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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
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Relatório Final de Estágio do Mestrado Integrado em Medicina Veterinária

INVESTIGATION OF CHANGES IN STALLION SPERM MITOCHONDRIAL MEMBRANE POTENTIAL DURING STORAGE

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ABBREVIATIONS

AI – artificial insemination

ALH – amplitude of lateral head deviation

AO – acridine orange

APSL – Associação Portuguesa de Criadores do Cavalo Puro Sangue Lusitano

Ar – argon

ART – assisted reproductive techniques

ATP - Adenosine triphosphate

BCF – beat cross frequency

BSE – breeding soundness examination

CASA – computer-aided sperm analysis

DNA - Deoxyribonucleic acid

DSL – straight line distance

EDTA - ethylenediaminetetraacetic acid

ET – embryo transfer

FC – flow citometry

FSC – forward scatter

GIFT – gamete intrafallopian transfer

h – hour

H_2O_2 - hydrogen peroxide

HCl - Hydrogen chloride

Hz – hertz

ICSI – intracytoplasmic sperm injection

IVF – in vitro fertilization

JC-1 – 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolyl carbocyanine iodide

LIN – linearity

LL – low left

LR – low right

M – molar

mM – millimolar

MI – membrane integrity

Min – minute

MitoSOX – MitoSOX Red

mL – milliliter

MMP – mitochondrial membrane potential

mW – milliwatt

NaCl - Sodium chloride

Na_2HPO_4 - sodium hydrogen phosphate

nm – nanometer

NT – nuclear transfer

OXPHOS – oxidative phosphorylation

P – P value

PA – plasminogen activators

PBS - phosphate buffer saline

PI – propidium iodide

PM – progressive motility

P3 – population 3

P4 – population 4

P5 – population 5

r – correlation

ROS – reactive oxygen species

s – second

SAS – Statistical Analysis Systems

SCSA – sperm chromatin structure assay

SLU – Swedish University of Agricultural Sciences

$SO^{\bullet}$ - superoxide

SSC – side scatter

STR – straightness

TM – total motility

TNE – Tris sodium EDTA

UL – up left

UR – up right

VAP – average path velocity

VCL – curviliniar velocity

VSL – straight live velocity

WOB – wobble

µL – microlitre

µm – micrometer

ºC - degree Celsius

%DFI – DNA fragmentation índex

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RESUMO

Devido ao crescente interesse na utilização da inseminação artificial na última década, têm-se observado uma intensa pesquisa sobre tecnologias referentes ao sémen equino. No entanto, a Inseminação Artificial não é tão amplamente utilizada nos equinos como em algumas outras espécies domésticas. Os cavalos são normalmente selecionados com base no seu pedigree e desempenho atlético. Devido a esta seleção, os garanhões não são escolhidos para reproduzir com base na sua fertilidade, demonstrando uma grande variação na qualidade do sémen entre indivíduos. Além disso, o processo de refrigeração de doses de Inseminação Artificial para transporte influencia a fertilidade, tornando-a diminuída em comparação com a fertilidade das amostras frescas. A motilidade espermática é o parâmetro mais utilizado para avaliar a qualidade espermática. No entanto, tem pouco valor preditivo para a fertilidade, precisando a avaliação da qualidade do sémen de garanhão de meios adicionais. O potencial de membrana mitocondrial tem sido associado à fertilidade em algumas espécies, mas é ainda pouco estudado em equinos. A produção de espécies reativas de oxigénio no garanhão é alta em comparação com outras espécies, uma vez que a produção energética dos espermatozóides de equino depende quase inteiramente da fosforilação oxidativa. No presente estudo, o potencial de membrana mitocondrial e produção de espécies reativas de oxigénio foram medidos em doses frescas e refrigeradas de sémen de garanhões. A relação entre estes dois parâmetros foi também investigada na mesma amostra. Houve uma diminuição significativa entre as 0 e 24 horas de refrigeração para alguns parâmetros de motilidade e as amostras de sémen fresco tiveram uma maior percentagem de células espermáticas com membrana e cromatina íntegra. A motilidade total teve uma correlação positiva com a integridade da membrana no sémen fresco e refrigerado. Não houve nenhuma associação clara entre a produção do radical superóxido e a motilidade, viabilidade ou dano na cromatina, nas amostras frescas e refrigeradas. Também foi observada uma diferença significativa no sémen refrigerado incubado com menadiona entre as 0 e 24 horas. Foi observada correlação entre o potencial de membrana mitocondrial e produção de espécies reativas de oxigénio nas amostras refrigeradas, mas não nas amostras frescas ou em amostras incubadas com menadiona (um estimulante da produção de ROS).

ABSTRACT

Due to the increasing interest in Artificial Insemination (AI), sperm technologies in equine species have developed in the last decades. However, AI is not as widely used in equids as in other domestic species, namely dairy cattle and pig. Horses are not selected for breeding on the basis of fertility. Instead, they are chosen for their pedigree and athletic performance. As a result there is a wide variation in sperm quality between stallions. Also, the process of cooling sperm doses for further transportation is known to adversely affect sperm quality and fertility compared with fresh samples.

Sperm motility is the most used parameter to assess sperm quality. However, it has poor predictive value for fertility. The evaluation of stallion sperm quality needs additional means. Mitochondrial membrane potential (MMP) has been linked to fertility in some species, but is still poorly understood in equids. The production of reactive oxygen species (ROS) by stallion spermatozoa is high compared with other species, since equine spermatozoa rely almost entirely on oxidative phosphorylation.

In the present study, MMP and ROS production were measured in doses of fresh and cooled stallion semen. The relationship between MMP and ROS production in the same sample was also investigated. The effect of storage in the stallion samples was shown, because there were significant differences in some sperm kinematics between 0 and 24h. Membrane Integrity and Chromatin Integrity were also higher in fresh semen. Total motility was positively correlated with MI in fresh and cooled stallion samples. There were no clear associations between superoxide production and motility, viability or chromatin damage at either 0h or 24h. Also a significant difference was observed in cooled semen incubated with menadione (a stimulant of ROS production). There was a correlation between MMP and ROS production in cooled samples, but not in fresh samples, and in samples incubated with menadione.

INTRODUCTION

This report aimed the description and discussion of a research carried out at the Department of Reproduction, in the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. My internship was focused on the area of Animal Reproduction and it gave me the chance to perform different experiences and cultural contexts.

The internship was divided into two stages, but only the work in the Swedish University of Agricultural Sciences will be presented in this report, starting with the following Literature Review.

The first three weeks were spent with the excellent veterinary team of Serviços Veterinários Associados, having Dr. Pedro Meireles as my supervisor. During this period I had the opportunity to get in touch with the reality of the milk production in farms in North and Central Portugal. I was involved in the area of fertility, clinic and surgery, nutrition, husbandry and welfare, technical and economic management, milk quality and data analysis, especially focusing in the cases of animal reproduction.

In the next thirteen weeks, I worked in the Department of Reproduction, the Swedish University of Agricultural Sciences. I had the opportunity to research on the mitochondrial membrane potential during storage and its relationship with fertility. I was responsible for measuring the concentration, motility and the staining of the samples received at the laboratory, to be evaluated at Flow Cytometry. Data analysis, results and discussion were also part of my work, always with the support of Dr. Jane Morrell and Dr. Anders Johannisson. At the end of the thirteen weeks I presented my work at SLU Reproduction department.

