (**a**) Total activity; (**b**) Specific activity. No statistical differences were observed.

3.4. Activity of glutathione peroxidase in seminal plasma of good and poor freezability ejaculates

Neither total nor specific activities of GPX in seminal plasma differed between GFE and PFE (Figs. 1 and 2). Furthermore, no correlation between total and specific GPX activities and post-thaw sperm quality parameters was found (Tables 2 and 3).

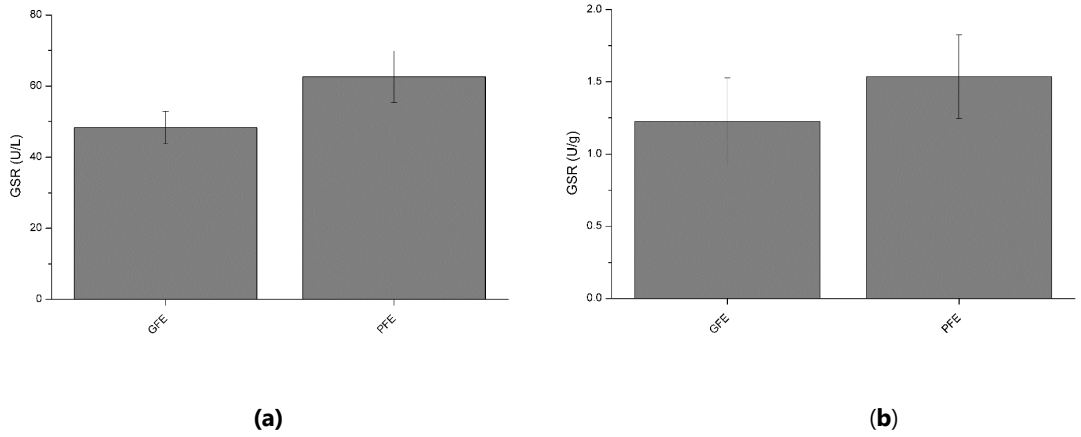


(a) (**b**)

Figure 3. Glutathione peroxidase activities in jackass seminal plasma (mean ± SEM): (**a**) Total activity; (**b**) Specific activity. No statistical differences were observed.

3.5. Activity of glutathione reductase in seminal plasma of good and poor freezability ejaculates

Total and specific activities of GSR in the seminal plasma of GFE and PFE (as mean ± SEM). are shown in Figs. 1 and 2, respectively in a similar fashion to CAT and GPX, no significant differences between GFE and PFE were observed. Nevertheless, total GSR activity was negatively correlated ($P<0.05$) with post-thaw VCL and VAP (Table 2). Moreover, specific GSR activity was negatively correlated with post-thaw percentages of STR ($P<0.01$) and BCF ($P<0.05$) (Table 3).



(a) (**b**)

Figure 4. Glutathione reductase activities in jackass seminal plasma (mean ± SEM): (**a**) Total activity; (**b**) Specific activity. No statistical differences were observed.

4. Discussion

The present study evidences that total and specific activities of SOD, CAT and GSR in seminal plasma are correlated with specific kinematic parameters of frozen-thawed jackass spermatozoa. The main finding, however, is that total and specific activities of SOD in jackass seminal plasma are positively correlated with sperm cryotolerance.

Cryopreservation is a practical approach for long-term sperm storage and allows setting germplasm banks for subsequent genetic improvement. However, this technology inflicts damage on sperm cells, due to cold shock,

osmotic stress, formation of intracellular ice crystals, and over production of ROS [47,37,51]. As a result, motility, viability and DNA integrity of frozen-thawed spermatozoa are lower than that of fresh semen. Unfortunately, conventional spermiogram parameters of raw/diluted semen are not usually indicative of the sperm resilience to withstand cryopreservation, i.e. post-thaw sperm quality [16].

Because of the little content of cytoplasm in spermatozoa, seminal plasma is known to be the main defense against ROS-induced damage on sperm cells [5]. Antioxidant defense in seminal plasma relies on enzymatic and non-enzymatic components. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and the glutathione peroxidase – glutathione reductase system (GPX/GSR) [8,34]. SOD converts superoxide anion into hydrogen peroxide, which is catabolized into water and oxygen by CAT [19]. GPX reduces hydrogen peroxide into two molecules of water, in the presence of reduced glutathione, which oxidized form can be regenerated by GSR. These four antioxidant enzymes are present in the seminal plasma [34], however, not only do the composition and volume of seminal plasma differ between species, but differences also exist between individuals from the same species and even between ejaculates from the same animal [25,38].

To the best of our knowledge, this is the first report addressing the relationship between the activities of the most important antioxidant enzymes in jackass seminal plasma (SOD, CAT, GPX and GSR) and the sperm recovery after freeze-thawing. Interestingly, our results demonstrate that specific activity of SOD in seminal plasma is positively correlated with post-thaw sperm viability. Moreover, total and specific activity of SOD in seminal plasma appear to be higher in GFE than in PFE. From these considerations, it is reasonable to suggest that the resilience of jackass sperm to cryodamage may be partially determined by the high activity of SOD in seminal plasma and that the quick contact of spermatozoa with seminal plasma before its removal is enough to exert a positive impact upon sperm cells. In human, protein expression of SOD in seminal plasma, including CuZnSOD and MnSOD, has been positively related with quality parameters of fresh semen [33]. In addition, Buffone et al. also

observed that relative levels of SOD in human semen are significantly and positively correlated with post-thaw sperm motility recovery, although, in that case, SOD content was not only determined in seminal plasma but also in spermatozoa [13]. These results indicated that human ejaculates with higher SOD content better withstand freezing and thawing procedures. In livestock sires, has been related to sperm quality parameters. Indeed, in boar seminal plasma total SOD activity has been positively correlated with fresh sperm motility and H2O2 generation in viable spermatozoa [10], and with cryotolerance [23]. Furthermore, addition of SOD to cryopreservation medium has been shown to improve significantly post-thaw sperm survival in pigs [39]. In buffalo-bulls, influence of total SOD activity in seminal plasma on functional competence of cryopreserved spermatozoa has also been observed [46].

The relevance of CAT as a ROS scavenger has been described in several mammalian species. Indeed, supplementation of semen with CAT counteracts detrimental effects of oxidative stress in human [3], mouse [7], stallion [11], and boar spermatozoa [18]. Moreover, addition of CAT to the freezing medium also improves post-thaw sperm motility and viability and reduces ROS generation in boars [39]. In the donkey, total and specific activities of CAT in the seminal plasma from raw ejaculates have been shown to be positively correlated with percentages of LIN and STR [34]. In this context, it was not surprising that, in this study, positive correlations between CAT activities and post-thaw sperm kinematic parameters (STR and BCF) were observed. However, it is also worth mentioning that, controversially, total activity of CAT has been negatively correlated with the fertilizing ability of Arabian horse spermatozoa [45]. Further research is thus warranted to address whether the addition of CAT to frozen-thawed jackass spermatozoa has any impact on their reproductive performance.

