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"Beneficial effects of antioxidants in canine semen quality"

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List of abbreviations

AI Acrosome intact
AI Artificial insemination
AIF Apoptosis inducing factors
ALH Amplitude of lateral head displacement
AR Acrosome reacted
ATP Adenosine triphosphate
AV Artificial Vagina
BCF Beat cross frequency
BHA Butylhydroxyanisole
BHT Butylhydroxytoluene
C Control group
CAT Catalase
CASA Computer Assisted Semen Analysis
CCP corpora cavernosa penis
CSP corpus spongiosum penis
CLCs Cholesterol-loaded cyclodextrins
CRISPs Cysteine-rich secretory proteins
DFI DNAfragmentation index
EDRF Endothelium-derived relaxing factor
EDTA Ethylenediaminetetra-acetic acid
EE Electro-ejaculation
EIU Endoscopic-assisted transcervical insemination
G6PD Glucose-6-phosphate dehydrogenase
GMP Guanosine monophosphate
GPX Glutathione Peroxidase
GPX/GRD Glutathione peroxidase/ glutathione reductase
GSH Reduced glutathione
GSSG Oxidized glutathione
GWS Glass wool-Sephadex
H2O Water
H2O2 Hydrogen peroxide
LDL Low density lipoproteins
LIN Linearity
LPO Lipid peroxidation
M Treatment group (Maca supplementation)
NADPH Nicotinamide adenine dinucleotide phosphate
NFκB Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS neural nitric oxide synthetase
NOX5 NADPH oxidase system

List of abbreviations

NPPC Native phosphocaseinate
O2 Dioxygen molecule
O2- Superoxide anion radical
OH Hydroxyl radical
OS Oxidative stress
PS Phosphatidylserine
PUFA Polyunsaturated fatty acid
PVDF Polyvinylidene difluoride
RNS Reactive nitrogen species
ROS Reactive oxygen species
RT Room temperature
SCA Sperm Class Analyzer
SCSA Sperm chromatin structure assay
SD Standard deviation
SD Standard deviation
Se Selenium
SUI surgical uterine implant
SOD Superoxide dismutase
STR Straightness
T Treated semen
TBARS Thiobarbituric acid reactive species
TCI Transcervical Insemination
TNE Tris-NaCl-ethylenediaminetetra-acetic acid
TSC Total sperm count
TUNEL Terminal deoxynucleotidyl transferase-mediated
dUDP nick end-labeling
VAP Average path velocity
VCL Curvilinear velocity
VSL Straight-line velocity
WOB Wobble

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Abstract

Artificial insemination is a routinely performed method in canine breeding programme and semen evaluation results crucial for the successful achievement of a pregnancy. Insemination can be performed by using fresh, chilled or frozen semen. However, semen storage should be performed with the presence of antioxidants, which helps in preventing damaging processes to spermatozoa. Recent studies have been conducted to investigate the feasibility of different antioxidants in several species, to improve semen viability during storage. The aim of this study was to evaluate the effect of two antioxidants, Maca and Crocin, in the supplementation of semen extender on quality-related canine semen parameters during cooling and freezing. For the first experiment ejaculates from nine dogs were cooled in the absence (control group) or the presence of 10, 20 and 50 µL/mL of an aqueous extract of Maca. Sperm were evaluated for sperm viability, motility, DNA fragmentation and lipid peroxidation after 3 h, 24 h, 4 days and 7 days of storage for chilled semen and immediately after thawing, after 1h and 2 h at 37°C. For the chilled semen, the addition of 10 µL/mL of Maca preserved sperm DNA and plasma membrane integrity at 3 h and increased sperm curvilinear velocity after 24 h. Treatment with 20 and 50 µL/mL of Maca increased the percentage of hyperactivated sperm after 3 h. Moreover, semen treated with 20 µL/mL of Maca decreased lipid peroxidation at 24 h. A significant reduction of sperm DNA and plasma membrane integrity as well as of kinetics parameters between 3 and 24 h of refrigerated storage with the higher concentration tested was observed. The second experiment aimed to evaluate the effect of Maca on frozen-thawed sperm quality in canine semen. Ejaculates from ten dogs were frozen in the absence (control group) or the presence of 10, 20 and 50 µL/mL of an aqueous extract of Maca and were evaluated immediately after thawing and after 1h and 2 h at 37 C° for sperm viability, motility, DNA fragmentation and lipid peroxidation. Canine sperm cells frozen with an extender supplemented with Maca exhibited higher total motility, especially the subpopulation of sperm with medium velocity 1 hour after thawing than control semen. Canine frozen semen with the supplementation of Maca is responsible for a surge in hyperactivation and WOB of sperm cells after one hour at 37°C. Movements of hyperactivation are considered part of the capacitation process and it is an event crucial for acrosome reaction and fertilization. What emerges from this study is the protective role of Maca against lipid membrane peroxidation of canine spermatozoa, which is a primary marker of oxidative stress. In conclusion, supplementation of the frozen extender with 10 µl/mL of aqueous extract of Maca improves the cold shock

resistance of spermatozoa, protecting sperm against lipid peroxidation during the frozen-thawed process, and activates canine sperm motility and hyperactivation after thawing, improving the fertility. The third experiment was aimed to evaluate the effect of Crocin supplementation extender at three different concentrations (C0,5, C1 e C2) on quality-related canine semen parameters after cooling. Ejaculates from ten dogs were evaluated for sperm viability, sperm motility, membrane integrity and lipid peroxidation after 3 h, 24 h, 4 days and 7 days of storage at 4 C°. The most interesting findings of the present study regard the improvement of semen quality obtained with 0.5 mM crocin. Indeed, the addition of 0.5 mM crocin in the extender significantly increased the proportion of spermatozoa with intact membranes at both 4 and 7 days compared to the control group and despite similar values of total motility and progressive motility most of the sperm kinetic parameters improved in C0.5 group compared to the control after 4 days of storage. In conclusion, we demonstrated that the enrichment of the extender with the crocin improves to a certain improved canine semen quality, particularly after 4 days of storage at 4 °C.

Chapter 1

Artificial insemination in canine species

1.1 Evolution and current status of AI in Europe

Artificial insemination (AI) consists of collecting semen from a male breeding animal, which is then introduced into the female reproductive tract, after appropriate dilution, so that fertilisation can occur in the absence of natural mating. Astonishing progress has been made since the first successful artificial insemination with fresh semen, resulting in the birth of 3 pups, reported by Spallanzani in Italy in 1780 (Heape W. et al 1987). AI can result in several advantages, such as the international exchange of semen without the transport stress to the animals, improvement of genetics, and also prevention of sexually transmitted diseases spread, like Brucella canis and Herpes virus (Linde Forsberg, 2005; Farstad, 2010). AI can be performed with fresh, fresh chilled or frozen-thawed semen, using different techniques, which will be mentioned later. The use of chilled semen is an excellent way to accomplish international breeding, without costs and stress related to dog transport. The success of AI depends on the proper selection of male and female dogs, proper preparation of semen, semen quality, accurate ovulation timing, the correct insemination timing and technique, and good communication between the veterinarian and the client. Indeed, AI with cooled-stored semen is a common practice in dog breeding programmes. AI may be performed, according to breeder request, in a variety of situations such as, when a successful natural mating cannot be achieved (including slip matings), or for conformational inability to mate, or when fresh chilled or frozen semen should be used. The dog's ejaculate consists of three distinct fractions: pre-sperm, sperm rich and post-sperm. The first fraction is usually produced before intromission during foreplay and is probably used to flush urine and debris from the urethra. The second fraction is ejaculated after intromission and deposited in the cranial vagina. The third (prostatic) fraction is produced during the copulatory 'tie' and it is likely that this flushes the second fraction forwards into the uterus through the open cervix. Although the deposition occurs inside the vagina, spermatozoa are rapidly transported inside the uterine body. Furthermore, the third fraction has an important effect on regulating sperm fertility capacity and should be used as a seminal extender in AI, whenever possible and in the absence of prostatic disorders (England and others 2012). Successful natural mating requires the full intromission of the penis into the vagina. The dog achieves intromission by vigorous thrusting of the hindquarters. Once intromission occurs, and the rate of thrusting increases, the bulbus glandis fully erects, while constrictor vestibuli muscles of the

female contract behind it, thus forming a unique canine event, the "copulatory tie" or "lock". After the sperm-rich fraction is ejaculated, the dog dismounts but keeping the lock and faces away from the bitch. The third fraction of semen is ejaculated during this stage. Second-stage coitus may last from 5-45 minutes. It is believed that the purpose of second-stage coitus is to promote uterine rather than vaginal insemination. Turning around prevents the penis from swelling down, thus maintaining high vaginal pressure. The dog constantly ejaculates up to 30 ml of seminal fluid, which is delivered through the cervix into the uterus. The main disadvantage of chilled semen is the limited lifespan of spermatozoa, as the extended sample should be used within approximately 4.9 days after collection (England and Ponzio, 1996). If the storage period exceeds this time, it would be suitable to freeze the semen samples. Although good fertility rates are obtained in dogs with chilled semen, improvement in extended semen could reduce the required insemination dose of the chilled transported sperm, which should be between 150 and 200 × 106 spermatozoa/insemination (Linde-Forsberg, 1991). High-quality chilled semen may allow multiple inseminations by using a single ejaculate, resulting in an increase in the number of puppies per litter than the equivalent obtained after natural mating. Although semen quality progressively decreases when stored at 4 °C, the quality of cooled semen up to 2 days after collection is undoubtedly better than that of frozen-thawed spermatozoa (England and Ponzio, 1996). A new potential strategy for cryopreservation of canine sperm concerns the possibility to freeze already chilled semen, which may save dog owner's time and money, in case semen is collected close to home, refrigerated, and then sent to a semen bank for freezing and long-term storage (Hermansson and Linde-Forsberg, 2006). However, in any of these cases, semen quality improvement may be achieved by enriching the extender with antioxidants.

The anatomy of the bitch makes the deposition of semen in the uterus during artificial insemination a major challenge. The vagina is long and considerably narrowed cranially by the dorsal median mucosal fold. In addition, the cervix is angled craniodorsally, so that when it is examined from the vagina the dorsal surface of the caudal cervix is revealed and, commonly, the cervical os is not visible, even when open. As a result of this anatomical structure, veterinarians conceived several techniques for AI, depending on the type of semen used and timing availability: deep vaginal insemination and intrauterine insemination, either via non-surgical transcervical insemination or surgical insemination, by laparotomy or laparoscopy (Payan- Carreira, 2011).

Deep vaginal insemination is one of the most commonly used methods for insemination, mainly with fresh semen. It is performed with a plastic catheter or a commercially available catheter in a flexible latex tube which presents an inflatable balloon at the tip, whose goal is to prevent semen backflow when inflated (Linde Forsberg, 2005; Farstadt, 2010). Before AI, the perineal and peri-vulvar areas should be cleaned. Once the bitch is placed in a standing position, the insemination catheter is carefully introduced into the vagina, initially steeply upward until the pelvic brim is passed, and then gently pushed forward at a horizontal angle (Farstad, 2010). Care must be taken not to catheterize the urethra. Therefore, a gloved finger can be inserted into the vagina and secure the catheter while it is moved cranially through the cranial portion of the vagina, bounded by the dorsal medial folds. Once the catheter is in the paracervical area close to the cervical os, the semen may be slowly deposited (Payan-Carreira, 2011). During AI, the bitch should be held with hindquarters up and head down at an angle of 45-60°, to ensure that the semen will not be expelled with the backflow. It is also recommended for bitch to stay in this position for 5-20 minutes after AI (Payan-Carreira, 2011). Pinto et al. (1998) have found that shortening the time of hindquarter elevation has little impact on pregnancy rate and litter size. Greater success in pregnancy is achieved when vaginal massage is performed with the finger guiding the urethral opening, inducing vaginal contractions and mimicking natural breeding conditions.