I believe I have fulfilled the goals that I set out initially. This internship allowed me to gain autonomy, develop my communication skills and teamwork, my clinical reasoning, my laboratory techniques and finally to apply the knowledge acquired during the Veterinary course. It was a very enriching experience, especially for getting to know two different work areas, which contributed to my personal and professional training.

LITERATURE REVIEW

History of the Horse

The history of the horse begins 55 million years ago, when the dog-sized *Hyracotherium*, the first member of the horse family, was living in the forests that covered North America[1]. Curiously, when colonists first reached America, no equids were found, although most of the fossil evidence comes from there[2]. About 5000-6000 years ago, the Botai people localized on Central Asia were among the first humans to breed horses and put them to use[1,3]. The close relationship between horses and humans resulted in the creation of different horse breeds[1] adapted for different uses. The horse was utilised for work and transport until the development of the automobile and tractor. Since then, equine usage has declined, now being used mainly for sport and recreational activities.

Assisted Reproduction Techniques

In the past decades, with the main objective being the pregnancy and birth of a healthy foal, many Assisted Reproductive Techniques (ART) have been developed as a mean of avoiding disease transmission through contact at mating[4].

There are many techniques included in ART: (1) cryopreservation, where spermatozoa, embryos or oocytes are cryopreserved at very low temperatures in liquid nitrogen to maintain their viability for use at a later stage[4]; (2) artificial insemination (AI), where semen is injected into the female (2b) gamete intrafallopian transfer (GIFT), where spermatozoa are placed within the oviduct to be close to the site of fertilization; (3) embryo transfer (ET), where embryos from a donor female are removed and reimplanted in the uterus of the recipient female; (4) in vitro fertilization (IVF), where the oocyte is fertilized outside the body; (4b) intracytoplasmic sperm injection (ICSI) where a single spermatozoon is injected into an oocyte outside the body; (5) nuclear transfer (NT), where the DNA from an oocyte is removed and a nucleus with the DNA to be cloned is injected in the oocyte[5].

In equine clinics worldwide, the most used ART is AI, which will be further explained in this work. Embryo Transfer is a clinical procedure used occasionally to increase the number of foals produced from selected mares[6]. The use of ET in mares is limited because of the technical skill required and expense, and also because superovulation is still not effective in mares [7]. Intracytoplasmic Sperm Injection has been developed for fertilization of horse oocytes in vitro, since IVF has not been successful. This may be because spermatozoa fail to penetrate the zona pellucida in vitro[5]. Gamete intrafallopian transfer is not commercially viable, because it is a

more invasive procedure than AI and may only work with fresh, non-extended semen[7]. Nuclear Transfer still has low efficiency, and is not commonly used in equids[8].

Horse reproduction in Portugal

Horse reproduction technologies in Portugal have followed the same pattern as in other countries, such as USA, UK, Germany, The Netherlands, Belgium, France, Sweden or Switzerland, although at a slower rate of progress.

In 2001 AI with cooled semen was approved by the APSL (Associação Portuguesa de Criadores do Cavalo Puro Sangue Lusitano), followed by frozen semen in 2004[9]. In 2007, the first Credited Center of Portuguese Stallion Semen Collection and Freezing was created, although it was not until 2010 that APSL approved the registration of foals produced after ET[8].

Artificial Insemination

According to legend, in 1322, the horse was the first animal upon which AI was practised successfully[10]. To the present day, the interest in equine AI has increased and the technique is applied worldwide in most breeds and types of horse. Frozen semen is used less frequently than fresh semen, but even so it is used in a significant number of countries.

Pre-requisites for a successful AI are: the spermatozoa can survive outside the body; it can be introduced into the female genital tract in a way that results in an acceptable conception rate; the fertile period of the female can be identified[11].

Advantages:

- Helps to prevent the spread of diseases, especially the venereal ones;
- The rate of genetic development can be increased, if the semen comes from males of high genetic merit and is used for superior females;
- Breeding between animals in different geographic locations, or at different times, is made possible without having to move the animals themselves;
- Obstacles such as physical, physiological or behavioural abnormalities are transcended;
- Prevents trauma/injuries at mating;
- Sperm cryopreservation, sperm sexing and other reproductive biotechnologies can be linked to AI;
-It has being used in conservation of rare breeds or endangered species[4].

Disadvantages:

- Virus in semen can be shed without the male showing clinical signs of disease;

- Semen extenders with antibiotics may encourage the development of antibiotic-resistant bacterial pathogens;
-Loss of genetic variation and genetic faults can occur because of the focus on certain individuals[4,11];
- Detection of the fertile period in the female oestrous cycle can be one of the problematic aspects of AI programmes[11].

In a healthy male, the ejaculate itself should not contain microorganisms, but contamination can originate from external sources[11,12]. Therefore, general recommendations for good AI practice should be followed, and also semen extenders with antibiotics are used to limit bacterial growth and prevent disease in the inseminated female[11].
Some pathogens are transmissible via equine semen. Non-specific bacterial contaminants of semen may cause infertility in inseminated mares. *Equine Herpesvirus I, II and III, Beta-haemolytic streprococci, Haemolytic E.coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella spp., Taylorella equigenitalis, Trypanosoma equiperdum* are some of the principal pathogens[11].

Collection and Evaluation of the Semen

With the increasing popularity of insemination, using semen transported over long distances, mainly in Europe, semen quality, and its correct handling are crucial for positive pregnancy results[8].
In horses, an artificial vagina is used for semen collection, usually when the male mounts either an oestrous female or a phantom[13]. Collection with the stallion remaining on the ground i.e. without mounting is also possible.
Semen analysis is part of the breeding soundness examination (BSE), which reflects the suitability of the stallion as a breeding sire only at the time of the evaluation. One cannot rely on the evaluation from the previous breeding season since the animal can suffer some illness or other kind of problem that causes the semen quality to deteriorate[14].
In semen analysis, macroscopic features such as volume, colour, and opacity are recorded, as well as microscopic characteristics such as motility, concentration, integrity, morphology and sperm survival[13,14].
Semen quality can be affected by diverse factors, such as breed, age, nutrition, time of the year, number and frequency of ejaculate, sexual rest, arousal period, testicular size, physical and hormonal abnormalities and diseases[15,16]. Semen production and fertility fluctuate widely among stallions[17,18].

Seminal plasma contains fructose as an energy source, proteins and various ions such as calcium, magnesium and zinc. Although seminal plasma plays such an important role in sperm activation, it is detrimental to long-term sperm survival outside the body[4].

The volume and sperm concentration are used to calculate the total number of spermatozoa in the ejaculate, which is then used together with the motility to determine the number of insemination doses that can be produced. The progressive motility percentage is calculated immediately after the collection of the semen using a phase contrast microscope either for subjective assessment or for a more objective assessment using a computerized system. It can be done with either raw or extended semen. All the surfaces that come into contact with semen should be at an adequate temperature, approximately of 37-38ºC, to avoid causing cold shock to the spermatozoa[13].

To calculate the concentration, a counting chamber can be used e.g. the Neubauer chamber, or a photometer[14,15] or, more recently, fluorescence meters such as the Nucleocounter[19]. The Nucleocounter is a sperm cell counter considered to be very simple to operate and rapid. It's effective determination of total sperm concentration and sperm viability make it a viable choice[19]. Cell viability is measured in a two-step procedure with reagents and PBS (phosphate buffer saline), to count total and non-viable cells. The difference between the two populations provides the viable cell count[20].