Differences between species exist regarding to the functional role of GPX. In effect, while kinetic sperm parameters are negatively correlated to GPX activity of ram spermatozoa and seminal plasma [21], GPX activity in seminal plasma is positively correlated with the quality and fertilizing ability of fresh Arabian horses spermatozoa [45]. On the other hand, sperm cryotolerance in humans and boars has been positively correlated with the relative content of

GPX1 [27] and GPX5 [23], respectively, which are two enzymes that belong to the GPX family. A previous study in jackasses has demonstrated that neither total nor specific GPX activities are correlated with sperm parameters in fresh semen [34]. Matching with these previous findings, in the current study we observed that total and specific activities of GPX in seminal plasma are not correlated with post-thaw sperm quality parameters. Therefore, not only does GPX appear to play a marginal role in the antioxidant system of jackass semen, but the lack of differences between GFE and PFE indicates that it cannot be used as a sperm cryotolerance marker.

GPX activity is strongly linked to that of GSR. In humans, the GPX/GSR system represents an effective antioxidant system in normal spermatozoa [48] and GSR activity does not seem to be affected by cryopreservation [27]. With regard to jackasses, it is worth mentioning that while total GSR activity in seminal plasma has been found to be positively correlated with kinetic parameters of fresh semen [34], this activity is negatively correlated with VCL, VAP, STR and BCF of frozen-thawed spermatozoa. Further research is required to address which the role of GSR on regulating sperm motility is, both in fresh and frozen-thawed sperm.

Finally, individual variability in the sperm resilience to withstand cryopreservation has already been described in jackasses [16]. In horses, which are genetically closed to donkeys, Aurich et al. observed that supplementing PFE ejaculates with seminal plasma from GFE improves the sperm ability to withstand cryopreservation [12]. Although differences in the composition of seminal plasma of jackasses and stallions have been described [34,25], our results suggest that total and specific activities of GPX, CAT, and, especially, SOD have an influence in the capacity of spermatozoa to tolerate the cryopreservation process. Related with this, significant efforts have been made to identify ejaculate freezability markers, also in seminal plasma. In effect, the activities of alkaline phosphatase in the seminal plasma of stallions [12] and those of non-heparin-binding spermadhesin (PSP-II) and lipocalin enzyme (L-PGDS) in that of boars [37] have been identified as sperm freezability markers. Since, as aforementioned, our study also supports that total and specific SOD

activities in seminal plasma may be used as a cryotolerance marker for jackass sperm, further research addressing the relationship between these four antioxidant enzymes and sperm cryotolerance in other species is warranted.

5. Conclusions

In summary, the current study has confirmed, for the first time, that seminal plasma components with antioxidant properties are related with sperm cryotolerance in jackasses. Concretely, we found that total and specific activities of SOD in seminal plasma are higher in GFE than in PFE. Therefore, we suggest that that total and specific activities of SOD in seminal plasma may be used as cryotolerance markers for jackass sperm.

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Seminal plasma has limited counteracting effects following induction of oxidative stress in donkey spermatozoa

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Effects of single layer centrifugation through EquicollTM on donkey fresh spermatozoa and on its interaction with polymorphonuclear cells

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Animal Reproduction Science
To be submitted

Abstract

Prior to conducting assisted reproduction techniques, ejaculates are first processed to select spermatozoa with higher motility, intact plasma membrane and acrosome, normal morphology and lower DNA damage. However, sperm selection leads to the removal of seminal plasma components, which may affect the modulation of endometrial reaction following semen deposition. Against this background, the aim of the study was to determine whether single layer centrifugation (SLC) of fresh donkey semen has any impact on sperm quality parameters and on its interaction with neutrophils. For this purpose, a total of 15 ejaculates from five jackasses were obtained using an artificial vagina and subsequently diluted in a skim milk extender. Spermatozoa were then selected through centrifugation with SLC (Equicoll^{TM}, Sweden), and diluted semen was used as a control. Two experiments were performed. The first one consisted of incubation of selected and unselected spermatozoa at 37ºC under aerobic conditions. Sperm kinematic parameters and plasma membrane integrity were evaluated at 0 h, 1 h, 2h and 3 h. The second experiment referred to an *in vitro* model where PMNs from peripheral blood were mixed with selected and unselected spermatozoa. Interaction between spermatozoa and neutrophils was evaluated with a modified Wright's stain (Diff Quick®) at 0, 1, 2 and 3 hours of co-incubation at 37ºC. While previous centrifugation with Equicoll^{TM} likely selects donkey spermatozoa with higher viability, it seems to have lesser impact on motility parameters. In addition, donkey sperm selection through SLC increases phagocytosis, which supports the modulating role of seminal plasma proteins in sperm-PMN interaction. In conclusion, SLC of donkey spermatozoa allows selection of viable sperm cells, and seminal plasma proteins in this species inhibit the ability of PMN to trap spermatozoa.

Key words: Sperm, Donkey, Single Layer Centrifugatio

1. Introduction

Proper management of breeding and genetic diversity in equine species requires the use of assisted reproduction technologies (ART), which includes artificial insemination (AI), embryo transfer (ET), intracytoplasmic sperm injection (ICSI), and cryopreservation (Hinrichs, 2018; Alvarenga et al., 2016). Indeed, to obtain better ART outcomes and increase fertility rates, ejaculates must first be processed to select high quality sperm. Sperm selection is based on motility, morphology, plasma membrane, acrosome and DNA integrity. In addition, sperm selection allows removal of seminal plasma, non-viable sperm, pathogens and debris particles (Morrell, 2012). While seminal plasma has been described to have a detrimental effect on sperm motility and viability during storage, the presence of non-viable, morphologically abnormal spermatozoa may be sources of an increased production of reactive oxygen species (ROS), which can also be detrimental for sperm survival during storage.

Several sperm preparation techniques have been described to separate male gametes from the rest of the ejaculate and to select spermatozoa with better quality. Because simple sperm washing does not remove all seminal plasma (Morrell et al, 2017), sperm migration and centrifugation through colloids are the mostly used in techniques equine practice (Morrell et al., 2016). However, with the swim up technique, low recovery rate is obtained, and spermatozoa are selected only based on their motility (Morrell et al., 2017). Colloid centrifugation consists of silane-coated silica particles in a species-specific formulation that provides separation of heterogeneous cell populations into sub-populations according to their density. This technique selects better sperm quality regarding kinetic parameters, membrane and chromatin integrity, and normal morphology (Morrell et al., 2016).

Single Layer Centrifugation (SLC) is a simplification of the density gradient centrifugation since only one layer of colloid is employed (Morrell et al., 2009). Previous studies determined that this technique could optimize stallion sperm selection since SLC-selected spermatozoa display better motility parameters, less DNA fragmentation and increase pregnancy rates in subfertile stallions (Morrell et al., 2009; Morrell et al., 2011). With respect to jackasses,

Ortiz et al. found that SLC using Androcoll-E could improve sperm quality parameters, such as motility, viability and morphology, in jackass ejaculates evaluated after 24 h of cooled storage (Ortiz et al., 2014) and after thawing cryopreserved doses (Ortiz et al., 2015).