Various devices can be used to perform vaginal insemination in bitches: an AI pipette for dogs and a rigid plastic pipette with no cuff. The passage at the level of the cervical os is allowed by the rigidity and small diameter of the catheter. As there is no cuff, no vaginal stretching or prevention of semen backflow is possible.

Osiris catheter, a rigid catheter with an inflatable cuff at the distal opening, usually measures 25 cm in length and 5 mm in diameter. Once the catheter is properly positioned, the cuff is blown up with an external valve, inducing vaginal distention and preventing semen backflow around the catheter. The sperm is deposited through the lumen and the syringe should be left in place, again to avoid semen reflux. This type of catheter is good for small and medium-sized dog breeds, whereas it could result in a too short and a too small cuff for larger breeds (Mason, 2018).

Foley catheters are flexible, having an inflatable cuff near the distal opening. They are commercially available in different sizes and may be adapted to different dog sizes. The sperm is introduced into the vagina by using the same method described for the Osiris catheter, but its flexibility makes it

difficult to place the distal opening of the catheter close to the cervix (Mason, 2018).

The Mavic catheter is a plastic catheter with a stylet in the middle. It has an inflatable cuff at the distal opening, and the stylet, through which semen is inseminated, is characterized by a one-way valve, which prevents the semen backflow. The same goal is determined by the presence of an inflatable cuff, which allows vaginal stimulation. These catheters are available in 3 different sizes to suit all sizes of bitches (Mason, 2018).

Transcervical insemination is the most widely used technique for AI when using chilled and frozen-thawed semen. Two different techniques are described: the endoscopic-assisted transcervical and the Norwegian method. In both procedures, insemination can be performed more than once, resulting in increased offspring per litter (Mason, 2018).

Endoscopic-assisted transcervical insemination EIU, also known as transcervical insemination (TCI). Cystoscope was firstly used, whereas, nowadays, most operators use the ureterorenoscope, for its higher length and thinness compared to the cystoscope, and its wider suitability to all sizes of bitches. This procedure is performed with the bitch in a standing position but restrained on the table. In addition to the ureterorenoscope, an additional light source and a camera, which projects the image onto a monitor are needed. The endoscope is inserted into the vagina, and the air is introduced inside. The endoscope is slowly moved cranially past the dorsal median folds to the cervix. The cervical os is commonly located dorso-cranially to the cervical tubercle. Once the catheter is in the cervix, the stylet is removed, and the catheter is introduced into the uterine body. Then, the semen is slowly and carefully injected through the catheter, taking care that no leakage occurs outside the cervix. At the end of the procedure, the vulva is massaged to stimulate uterine contractions (Makloski, 2012). This method ensures an optimal place for semen deposition and results in very good pregnancy rates. It is simple to learn but each step of the process must be closely monitored (Mason, 2018).

The Norwegian (or Scandinavian) method, using a Norwegian catheter, different from those previously described is also used for transcervical/intrauterine insemination. It consists of an outer nylon sheet, with an inner metal stylet, and a blunted and rounded distal tip. This procedure can be used in bitches of any size, and no anesthesia is required. The bitch is restrained on the table in a standing position. The operator palpates and feels the cervix with abdominal palpation, and with the other hand inserts the catheter into the vagina. While holding the cervix, the stylet

is introduced through the cervical os, reaching the uterine body. Once the catheter is in place, the semen is injected through the stylet into the uterine lumen. This procedure is quick, simple, and inexpensive, and can be performed for fresh, chilled or frozen semen, but most published reports suggest its use when managing frozen semen (Makloski, 2012; Mason, 2018). The disadvantage may be the steep learning curve, and difficulties occurring for larger-sized or obese dogs (Mason, 2018).

Surgical insemination may include conventional, laparotomic intrauterine insemination or laparoscopic insemination (Makloski, 2012). These procedures have some welfare issues, as they are considered illegal in some countries (Norway, Sweden) (Mason, 2018). On the other hand, success rates of both methods can reach 100 %, including bitches with known reproductive issues as long, as the female's ovulation timing is properly detected (Brittain Et Al., 1995, Silva Et Al., 1995).

Conventional laparotomy AI, commonly also known as surgical AI or surgical uterine implant (SUI), is performed under general anesthesia by a classic laparotomic surgical approach. The bitch is positioned in dorsal recumbency, and a 25 mm midline incision is performed caudally to the umbilicus, toward the caudal direction. The uterus is exteriorized and inspected. A 22-gauge IV catheter can be used for insemination. After placement of the catheter, the uterus is occluded above the cervix, and the syringe with the semen is attached to a catheter, followed by a slow injection into the uterus. After insemination, the uterus is massaged to stimulate uterine motility, which may be usually reduced due to anesthesia. The catheter is then removed, and the abdomen is finally closed (Mason, 2018).

Laparoscopic insemination is not commonly used in clinical practice, due to the high costs, required knowledge and experience, and lack of advantages of this procedure over SUI (Mason, 2018). Semen is introduced into the uterine horn through an 18–22- gauge catheter under the visualization of a laparoscope, connected to an electronic light source and camera, inserted into the abdominal cavity. The uterine horns are visualized and elevated near the abdominal wall so that the catheter may be inserted. The procedure also requires general anesthesia. Compared to SUI, laparoscopic insemination is less traumatic and invasive for the bitch and allows for a faster recovery. It also provides better protection against the surgical risk of infection, as the abdominal cavity is not opened (Silva et al., 1995).

Intratubal insemination involves the deposition of semen into the oviduct. It is as invasive as other laparotomies for surgical insemination, allowing the use of reduced quantity of spermatozoa to be inseminated but results in

lower pregnancy rates compared to surgical or transcervical insemination (Tsutsui et al., 2003).

However, besides the great opportunities offered by AI in dogs, its use results limited in many cases. Probably the most common reason, especially for the amateur breeder, is the requirement of well-designed planning, the presence or not of a reproductive specialist veterinarian, and the increased cost and inconvenience associated with it. The bitch is a mono-oestrus breeder with a long inter-oestrus interval of approximately seven months, therefore failure to achieve pregnancy creates a real challenge to plan a breeding programme. Other limitations concerning the use of Ai include the variable day on which ovulation occurs during oestrus, so it is not appropriate to perform a 'blind' insemination on a specific day of the oestrous cycle. A detailed examination of the bitch is required to determine the optimal day for insemination, and this day varies individually, also within the same breed. Despite there are still several methods for the accurate determination of the ovulation timing, regular examination of the bitch is required, and this can result in substantial expense. Furthermore, in the bitch, it is technically difficult to place semen into the uterus and both the development of significant skills by the veterinarian and the use of specialist equipment are crucial. Finally, although fresh semen appears to have a long survival time within the bitch's reproductive tract, semen that has been preserved either by cooling (chilled-rewarmed semen) or by freeze-thawing has a significantly shortened life span in the female reproductive tract, which can significantly impact fertility (England et al. 2014).

1.2 Semen Collection Techniques from Male dogs

The indications for collecting semen from a male dog include artificial insemination, cryopreservation or diagnostic purposes. The specific method for collecting semen from a male dog depends on what the semen is to be used for. Only the first (pre-sperm) and second (sperm-rich) fractions of the ejaculate are needed for semen used for artificial insemination or cryopreservation. However, all three fractions should be evaluated when males are presented for breeding soundness examinations or overt evidence of reproductive disease. Semen collection performance is crucial for obtaining good quality semen. Tesi and colleagues found that semen volume and sperm concentration, for instance, may be influenced by semen collection performance. In case fresh semen should be used for AI, an incorrect procedure could affect semen samples, by having a higher volume, but with a consequently lower concentration. The author did not ascribe this

difference to the dogs, whereas to the operator procedure in the division of the three ejaculated fractions (Tesi et al. 2018).
The presence of an oestrous bitch especially for inexperienced dogs facilitates semen collection. However, the lack of an available estrous bitch should not automatically preclude an attempt to collect a semen sample from a dog. Semen should be collected on a non-slip surface and in a quiet area. It is imperative that any distractions or procedures that would induce anxiety to be eliminated or minimized. Fear and pain will prohibit a dog from attaining a complete erection and ejaculating. For excessively timid males, allowing the male to ‘‘play’’ with the teaser or the owner or the collector prior to the collection may improve the quality of the ejaculate (Kutzler et al. 2005).
Canine semen collection can be performed with three different methods: a) digital manipulations (with the finger), b) by using a conical rubber plastic and hand massage c) by using an electro-ejaculator (Baran, 2015) d) Urethral catheterization after pharmacological induction (Kuczmarski et al. 2020).

1.2.1 Physiology of erection and ejaculation

Erection is achieved by an increase in the haemostatic pressure of the penile erectile tissues. The process is mediated by a reflex that is coordinated predominantly at the spinal level (Andersson 2003), but it starts and is modulated centrally (Andersson & Wagner 1995). Erection is prompted via the parasympathetic system originating from the sacral plexus. Nitric oxide, produced by the action of parasympathetic–neural nitric oxide synthetase (nNOS) on L-arginine (Andersson & Wagner 1995), increases the concentrations of cyclic guanosine monophosphate (GMP) in smooth muscle cells, resulting in a reduced responsiveness to calcium and thus relaxation (Andersson 2003, de Tejada et al. 2004). Contraction of the ischiocavernosus muscles at the penile roots increases blood flow in the penile vasculature and occludes the veins that draining the corpora cavernosa penis (CCP). In the dog the levator ani, the coccygeus, and the internal obturator muscles also participate in venous occlusion (Ninomiya et al. 1989). However, venous occlusion is not complete as some drainage continues through smaller channels, when the dorsal vein of the penis is occluded. The increase in blood flow in the penile arteries and CCP and the interruption of the venous outflow determine the engorgement of the cavernous spaces in the blind-ending CCP, causing stiffening and lengthening of the penis. The ejaculatory reflex is stimulated by sensory

nerves within the glans penis, which transmit to the spinal cord via the dorsal nerve of the penis, a branch of the pudendal nerve (Johnson & Halata 1991). Ejaculation cosists of two components: (1) emission and accessory gland secretion, controlled by the autonomic nervous system; and (2) propulsion, controlled by somatic nerves. Uretral propulsion of semen is determined by rhythmic contractions of the bulbospongiosus muscle, located above the corpus spongiosum penis (CSP) in the bulb of the penis, and urethral smooth muscles (Giuliano & Clement 2005). This is accompanied by proximal closure of the bladder neck and intermittent relaxation of the external urethral sphincter and urogenital diaphragm. Contraction of the bulbospongiosus muscle results in increased haemostatic pressure in the CSP. Therefore, each contraction of the bulbospongiosus muscle causes a transient wave of increased pressure in the CSP, which propagates from the bulb to the glans, where it relieved by the dorsal venous drainage of the blood. As the CCP is swollen, the increased pressure in the CSP causes a wave of urethral occlusion. For this reason, assisted by the contraction of the muscle surrounding the extrapelvic urethra, allows the sperm to pass through the urethra. The dog is the only species among the domestic animals in having a penile bone (os penis), surrounding dorsally the urethra. During copulation the dog's penis is gripped by the levator vestibuli of the bitch's vagina, whereupon engorgement of the bulbus glandis occurs. Ejaculation occurs over a prolonged period of time, with the brief pre-sperm fraction phase and an 80-seconds sperm-rich fractions, followed by a very protracted deposition of prostatic fluid during the copulatory tie. The prostate is the only accessory sex gland present in the dog. The absence of the bulbouretral gland is the main reason why canine semen results particularly liquid, so that viscosity and liquefaction time are not evaluated (Parkinson, 2019).