Semen preservation

Semen is used either immediately after collection ("fresh"); after storage at a reduced temperature ("stored"); or after freezing and thawing ("cryopreserved")[4].

Cooled stored semen is the preferred type of semen for breeding horses, enabling the semen dose to be transported to different locations for insemination. With reduced temperatures, sperm metabolism and bacterial growth are slowed, helping to extend sperm life[4]. Frozen semen offers the possibility to use stallions simultaneously in breeding and competition without restrictions; time limitations are not a concern during shipment of frozen semen[21] and the life of semen is virtually indefinitely maintained. However, there is considerable variation in the quality of thawed semen and varying pregnancy rates after insemination. Hyperosmotic extenders draw intracellular water out of the spermatozoa while cryoprotectants, such as glycerol or dimethyl formamide, reduce intracellular ice formation, helping to preserve membrane integrity during the processes of cooling and re-warming[4,11]. Sperm motility must also be maintained, so that the thawed spermatozoa can reach the oocytes after insemination to fertilize them[4]. Since thawed spermatozoa have a shorter life within the female reproductive tract than fresh spermatozoa, the timing of insemination in relation to ovulation is critical when cryopreserved semen is used[11].

Oestrus detection and ovulation

It is stipulated that the universal birthday for all foals is the January 1st, independently of date of birth[22,23]. To obtain mature yearlings, owners are under pressure to time mating in such a way that the resulting foals are born as close as possible after January 1st, in any given year[24].
A successful outcome for AI depends on the deposition of spermatozoa at a suitable time relative to ovulation; thus accurate oestrus detection is crucial if the female is to be inseminated at the correct time.
Teaser males are used to identify the typical behaviour exhibited by oestrous mares[13].

Artificial Insemination and Pregnancy Rate

In horses, semen deposition is intrauterine: a catheter is passed through the cervix and the semen is deposited in the uterus[4]. With cooled semen from stallions with high fertility, acceptable pregnancy rates can be achieved by insemination within 48 hours of ovulation, but with semen from less fertile stallions insemination should take place within 12-24 hour of ovulation. With frozen-thawed semen the presence of an dominant follicle of suitable size for ovulation must be determined, and insemination of frozen-thawed semen performed within 6 hours of ovulation[25,26]. Pregnancy rates achieved with frozen-thawed semen are significantly lower than with other methods[27-30]. A contributing factor to this lowered pregnancy rate is that stallions are selected for breeding on the basis of their pedigree and athletic performance, instead of for fertility. Reproductive and cooling/cryopreserving traits, which have been shown to be heritable in the equine, have almost no selection pressure[31,32]. Thus, significant variation in semen quality exists between stallions. These individual differences in ejaculate quality also exist in cooled semen[33].
Stallion semen is transported in cooled insulated containers (4-6ºC)[4,34]; or frozen, depending on the availability of frozen semen or sometimes on the length of time between the sperm collection and the AI[34]. Frozen semen doses are used infrequently, although this trend may change with the development of better freezing protocols or better means of assessing sperm quality[35].

Portuguese Centres of Horse Semen Collection and Freezing

During the last few years, the use of ART in Portugal has been growing rapidly, with numerous national centres providing ART services. The first certificated center to open was the LusoPecus in 2007[36]. Nowadays, there are three European Union-approved Portuguese

centres for collection and freezing equine semen listed to the by the Portuguese Ministry of Agricultural[36].

Sperm Quality

To increase pregnancy rates, equine breeders would like to have more effective methods to analyse sperm quality in the hope of using only good-quality sperm doses for AI [37]. The proportion of motile sperm in a sample is most commonly used to evaluate semen quality[37,38]. However, it is not highly correlated with the fertilizing capacity of semen samples[38], since motility is only one of many attributes that a spermatozoon must possess to fertilize an oocyte. Semen samples consist of a heterogeneous mixture of spermatozoa of different stages of maturity and fertilizing ability. Therefore, evaluating an aliquot of the sample may not provide an accurate picture of the quality of the spermatozoon that succeeds in fertilizing the oocyte. Although flow cytometry (FC) is a powerful tool for evaluating many sperm attributes, it cannot evaluate all of the attributes a sperm cell requires to fertilize an oocyte.

In an effort to have more effective laboratory assays, FC analyses have been developed to evaluate spermatozoa. To conduct these analyses, spermatozoa are stained and the fluorescence emitted by each spermatozoa measured by the flow cytometer, so the presence or absence of fluorescence associated with cells can be determined[39]. Viability is a term often linked to an intact plasm membrane, since the sperm interactions with other cells and the environment depend on the plasmalemma[38].

Flow Cytometry is now a recognized methodology in the assessment of animal semen destined to breeding[38]. With FC, 50.000 sperm cells can be evaluated in less than 1 min at a reasonable cost[39,40].

Mitochondrial status

Mitochondria, located in the sperm mid-piece[40], generate a major part of the ATP required for sperm metabolism, membrane function and motility, together with anaerobic glycolysis in the cytoplasm[41-43]. In horse semen, the main source of ATP is provided by mitochondrial oxidative phosphorylation (OXPHOS)[24].

Measurement of sperm quality can also be analysed by metabolic activity of the spermatozoa[24], for example, by measuring mitochondrial membrane potential (MMP)[44]. A high MMP of spermatozoa was considered by some researchers to be related to fertility in AI[45,46]. Oxidative phosphorylation for energy production is considered to be the main role of sperm mitochondria[43]. Reactive oxygen species (ROS) are produced by all metabolising cells, so it

would be logical to assume that metabolic activity may be linked to ROS production[37]. Thus, the potential fertility of the stallion can be indicated by a combination of ROS content and MMP levels contained in semen samples.

The effect of ROS on Equine Sperm

The imbalance between the generation and degradation of ROS may be defined as oxidative stress[38,47]. The formation of ROS can modify cell functions of viability. Under physiological conditions, ROS in low levels appear to be important for normal sperm functioning[46], but excessive ROS-formation can affect cell viability[47-49]. Spermatozoa produce hydrogen peroxide (H_2O_2) and superoxide ($O_2^{-\cdot}$) and these may have a functional role in cell signalling, Ca^{2+} buffering[50,51], apoptosis[52,53], cell death[54], controlling capacitation[55] and sperm-oocyte fusion[47,48,56]. Immature, morphologically abnormal spermatozoa and seminal leukocytes are the main sources of ROS in ejaculates[24]. It has been suggested that ROS promote premature capacitation, resulting in decreased fertility[57,58].

For many sperm preparation methods associated with ART, seminal plasma is removed, decreasing the antioxidant protection for spermatozoa, allowing them to be susceptible to oxidative stress[47]. Also, sperm concentration can also influence ROS production in stored samples. Semen doses with low progressive motility will have higher sperm concentration, to compensate for the low motility. Lower quality and mitochondrial dysfunction may result in more ROS[59,60] production during storage of sperm doses, resulting in a negative relationship between the percentage of ROS in the sample and the foaling rate[46]. Mitochondrial damage during cryopreservation is suggested to be a major cause of low post-thaw semen quality, [45,61].

However, in some studies[24,62], significant correlations were found between oxidative stress parameters and a number of motility parameters, suggesting that the most fertile ejaculates were those exhibiting higher levels of ROS production. A possible explanation for the relationship between the generation of ROS and fertility might be that the most fertile sperm populations are those exhibiting the highest levels of OXPHOS, with ROS as a by-product of intense mitochondrial activity[24].