During SLC of mammalian semen, all seminal plasma components, including porcine spermadhesins PSP-I and PSP-II, as well as some cholesterol molecules of sperm plasma membrane are removed (Kruse et al., 2011). Removal of seminal plasma has beneficial effects on sperm cryopreservation (Moore et al., 2005) and allows better stabilization of plasma membrane in fresh and cooled stallion spermatozoa (Barrier-Battut et al., 2013). However, several seminal plasma factors play an important function in the female reproductive tract regarding the inflammatory response. In effect, the presence of seminal plasma reduces chemotaxis, sperm-neutrophil binding, phagocytosis, complement activation, and the formation of DNA-based neutrophil extracellular traps (NETs) (Alghamdi et al., 2009). In jennies, addition of seminal plasma after artificial insemination with frozen-thawed jackass sperm reduces *COX-2* expression (Vilés et al., 2013). Moreover, *in vitro* models suggest that seminal plasma can suppress sperm-PMN attachment (Miró et al., 2013).

Against this background, this study aimed to elucidate the effects of SLC of jackass spermatozoa with Equicoll™ on their quality parameters, such as motility and viability. In addition, because seminal plasma has been shown to be involved in the modulation of sperm-PMN attachment, the interaction between spermatozoa and neutrophils following SLC with Equicoll ™ was also investigated.

2. Materials and Methods

2.1 Animals

Animals involved in the current study consisted of five jackasses of proven fertility, aged 3 to 11 years old, individually housed at the Equine Reproduction Service, Autonomous University of Barcelona (Bellaterra, Cerdanyola del Vallès, Spain), an EU-approved equine semen collection center (ES09RS01E). All procedures were performed under strict protocols of animal welfare and health

control, in accordance with the European Union Directive for animal experiments (2010/63/EU) and the Animal Welfare Act from the Regional Government of Catalonia (Spain). All animals received a standard diet based on concentrate and mixed hay, with water being provided *ad libitum*. Jackasses underwent a regular semen collection, every other day during the week.

2.2 Experimental design

2.2.1 Experiment 1

The goal of this experiment was to determine the effects of SLC with Equicoll™ of jackass spermatozoa on sperm quality parameters, such as motility and viability. Effects on selection of jackass sperm subpopulations were also considered. With this purpose, five ejaculates were collected from three jackasses through a Hannover artificial vagina (Minitüb GmbH, Tiefenbach, Germany) with an in-line nylon mesh filter to remove gel and debris. Immediately after collection, gel-free semen was extended at a ratio of five volumes of skim milk extender (4.9% glucose, 2.4% skim milk, 100 mL double distilled water), previously warmed at 37°C, and one volume of semen. After assessing sperm concentration, viability, morphology, and motility, each ejaculate was split into two aliquots. The first one was used as a control and directly adjusted to a final concentration of 25×10^6 spermatozoa/mL. The second one was centrifuged through a single layer of a silane-coated silica based colloid formulation (Equicoll™, Sweden) according to the protocol for small centrifuge tubes described by Morrell et al. (2011). Briefly, 1.5 mL of extended semen containing up to 100×10^6 spz/mL was pipetted on top of 4 mL of Equicoll™ in a 15-mL tube. After centrifugation at 300×g and room temperature for 20 min, the supernatant and most of the colloid was discarded and the sperm pellet was transferred to a clean centrifuge tube containing 0.5 mL of the same skim milk extender. Sperm concentration was evaluated and subsequently adjusted to a final concentration of 25×10^6 spermatozoa/mL. Aliquots were incubated at 37ºC under aerobic conditions for 3 h. Kinetic parameters and viability of spermatozoa were evaluated at 0 h, 1 h, and 3 h.

2.2.2 Experiment 2

This experiment aimed to determine the effects of SLC with Equicoll™ of jackass spermatozoa on the interaction between spermatozoa and neutrophils. With this purpose, 10 ejaculates were collected from four jackasses at the same conditions of experiment 1. Each ejaculate was split into two fractions. One fraction was used as control and its sperm concentration was adjusted to 500×10^6 spermatozoa/mL. The other fraction was centrifuged through a single layer of a silane-coated silica based colloid formulation (Equicoll™, Sweden) as described in experiment 1. After centrifugation, sperm concentration was evaluated and subsequently adjusted to a final concentration of 500×10^6 spermatozoa/mL. Treatments consisted of *in vitro* co-incubation of sperm cells with PMNs-rich samples 1:1 (v:v) in water bath at 37ºC for a 3-h period, as described previously by Marín et al. (Marín et al., 2015). Briefly, peripheral blood neutrophils were isolated from healthy jennies. Following an incubation in a water bath at 37ºC for 30 min, heparinized blood was subjected to centrifugation at 400×g and 4ºC for 5 min and plasma was then removed. The buffy coat was mixed with an isotonic saline solution (PBS) and again centrifuged at 400×g and 4ºC for 5 min. The PMNs-rich buffy coat was collected and re-suspended in PBS to a final concentration of 100×10^6 PMN/mL. Samples from each of the treatments were examined for sperm phagocytosis at 0 h, 1 h, and 3 h.

2.3 Evaluation of sperm motility

Kinetic parameters of jackass sperm were assessed using a computer-assisted sperm motility analysis (CASA) system (ISAS: Proiser, Valencia, Spain) and an Olympus BX41 microscope (Olympus 20x 0.30 PLAN objective; Olympus Europe, Hamburg, Germany). With this purpose, 5 µL of each sample were placed into a Neubauer Chamber and then observed using a phase contrast microscope with a pre-warmed stage (37ºC). For each sample, three fields per drop and at least 1,000 spermatozoa were assessed. The following variables were analysed: percentage of total motile spermatozoa (TMOT), percentage of progressively motile spermatozoa (PMOT), curvilinear velocity (VCL), average path velocity

(VAP), straight line velocity (VSL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (%LIN), straightness (%STR), and motility parameter wobble (%WOB). A spermatozoon was considered to be motile when showed a VAP >10 μm/s, and progressively motile when STR > 75%.