1.2.2 Collection of Semen with Digital Manipulation

A conical rubber plastic (contact funnel) that provides the connection between the artificial vagina and the graduated collection glass may be used. The practitioner should stand to the right of the male, holding the conical semen collection device with the left hand. The operator stimulates the erection by sliding his right hand back and forth on the prepuce. The bulbus glandis should begin to swell being the first sign of erection, as the cavernous penile body fills with blood. The prepuce is then withdrawn to expose the bulbus glandis before the erection is fully achieved, with the conical spermatic collector passing over the penis. If a full erection occurs

before the bulbus glandis protrudes from the prepuce, the animal could feel pain and the erection then quickly disappears. The thumb and forefinger of the hand holding the collection funnel should begin to apply light and rhythmic pressure to the back of the bulbus glandis. After the pelvic thrusts have reached their peak (after about 15-30 seconds), the penis should be turned caudally with the rubber funnel. The first and second fractions of ejaculate are obtained in the first 1-2 minutes. The second sperm-rich fraction of semen has a milky appearance and an average volume of 0.5-5 ml (Ucar, 2000; Baran, 2015). About 1-3 minutes after ejaculation, the prostate fraction is ejaculated, presenting as a clear fluid (3rd fraction). This part is the last one and may have a total volume of 5-40 ml, obtained within 5-45 minutes. With the present method, the detection of the first drops of sperm-rich fraction could result more difficult, and often parts of the ejaculate could mix unintentionally. Moreover, smegma preputi contained in the prepuce during semen collection may contaminate semen samples with this method. The most convenient way to collect semen is by finger movement, using a glass funnel and a measuring cup (Fig.1.2.1). Most dogs are trained for semen collection by digital manipulation. Semen can be collected while the animal is standing on the ground. During semen collection, contact of the rubber glove with the collected ejaculate may affect motility, therefore it should be avoided. During semi-erection, the prepuce sheath is retracted. It is usually recommended that samples containing blood or pus should be discarded, and either another sample taken later, or another stud dog should be used. There are several published studies conducted on the use of sperm separation media (usually single-layer centrifugation), that show favourable results in separating erythrocytes from sperm and improving the motility, morphology, and viability of fresh, frozen or chilled semen (Phillips TC. et al. 2012). The semen collection goblet is kept away, to avoid haemorrhage and trauma. In case the male is reluctant to semen collection, ejaculation can be stimulated by holding the collection cup and placing it against the urethral opening (orificium urethrale) of the glans penis. After the pelvic thrusts are completed, the penis is rotated backwards between the dog's hind legs. At this time, rhythmic pressure should be applied to the posterior portion of the bulbus glandis between the thumb and index finger. After completion of sperm collection, the penis should be lubricated and washed with an antibiotic solution and inserted into the prepuce (Baran, 2015).

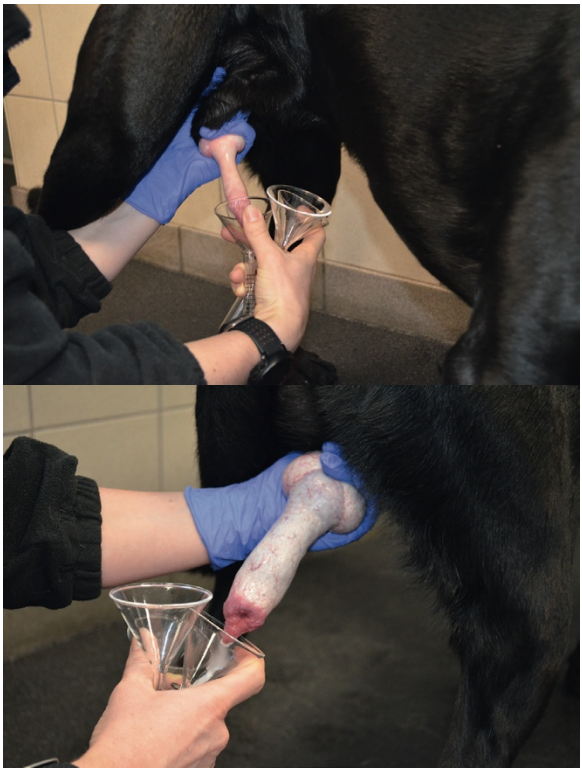

*Fig1.2.1 Manual collection of semen from the dog. (A) The penis is grasped behind the bulbus glandis, simulating the lock of the levator vestibuli of the bitch. (B) The different fractions of semen are collected into separate collecting vessels to minimize the dilution of the sperm-rich fraction with accessory gland fluids. (**Mcgowan M., Evaluation of the Fertility of Breeding Males; Veterinary Reproduction and Obstetrics, 10th ed. Noakes DE, Parkinson TJ, England GCW eds; Publ. 2018)***

1.2.3 Semen Collection with Conical Rubber Plastic and Hand Massage

A collection goblet is placed on the conical rubber plastic end and vaseline or similar lubricants are placed within the plastic. The operator stands on the male's right side, holding the conical rubber plastic in his right hand. By gently massaging the ensheated penis, it is inserted into the conical rubber plastic after the erection is achieved. After the thrusting movements of the pelvis, the rubber is held in the palm of the hand and the penis is rotated backwards by applying pressure to the posterior part of the bulbus glandis.

However, this method precludes to separate the sperm fractions (Baran, 2015).

1.2.4 Collection of Semen with Electrical Stimulations (Electro-Ejaculator)

It is possible to obtain sperm from dogs by inducing an electro-ejaculation (EE) with an electro-ejaculator. However, this method should be performed under general anaesthesia and just when absolutely necessary (e.g. aggressiveness of the stud dog).
For the collection a rectal probe, an electrostimulator and general anaesthesia are required. Once the bipolar rectal probe is inserted into the rectum, the ejaculation centre is stimulated at regular intervals with an electric current of 140-180 mA and a voltage of 10-20 volts and therefore seminal fluid is extracted. In the electro-ejaculation method, the volume of semen extracted is greater than in natural mating because of the over-stimulation of the prostate. However, it does not represent the first choice, because urine may mix with the ejaculate obtained. It could be a good option, for collecting semen from very valuable stud dogs cannot be performed with other methods (Baran, 2015). Christensen and collegues found that, semen samples collected manually from dogs, without extender addition, consistently maintained significantly higher motility after 4 h compared to samples collected by electroejaculation samples. Moreover, exposure of sperm to more prostate fluid following EE seemed the more probable cause of impaired motility, as during EE, the prostate gland is directly stimulated (Ohl et al. 1994), which may result in release of more prostatic fluid than during a natural ejaculation. That could affect semen samples parameters, by decreasing sperm concentration and viability (Christensen et al. 2011).

1.2.5 Collection of Semen by urethral catheterization after pharmacological induction

The a-adrenergic agents are known to influence erection and ejaculation, and ejaculatory reflex is a primarily a-adrenergically mediated event. This method is widely used for semen collection in several species (Mcdonnel & Love, 1991; Zambelli et al. 2008; Silinski et al. 2002). Kuczmarski and collegues demonstrated that the urethral catheterization after pharmacological induction using dexmedetomidine-ketamine association can be performed for canine semen collection, enabling new perspectives

for reproductive biotechnology application in domestic dogs and endangered wild canids. After a routine clinical examination, dogs should be catheterized before the anesthetic procedure for urinary bladder emptying. Twenty minutes after induction with dexmedetomidine and ketamine, semen could be collected with the aid of a round and fenestrated urethral catheter. After 1 min, the catheter should be removed from the urethra. Approximately 13 cm (12.7 cm) of length of the urethral catheter has been recorded for dogs weighing from 5 to 10 kg and established as a standard to be used in all animals during the experiments. Blood semen contamination was not observed in the present study. However, urine contamination was noticed in 44% of the samples. Urethral catheterization after pharmacological induction for semen collection in dogs showed lower volume (0.092 ± 0.03 mL) but higher sperm concentration (1186.67 ± 304.67 x 106 sperm/ mL) (Kuczmarski et al. 2020).

1.3 Cryobiology of Sperm

Sperm cryopreservation has become an indispensable tool in reproductive biology. It has become the best performing technique for long-term conservation, delivery, and dissemination of valuable animal genetic resources worldwide (Akhtar et al. 2022). In addition, cryopreservation of spermatozoa for artificial insemination can prevent sexually transmitted diseases, such as brucellosis and herpes virus infections. Artificial insemination performed with cryopreserved spermatozoa provides several potential advantages, including avoidance of transport-related stress, breeding problems such as copulation failure due to behavioral issues (including female aggressiveness and male indifference), and the quarantine placed on live animals (Suzuki et al. 2022).

Cryopreservation preserves semen fertility virtually indefinitely, though a large proportion of individual spermatozoa do not survive the significant stresses of freezing and thawing. Actually, cells are exposed to several damaging processes during freezing process, which subsequently impairs cells or tissue function. In particular, cryopreservation can damage the DNA of the spermatozoa and/or the acrosomal cap, resulting in reduced fertilization ability or embryonic development. Therefore, post-thaw motility, membrane integrity (via HOST) and acrosomal staining techniques should be always assessed to determine sperm quality (Lopate, 2022, Manual of Andrology). Several factors can influence semen cryopreservation: the fertility and age of the bitch and stud dog, semen

quality before and after freezing, the accuracy of ovulation timing, the site of semen deposition and semen handling techniques during collection, processing, freezing, and thawing (Lechner et al. 2022). Cooling survival and storage can be improved by adding extender solutions to semen. Actually, extenders cannot just be considered as protecting substances for semen preservation but also as cryoprotectants. The presence of glycerol protects spermatozoa from the harmful effect of ice crystal formation (Hermansson et al. 2021). More specifically, the use of extenders improves pH stabilization and energy reservation and helps protecting the sperm membrane from injury caused by shaking and temperature variations during transport. Higher pregnancy rates are achieved when inseminations are performed with extended semen compared to raw chilled semen (Suzuki et al. 2022). The potential fertility of chilled canine semen is maintained by the following three factors:

- Reduced sperm metabolism at very low temperatures;
- Protection from cold shock provided by the use of extenders;
- The relatively high resistance of canine sperm cells to cold shock.

Semen extenders should have specific general features such as adequate osmotic pressure, nutrient medium, absorption of metabolic residues and protection from cold temperatures. Cooling the semen to +5°C requires special care, as a sudden drop in temperatures below 17°C can determine irreversible changes, especially in the acrosome (Ucar et al.2000).

1.3.1 Processing of semen for cooled storage

Semen can be stored in liquid form if the metabolic activity of the spermatozoa is reduced by cooling. The process of cooling from body temperature to 5°C can result in significant damage to the cells unless they are protected from the effects of 'cold shock'. Semen processing steps, such as the addition of extenders, centrifugation, dilution, cooling and storage, contribute to a decrease in motility and fertilizing ability (Aurich, 2005). Semen processing techniques should be adapted to reduce these losses as much as possible. The development of cold shock can be induced by rapid cooling rates, even though it cannot be entirely prevented if slow cooling is performed. Cold shock causes damage to cell membranes, leading leakage of intracellular potassium, enzymes, lipids, cholesterol, lipoprotein, and adenosine triphosphate (ATP). Lowering the temperature determine a transaction of membrane phospholipids from a liquid to a gel status, which can lead to phase separation as this occurs at different temperatures for

different structural lipids (Drobnis et al. 1993; Maldjan et al. 2005; Mahiddine et al. 2021). As a result, the membrane proteins become irreversibly clustered, leading to a loss of function. These changes are also associated with an influx of intracellular calcium, that triggers protein phosphorylation and subsequent sperm capacitation-like changes, also known as cryocapacitation (Singh et al. 2012; Kumar et al. 2012). However, membranes of canine spermatozoa are less sensitive to cold shock than those of other species, due to a relatively high cholesterol:phospholipid ratio (Amann et al. 1987) and increased polyunsaturated fatty acids (PUFA) content in the membrane. Indeed, it has been reported that DNA integrity is not affected by freezing during thawing (Urbano et al.2013), unlike other species, such as horses (Yeste et al.2015). The most effective way of protecting spermatozoa against the harmful effects of cooling is by the addition of extenders, such as egg yolk or milk to the diluent.