Seasonality

Timing of seasonal reproduction is mediated by circadian mechanisms[63]. With increasing daylight, as the conditions become more favourable for the progeny to survive, equids start their reproductive season during the early spring[64]. Being the mares' gestation length of 11 months, the peak in births occurs at the end of winter-early spring in the following year[63].

Reproductive activity is mediated by secretion of melatonin from the pineal gland, creating seasonal patterns[65]. Light controls both the timing and duration of the nightly peak in melatonin secretion, so the duration of melatonin secretion is extended during the longs night of winter compared to summer [63].

A photoperiodic treatment, that consists of extra-light applied during natural short days, may be applied in mares to advance the annual breeding season[63]. Those treatments allow mares to have their foals approximately 2-3 months earlier than females without treatment[22,66].

Males in general show a great variation in their reproductive characteristics during the breeding season. Decreased melatonin production leads to an increase of: testicular size[67], germinative testicular function[67,68], reproductive behaviour[64] and hormone concentrations[68,69]. Plasminogen activators (PAs) are also among the enzymes controlled by the melatonin secretion. These enzymes convert plasminogen into plasmin and play an essential role in spermatogenesis, spermatozoa capacitation and fertilization[70]. Clear significant differences exist in sperm concentration, motility, viability, sperm morphology, acrosome integrity and IMM during winter and may be maintained at reduced level outside the breeding season[71,72]. On the other hand, during the breeding season it is possible to observe high MMP, intact acrosomes and membrane stability[71].

Stallion fertility depends on many factors, the most important one being the initial quality of the ejaculate. Stallions with fertility problems have a higher percentage of spermatozoa instable membranes and sperm morphologic alterations. Better fertility results are normally obtained from stallions with a higher percentage of live and acrosome intact cells with high MMP[71].

The aims of the present study were to evaluate the influence of ROS on equine sperm motility, membrane integrity and %DFI in stored semen.

A further aim of the study was to investigate the possibility of performing MMP analyses simultaneously with ROS on the same spermatozoa in fresh and cooled stallion semen doses and to investigate a potential relationship between MMP and ROS in stallion spermatozoa.

MATERIALS AND METHODS

Semen collection

Commercial semen doses were obtained from 8 fertile Warmblood stallions, 4-18 years old, kept on a commercial stud in Sweden. Semen was collected up to three times per week during the breeding season; four ejaculates from each of three stallions and three ejaculates from each of four stallions were obtained in March and April 2016. The semen was collected using an artificial vagina, Missouri model, when the stallion had mounted a phantom. Gel was removed using an in-line filter.

Sperm analysis

Sperm concentration

The concentration of spermatozoa in raw semen was measured immediately after ejaculation using a Nucleocounter SP-100 (Chemometec, Allerød, Denmark). Semen doses were prepared by adding warm (37ºC) semen extender without antibiotics (Equiplus). Then antibiotics (Bensylpencellin and dihydrostreptomycin) were added to provide AI doses of one billion motile spermatozoa (the standard dose for cooled semen in Sweden). The extended semen was aspirated into 20-mL syringes. Immediately after collection, the fresh semen doses were sent to the laboratory in a Styrofoam box containing a cold pack, maintaining the temperature of semen doses at approximately 7ºC for 24h when the ambient temperature is 20ºC[73]. Once at the laboratory, following the initial fresh semen analysis, the semen was placed in a refrigerator; and, the analyses were repeated after 24h.

On arrival at the laboratory at SLU, the sperm concentration was again measured using the Nucleocounter SP-100 to establish the sperm concentration for staining the spermatozoa for flow cytometry.

Computer-aided sperm analysis (CASA)

Motiltiy analysis (CASA) was performed using a SpermVision (Minitüb, Tiefenbach, Germany), which was connected to an Olympus BX 51 microscope (Olympus, Japan), when the samples arrived and again after 24 h. Aliquots (6 µL) of sperm samples were placed on a warm glass slide covered with an 18 x 18-mm coverslip. Motility in eight fields (~1000 spermatozoa) was evaluated at 38ºC using the SpermVision software program with previously established

settings[74,75]. The cell identification area was set at 14-80 μm^2 and spermatozoa were classified as follows: (1) immotile spermatozoa were defined as those with an average change in the orientation of the head of less than 17º; and (2) local (i.e. non-progressive) motile spermatozoa were defined as those covering a straight line distance (DSL) < 6 µm or having a circular movement with a radius <35 µm and DSL <30 µm. The kinematics measured were curviliniar velocity (VCL), straight line velocity (VSL), average path velocity (VAP), straightness (STR), linearity (LIN), Wobble (WOB), amplitude of lateral head deviation (ALH) and beat cross frequency (BCF).

Sperm chromatin structure assay (SCSA)

The method used for the sperm chromatin structure assay (SCSA) was based on Everson et al.[76]. Equal volumes (50 µm) of sperm samples and buffer containing 0.01 M Tris-HCl, 0.15 M sodium chloride and 1 mM EDTA (pH 7.4; TNE) were mixed to give a final sperm suspension of approximately 2 x 10^6 cells mL^{-1}; samples were snap-frozen in liquid nitrogen before being transferred to a -80ºC freezer for storage until subsequent evaluation by flow cytometry. Samples were thawed on crushed ice immediately before staining as follows: 90 µl of TNE-buffer was added to 10 µl of each thawed sample. The TNE-extended sperm suspensions were subjected to partial DNA denaturation in situ by mixing with 0.2mL of a low-pH detergent solution containing 0.17% Triton X-100, 0.15 M NaCl and 0.08 M HCl (pH 1.2), followed 30s later by staining with 0.6 mL acridine orange (AO) (6 µg mL^{-1} in 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, pH 6.0). Measurements were made with an LSR flow cytometer (BDBiosciences, San José, CA, USA) equipped with standard optics. Acridine Orange is a stain for the sperm DNA. The ratios of single-stranded (abnormal) and double-stranded (normal) DNA present in individual spermatozoa are evaluated with this stain. When the DNA stains green, it is stable, double-stranded DNA, and when it stains red, it is denaturated, single-stranded DNA. AO was excited with an Ar ion laser at 488nm, running at 200mW. From each sample, a total of 10 000 events was measured at a flow rate of approximately 200 cells s^{-1}. Green fluorescence from AO was detected through a 530/30 bandpass filter, whereas red fluorescence was detected through a 660/20 bandpass filter. Data were collected using Cellquest version 3.3. In Figure 1 and 2, the FC graphics for SCSA can be seen. Figure 1 shows the sperm population (Gate 1) and Figure 2 shows the sperm population M1 and the sperm population with DNA fragmentation (M2). Further calculations of SCSA

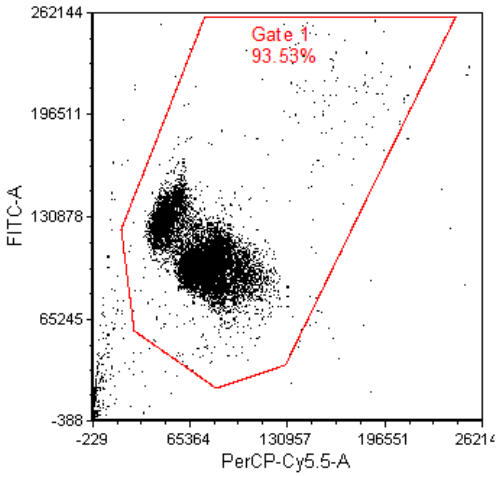


Figure 1. Sperm chromatin analysis by acridine orange staining. Spermatozoa can be seen inside the red lines as Gate 1 and debris outside the red lines.