2.4 Evaluation of sperm viability

Sperm viability was assessed using flow cytometry (Cell Laboratory QuantaSC cytometer, Becjman Coulter; Fullerton, CA, USA) and a combination of two fluorochromes (SYBR14 and propidium iodide, PI; Live/Dead Sperm Viability kit; Molecular Probes, Life Technologies, USA). After processing, sperm samples, with a final concentration of 1×10^6 spermatozoa/mL, were incubated at 38.5ºC for 10 min with 100 nM of SYBR-14. Then, 12 μM of PI was added before incubating the mixture for further 5 min. Samples were excited with an argon ion laser emitting at 488 nm and set a power of 22 mW. The sheath low rate was set at 4.17 μL/min. The fluorescence from SYBR-14 was detected through FL1 photodetector (green fluorescence; Dichroic/Splitter, DRLP: 550 nm, BP filter: 525 nm) and PI through FL3 photodetector (red fluorescence; LP filter: 670/730 nm). The following three categories of spermatozoa were identified: (i) viable, with intact membrane spermatozoa, emitting green fluorescence ($SYBR14^+/PI^-$); (ii) non-viable spermatozoa showing red fluorescence ($SYBR14^-/PI^+$) and (iii) moribund spermatozoa that were stained both green and red ($SYBR14^+/PI^+$). Non-stained particles, either for SYBR14 or PI ($SYBR14^-/PI^-$), were considered as non-sperm or debris cells. The selection of sperm cells was based on the electronic volume (EV) and the side scatter (SS). All the non-sperm specific events were discarded. From each sample, a total of 10,000 events were collected, and three technical replicates were evaluated. Compensation SYBR14-spill over into the PI-channel was performed (2.45%). In order to quantify each sperm population in the dot-plots, all data were analyzed using the Cell Lab Quanta® SC MPL Analysis Software (version 1.0; Beckman Coulter).

2.5 Evaluation of sperm-PMN phagocytosis

Smears were prepared on microscope slides for each treatment and stained with a modified Wright's stain (Diff Quick®). Phagocytosis was determined at

1,000× magnification (Olympus Europe, Hamburg, Germany) with immersion oil, and are expressed as the percentage of PMNs that ingested at least one spermatozoon. A total of 200 PMNs per slide were counted, and three technical replicates were made.

2.5 Statistical analysis

Data obtained from the analysis of all sperm parameters were first tested for normality and homoscedasticity using the Levene's test. Following this, all variables except the effects of SLC-filtration upon sperm motile populations were evaluated through a mixed model (intra-subjects factor: time of incubation; inter-subjects factor: control *vs.* equicoll), followed by a post-hoc Sidak's test.

Sperm subpopulations were set according to the procedure described by Luna et al. (2018) with minor modifications. In brief, the individual kinematic parameters obtained for each spermatozoon after CASA assessment (VSL, VCL, VAP, %LIN, %STR, %WOB, ALH and BCF) were used as independent variables in a Principal Component Analysis (PCA). These kinematic parameters were sorted into PCA components and the obtained data matrix was rotated using the Varimax procedure with Kaiser normalization. As a result, regression scores for each of the PCA components were calculated per spermatozoon.

Cluster analyses were conducted using regression scores from all evaluations made (photo-stimulation patterns and control). Sperm subpopulations were set through a two-step cluster analysis based upon log-likelihood distance and the Schwarz's Bayesian Criterion. A total of four motile sperm subpopulations were obtained. Proportions of sperm cells belonging to each subpopulation (SP1, SP2, SP3 or SP4) were calculated in each assessment (i.e. independent replicate consisting of a combination between treatments and incubation time).

The effects of SLC-filtration upon sperm motile populations were evaluated through a Scheirer-Ray-Hare ranked ANOVA followed by Mann–Whitney test. Percentages of SP1, SP2, SP3 and SP4 spermatozoa were considered as dependent variables.

Data are presented as mean ± SEM, and the minimal level of significance was set at $P < 0.05$ in all cases.

3. Results

3.1 Experiment 1

3.1.1 Sperm plasma membrane integrity

Figure 1 shows (mean ± SEM) percentages of viable spermatozoa (SYBR14$^+$/PI$^-$). Spermatozoa selected through SLC presented significantly (P<0.05) higher sperm plasma membrane integrity than those not selected until 120 min of incubation. At 180 min of incubation, no significant differences between treatments were observed.

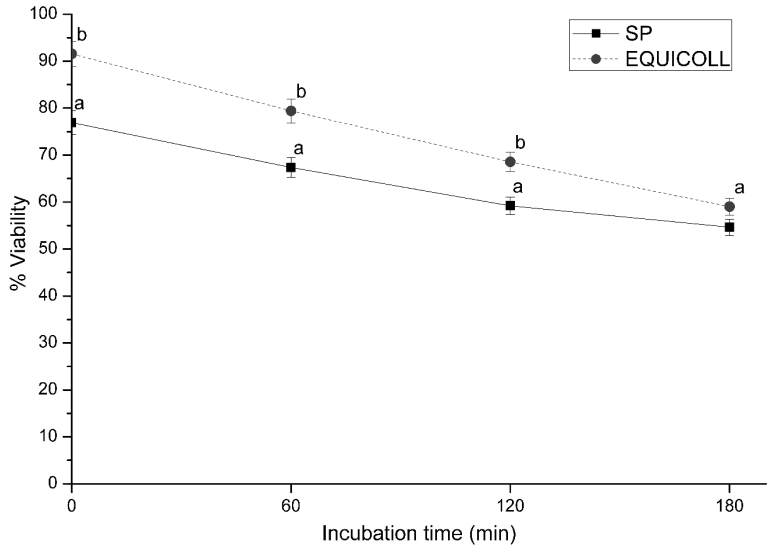
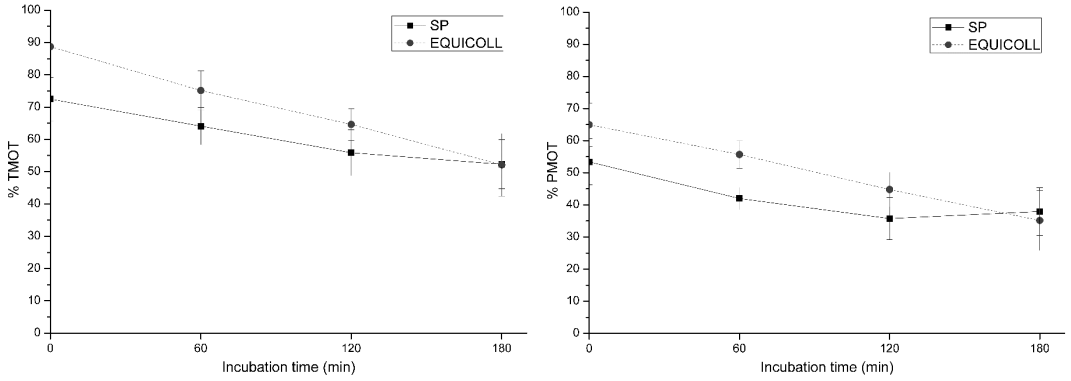


Figure 1: Percentages of viable spermatozoa (SYBR14$^+$/PI$^-$; mean ± SEM) throughout incubation time in the presence of seminal plasma and without selection (SP) or after selection through centrifugation with Equicoll^{TM} (EQUICOLL). Different superscript letters (a-b) indicate significant differences (P≤0.05) between treatments within a given time point.

3.1.2 Sperm motility

Percentages of total and progressive sperm motility during incubation for samples with seminal plasma and samples centrifuged with Equicoll^{TM} are shown in Figure 2 (mean ± SEM). No significant differences were observed regarding total and progressive motilities between semen with seminal plasma

and semen after SLC, which indicated that the effects of sperm selection through Equicoll™ did not improve this parameter.



a. b.