1.3.2 Extenders

Specific membrane changes that occur during cooling, storage and cryopreservation of the semen could be the reason why different extenders are needed. Experimental results reported high individual variability in terms of fertility when using different extenders, or, in some cases differences in the concentration of individual components of the extenders (Akhtar et al 2022). Semen extender should: provide nutrients as a source of energy for the spermatozoa and buffer(s) to prevent harmful shifts in pH determined by the metabolic product such as lactic acid; maintain adequate osmotic pressure and electrolyte balance for the spermatozoa; inhibit bacterial growth; protect against harmful effects of storage, refrigeration, freezing and thawing; increase volume of raw semen so that multiple inseminations with suitable numbers of spermatozoa can be performed (Mahiddine et al. 2021). Whole or semi-skimmed milk, coconut milk and chicken egg yolk are often used as extenders for sperm preservation. Extenders are prepared to contain a desired percentage of egg yolk, usually from chicken, although yolk from other species may also be used. Membranes-free egg yolk should be chosen since spermatozoa bind or adhere to membranes. Therefore, an albumin-free and membrane-free yolk should be collected. This can be accomplished using commercially obtained hand-held devices used for baking, or by using a simple laboratory procedure (Lopate, 2022).

Egg yolk (EY) proved to be able in providing an excellent protection for sperm against cold shock. The cryoprotective properties of EY are due to the presence of low-density lipoproteins (LDL) (Foulkes, 1977; Moussa, Martinet, Trimeche, Tainturier, & Anton, 2002; Pace & Graham, 1974; Quinn et al., 1980). Besides EY is a widely and routinely used cryoprotectant for canine semen extenders composition, there are some concerns and risks associated with its use including the risk of bacterial contamination and the potential risk of causing diseases (Hermansson et al. 2021). Moreover, the presence of granules impairs sperm respiration (due to high density lipo-proteins; Amirat et al, 2004) and in vitro analysis of their motility properties. Actually, EY granules may negatively influence objective evaluation performed with Computer Assisted Semen Analysis (CASA) as they may be counted as dead spermatozoa. However, several studies have been carried out to isolate the cryoprotective element of chicken egg yolk in order to obtain a specific extender, without hygienic risks and the reported disadvantages, but keeping its beneficial properties on semen (Bousseau et al., 1998; De Leeuw, Leeuw, Daas, Colenbrander, & Verkley, 1993).

Recently, it has been proposed to replace egg yolk with low-density lipoproteins (LDL), molecules responsible for the cryoprotective effect of egg yolk, and to study their effects on sperm survival during freezing and thawing in dogs. The study conducted by Bencharif et al. showed that a 6% LDL concentration in a Tris-citric acid-fructose medium provided good motility after thawing compared to an egg yolk medium in canine semen (49.9% vs. 47.9% vs. 27.7% for 6% LDL-based media, Equex and conventional egg yolk medium) (Bencharif et al., 2008). Skimmed milk, whole milk, and coconut milk have also been used successfully, although egg yolk is by far the most commonly used additive (Vishwanath & Shannon 2000). Whole milk contains a protein, lactenin, which is spermicidal, so milk for use as a semen diluent must be heat-treated (e.g., in the skimming process) to inactivate this toxic factor. Chemically defined extenders containing phosphocaseinate have now been developed (Batellier et al. 1998) and are tending to replace skimmed milk extenders. Antibiotics are added to most semen diluents as a prophylactic measure against the transmission of pathological bacteria and to reduce the load of non-pathogical organisms that contaminate the semen. The main antibiotic activity is normally given at a temperature above 15 °C, and thus it should be present during the semen cooling process from collection to storage (McCue et al., 2014).

1.3.3 Seminal plasma removal/reduction

The exact role carried out by seminal plasma during the cooling of canine semen remains controversial. Seminal plasma may be a source of ROS, due to the presence of defected and/or dead spermatozoa, which has been demonstrated to affect semen viability (Morrell et al., 2009). However, Araujo and colleagues demonstrated that the presence or lack of seminal plasma during cooling the semen of dogs does not influence sperm quality at 5°C. Moreover, the components of the semen extender may contribute to maintaining good sperm quality and low reactive oxygen species production during the long period of the dog's semen cooling, even after semen centrifugation (Araujo et al. 2022). Nevertheless, the present study was conducted just on eight dogs, therefore, further studies are needed on higher number of specimens, to determine how and how much can seminal plasma influence sperm quality during cryopreservation process.
In order to remove prostatic fluid, Rijsselaere demonstrated that the loss of sperm cells is acceptable by centrifuging canine semen for 5 min at 720 g (Rijsselaere et al. 2012). Higher centrifugation forces may damage spermatozoa and subsequently reduce sperm quality and motility (Dorado et al.2013). If too many spermatozoa remain in the supernatant, centrifugation can be repeated. Pena and colleagues reported using diluted sperm 1:1 in a tris-glucose extender before separating the seminal plasma by centrifugation (Pena et al. 2006). Sperm were then resuspended in another Tris-glucose-yolk diluent, with two-step glycerolization. However, with the "cushion" technique, a higher sperm centrifugation speed can be used (Bliss et al., 2012). A high specific gravity solution should be layered under the diluted sperm, to avoid sperm packing at the bottom of the centrifugation tubes. The use of cushion technique allows the semen to be centrifuged at 1000 g for 20 min, being not affected in terms of sperm motility (Knop et al., 2005). The major disadvantage of standard centrifugation is the loss of approximately 25% of the spermatozoa, when the supernatant is removed. In addition to the padded centrifugation technique, many new methods of semen processing have been recently proposed. The best options for semen centrifugation are sperm separation by density gradient or a simplified single-layer centrifugation method. The latter showed favorable results in separating erythrocytes from sperm (Philpps et al. 2012) and in improving motility, morphology, and viability (Dorado et al.2013).

1.3.4 Cooling rates

The “cold shock” phenomenon can be induced at a specific temperature range between 18°C to 5°C during the cooling processes of extended semen (Watson et al. 2000). Cryopreservation of canine spermatozoa requires high adaptability of the cell to changing osmolarity and temperature. As in other species, the fluidity of the membrane changes significantly during the freeze-thawing procedure, which coincides with a rearrangement of membrane phospholipids. The membrane fluidity is dependent on the cholesterol content and the amount of disulfide bonds, the acyl chain length saturation and the temperature of the surrounding milieu (Schäfer-Somi et al. 2022). When the temperature decreases, the membrane lipids change toward the crystalline phase with lateral segregation, lipid peroxidation, loss of lipids and formation of reactive oxygen species (Liu et al. 2021). This finally leads to membrane destabilization and may cause membrane damage, especially when the cooling rates during freezing are too high or too low (De Leeuw et al. 1990; Ricker et al. 2006). Although canine spermatozoa are considered relatively resistant to cooling at 5°C (Baptista et.al 2012), slow cooling rates prior to freezing are generally used for this species. Cold shock damage in canine semen is caused by cooling rates greater than -0.3°C/min and is due to lipid phase transitions that cause lateral lipid rearrangement and may even involve loss of lipid fractions from the plasma membrane (Rodenas et al.2014). The rearrangement of membrane components predisposes the cells to membrane lipid peroxidation as a result of the formation of ROS, which may compromise membrane integrity (Ricker et al., 2006). One of the most used cryopreservation protocols for canine spermatozoa is the Uppsala method, which consists of two dilutions before freezing separated by a long cool-down step from room temperature 23°C to 5°C, over 1 to 2 h (Schäfer-Somi S, et al. 2006; Hermansson et al. 2006; Peña et al. 2012). These equilibration periods correspond to estimated mean cooling rates of approximately 0.15–0.3 C/min. Although slow cooling rates are considered optimal, few studies have been performed to evaluate the effects of a rapid cooling rate and of reducing the cool-down time in the Uppsala protocol. Rodenas and colleagues provided the first evidence that dog spermatozoa are able to survive rapid cooling rates (2.25 C/min) before freezing with the Uppsala method. While in the traditional Uppsala protocol, semen reaches 5°C in approximately 90 min, this rapid cooling protocol would support a considerable reduction in the time required for the process of freezing dog spermatozoa, as it allows an interval of

approximately 8 min between 23 and 5°C (Rodenas et al. 2014). The use of programmable freezers has the potential to significantly improve post-thaw recovery rates. Therefore, it is difficult to generalize about optimal cooling rates, as the best ones are determined by the concentration of glycerol and other components of the diluent. Some methods used in equine breeding, such as the Equitainer system, for chilling and transporting semen, can also be successfully employed in dogs (Pinto et al. 1999) (Fig.1.3.4).

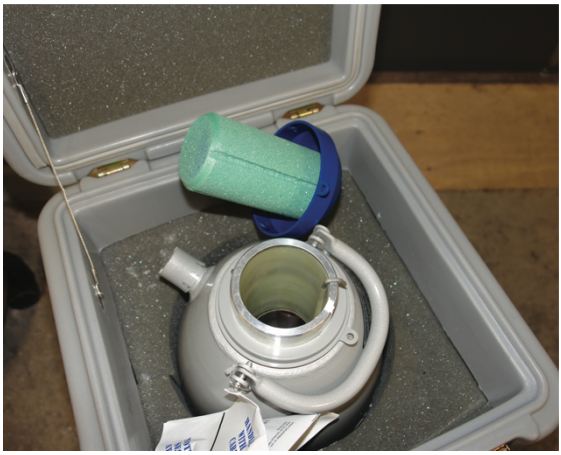

Fig.1.3.4 Traditional shipping vessel and case for transportation of frozen semen. In this example, the shipper is described as 'dry' because the liquid nitrogen is absorbed into an absorbent pad surrounding the semen; in this way there is no free liquid, and the container is described as non-hazardou (England et al. 2014)

1.3.5 Storage temperature

A temperature range between 4°C and 6°C has been defined as the optimal storage temperature for maintaining sperm motility and fertility (Bencharif et al 2022). Cold inhibits microbial growth and reduces sperm metabolism, extending their life span (Yoshida, 2000). Appropriate packaging should be used to maintain a constant temperature during sperm transport (Bradecamp, 2014). Refrigerated semen can be packaged in plastic bags, heat-sealed plastic bags, all-plastic syringes (without rubber plungers), or plastic containers such as conical centrifuge tubes. Then, these packages should be placed in a commercial shipping container. Several methods for refrigerated semen transport have been evaluated. Currently, canine chilled semen samples can be shipped in different containers, including thermo flasks,

styrofoam boxes or even in containers designed for stallion sperm, such as Equitainer (Lopes et al. 2009). Among these options, the Neopor canine transport box provided by Minitübe is one of the most used in dogs due to the reduced cost and easy handling (Rijsselaere et al. 2011). There are several reusable and disposable containers for shipping refrigerated semen.