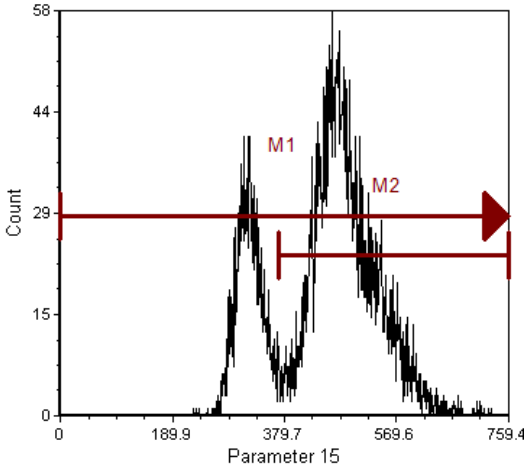


Figure 2. DNA fragmentation index evaluation. Histogram shows the αt value, calculated as red/red-green fluorescence of all cells inside Gate 1 (marker M1) and spermatozoa with high %DFI percentage (marker M2).

parameters, namely %DFI and the mean[76], were performed using FCSExpress version 2 (DeNovo Software, Thornthill, Ontario, Canada).

Mitochondrial membrane potential and measurement of ROS

Measurements of MMP were made by staining spermatozoa with the lipophilic substance 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolyl carbocyanine iodide (JC-1). JC-1 is transported into the interior of functioning mitochondria. This dye differentially labels mitochondria according to their membrane potential, emitting in the high orange wavelength for high MMP and in the green wavelength for low MMP, when excited at 488 nm[77].

Measurements of ROS were made by staining spermatozoa with MitoSOX Red (Invitrogen, Carlsbad, CA, USA) (MitoSOX), MitoSOX Red is a specific fluorescent probe for $SO^{\bullet}$ produced by mitochondria in the cell population[78]. The probe is composed of dihydroethidium, which reacts with $SO^{\bullet}$, coupled with a triphenylphosphonium cation that directs the probe to mitochondria. Fluorescence is created after binding between DNA and the reaction product[79].

The samples were stained with Hoechst 33258 to indicate viability and one of two samples was also incubated with menadione, a stimulant of ROS production.

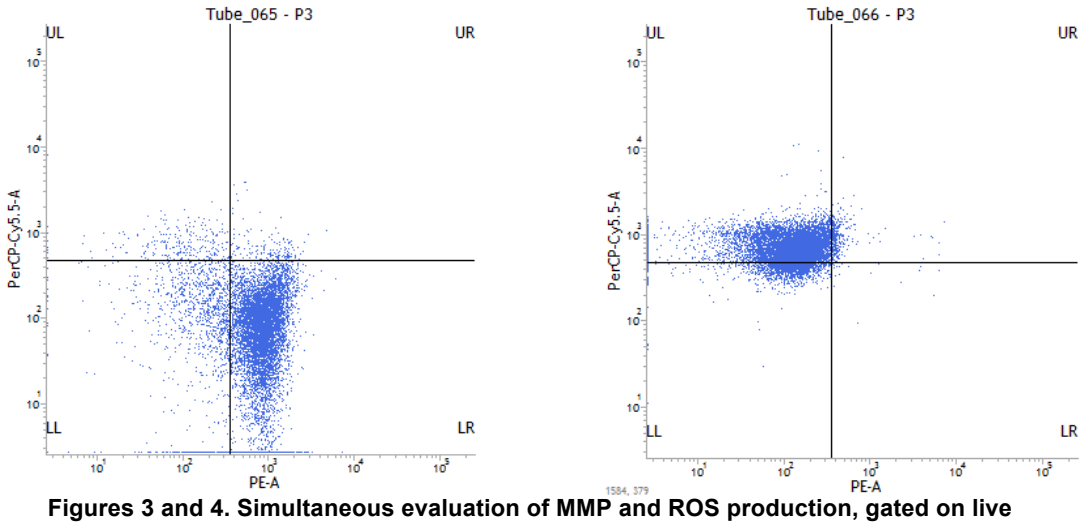
A cell suspension with 2 million sperm/mL (final volume of 300 µL) was stained. The samples were divided in two groups: both groups were incubated with a final concentration of 1.5 µM of JC-1, 3 µM of MitoSOX and Hoechst 33258 (1.2 µM); to the second group menadione (200 µM) was added.

After incubation for 30 min at 37ºC, the samples were analysed using a FACSVerse flow cytometer.

Samples were excited with a blue laser (488 nm) and a violet laser (405 nm). Green fluorescence was detected with a bandpass filter (527/32 nm), orange fluorescence was detected using bandpass filters (586/42 nm), red fluorescence was measured using a bandpass filter (700/54 nm) and blue fluorescence was detected with a 528/45 nm bandpass filter.

In Figure 3 and 4, Flow Cytometry graphics for this assay can be seen. Figure 3 shows a sperm population with high MMP and low $SO^{\cdot}$ production (LR quadrant) and Figure 4 shows a sperm population with low MMP and high $SO^{\cdot}$ Production (UL quadrant).

A total of 30 000 events was evaluated and calculated as percentages of spermatozoa with high or low mitochondrial membrane potential, live or dead superoxide negative and live or dead superoxide positive, after gating for sperm cells in the forward scatter (FSC)-side scatter (SSC) dot-plot. Cells were classified as having either high or low MMP and high or low ROS production.



Figures 3 and 4. Simultaneous evaluation of MMP and ROS production, gated on live spermatozoa, based on Hoechst 33258. LL shows low MMP and low $SO^{\cdot}$ production, LR shows high MMP and low $SO^{\cdot}$ production, UL shows low MMP and high $SO^{\cdot}$ Production, UR shows high MMP and high $SO^{\cdot}$ Production. In Figure 4., the sample is also incubated with Menodione.

Membrane integrity

Membrane integrity (MI) was analysed with a mixture of SYBR-14 and propidium iodide (PI; Live-Dead Sperm Viability Kit L-7011; Invitrogen, Eugene, OR, USA)[80,81]. PI is a fluorescent probe that binds to DNA. Cells having an intact plasma membrane will prevent PI from entering into the cell and staining the nucleus. In Figure 5, the Flow Cytometry graphics for SYBR-14/PI can be seen. With this combined stain, PI is prevented from entering cells having an intact plasma membrane, so the cells fluoresce green (P3) while the ones with a damaged plasma

membrane will permit PI to enter into the cell and bind to the DNA, and they will fluoresce red (P5). A moribund cell subpopulation stains green-red (P4)[82,83].

Aliquots with cell suspension and CellWash with 2 million sperm/ml (final volume of 300 µL) were stained with 0.6 µL SYBR-14 stock solution (diluted 1 : 50 in CellWash) and 3.0 µL PI. After incubation at 37ºC for 10 minutes, spermatozoa were analysed using a FACS Flow Cytometer with standard optics. A total of 30 000 events was collected and quantified as percentages of sperm populations. Samples were excited with an Ar ion laser (488nm). Green fluorescence was detected with an FL1 bandpass filter (527/32 nm), whereas red fluorescence was measured using an FL3 bandpass filter (700/54 nm). The spermatozoa were classified as live spermatozoa with an intact membrane ($SYBR^{+}$-14/PI^{-}), moribund ($SYBR^{-}$-14/PI^{+}), and dead ($SYBR^{+}$-14/PI^{+}).