Figure 2: Percentages of a. total motile donkey spermatozoa (TMOT; ± SEM) and b. progressive motile donkey spermatozoa (PMOT; ± SEM). SP: diluted semen, Equicoll: semen selected through single-layer centrifugation. No significant differences between SP and Equicoll were observed.

Sperm velocity parameters are shown, as mean ± SEM, in Table 1. After 120 min of incubation, significantly (P<0.01) lower values of VCL were observed in samples selected through SLC. Between 60 and 180 min, VSL and VAP were significantly (P<0.01) higher in samples without selection through SLC than in non-filtered samples. At 180 min, significantly (P<0.05) lower values of WOB were observed in samples selected with Equicoll™ than in the other samples.

Table 1. Sperm kinematic parameters (as mean ± SEM) throughout incubation time with and without selection with SLC.

	0 min		**60 min**		**120 min**		**180 min**	
	SP	**Equicoll**	**SP**	**Equicoll**	**SP**	**Equicoll**	**SP**	**Equicoll**
VCL	155.0 ± 7.8	138.4 ± 9.3	143.4 ± 12.2	102.5 ± 11.2	136.3 ± 14.6**	84.8 ± 5.4**	108.5 ± 10.7	67.7 ± 4.7
VSL	115.3 ± 6.0	96.8 ± 6.9	112.4 ± 5.3**	80.6 ± 6.0**	109.1 ± 9.0**	68.5 ± 4.2**	106.9 ± 3.8**	53.9 ± 4.2**
VAP	141.6 ± 5.7	121.9± 7.6	130.0 ± 9.5**	91.9 ± 9.2**	124.7 ± 12.0**	76.3 ± 5.2**	100.1 ± 9.1**	58.9 ± 4.5**
LIN	74.7± 3.4	70.2± 2.3	79.6± 3.8	79.8 ± 3.1	81.6 ± 4.1	81.0 ± 1.2	84.5 ± 2.3	79.5 ± 1.2
STR	81.4 ± 2.6	79.4 ± 2.5	87.2± 3.0	88.6 ± 2.5	88.4 ± 3.0	90.1 ± 1.9	91.3 ± 1.6	91.5 ± 0.8
WOP	91.6 ± 1.7	88.2 ± 1.0	91.1 ± 1.6	90.0 ± 1.1	92.2 ± 1.6	89.8 ± 0.7	92.4 ± 1.4*	86.8 ± 1.2*
ALH	3.2 ± 0.4	3.1 ± 0.3	3.0± 0.4	2.4 ± 0.2	3.0 ± 0.4	2.1 ± 0.1	2.5 ± 0.3	2.1 ± 0.1
BCF	9.5 ± 0.8	10.1± 1.1	10.0 ± 10.9	9.9 ± 0.6	10.1± 1.0	9.1 ± 0.2	8.7 ± 0.1	9.7 ± 0.3

Abbreviations: SP: diluted semen; Equicoll: semen selected through single-layer centrifugation; VCL: sperm curvilinear velocity; VSL: sperm linear velocity; VAP: mean velocity; LIN: linear coefficient; STR: straightness coefficient; WOB: wobble coefficient; ALH: mean lateral head displacement; BCF: frequency of head displacement. *P < 0.05; **P < 0.01.

3.1.3 Sperm subpopulations

A two-step clustering procedure based on log-likelihood and Schwarz Bayesian criterion was run with the 24,399 motile spermatozoa evaluated and four sperm subpopulations (SP1, SP2, SP3, SP4) were identified. SP1 and SP2 exhibited the highest average path velocity (VAP), SP3 was characterized by moderate VAP and SP4 showed the lowest VAP (Table 2).

Table 2: Sperm subpopulation (as mean ± SEM) and motility descriptors in fresh donkey semen.

	SP1	**SP2**	**SP3**	**SP4**
n	8422	8133	6152	1692
VCL	165.8±0.2	103.8±0.3	120.1±0.1	0.6±0.1
VSL	132.8±0.3	90.7±0.3	46.4±0.3	2.0±0.1
VAP	150.5±0.2	97.5±0.3	94.4±0.6	0.3±0.0
LIN	80.2±0.1	87.3±0.1	40.6±0.2	0.3±0.1
STR	88.2±0.1	93.2±0.1	53.2±0.3	0.67±0.1
WOB	90.6±0.1	93.4±0.1	767.0±0.1	1.0±0.2
ALH	3.6±0.0	2.1±0.0	3.6±0.0	0.1±0.0
BCF	11.0±0.0	8.4±0.0	7.8±0.0	0.1±0.0

Abbreviations: SP1: subpopulation 1; SP2: subpopulation 2; SP3: subpopulation 3; SP4: subpopulation 4; VCL: sperm curvilinear velocity; VSL: sperm linear velocity; VAP: mean velocity; LIN: linear coefficient; STR: straightness coefficient; WOB: wobble coefficient; ALH: mean lateral head displacement; BCF: frequency of head displacement. *P < 0.05; **P < 0.01.

After 60 and 120 min of incubation, proportions of motile spermatozoa belonging to SP1 and SP2 were significantly (P<0.05) lower and higher, respectively, in the SLC-selected sample than in the control (Figure 3). No significant differences were observed regarding SP3 and SP4 throughout the incubation time.