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Chapter 2

Oxidative stress and free radicals

2.1 Reactive oxygen species in canine spermatozoa

Mitochondria are not only energy producers but are also the source of reactive oxygen species (ROS) necessary for normal sperm function in all mammals (Fig.2.1). Unfortunately, when exceeding they can cause sperm damage. Oxidative stress is characterized by an imbalance between the levels of ROS and antioxidants (Vieira et al., 2018). Oxidative stress in canine spermatozoa can lead to deleterious effects on sperm structure and function, including lipid peroxidation, DNA damage, and sperm apoptosis (Kothari, Thompson, Agarwal, & Plessis, 2010). Vieira et al. (2018) found in their studies on dog sperm that the most harmful and toxic ROS were hydrogen peroxide (H2O2) and the hydroxide ion (OH -). Hydrogen peroxide could penetrate cell membranes unhindered and can inhibit the enzymatic activity and functions of various cells. Spermatozoa are particularly susceptible to oxidative stress due to the lack of antioxidants caused by the reduced cytoplasm and the high levels of polyunsaturated fatty acids in the plasma membrane (Luvoni & Morselli, 2017). In contrast, mild oxidative stress is considered a normal physiological response and is necessary for several sperm functions, including sperm maturation, capacitation and hyperactivation, acrosome reaction and sperm-oocyte fusion (Kothari et al., 2010). For example, ROS promotes capacitation through redox regulation of tyrosine phosphorylation (Aitken, Jones, & Robertson, 2012). It has been established that the ROS presence in the male genital tract and semen is not a negative phenomenon. In fact, low concentrations of ROS are essential for the physiological function of spermatozoa, i.e., for the activation of their fertilizing ability. Both spermatogenesis and steroidogenesis of Leydig cells have been found to be sensitive to excessive concentration of ROS in the testis. Consequently, ROS is involved in the production of immature and defective sperm (Hales et al., 2005). High concentrations of ROS lead to the previously mentioned situation of oxidative stress (OS), a condition associated with an increased rate of cellular damage (Sikka et al., 1995). All sperm cell biomolecules, including membrane lipids and proteins and DNA, can be the target of oxidative stress-induced sublethal cell damage (Aitken et al., 2010). Therefore, attention has focused on the role of ROS in sperm and the relationship between OS and male infertility (Aitken and Baker, 2004). To avoid excessive concentration of ROS, the testis has an enzymatic and a non-enzymatic antioxidant system (Aitken and Roman, 2008). In addition, human and canine seminal plasma have also been found to be endowed with

antioxidants that can protect sperm from the time of ejaculation to the oviduct (Viera et al.2018). Oxidative stress in the testis, epididymis, and ejaculate is caused by either an inefficient antioxidant barrier or excessive production of ROS by immature and damaged spermatozoa (Roca et al., 2013). The balance between the production and degradation of ROS should be maintained during sperm processing and storage to preserve sperm motility and fertilizing ability. During refrigeration or cryopreservation of sperm, the production of ROS increases dramatically and exceeds the antioxidant capacity of seminal plasma, leading to oxidative stress (reviewed in Ball, 2008).

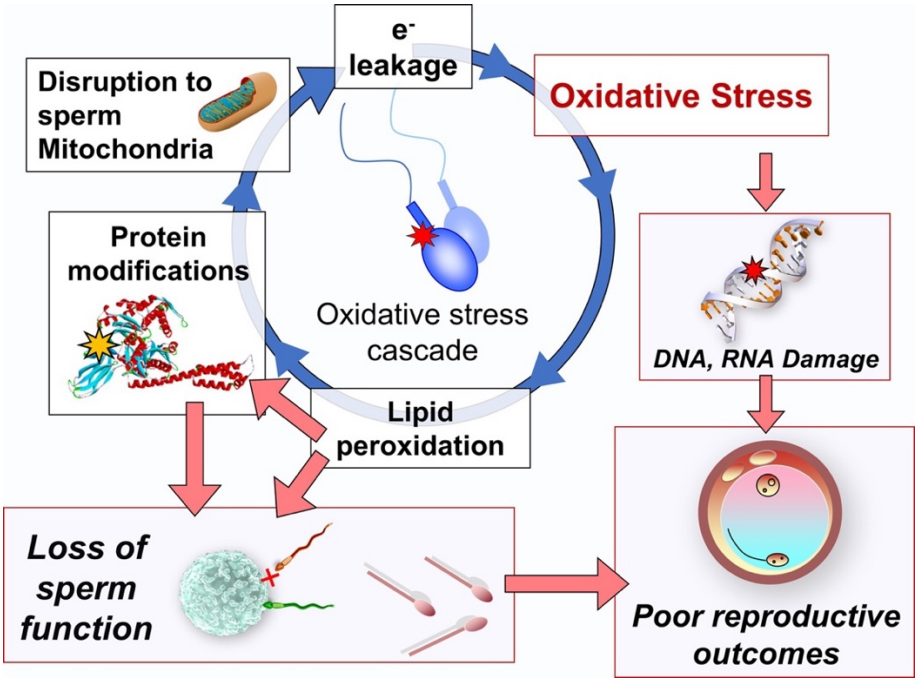

Fig.2.1 Sperm mitochondria can propagate oxidative stress in the male germline inducing protein, lipid and DNA damage, resulting in a loss of sperm function.Z. Gibb, et al. Animal Reproduction Science 220 (2020) 106456

2.1.1 Source of ROS in canine semen

The sources of ROS in semen are epithelial cells, leukocytes, and especially spermatozoa (Makker et al., 2009; Mathur and D'Cruz, 2011). The ROS production is markedly different among spermatozoa. This is probably related to different activities of enzymes such as the cytosolic enzyme glucose-6-phosphate dehydrogenase (Esfandiari et al. 2003, Aziz et al. 2004). This enzyme is involved in the formation of nicotinamide adenine dinucleotide phosphate (NADPH). NADPH appears to be the major source of ROS in sperm. A sperm-specific NADPH oxidase system (NOX5) and a NADP-dependent oxidoreductase (diaphorase) are located in the plasma membrane of the sperm head and at the mitochondrial level, respectively (Gavella and Lipovac, 1992). The number of mitochondria in spermatozoa is high to provide the energy required for their motility. Morphologically abnormal spermatozoa contain defective mitochondria that produce increased ROS. ROS may affect the membranes of other mitochondria, causing a further increase in ROS production (Evenson et al., 1982). Production of ROS has been found to vary from cell to cell in subsets of spermatozoa at different stages of maturation (Gil-Guzman, 2001). Immature germ cells produce higher amounts of ROS than mature germ cells. Spermatocytes, round and elongated spermatids produce low amounts of ROS (Gil-Guzman, 2001). Several authors suggest that leukocytes, particularly neutrophilic granulocytes and macrophages, are an important source of ROS in the male reproductive tract and ejaculated semen because leukocytes possess a membrane-bound NADPH oxidase similar to that of spermatozoa (Agarwal et al., 2004). However, other studies do not confirm a high correlation between leukocyte concentration and levels of ROS (Fedder et al. 1996).

2.2 Physiological role of ROS in spermatozoa

Low concentrations of ROS are a prerequisite for spermatozoa to obtain their full fertilizing capacity. ROS are involved in keeping a good motility, capacitation, hyperactivation, acrosome response, and fertilization (DeLamirande et al., 1998). It has been suggested that ROS may influence the physiological changes associated with sperm capacitation (de Lamirande et al., 1998). ROS promotes capacitation through redox regulation of tyrosine phosphorylation in several species, including males (Leclerc et al. 1997; Aitken et al., 1998) and dog (Viera et al., 2018). ROS may also be

involved in other mechanisms of sperm capacitation, i.e., stimulation of cyclic adenosine monophosphate production and activation of protein kinase A, activation of extracellular signal-regulated kinase-like proteins and upregulation of tyrosine phosphorylation in the sperm tail, or in the induction of sterol oxidation (reviewed by Aitken, 2017). Superoxide anion and small amounts of hydrogen peroxide have been shown to be involved in signal transduction and tyrosine phosphorylation of sperm membrane proteins, thereby stimulating capacitation and acrosome response (Griveau et al., 1994). Superoxide anions and hydrogen peroxide also cause tyrosine phosphorylation, which in turn promotes sperm membrane binding to ZP-3 protein in the zona pellucida (Aitken et al., 2010).
ROS provoke lipid peroxidation in the plasma membrane, which is usually associated with reduced sperm function and viability (Griveau et al., 1994). However, a low level of lipid peroxidation is necessary to facilitate the adhesion process of spermatozoa to homologous and heterologous zonae pellucidae (Aitken et al., 1989). In contrast, nitric oxide has no effect on zona pellucida binding but is essential for activating the ability of sperm to fuse with oocytes (Zini et al., 1995; Francavilla et al., 2000).

2.2.1 Effect of oxidative stress on spermatozoa

Spermatozoa are highly susceptible to oxidative stress and especially lipid peroxidation due to their high content of polyunsaturated fatty acids in the plasma membrane. Fatty acids are essential for the male germ cell to maintain sperm functions, and spermatozoa are unable to resynthesize their membrane components (Henkel, 2005). ROS may also indirectly generate oxidative stress by reducing sperm enzymatic defenses. On the other hand, oxidative mechanisms play a key role in the physiological control of mammalian sperm functions (Kodama et al., 2013). Small amounts of ROS are necessary for sperm to become fertilizable (Saleh and Agarwal, 2002). ROS is normally involved in sperm kinetic function (Griveau and Le Lannou, 1997) and in capacitation and hyperactivation processes (Agarwal et al., 2006) by stimulating intracellular cAMP production and tyrosine phosphorylation. Hydrogen peroxide appears to be the most dangerous ROS for semen because it is membrane permeable and can damage DNA, whereas another extrinsic ROS can mainly cause lipid peroxidation (Henkel et al., 2005). The deleterious effects of oxidative stress depend on the amount of ROS and duration of ROS exposure (de Lamirande and Gagnon, 1995; Agarwal and Prabakaran, 2005). Extracellular factors such as

temperature, oxygen tension, and environmental composition influence the extent of OS (Aitken and Fisher, 1994). The extent of OS in the seminal plasma depends on composition, such as the concentration of ions, proteins, and especially ROS scavengers (Agarwal and Saleh, 2002). Reduction in sperm motility is a sensitive indicator of oxidative stress. Motility is the first parameter to be affected by excessive amounts of ROS (Baumber et al., 2000). The exact mechanism underlying the relationship between OS and the reduction in motility is not clear. It has been hypothesized that extracellular hydrogen peroxide enters spermatozoa and inhibits the activity of several enzymes, such as glucose-6-phosphate dehydrogenase. G6PD regulates intracellular glucose concentration and consequently the availability of NADPH (Aitken et al., 1997). Decreased availability of NADPH and accumulation of oxidized and reduced glutathione impair sperm antioxidant defenses and peroxidation of membrane phospholipids. Lipid peroxidation is associated with a loss of motility due to the release of enzymes and ATP, followed by a decline in sperm metabolic activity (Storey, 1997). Any impairment of the ATP production process may have a negative effect on motility. Such ATP depletion decreases the available energy supplied by mitochondria, resulting in decreased axonemal protein phosphorylation and sperm immobilization (deLamirande and Gagnon, 1992). ROS also has a direct effect on mitochondria by destroying the inner and outer mitochondrial membranes and inducing the release of apoptosis-inducing factors (AIF). The AIFs released are the cytocrome C protein and proteases, i.e., caspases 3 and 9, which interact directly with DNA and lead to DNA fragmentation and apoptosis (Candé et al., 2002; Paasch et al., 2004). In addition, ROS plays a physiological role in the fusogenic function of sperm, allowing them to bind to the zona pellucida, undergo the acrosome reaction, cross the zona pellucida, and fuse with the oocyte membrane (Griveau and Le Lannou, 1997). When ROS is present in low concentrations, they act as mediators of normal sperm functions, whereas when produced in excess, they are highly toxic to the cell. The sperm has three protective enzyme systems against ROS damage, including superoxide dismutase (SOD), catalase, and the glutathione peroxidase/reductase system (Griveau et al., 1995). ROS effects on semen are summarized in Fig. 2.2.1.