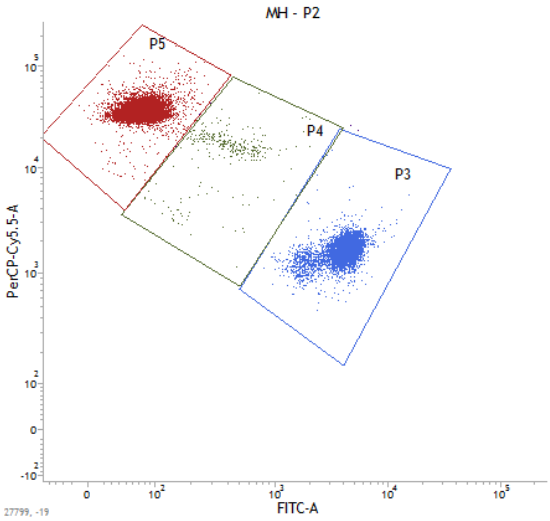


Figure 5. Evaluation of sperm viability (SYBR 14/PI labeling). Color dot plot shows viable (P3 – $SYBR^{+}$-14/PI^{-}, blue color), dying (P4 – $SYBR^{-}$-14/PI^{+}, green color) and dead spermatozoa (P5 – $SYBR^{+}$-14/PI^{+}, red color).

Statistical analysis

All statistical analyses were performed with SAS® software version 9.3 (2002-2011 by SAS Institute Inc., Cary, NC, USA). Data was tested for normal distribution by using PROC UNIVARIATE. Variables were plotted against the other using PROC SCATTERPLOT and then visually inspected to check for monotonicity. The strength and direction of association that exists between variables was measured using the Spearman's correlation. Means were analyzed by paired Student t-test (0,24h); the differences were considered significant at $p<0.05$. Results are presented as mean ±SE[84].

RESULTS

Sperm kinematics from the fresh semen samples (0h) and cooled samples (24h) are given in Figure 1 and Table 1. There were significant differences between hour 0 and 24 in total motility (P=0.0006), progressive motility (P=0.0016), linearity (P=0.003), wobble (P=0.001) and beat cross frequency (P=0.02).

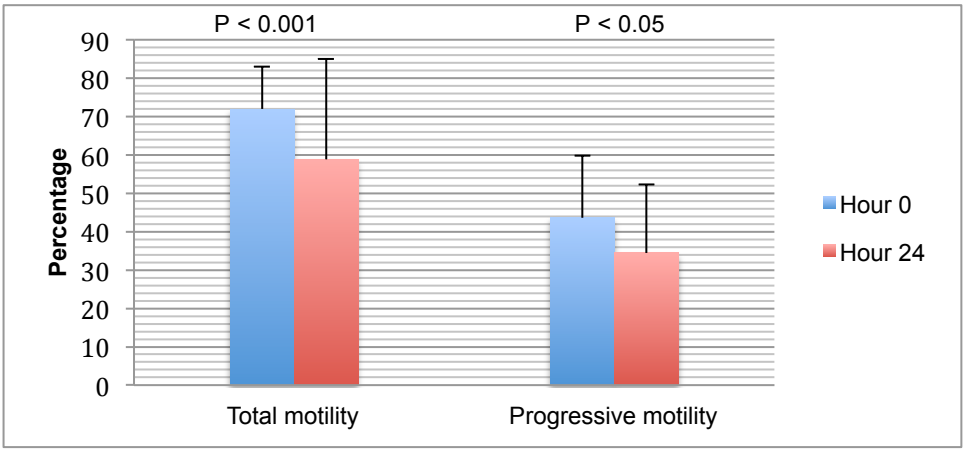


Figure 1. Mean (±s.d.) values for total and progressive motility for fresh (Hour 0) and cooled (Hour 24) of stallions spermatozoa (n = 26)

	0h	24h
TM(%)	71.96±11.12[a]	58.87±26.23[a]
PM(%)	43.6±16.26[b]	34.45±17.86[b]
VAP(μm s^{-1})	83.57±9.06	83.62±10.18
VCL(μm s^{-1})	141.03±16.28	147.28±19.89
VSL(μm s^{-1})	62.53±7.52	61.74±8
STR	0.74±0.03	0.73±0.04
LIN	0.44±0.03[c]	0.42±0.05[c]
WOB	0.59±0.03[d]	0.57±0.04[d]
ALH(μm)	4.4±0.52	4.31±0.46
BCF(Hz)	32.85±2.56[e]	31.15±3.66[e]

Table 1. Mean (±s.d.) values for sperm kinematics for fresh (0h) and cooled (24h) selected sperm samples (n=26). Same superscript within a row indicates a significant difference P<0.05

TM, total motility; PM, progressive motility; DAP, average path distance; DCL, distance curved line; DSL, distance straight line; VAP, average path velocity; VCL, velocity curved line; VSL, velocity straight line; STR, straightness (VSL/VAP); LIN, linearity (VSL/VCL); WOB, wobble (VAP/VCL); ALH, amplitude of lateral head displacement; BCF, beat cross frequency

	0h	24h
Living spermatozoa (%)	63.05±20.04[a]	57.68±23.82[a]
Dying spermatozoa (%)	1.55±1.15[b]	1.01±0.51[b]
Dead spermatozoa (%)	35.13±19.95[c]	41.26±23.41[c]
% DFI	26.5±18.86[d]	31.71±21.24[d]

Table 2. Membrane integrity indicated by the mean (±s.d.) values of living, dying and dead spermatozoa and DNA fragmentation index (%DFI) for fresh (0h) and cooled semen (24h) (n = 26) Within a row values with the same superscript, a significant difference was observed. P<0.05

Membrane Integrity and Chromatin integrity were higher in fresh semen samples than in cooled samples (P<0.05; Table 2).

As seen on Table 3, there was no significant difference between the mean values for MMP at the time timepoints (0h and 24h); similarly there was no difference in mean Superoxide production when not stimulated with menadione. There were also no significant differences in means between the two timepoints for the unstimulated samples: for high MMP and high $SO^{\bullet}$ production, high MMP and low $SO^{\bullet}$ production, low MMP and high $SO^{\bullet}$ production, low MMP and low $SO^{\bullet}$ production. However, for the samples stimulated with menadione, the proportion of spermatozoa producing superoxide was significantly increased at 24h compared to 0h. (P<0.05; Figure 2).

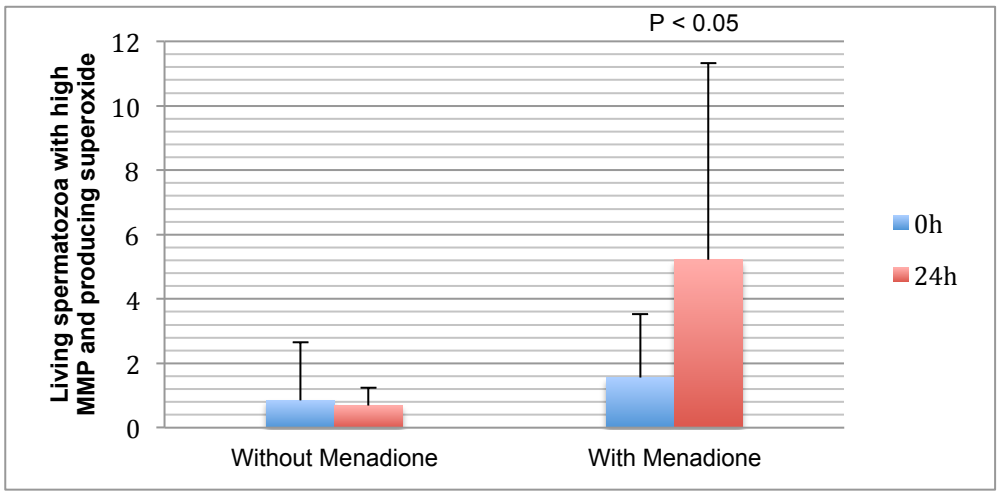


Figure 2. Effect of Menadione on $SO^{\bullet}$ production and MMP of the 0h and 24h samples Data are the mean ± s.d. (n= 22).