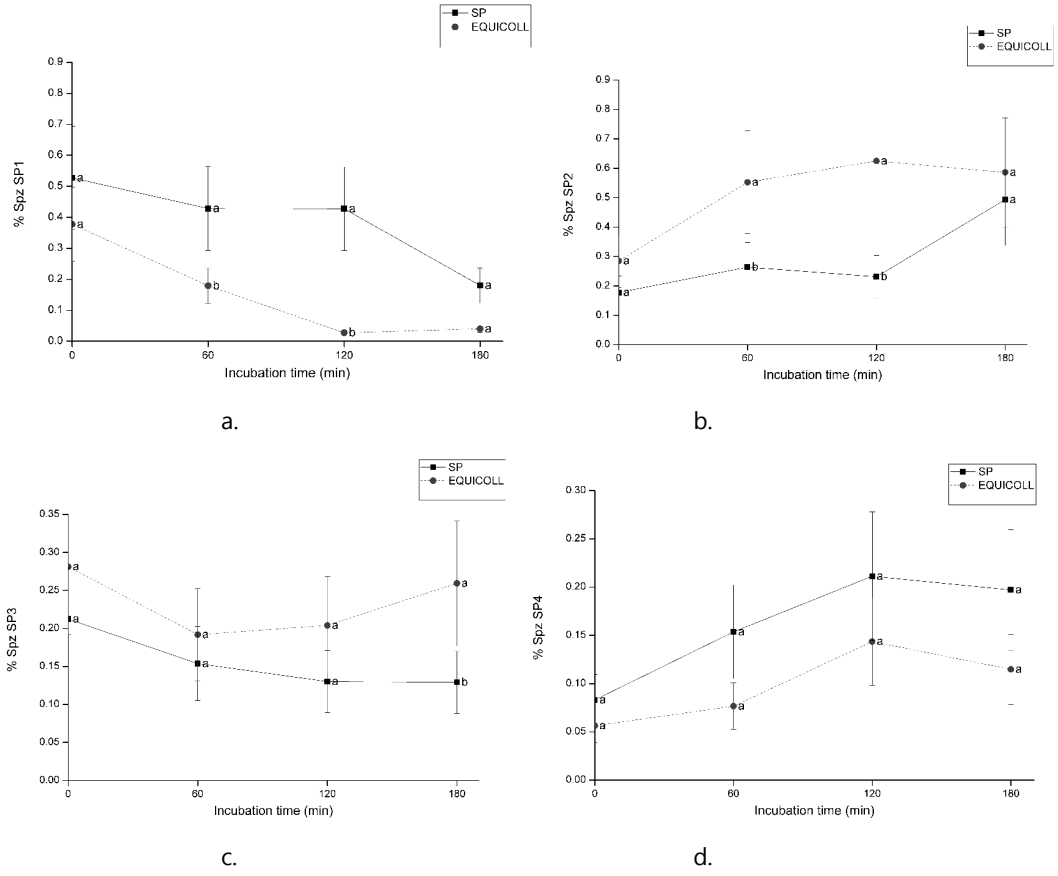


Figure 3: Percentages of donkey sperm subpopulations throughout incubation time (as mean ± SEM). a. subpopulation 1; b. subpopulation 2; c. subpopulation 3; d. subpopulation 4. SP: diluted semen, Equicoll: semen selected through single-layer centrifugation. Different letters (a, b) mean significant (P<0.05) differences between groups.

3.2 Experiment 2

3.2.1 Phagocytosis

Percentages of sperm-PMNs phagocytosis are shown in Figure 4. No phagocytosis of spermatozoa by PMNs was observed in *in vitro* culture with diluted semen. When semen was selected through SLC, phagocytosis of spermatozoa was observed after 60 min of incubation and was significantly (P<0.05) increased after 120 min of incubation.

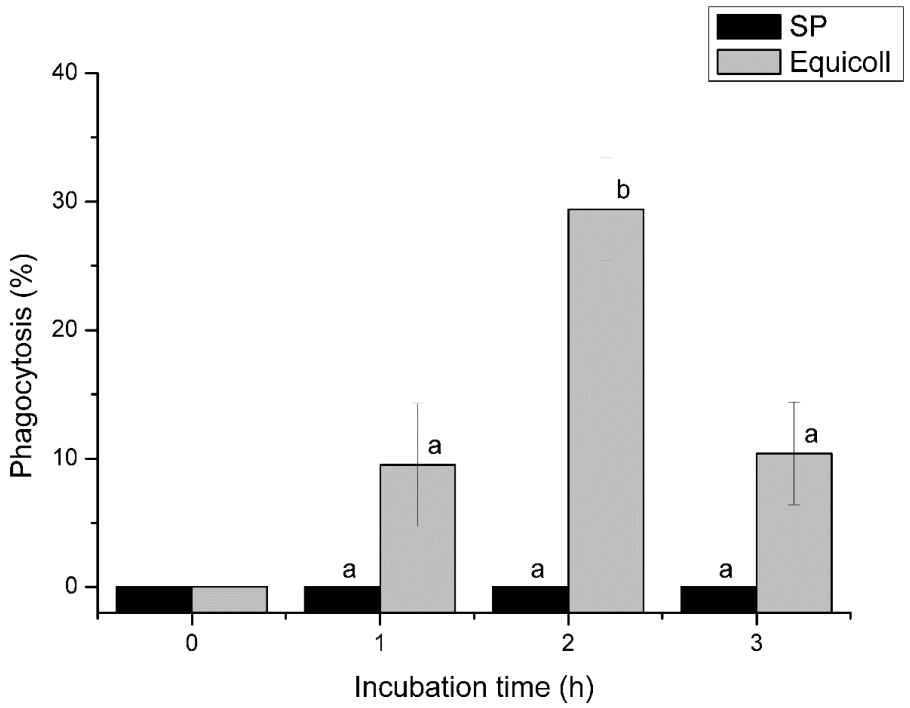


Figure 4. Percentage sperm-PMNs phagocytosis (as mean ± SEM). Different letters (a, b) mean significant (*P*<0.05) differences between groups.

4. Discussion

Pregnancy rates after artificial insemination are affected by sperm quality and by the ability of the female reproductive tract to receive an embryo. Consequently, selection of high-quality sperm represents a challenge in equine assisted reproduction. Previous studies have already shown that SLC with fresh and frozen-thawed semen significantly improves sperm quality parameters in both stallions (Morrell et al, 2011) and jackasses (Ortiz et al., 2014).

In the current study, beneficial effects of sperm selection through Equicoll™ centrifugation were mainly observed regarding sperm membrane integrity and on the distribution of sperm subpopulations. Since our experiment was mainly conducted to determine the effects of sperm selection throughout three hours of incubation at 37ºC, it is difficult to compare with results from previous studies. Indeed, most of the previous works investigating the effects of sperm selection with single layer centrifugation were focused on immediate evaluation of fresh semen or on stored semen (cooled and frozen). In jackasses, Ortiz et al., obtained significantly higher values in sperm quality parameters with colloid

centrifugation using Androcoll-E after 24 hours of cooled storage (Ortiz et al., 2014) and after freezing-thawing procedures (Ortiz et al., 2015).

It is also worth highlighting that sperm samples assessed in this study were initially within the physiological values for jackass semen evaluation (Miró et al., 2005). In this regard, if SLC-selected spermatozoa had the higest quality parameters, it would be difficult to observe significant differences between control samples and samples selected through colloid centrifugation. Under this perspective, our results are in agreement with those obtained by Ortiz et al. who observed that, to improve post-thaw sperm quality of cryoprserved donkey semen, SLC procedure was more suitable for ejaculates with poor freezability (Ortiz et al., 2015).

The emerging finding of this study is the susceptibility of jackass spermatozoa to be phagocytosed by neutrophils while those with seminal plasma seem to be protected. Sperm separation methods seek to mimic *in vitro* natural selection in the female reproductive tract (Beydola et al., 2014). However, semen centrifugation involves removal of seminal plasma. Specific components of seminal plasma, particularly proteins, are adsorbed onto the surface of ejaculated spermatozoa during their passage throughout the male reproductive tract. These sperm coating components confer important properties to the sperm membrane, such as delaying sperm capacitation. Furthermore, previous studies have described that seminal plasma components are able to regulate the endometrial inflammatory reaction (Troedsson, 2006; Miró et al., 2013). Matching with our results in jackasses, the seminal protein CRISP 3 has been reported to inhibit the binding mechanism between viable spermatozoa and PMNs involved in phagocytosis in the horse (Doty et al., 2011), which improves the fertility in mares (Alghamdi et al., 2004). In jennies, suppression of sperm-PMN binding by the presence of seminal plasma has also been described (Miró et al., 2013), which would also be in agreement with the observed effect of seminal plasma in this study. Another work already described that SLC may remove some seminal proteins and cholesterol from the sperm membrane (Kruse et al., 2011). We can thus hypothesize that sperm selection

with Equicoll™ centrifugation leads to the removal of seminal plasma components involved in the protection of spermatozoa from phagocytosis.