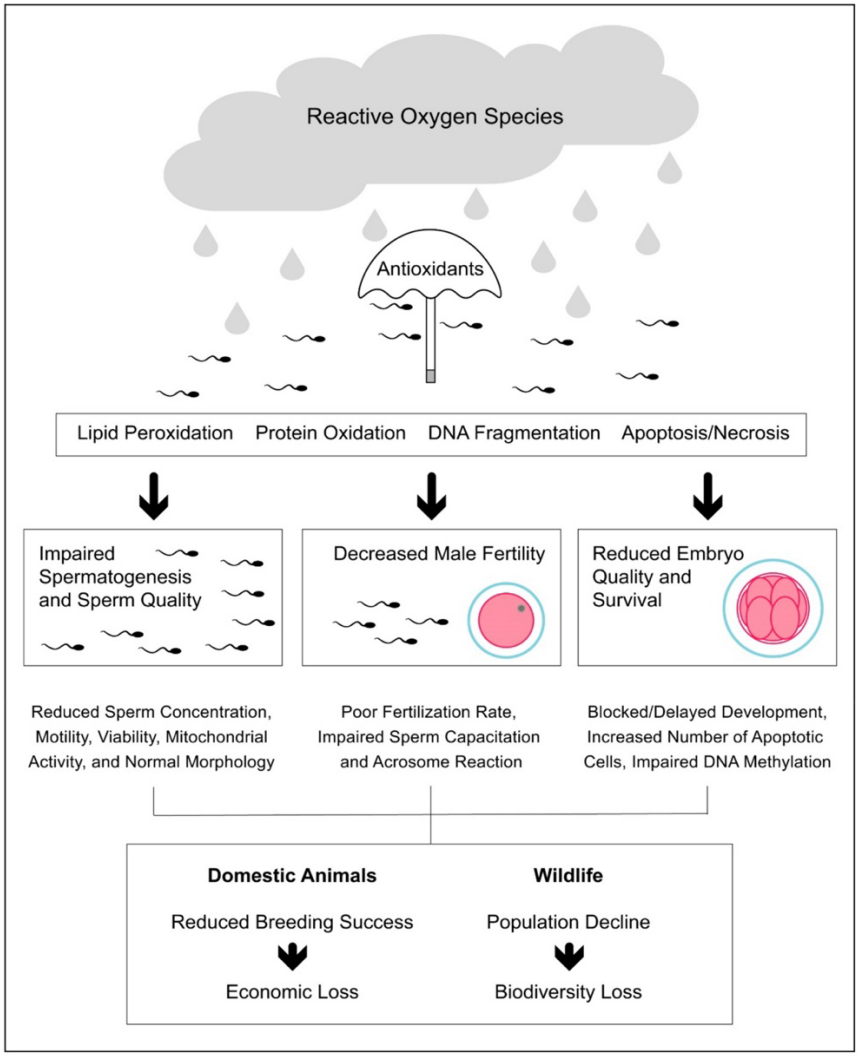

Figure 2.2.1 *Impact of oxidative stress on male reproductive performance in domestic and wild animals. (Pintus et al.2021)*

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Chapter 3

Antioxidants and male fertility

3.1 Role of antoxidants on semen

Mammals have evolved antioxidant defense systems playing a role in preventing the formation of ROS, inactivating oxidants, and limiting the harmful effects of oxidants by allowing repair of oxidative damage (Cheeseman and Slater, 1993). The male reproductive tract has enzymatic and nonenzymatic antioxidant strategies, to mitigate the effects of oxidative stress on spermatogenesis and steroidogenesis and to protect spermatozoa from excessive oxidative stress (reviewed in Vernet et al., 2004). After ejaculation, in the female genital tract, or during storage/dispatch, sperm rely on antioxidants, metal ions, and proteins derived from seminal plasma and sperm extender for protection (Wai-Sum et al., 2006). Understanding the mechanisms that protect spermatozoa from oxidative stress (OS) by affecting intrinsic antioxidant mechanisms may provide an ideal strategy for better preservation of sperm function. Human studies suggest improved antioxidant defense by exogenous enhancement against OS with two different approaches: antioxidant dietary supplementation to improve antioxidant status in tissues, seminal plasma, and spermatozoa or addition of antioxidants to seminal supplements to increase antioxidant status of seminal plasma (Agarwal et al., 2004). Substances with the antioxidative properties, which are a part of dog semen composition, are localized mainly in a small cytoplasmic area in the sperm midpiece and in the prostatic fluid (Luberda, 2001). According to the study conducted by Strzeżek (2009), the antioxidative system of dog semen is mostly represented by superoxide dismutase, and to a lesser extent by glutathione peroxide and phospholipid hydroperoxide glutathione peroxidase. Catalase, on the other hand, the enzyme responsible for the degradation of hydrogen peroxide, was not found in dog seminal plasma (Hatamoto et al., 2006; Strzeżek et al. 2009) observed the deficient activity of catalase in dog sperm. This is in contrast with the studies by Kawakami et al. (2007), who demonstrated catalase activity in dog ejaculates and showed that the addition of catalase and superoxide dismutase in the dilution extender of canine semen improved sperm quality. Michael et al. (2007) studied the protective activity of certain antioxidative factors in canine semen, demonstrating that the best protective properties were found in catalase, taurine and N-acetylcysteine (NAC) in the first, second and third place, respectively. Brito et al. (2018) did not find any differences on sperm oxidative stress between young and senile dogs. Antioxidants are represented by two groups of compounds: enzymatic

antioxidants, which include superoxide dismutase, catalase and glutathione peroxidase, and non-enzymatic antioxidants, for example vitamin E, vitamin A, vitamin C and uric acid (Del Prete et al., 2018).

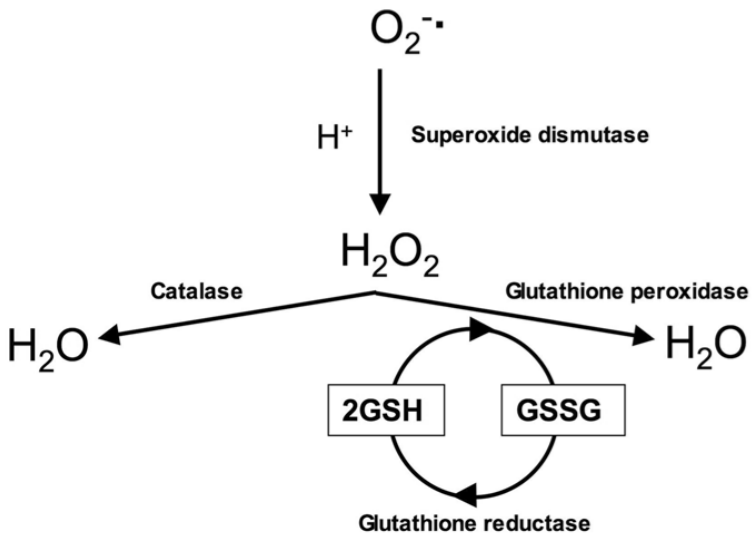

Figure 3.1 *Antioxidant scavenging pathways of free radicals by superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX); Glutathione reductase catalyzes the reduction of oxidized glutathione (GSSG) to regenerate glutathione (GSH; modified by Aitken and Roman, 2008).*

3.1.1 Enzymatic antioxidants

Oxidative stress in the testis triggers a response characterized by NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) mediated induction of mRNA species for superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities (Kaur et al., 2006). The enzyme superoxide dismutase (SOD) in the cytoplasm (Cu, Zn- SOD) and mitochondria (Mn- SOD) is responsible for association with two common ROS molecules, including the superoxide anion (O2-) and hydrogen peroxide (Amidi et al. 2016). However, there are controversial results regarding the beneficial effects of SOD. While the addition of SOD to extenders resulted in higher sperm motility during cooling and freeze preservation (Forouzanfar et al.2013), Silva et al. found no increase in sperm

kinematic parameters after addition of SOD to the ram sperm extender (Silva et al. 2011). Nevertheless, acrosome integrity and mitochondrial activity were improved in the presence of SOD (Silva et al.2011). Roca et al. reported that catalase improved the fertilization potential of boar sperm after thawing when catalase was used alone or in combination with SOD (Roca et al. 2005). In addition, Fernandez-Santos et al. demonstrated that catalase inhibits DNA damage during oxidative stress in cryopreservation (Fernandez-Santos et al. 2009). There are also numerous studies describing the beneficial effects of glutathione on semen cryopreservation in a variety of animal species. In fresh semen, glutathione did not affect the quality of ram semen (Silva et Al. 2009). In another study, Silva et al. demonstrated that glutathione (2 mM and 5 mM) preserved the integrity of ram sperm acrosomes (Silva et al. 2009). Moreover, 5 mM oxidized glutathione increased the movement and velocity properties of spermatozoa after freezing and thawing (Uysal et al.2007), and similar results were found at lower concentrations in turkey spermatozoa (Izanloo et al. 2022).Similar results were obtained with the multifunctional antioxidant melatonin, where positive effects on sperm cryopreservation were found in pig (Pezo et al. 2021), ram (Pool et al.2021) , goat (Tanhaei et al.2022), dog (Divar et.al 2022), fish (Felix et al.2021), bovine (Su et al.2021), and human (Minucci et al.2022).

3.1.2 Non-enzymatic antioxidants

Alpha-tocopherol (vitamin E) is one of the most important compounds with antioxidant properties, commonly found in the plasma membrane and seminal plasma. This lipophilic antioxidant protects the fatty acid content of membranes from peroxidation (Aikten et al. 2012) and has a dose-dependent effect (Hull et al.2000). The water-soluble Trolox analogue of vitamin E improved several quality indicators of boar semen during cold storage (Cerolini et al.2000). In addition, it increased fertilisation ability and decreased the amount of hydrogen peroxide in bull semen when added to the extender (Dalvit et al.1998). The beneficial effects of vitamin E on reproductive ability were also demonstrated in chickens (Khan et al.2012), rabbits (Yousef et al.2010) rams (Masoudi et al.2016), cattle (Majid et al.2015) and dog (Kirchhoff et al. 2017) when it was added to the diet. Ascorbic acid (vitamin C) is another water-soluble vitamin associated with reproduction; however, its exact mechanism is still unclear (Sonmez et

al.2005). Vitamin C may have a significant impact on DNA protection during cryopreservation (Fraga et al.1991) Azawi and Hussein reported an improvement in motility and viability of ram spermatozoa supplemented with 0.9 mg/mL vitamin C during room temperature preservation (Azawi et al.2013). In a conflicting report, vitamin C decreased the motility of ram spermatozoa when the extenders were supplemented with 50 mM or 100 mM compared to the control group (Sanchez-Partida et al.1997) Vitamin C could be a pro-oxidant compound in the presence of iron ions in the extender (Amidi et al.2016), as it converts Fe3+ to Fe2+, leading to a reaction with oxygen or hydrogen peroxide, which then triggers lipid peroxidation (Rietjens et al.2002).

3.1.3 Metal chelators or metal binding proteins

Transitional metal ions are involved in the generations of highly reactive oxygen species (Ochsendorf, 1999). Metal chelators and metal-binding proteins (e.g. albumin, metallothionein) reduce the formation of new ROS, by inactivation of transitional ions. In the seminal plasma, metal chelators such as transferrin, lactoferrin, and ceruloplasmin protect sperm integrity by controlling lipid peroxidation of plasma membrane (Sanocka and Kurpisz, 2004). The addition of other metal chelators to semen extender such as ethylene diamine tetraacetic acid, 1,10-phenanthrolin, DL-penicillamine and neocuproine have been shown to reduce sperm DNA damage and increase sperm motility (Henkel and Schill, 2003; Wroblewski et al., 2003; Agarwal et al., 2005).

3.1.4 Other non-enzymatic antioxidants

Amino acids are present in the seminal plasma and are categorised as non-enzymatic scavengers with antioxidant properties. Different types of amino acids such as hypotaurine, glutamine, cysteine, taurine, histidine, proline, and glycine were found to reduce DNA fragmentation and improve various post-thaw parameters of ram sperm (Sanchez-Partida et al.1997). Sangeeta et al. found that the addition of 25 mM l-proline and 20 mM l-glutamine in a Tris-based medium reduced lipid peroxidation and improved the integrity of sperm acrosomes (Sangeeta et al.2015). Fattah et al. found that the addition of 1 mM and 2 mM l-carnitine to cryopreservation media improved sperm mitochondrial function and resulted in higher progressive motility

after thawing (Fattah et al 2017). However, the combination of these amino acids may have a negative effect on semen quality, as Zhandi and Sharafi found that the combination of cysteine and glutathione in a soybean lecithin-based extender increased apoptosis in their analysis of ram sperm after thawing (Zhandi et al.2015). Another amino acid commonly used in the different types of extenders is bovine serum albumin (BSA), which can protect sperm membrane integrity, especially during heat stress (Lewis et al. 1997). In some experiments, 10% or 15% BSA was used as a substitute for egg yolk in ram sperm diluents, and these showed an equivalent cryoprotective effect compared to egg yolk (Fukui et al.2007). Finally, in a study by Coyan et al, the addition of 1, 2, and 4 mM ergothioneine decreased the percentage of DNA fragmentation in ram sperm after thawing (Coyan et al.2012). Ergothioneine is a low molecular mass thiol found in some tissues. It scavenges oxygen hydroxyl radicals and peroxyl radicals and acts as a regulator of iron metabolism. Ergothioneine has been shown to protect spermatozoa from oxidative stress and to improve motility of ram (Coyan et al.2012) and dog (Usuga et al.2021) spermatozoa after thawing. High concentrations of carnitine have been found in human semen produced by the seminal vesicle and epididymis (Lewin et al., 1976). Carnitine plays an important role in sperm maturation and development (Lenzi et al., 2003; 2004). Dietary supplementation with carnitine promotes membrane stability and protects sperm from ROS damage and apoptosis (reviewed in Lombardo et al., 2011).