	Menadione	0h	24h
%Jc1+	-	90.74±4.38	92.61±4.63
%Jc1-	-	5.52±4.55	3.34±3.13
%MSox+	-	0.87±0.69	1.04±0.83
%MSox-	-	99.09±0.68	98.96±0.83
%MSox+/Jc1-	-	0.30±0.25	0.29±0.45
%MSox+/Jc1+	-	0.49±0.43	0.69±0.56
%MSox-/Jc1-	-	5.39±3.75	3.42±2.83
%MSox-/Jc1+	-	93.78±3.80	95.58±3.56
%Jc1+	+	13.07±21.28	19.44±22.81
%Jc1-	+	78.33±21.82	71.57±24.32
%MSox+	+	18.01±19.30	18.89±21.06
%MSox-	+	81.99±19.30	81.11±21.06
%MSox+/Jc1-	+	16.04±19.49	14.52±19.61
%MSox+/Jc1+	+	1.92±2.47[a]	5.21±6.12[a]
%MSox-/Jc1-	+	69.30±25.70	62.96±27.91
%MSox-/Jc1+	+	12.74±20.95	17.30±21.69

Table 3. Superoxide ($SO^{\bullet}$) and mitochondrial membrane potential (MMP) in fresh (0h) and cooled (24h) selected sperm samples (n=22) Within a row values with the same superscript, a significant difference was observed. P<0.05. Jc1+ refers to high MMP, Jc1- refers to low MMP, MSox+/Jc1- refers to high $SO^{\bullet}$ production and low MMP, MSox+/Jc1+ refers to high $SO^{\bullet}$ production and high MMP, MSox-/Jc1- refers to low $SO^{\bullet}$ production and low MMP, MSox-/Jc1+ refers to low $SO^{\bullet}$ production and high MMP. Data are the mean ± s.d.

The total motility was positively correlated with the percentage of living spermatozoa (SYBR14/PI test) at 0 and 24h (0h: r = 0.659 P<0.001; 24h: r = 0.699, P<0.001). However, there were no clear associations between $SO^{\bullet}$ production and motility, viability or chromatin damage at either 0h or 24h.

Regarding MMP and $SO^{\bullet}$ production, positive correlations were seen as follows: low MMP and $SO^{\bullet}$ production at 24h (Figure 3, Letter A; r = 0.892, P<0.001); high MMP and no $SO^{\bullet}$ production at 24h (Figure 3, Letter D; r = 0.745, P<0.001). Negative correlations were seen between high MMP and $SO^{\bullet}$ production at 24h (Figure 3, Letter B; r = - 0.746, P<0.001) as well as low MMP and no $SO^{\bullet}$ production at 24h (Figure 3, Letter C; r = - 0.892, P<0.001).

No correlation was observed between MMP and $SO^{\bullet}$ production at 0h and also when the samples were incubated with menadione.

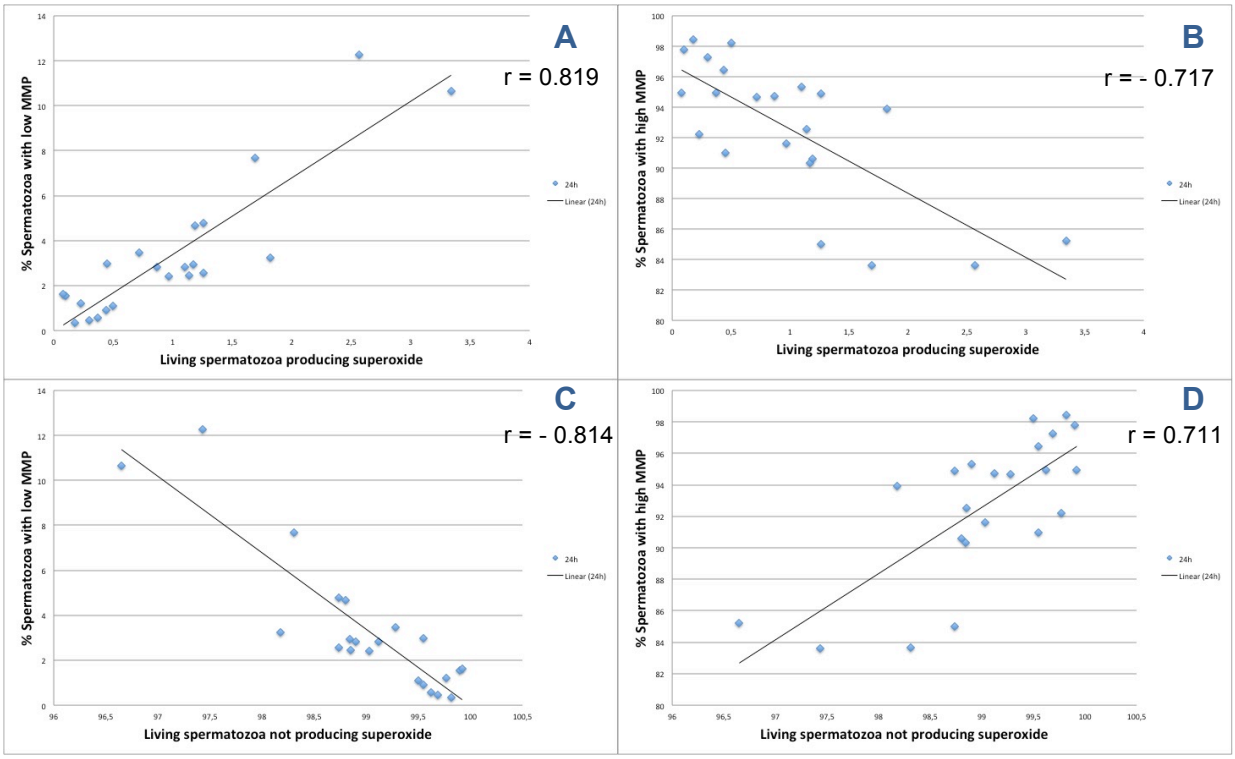


Figure 3. Relationship between $SO^{\cdot}$ production and MMP in cooled semen (24h) without the addition of menadione (n = 22) A correlation between low MMP and positive $SO^{\bullet}$ production (r = 0.819, P<0.001); **B** correlation between high MMP and positive $SO^{\bullet}$ production (r = - 0.717, P<0.001); **C** correlation between low MMP and negative $SO^{\bullet}$ production (r = - 0.814, P<0.001); **D** correlation between high MMP and negative $SO^{\bullet}$ production (r = 0.711, P<0.001).

DISCUSSION

The aims of the present study were to evaluate the influence of ROS on equine sperm motility, membrane integrity and %DFI in stored semen.