5. Conclusion

We can conclude that previous centrifugation with Equicoll™ likely selects jackass spermatozoa with higher viability, although selection regarding motility parameters seems to be limited. Interestingly, since selection of jackass sperm through SLC increases phagocytosis, further studies should be performed to analyze effects of SLC on sperm-PMN interaction, particularly NETosis.

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DISCUSSION

This Dissertation aimed to evaluate how spermatozoa can be protected by seminal plasma in both stallions and jackasses. Although these two species are closely related genetically, the role of seminal plasma in the regulation of sperm function might be different. Seminal plasma is the fluid portion of the ejaculated semen consisting of the secretions from the testis, the epididymis, and the accessory sex glands. In mammals, this fluid is known to play an important role for sperm function in both male and female reproductive tracts (Senger, 2012). Indeed, seminal plasma components are clearly involved in essential steps for the success of fertilization, such as sperm transport, capacitation, establishment of the sperm reservoir in the oviduct, modulation of the endometrium inflammatory response to sperm, and gamete interaction. In addition, seminal plasma has a highly specialized scavenger system, made up of enzymatic and non-enzymatic components, that defends from ROS-induced damages (Lenzi et al., 2002). The antioxidant enzyme defense system includes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GSR).

The composition of seminal plasma is complex and species-specific (Druart and de Graaf, 2018). In the current Dissertation, we tried to compare the activities of the seminal plasma antioxidant and enzyme defense system (SOD, CAT, GPX, GSR) between stallions and jackasses. Interestingly, the activities of all these enzymes were much higher in jackass than in stallion seminal plasma. A possible explanation for these differences could be related to the size of the accessory glands, which are involved in the production of seminal plasma and larger in jackasses than in stallions (Miragaya et al., 2017). One could also purport that the much higher levels of antioxidant enzymes in jackass seminal plasma have physiological implications and are related to the reproductive strategy in this species. In effect, in mare as in jennies, seminal plasma components play a key role in the regulation of the immune system of the female reproductive tract and represent an important factor for the modulation of post-breeding induced endometritis (Troedsson et al., 2005; Vilés et al., 2013). It is a fact that through a physiologic and transient inflammatory reaction

in response to semen deposition, a rapid migration of PMN into the uterus occurs both in mares (Troedsson, 2006) and jennies (Miró et al., 2013). The chemotactic signal to neutrophils aims to eliminate spermatozoa by phagocytosis and/or forming neutrophil extracellular traps (Troedsson, 2006). In that regard, activated leukocytes and subsequent inflammation increase ROS levels inducing sperm oxidative damage (Shi et al., 2011), which could be regulated by the antioxidants contained in the seminal plasma. However, when artificial insemination is performed with frozen-thawed semen, the seminal plasma is not present. Interestingly, and even when jackass sperm show good viability and motility at post-thaw (Canisso et al., 2019), pregnancy rates using frozen-thawed semen are much lower in jennies than in mares (Oliveira et al., 2006; Vidament et al., 2009). Indeed, the more pronounced post-breeding inflammatory response in jennies may explain the poor pregnancy rates. In addition, when seminal plasma is added prior to artificial insemination with frozen-thawed semen, conception rates of jennies are improved (Rota et al., 2012). All these previous results suggest that the presence of seminal plasma at the moment of the insemination plays a crucial role for fertility of the jennies. Such a role appears to have less importance in mares, since pregnancy rates after insemination without seminal plasma are higher. Therefore, we can hypothesize that the higher enzyme activities in jackass seminal plasma are needed to regulate the post-breeding endometritis in jennies.

Seasonality of domestic jennies can be disputed depending on areas of the world and authors (Canisso et al., 2019). However, since equine (*Equus* genus) is a seasonal polyestrous species with the onset of the breeding season being associated with the increase of day length, we investigated the impact of seasonal variation on the antioxidant composition of seminal plasma. While we found no differences in the activities of the four enzymes between seasons in stallion seminal plasma, SOD and GPX activities in jackass seminal plasma were higher during the summer than in the other seasons. These data indicate a clear influence of season on jackass seminal plasma and a higher level of antioxidant enzyme activities in jackasses than stallions. These differences between species highlight, once again, the relevance of the reproductive strategy in each

particular case and supports that both the composition and the activities of antioxidant enzymes in seminal plasma are species-specific.

In order to address whether the activities of these enzymes are related with sperm quality parameters in fresh semen, we determined linear correlations. Interestingly, we found that while the activities of some enzymatic components in jackass seminal plasma were correlated with spermiogram parameters, no significant correlations were found in the case of stallions. Specifically, the activity of CAT was correlated with LIN and STR, and SOD activity was correlated with the percentage of progressively motile spermatozoa.

As aforementioned, seminal plasma can be considered to be beneficial for sperm function; however, it can be detrimental for sperm survival during liquid and frozen storage (Morrel and Rodriguez-Martinez, 2009). As a consequence, prior to preservation and/or use for assisted reproductive technologies, seminal plasma is often removed from stallion ejaculates through simple washing or other sperm selection techniques. During semen handling and manipulation in the laboratory, semen can be exposed to variations in temperature, osmotic pressure and by-products of metabolism during incubation (Loomis, 2006). In addition, elimination of seminal plasma during semen storage may reduce the protective enzyme capacity and make sperm more susceptible to oxidative stress. Since sperm cryopreservation allows the international trade of equine semen and the banking of gametes from high-merit stallions, the use of frozen semen is widening in equine breeding industry. However, pregnancy rates with cryopreserved semen are often lower than with fresh semen in both mares (Vidament, 2005) and jennies (Vidament et al., 2009). Furthermore, stallions are not selected for their semen quality and ability to withstand freezing and thawing procedures, but for their conformation, lineage, and sport ability. Finally, another important issue in equine reproduction management is the large inter-individual variability, which can be very apparent in the quality of frozen-thawed semen (Loomis and Graham, 2008). Against this background, improvement of freezing protocols and of the prediction of sperm cryotolerance is of great importance. While the quality of frozen-thawed sperm

is mostly evaluated based on post-thaw motility values (Kirk et al., 2005; Vidament, 2005), Barrier Battut et al. purported that a combination of computer-assisted motililty analysis, microscopy assessment, and flow cytometry analysis provides a more acurate prediction of their fertilizing ability (Barrier-Battut et al., 2017).