Since the deleterious effect of oxidative stress on sperm is considered to be the main cause of subfertility in men (Tremellen, 2008), antioxidant strategies have been proposed to reduce oxidative stress and thereby improve male fertility (Agarwal et al., 2004; Agarwal and Sekhon, 2010; Lombardo et al., 2011; Elmussareh et al., 2015). Although a general positive effect of antioxidants on semen quality has been demonstrated, few studies reported improvement in pregnancy rates (reviewed in Ross et al., 2010; Showell et al., 2011). The goal of antioxidant supplementation is to improve antioxidant defense mechanisms in the male genital tract and in seminal fluid and sperm after ejaculation. Antioxidant strategies may include antioxidant dietary supplementation (in vivo studies) as well as the addition of antioxidants to semen diluents used to produce fresh, chilled, and frozen semen (in vitro studies) (Lombardo et al., 2011).

3.2. Antioxidant strategy

Antioxidants can be administered by using several strategies such as: addition to diet supplementation and addition to fresh, chilled or frozen semen.

3.2.1 Dietary supplementation of antioxidants

Nutritional deficiencies can have a significant impact on sperm quality; low intake of antioxidants in nutrients such as selenium (Se) and vitamin E can cause poor sperm quality or even loss of fertility (Ahsan et al., 2014; Boitani & Puglisi, 2008; Zubair, 2017). Dietary supplementation with antioxidants has been attempted to improve male fertility and to try to influence freezing success. An improvement in semen quality after dietary supplementation of antioxidants in humans, rats, pigs, turkey, sheep, fish and dog has been detected (Audet et al., 2004; Eskenazi et al., 2005; Sönmez et al., 2005; 2007; Akmal et al., 2006; Eid et al., 2006; Yue et al., 2010) The success of antioxidant supplementation is due to an enhancement of antioxidant protective mechanisms in the epithelial mucosa and secretions of the male reproductive tract. This results in less ROS-induced damage to sperm in testes, epididymis and in ejaculated and processed semen. Dietary supplementation with PUFA in combination with vitamin C or E as antioxidants in the diet has been shown to improve sperm quality in e.g. rabbits (Castellini et al., 2004), broilers (Surai, 2000), roosters (Cerolini et al., 2005), boars (Liu et al., 2015), Japanese quails (Al-Daraji et al., 2010), rams (Alizadeh and Shaabani, 2014; Jafaroghli et al., 2014), goats (Dolatpanah et al., 2008), and bulls (Kaka et al., 2015). Dietary supplementation with supplements containing antioxidants is now routinely used in male infertility because it is widely available.
Oral administration of vitamin E to male dogs prevented some of the negative stress effects of oral administration of dexamethasone on sperm quality (Hatamoto et al. 2006). Dietary supplementation with a number of antioxidants was found to have no effect on fertility in stallions (Deichsel et al. 2008). As for dietary supplementation with antioxidants to improve sperm quality and fertility in males, astaxanthin is an antioxidant without vitamin A activity. Astaxanthin belongs to the carotenoid family and has 10 times higher antioxidant activity than B-carotene (Naguib et al. 2000). Recent scientific evidence suggests that astaxanthin is a potent antioxidant

and that its potent antioxidant activity on the surface and inside the phospholipid membrane is responsible for its highly potent antiperoxidative activity (Goto et al. 2001). Dietary supplementation with astaxanthin has been shown to improve semen quality in subfertile males (Comhaire et al. 2005). Most herbs, fruits, and vegetables have been shown to have antioxidant properties and can affect sperm production and function (reviewed in Ko EY and Sabanegh, 2014). Several nutraceuticals have also been tested in animal reproduction (review in Arruda et al., 2010). For example, the use of the Andean plant Lepidium meyenii (maca) has been tested in various animal species, such as humans, rodents, and cattle. Lepidium meyenii (Maca) grows between 3,800 and 4,500 m altitude. Clinical studies have shown that administration of maca increases sperm count and motility and improves sexual function in humans (Gonzales et al., 2001; 2002). Several studies conducted in rats have shown the positive effect of Maca administration on spermatogenesis, improving sperm count and motility (Gonzales et al., 2003; 2004; 2006; 2013; Chung et al., 2005; Gasco et al., 2007; Yucra et al., 2008). Dietary supplementation with maca meal improved sperm count and motility and decreased the percentage of DNA fragmentation index in bulls (Clément et al., 2010). This plant has beneficial effects on the reproductive tract, sperm quantity and quality in mammals (Clément et al., 2012; and by Gonzales, 2011).

3.2.2 Antioxidant addition in semen extender

The presence and availability of antioxidants is important to prevent damage to cell membranes. Although semen dilution prior to freezing removes most of the seminal fluid prior to dilution, residual components of the seminal fluid are still present, although possibly in less than optimal amounts. EY the seminal fluid commonly found in semen diluents contains several antioxidants, including phosvitin, vitamins E and C, which protect sperm from oxidative damage. More and more publications point to the need to protect sperm from oxidative damage during processing and freezing. The addition of antioxidants to media for seed handling and storage aims to compensate for the decrease in antioxidant defense capacity of semen in vitro that occurs during semen processing and chilled or frozen semen storage. Whether the addition of antioxidants to seed extenders has a positive or negative effect depends on the dosage, the type of antioxidant, the combination of antioxidants, and the context in which the antioxidant is

used. Several antioxidants have been previously tested in in vitro studies using human, bovine, porcine, rabbit, and equine semen (Alvarez and Storey, 1983; Beconi et al., 1993; Kessopoulou et al., 1995; Baker et al., 1996; Aurich et al., 1997; Donnelly et al., 1999; Ball et al., 2001a, 2001b; Bilodeau et al., 2001; Peña et al., 2003), with controversial efficacy and benefits. For example, vitamin E has a detrimental effect on fresh human semen (Donnelly et al., 1999) and liquid ram semen (Upreti et al., 1997) and little or no effect on chilled horse semen (Aurich et al., 1997; Ball et al., 2001b) and on frozen, thawed human semen (Askari et al., 1994). In contrast, the addition of vitamin E or B16 to chilled or frozen dog semen improves semen parameters and inhibits the production of ROS (Michael et al., 2007; 2009). In boars, the addition of vitamin E to semen has a positive effect on sperm motility, mitochondrial membrane potential, and membrane integrity after cryopreservation, but the effect depends on the proportion of ejaculate (Peña et al., 2003; 2004). The addition of diluents containing enzymatic antioxidants has been tested individually to counteract oxidative stress during chilling and freezing of semen. In previous studies, the addition of CAT to semen diluents was shown to improve semen functions after thawing in deer (Fernández-Santos et al., 2007), dogs (Michael et al., 2007), boars (Roca et al., 2005), and bulls (Asadpour et al., 2012). In addition, CAT provides a protective effect against DNA damage in horse spermatozoa (Baumber et al., 2003a). SOD Addition to sperm extenders leads to controversial effects. A protective effect of SOD on sperm has been reported in males and mouflons (Kobayashi et al., 1991; Berlinguer et al., 2003), but a damaging effect has been found in horse sperm during cryopreservation (Baumber et al., 2005).The effects of the enrichment of extender with various antioxidants (vitamin C, vitamin E, vitamin B16, N-acetyl-L-cysteine, taurine, and catalase) were studied on the quality of chilled and thawed dog semen (Michael et al. 2007).Of the antioxidants tested, vitamin E and B16 were found to be more effective in improving the quality of chilled semen by reducing the levels of ROS (Michael et al. 2009), while catalase had the most pronounced effect in improving the quality of dog semen after thawing (Michael et al. 2007).

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Chapter 4

Effect of Aqueous Extract of Maca Addition to an Extender for hilled Canine Semen

4.1 Introduction

The practice of artificial insemination with cooled-stored semen is widely utilized in dog breeding. The shipment of semen reduces the movement of animals and increases genetic variation, expanding the number of males available for breeding. The main issue with the use of chilled semen is that during storage at 4 ◦C, spermatozoa undergo changes that could affect their fertilizing ability (Silvestre et al.2021). This detrimental effect is related to oxidative stress due to an excess of reactive oxygen species (ROS) and a decrease in antioxidants (Silvestre et al.2021, Henkel et al.2005). Oxidative stress significantly damages sperm functions such as motility, fluidity of the sperm plasma membrane and the integrity of DNA due to lipid peroxidation induced by ROS (Verstengen et al.2005, Kasimanickam et al. 2012). Therefore, a feasible new strategy to improve the long-term preservation of semen is the use of antioxidants that keep only a small amount of ROS necessary to maintain normal sperm function (Silvestre et al.2021; Maneesh et al.2006; Ciani et al.2021). The antioxidant supplementation has been proposed to reduce the impact of oxidative stress during the canine sperm storage process and slow or prevent semen deterioration (Beccaglia et al.2009; Del Prete et al.2018).

In the last years, there has been a growing interest in natural antioxidants found in plants, and among the most popular supplements, Maca (*Lepidium meyenii* Walpers) has attracted global attention. Maca is an Andean edible root that grows exclusively between 3500 and 4500 m above sea level. Maca is classified into three ecotypes according to the color of the hypocotyls (red, yellow and black) that show different concentrations of metabolites and different biological activities (Gonzales et al.2012). Yellow Maca contains several bioactive secondary metabolites, such as glucosinolates and specific alkaloids, called Macamides, that are responsible for its antioxidant effect (Tafuri et al.2019; Tafuri et al.2021). Several studies reported the effectiveness of the oral supplementation of Maca in improving fresh semen quality and quantity in humans, mice, bovines and stallions (Tafuri et al.2019; Clément et al. 2012; Del Prete et al.2018). Moreover, semen of animals fed with Maca supplementation diets showed an improved cooling and freezing ability (Del Prete et al.2019; D'Anza et al.2021). It has been recently demonstrated that Maca extract improves in vitro fertilization rates in mice by inducing an acrosome reaction and increasing sperm motility (Aoki et al.2019). To the best of our knowledge, the supplementation of the

semen extender for cooling or freezing with Maca has not yet been investigated.
Therefore, the aim of this study was to test for the first time the effect of different concentrations of aqueous extract of Maca on canine quality-related semen parameters (viability, motility, DNA fragmentation and oxidative stress) during storage at 4 ◦C for 7 days.

4.2. Materials and Methods

4.2.1. Animals

The semen samples included in this study were collected from dogs of the FOOF breeder center through their routine practice in the framework of breeding programs. The research involved nine dogs, 8 small breed (2 French bulldogs, 2 Jack Russel, 1 Pug, 1 Shih Tzu, 1 Poodle and 1 Cavalier King Charles Spaniel) and 1 large breed (Golden Retriever), with ages ranging from 1.5 to 8 years (median age was 6). Dogs received a standard commercial dog food twice daily and water ad libitum. All dogs received routine deworming treatments and vaccinations and shared the same environment for at least 6 months before the study.
The experiment was conducted in accordance with the code of ethics (D.lgs. 26—04/03/2014), and it was approved by the Ethics Committee of the Department of Veterinary Medicine and Animal Productions at the University of Naples Federico II, Italy (prot. no. PG/2021/0057934 of 07/06/2021).