A further aim of the study was to investigate the possibility of performing MMP analyses simultaneously with ROS on the same spermatozoa in fresh and cooled stallion semen doses and to investigate a potential relationship between MMP and ROS in stallion spermatozoa.

Although there was no correlation between $SO^{\bullet}$ production and %DFI, %living spermatozoa, total and progressive motility in fresh and stored semen; total motility was positively correlated with the MI, at 0 ($r = 0.659$; $P<0.001$) and 24h ($r = 0.699$; $P<0.001$). Also, all the sperm kinematics of the samples observed in this study decreased after 24h of cooling. Motility is the most easily evaluated parameter of sperm quality, and is commonly used as an acceptance criterion to use the semen for AI of not[54]. However, it has poor predictive value for fertility[37], especially since some motile spermatozoa may have morphologic or chromatin abnormalities. Therefore, it is important to evaluate other parameters in addition to motility[85]. However, more extensive assays are impractical because they are time-consuming and expensive[39]

Evaluation of longevity of sperm motility at samples for cooling is also important, because over the years, there has been an increasing use of transported, cooled semen doses for AI[80,86]. The storage temperature[87] and sperm DNA denaturation of the sperm samples are important parameters, and influence the fertility status[88]. In fertile stallions, when the semen is stored at 5ºC, chromatin quality decrease is significantly lower than in subfertile stallions, where the sperm DNA may have greater rate of denaturation after 20-30 hours[85].

Although the main purpose is to breed and impregnate mares, it is known that stallions are selected based mainly on performance, conformation and pedigree; their reproductive potential is not considered[89] Therefore, there is a considerable variation in sperm quality between stallions[90], and an similar variation between sperm quality and ability of the spermatozoa to endure cold temperatures.

The sperm membrane is related with many sperm functions, warranting the capability of the cell to maintain homeostasis and the capacity to interact with the environment, including the lining epithelium of the female genital tract or the oocyte-cumulus cell complex[90]. The combination of dyes used in this study (SYBR-14/PI) is able to simultaneously evaluate sperm cell viability together with plasma membrane integrity[38]. In horses, particularly when spermatozoa are stored or processed for later use, lipid peroxidation occurring in the plasma membrane is a major factor causing differences in sperm quality[91]. This damage, caused by ROS, among other factors, can alter the fluidity of the sperm membrane and the activation of signal transduction pathways, critical for sperm function[92].

Chromatin integrity is one of the sperm characteristics associated with fertility. The evaluation of sperm DNA integrity is of utmost importance since early embryo development depends on the integrity of the DNA[93]. Chromatin abnormalities and DNA damage are derived from many variables, including damage induced by ROS[24]. The SCSA has been used to evaluate the in vitro susceptibility of DNA to denaturation in many species, including stallions[94]

In this study, the samples incubated with menadione had a higher value of MMP and $SO^{\cdot}$ production after 24h of cooling. Also, the motility results significantly decreased after 24h of cooling. Stallion spermatozoa have a great ROS production comparing with other species, since sperm ATP production comes majorly from OXPHOS[24]. Cooling procedures causes damage to spermatozoa, including stallions', as reported in the literature[85] and confirmed by our results, since we observed that the membrane integrity and chromatin integrity were lower in cooled samples. In this study, the percentages of viable spermatozoa with high level of $SO^{\bullet}$ were increased in cooled samples, presumably reflecting a higher mitochondrial activity, since mitochondria are considered the main source of ROS within the cell. Kothari[95] suggests that the role of ROS in the initiation of hyperactivation is very important, $SO^{\bullet}$ being crucial to the hyperactive motility.

Capacitation can be measured by the CTC-staining assay[88,96], the most commonly used stain, or by loading spermatozoa with the lipid dye Merocyanine 540, when coupled with YO-PRO-1 and Hoechst 33342[96]. It would be interesting to investigate the relationship between the sperm hyperactivity, a sub-category of capacitation[95], and ROS production stimulated by menadione. Based on an investigation made by Rathi[96], by plotting the VCL and ALH of the motile cells on a two-dimensional scatter graph, motile spermatozoa are considered hyperactive if their VLC is ≥ 180 and ALH ≥ 12. It would be useful to measure sperm motility samples with CASA system with different times of incubation with menadione, to observe ROS-production effects on sperm hyperactivity.

In a study with hamsters made by Yeoman et al.[97], hyperactivation was inhibited in presence of superoxide dismutase (SOD), indicating the potential activity of $SO^{\bullet}$ as a hyperactivation activator. However, we would like to explore another explanation: hyperactive spermatozoa, with increased mitochondrial activity, are responsible for the higher ROS production. It would be interesting to investigate the relation between ROS and hyperactivity, analysing two different populations: one with spermatozoa samples incubated with menadione and another one incubated with SOD and menadione, to evaluate the percentage of hyperactive spermatozoa.

It has also been suggested that ROS promote premature capacitation, resulting in decreased fertility, but it was not possible to investigate the fertility levels with this samples, due to lack of information from the mares inseminated with cooled semen doses from these stallions.

This was the first study to use simultaneous measurement of MMP and ROS production on the same cell, in fresh (0h) and cooled (24h) samples. In our results, the correlation between MMP and ROS production was significant in cooled samples, without correlation in fresh samples. There were two different subpopulations in semen samples, with respect to JC-1/MitoSOX staining. It is known that different subpopulations of spermatozoa coexist in mammalian ejaculate, but few bibliographical references to the study of identified subpopulations in equines are available[98]. However, with better semen analysis, equine semen doses with greater guarantees of quality could be commercialized, having a significant economic repercussion.

In one subpopulation, spermatozoa had high MMP associated with a low production of superoxide. This results were in contrast with those reported by Morrell et al.[37] in the same species and Espinoza et al.[99], in a human study, who showed no association between intracellular ROS and high MMP. A reason for this may be methodological differences, since in the present study JC-1 and MitoSOX were analysed simultaneously on living spermatozoa, whereas they were measured in different aliquots in the previous studies. Conventional thinking is that mitochondria are the main source of ROS within the cell and superoxide is rapidly converted into hydrogen peroxide[37]. Such explanation can justify the results of the first subpopulation, where we find a high MMP, but a low $SO^{\bullet}$ concentration. This low $SO^{\bullet}$ could be converted to other by-products, eg. hydrogen peroxide. In our study, hydrogen peroxide was not measured; therefore it is not possible to know the correlation between $SO^{\bullet}$ and H_2O_2 concentration in the same sample.

In another subpopulation, a low MMP was found associated with a high $SO^{\bullet}$ production. It is known that oxidative stress is associated with an increased rate of cellular damage induced by ROS[100]. According to a study made by Baumber[47], ROS inhibits one or more enzymes of OXPHOS, glycolysis, or both, thus limiting ATP generation by the sperm cell. ROS may also affect sperm motility, via alterations in mitochondrial function, observed by MMP measurements, used as a measure of mitochondrial function[47].

CONCLUSION

In conclusion, MMP and $SO^{\bullet}$ production are highly correlated in cooled samples. Two different sperm subpopulations were found, with respect to JC-1/MitoSOX staining: one with spermatozoa presented high MMP associated with a low production of superoxide; in the other one a low MMP was found associated with a high $SO^{\bullet}$ production. Further studies are needed to establish a link between ROS-production and hyperactivity, and to investigation a possible relation between MMP and ROS-production and fertility in cooled stallion semen doses.

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