In two separate studies included in this Dissertation, we tried to determine whether the activities of SOD, CAT, GPX, and GSR in fresh seminal plasma are related to the sperm ability to withstand freezing and thawing, in both stallion and jackasses. Interestingly, we found that, in stallions, the specific activity of SOD in fresh seminal plasma was higher in GFE than in PFE. In jackasses, total and specific activities of SOD in seminal plasma were positively correlated with sperm cryotolerance. In human semen, seminal SOD activity is positively correlated with the amount of PUFA in sperm membrane (Tavilani et al., 2008). Furthermore, PUFA are positively correlated with the sperm resilience to cryopreservation, and clear differences on lipid composition of the sperm membrane have been described between 'good' and 'bad' freezers (Martínez-Soto et al., 2013). In stallions, similar results have been observed since percentages of highly PUFA in the sperm membrane are positively correlated with membrane integrity after thawing (Macías García et al., 2011). Since superoxide radicals induce lipid peroxidation and because optimal concentration of SOD scavenges superoxides, thus reducing lipid peroxidation (McCord, 2008), our results are consistent with previous reports highlighting the importance of SOD on the sperm resilience to withstand cryopreservation. It is worth mentioning that the results of this Dissertation, in regard to SOD activity as a freezability marker, are based on the ejaculate rather than on the animal. Indeed, one ejaculate was taken from the majority of individuals and, consequently, the potential inter-ejaculate (i.e. intra-individual) variability was not considered. As a matter of fact, considering the single ejaculate (and not the animal) as the experimental unit was thought to be a good start to evaluate the relationship between antioxidant enzyme activities and sperm cryotolerance. Our results, however, warrant further research on the reliability of SOD activity as a freezability marker for individual animals.

Matching with the differences between GFE and PFE in both species, it was also observed that seminal antioxidant enzyme activities are correlated to some extent, negatively or positively, with post-thaw sperm quality parameters. Surprisingly, while total and specific activities of CAT were negatively correlated with post-thaw stallion sperm parameters, a positive correlation was seen in the case of jackasses. These results can be explained by a different role played by CAT in the modulation oxidative stress induced by ROS. Indeed, while hydrogen peroxide is usually considered to be the most detrimental ROS in stallion spermatozoa (Baumber et al., 2000), it can be eliminated by either CAT or GPX. One could hypothesize that in jackasses, CAT represents the main important scavenger of hydrogen peroxide. In addition, total activities of GPX and GSR were found to be negatively correlated with post-thaw stallion sperm quality parameters. The results obtained in this Dissertation also match with a previous study from Aurich et al. in which the addition of seminal plasma from GFE was found to improve the resilience of PFE to withstand cryopreservation, as it better maintained post-thaw sperm viability and progressive motility (Aurich et al., 1996). In jackasses, we also observed that total and specific activities of GSR in seminal plasma were negatively correlated with specific kinematic parameters of frozen-thawed spermatozoa. Therefore, the role that previous exposure of stallion and jackass spermatozoa to seminal plasma plays with regard to their ability to withstand freeze-thawing differs between stallions and jackasses, since while SOD is the only one involved in the former, CAT, GPX and GSR are also implied in the latter.

During storage, accumulation of ROS must be avoided to maintain sperm membrane stability and acrosome integrity. Since seminal plasma represents the major source of antioxidant scavengers and because the activities of CAT were previously observed to be important in jackasses (Article 1), we hypothesized that it could be able to modulate the sperm response to oxidative stress exogenously induced by hydrogen peroxide in jackasses. However, regarding the protective effect of seminal plasma, it could only counteract the cytotoxic effects of induced oxidative damage partially. Indeed, while protection against the loss of total motility was not apparent, some kinetic parameters and

the relative levels of superoxides in the population of viable spermatozoa with high levels of superoxides were better maintained when seminal plasma was present in the induced oxidative damage environment. Although these results were not expected, the protective effect of seminal plasma on the sperm cell in front of an induced oxidative stress appeared to be marginal. This may bring us to think that seminal plasma exerts its protecting role on the uterus environment rather than on the sperm cell. This conclusion was already reached by Sabatini et al., who observed that supplementing post-thaw spermatozoa with seminal plasma did not improve their quality parameters, such as motility, plasma membrane and chromatin integrity (Sabatini et al., 2015). In that regard, further research is needed to investigate whether antioxidant levels of seminal plasma might modulate the interaction of spermatozoa with endometrium cells.

Upon ejaculation, the surface of sperm plasmalemma is bound by seminal plasma proteins that are progressively removed during the transit of the male gamete throughout the female reproductive tract. These seminal plasma proteins protect spermatozoa from the harsh female environment and delay premature capacitation (Troedsson et al., 1998). Indeed, the endometrium reacts to the sperm deposition attracting lymphocytes and natural killer cells. This inflammation, physiologic and transitory in the mare, may be increased by the removal of seminal plasma (Bromfield, 2016). Specific proteins of seminal plasma selectively promote elimination of dead spermatozoa and protection of live spermatozoa from PMNs binding and phagocytosis (Loomis, 2006). Some of these seminal proteins coating the spermatozoa can be removed through sperm selection protocols (Kruse et al., 2011). In this context, a sperm handling technique, single layer centrifugation, separates spermatozoa from seminal plasma and extender, thereby selecting those spermatozoa with superior quality from the rest of ejaculate (Morrell et al., 2009). In the fifth study of this Dissertation, it was observed that previous centrifugation with Equicoll^{TM} likely selects jackass spermatozoa with higher viability although selection regarding motility parameters seems to be limited. Interestingly, jackass sperm selection through SLC increases sperm phagocytosis. However, further studies should be

performed to investigate, in more depth, the effects of SLC on sperm-PMN interaction.

In summary, the results of the five studies included in this Dissertation highlight the great importance of seminal plasma in both horse and donkey reproduction. Since levels of antioxidant enzymes are much higher in the seminal plasma of jackasses than in that of stallions, we suggest that seminal plasma plays a key role in donkey reproductive strategies. In addition, sperm susceptibility to oxidative stress and subsequent membrane lipid peroxidation may be species-related and may result from the differences in terms of antioxidant capacity in both seminal plasma and spermatozoa. On the other hand, SOD activity in seminal plasma is related to sperm cryotolerance in both stallions and jackasses. Finally, considering that seminal plasma is removed just after collection and before cryopreservation, the beneficial effect of seminal plasma components on sperm function appears to need a very short period of time.

CONCLUSIONS

The conclusions of this Dissertation are:

1. Activities of the seminal plasma antioxidant (SOD, CAT, GPX, and GSR) are higher in jackasses than in stallions.
2. Activities of seminal plasma antioxidants SOD and GPX vary by seasons in jackasses but not in stallions.
3. In fresh ejaculates, sperm motility parameters are correlated with the activities of SOD and CAT in jackass seminal plasma, but not in stallions. In frozen-thawed sperm however, activities of CAT are negatively correlated with sperm motility in stallions and positively correlated with sperm motility in jackasses.
4. Activities of SOD in fresh seminal plasma are related to the sperm resilience to withstand cryopreservation in both stallions and jackasses.
5. Seminal plasma has limited, counteracting effects on the oxidative stress exogenously induced in jackass spermatozoa.
6. Single Layer Centrifugation of jackass sperm with Equicoll^{TM} increases the percentage of viable spermatozoa but has a moderate impact on their motility.
7. While sperm selection of jackass sperm with SLC-Equicoll^{TM} improves their viability, it removes the protection against phagocytosis by PMN.

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