4.2.2. Semen Collection and Processing

Semen collection was performed with an artificial vagina. Raw semen was evaluated for volume, color and concentration using a Burker's counting chamber. Each ejaculate was split into 4 aliquots that were diluted to reach a final concentration of 100 × 10s sperm/mL respectively in egg-yolk TRIS-citrate glucose (EYT-G: Tris 2.4 g, Citric Acid 1.4 g, Glucose 0.8 g, Penicillin G Sodium Salt 0.06 g, Streptomycin 0.1 g, 20 mL of egg yolk and distilled water to 100 mL), i.e., the control (CTRL group) and in YET-G supplemented with 10, 20, 50 µL/mL of maca extract (M10, M20 and M50 groups). All aliquots were placed in a syringe without air, transported to the laboratory at 4 ◦C within 3 h and then stored in the fridge at 4 ◦C for 7 days.

Control and treated semen samples were evaluated for sperm viability, sperm motility, DNA fragmentation and lipid peroxidation after 3 h, 24 h, 4 days and 7 days (4 d and 7 d) of storage.

4.2.3. Maca Source and Preparation of Aqueous Extract of Maca

The yellow ecotype of Maca used in this study was acquired from the district of Junín, Andean highlands of Peru (4100 m above sea levels), with a taxonomic identification by Professor Carotenuto D. at the Universidad Nacional Mayor de San Marcos, Lima, Peru. Roots were treated according to the traditional open-field method of drying: exposition of the hypocotyls for two months at extreme temperature cycles, under intense light conditions, and atmospheric pressure typical of the high-altitude environment (>3500 m). After drying, hypocotyls were selected, washed and milled to flour with a particle size of 0.8 mm.

Aqueous extract of Maca was prepared in accordance with the method described by Fei (Fei et al. 2020). Fifty grams of Maca powder were mixed with 1000 mL of water and auto- matically stirred in a water bath at 70 ◦C for 3 h. After that, the solution was centrifugated at 4000 RPM for 10 min, and then, the extraction within a water bath at 70 ◦C for 2 h was repeated. The final solution was placed in small vials and stored in a refrigerator at 4 ◦C for further use. The final aqueous extract of Maca should have concentrations of 750 mg/mL.

A chemical analysis of the powder and the aqueous extract of Maca were performed through liquid Chromatography with tandem mass spectrometry (LC-MS-MS) at the Interuniversity Consortium Biostructures and Biosystems National Institute (INBB). Con- centrations of different metabolites specific to Maca, such as Macaenes (polyunsaturated fatty acids), Macamides (a series of nonpolar and long-chain fatty acid N-benzylamides) and Macalines or Lepilidines were reported in Table 4.3.

Tab. 4.3. Concentrations of Maca metabolites in Maca powder and Aqueous extract of Maca.

Chemical structures of Maca metabolites	Maca powder (μg/L)	Aqueous extract of Maca (μg/L)
5-oxo-6E,8E-octadecadienoic acid (Macaen)	69,53	17,89
N-(3-hydroxy-benzyl)-2Z-fivecarbon acrylamide	614,29	157,99
N-benzyl-5-oxo-6E,8E-octadecadienamide (MI 7)	46,08	61,81
N-benzyloctadecanamide (MI 16)	53,96	28,89
1,3-dibenzyl-2, pentyl-4, 5-trimethylimidazilium (Lepilidine A)	59,03	13,31
(1R,3S)-1-methyltetrahydro-beta-5,6-hydridecarboline-3-carboxylic acid (MTACA)	47,17	3,63
1-dibenzyl-2-propane-4,5-dimethylimidazilium	19,52	1,25

4.2.4. Membrane Integrity (Hypo-Osmotic Swelling Test)

The hyposmotic swelling (HOS) test was carried out at each time point for the as- sessment of the functional integrity of the sperm plasma membrane in control and treated groups. Twenty microliters of semen were incubated at 37 ◦C for 45 min with 80 μL of pre-warmed HOS solution (0.73 g sodium citrate and 1.35 g fructose in 100 mL of distilled water, 150 mOsm). After incubation time, a volume of 10 μL was placed on a glass slide and covered with a cover slip. Evaluations were conducted under phase-contrast microscopy (40×; Eclipse E200, Nikon, Tokyo, Japan) by operators unaware of the experimental design. The cells were classified as positive (damaged membrane) or negative (intact membrane) according to the presence or absence of coiled tails, respectively. A total of 200 spermatozoa were counted.

4.2.5. Motility Assessment

Sperm motility parameters (total and progressive motility, sperm subpopulations and semen kinetic parameters) were assessed by Sperm Class Analyzer (SCA) system (Microptic SL, Veterinary Edition, Barcelona, Spain) installed on a camera-equipped light microscope system (Eclipse E200, Nikon, Tokyo, Japan). The following parameters were considered for the assessment: total motility (%), progressive motility (%), the percentage of sperm subpopulations (rapid and medium progressive), average path velocity (VAP; µm/s), straight-line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), straightness (STR; %) and linearity (LIN; %).

SCA system settings for dog semen classified as spermatozoa all the particles sized between 10 and 80 µm2 and as progressively motile spermatozoa with 75% STR. The minimum velocity (VCL) values considered for slow-medium and rapid spermatozoa subpopulations were 50 and 100 µm/s; spermatozoa with VCL below 10 µm/s were considered static and spermatozoa with VCL > 150 µm/s and ALH >3.5 µm as hyperactive. Sixty frames per second with minimum contrast of 35 were acquired.

For the evaluation, an aliquot of control or treated (M10, M20 and M50) semen at each time point (3 h, 24 h, 4 d and 7 d) was diluted 1:3 with TRIS-glucose-citrate in order to reach a concentration of 30 × 106 sperm/mL as required by SCA system and incubated at 37 ◦C for 10 min before evaluation. Then, 5 µL were spotted onto a pre-warmed glass microscope slide, covered with a glass coverslip (22 mm × 22 mm). At least 500 sperm cells in five randomly selected fields were evaluated.

4.2.6. DNA Fragmentation

Sperm DNA fragmentation was examined in each sample by using the terminal de- oxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay or in-situ Cell Death Detection Kit (Sigma-Aldrich, St. Louis, MO, USA), as previously described by Longobardi et al. (2022). Briefly, 40 µL of cooled semen was fixed with 250 µL of 4% (*w*/*v*) paraformalde- hyde in phosphate-buffered saline (PBS; pH 7.4) for 45 min at room temperature. After incubation, sperm cells were washed twice (300 g × 15 min) with PBS with Polyvinylpyrroli- done (PVP;1 mg/mL). After supernatant aspiration, the pellet was diluted at 1:10 with PBS. A drop of semen (approximately 20

µL) was smeared on an object glass, dried, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 10 min. Then, slides were washed twice with PBS, air-dried, and incubated with a TUNEL reaction mixture for 1 h at 37 ◦C in a humidified atmosphere in the dark. The negative control was made by adding all components of the label solution (except for the terminal deoxynucleotidyl transferase enzyme), and the positive control was made by incubating the samples with DNAse recombinant to induct DNA separation for 10 min before incubation with the Tunel reagent. After one h of incubation, slides were stained with PBS -PVP labeled with 1 mg/mL Hoechst 33342, for 30 min, at room temperature and then rinsed with PBS. The results were examined using a fluorescent microscope (Eclipse E-600; Nikon, Tokyo, Japan) under ultraviolet light; the excitation wavelength was 460 nm for the blue fluorescence and 520 nm for the green fluorescence.
TUNEL assay evaluates the presence of free 3′-hydroxyl ends, which are identified by terminal deoxynucleotidyl transferase (TdT) enzyme and catalyze the addition of fluo- rescently labeled deoxyuracil triphosphate breaks in DNA strands. Spermatozoa in blue (Hoechst+) in a bright green fluorescence (Tunel+) showed damaged (fragmented) DNA, while spermatozoa in a dull green fluorescence showed normal DNA.

4.2.7. Lipid Peroxidation

Sperm lipid peroxidation of control and treated samples at each time point was determined by assaying the Malondialdheyde (MDA) concentration by means of the thio- barbituric acid (TBA) test (Esterbauer et al.1990). In order to precipitate proteins, 100 µL of each sample was treated with 0.5 mL of cold 30% (*w*/*v*) trichloroacetic acid and centrifugate. One millimeter of supernatant was reacted with 1.3 mL of 0.5% (*w*/*v*) TBA at 85 ◦C for 40 min. In the TBA test reaction, each molecule of MDA reacts with two molecules of TBA with the production of a pink pigment having maximal absorbance at 532–535 nm. After cooling, the fluores- cence was read at wavelengths of 536 nm for excitation and 557 nm for emission using a SPEX Fluoromax spectrophotofluorimeter (GloMax®-Multi Detection System, Promega, Madison, WI, USA). Concentrations of MDA calculated using a calibration curve ranged between 0.5–2 pmoles/mL and were expressed as nmol/L of proteins.

4.2.8. Statistical Analysis

Data were first recorded using a computerized spreadsheet (Microsoft® Excel® 2021, Redmond, WA, USA) and then imported into Statistical Package for Social Sciences (SPSS IBM® Statistics version 27.0, IBM Corporation, Armonk, NY, USA) for statistical analysis. The Kolmogorov–Smirnov test was utilized for normality analysis of the parameters. All data were expressed in median and interquartile ranges (IQR) for the violation of normality. Non-parametric tests were used for evaluation. The effect of storage time on sperm analysis data was evaluated in each group (control, M10, M20 and M50) using a Friedman test, and in case of significance, post hoc analysis with Wilcoxon's signed-rank test was conducted to compare individual storage times; differences between groups at each time point were also evaluated with Wilcoxon's signed-rank test. Differences were considered statistically significant when $p \leq 0.05$.

4.3. Results

4.3.1. Fresh Semen

All ejaculates collected were white and milky in consistency. The median (IQR) of the volume of the sperm-rich fraction was 2.5 (1.5–5.0) mL with a sperm concentration of 230 (174–396) × 106 sperm/mL.

4.3.2. Membrane Integrity (HOS)

Regarding storage time, a decrease ($p \leq 0.05$) of membrane integrity was only observed at 7 days in the M50 group. No differences in membrane integrity were found between the treated groups and the control at 3 h, 4 d and 7 d. In M50-treated group, a decrease (76.5 (76–85)%; $p \leq 0.05$) was recorded compared to M10 (80.5 (80–88)%) and M20-treated groups (80 (77–84.5)%) at 3 h. Likewise, at 24 h a reduced sperm integrity was found in M50 (76.5 (72–83.7)%; $p \leq 0.05$) compared to CTRL group (83 (77–84.5)%) and M20-treated group (77 (75.6–86.2)%).

4.3.3. Motility

Figure 4.3 represents the results of total and progressive motility during storage time and the differences between time points inside each group and among groups at each time point. Semen motility gradually reduced during preservation at 4 ◦C in the control, as well as in the Maca-treated groups, as shown in Figure 1. After 4 days of cooling, total motility was significantly lower in the M10-treated group than in the CTRL group and in the M50- than in the M20-treated group ($p \leq 0.05$). Moreover, at the same time point, progressive motility was lower in the M50-treated group compared with the CTRL group ($p \leq 0.05$).

Analysis of sperm subpopulations is reported in Table 4.3.1. After 24 h, semen stored at 4 ◦C with the addition of 10 µL/mL of Maca had an increase in rapid sperm cells, although not significant. The percentage of rapid sperm cells remained constant until 4 days of storage at 4 ◦C in the CTRL group; meanwhile, at 4 days, there was a decrease in treated groups ($p \leq 0.05$). In all groups, the proportion of rapid sperm cells declined at 7 days ($p \leq 0.05$). On the other hand, rapid progressive sperm were lower in the M50-treated group than in all the other CTRL and treated groups at 4 days of sperm storage ($p \leq 0.05$). The percentage of medium progressive spermatozoa decreased after 4 d in all groups.

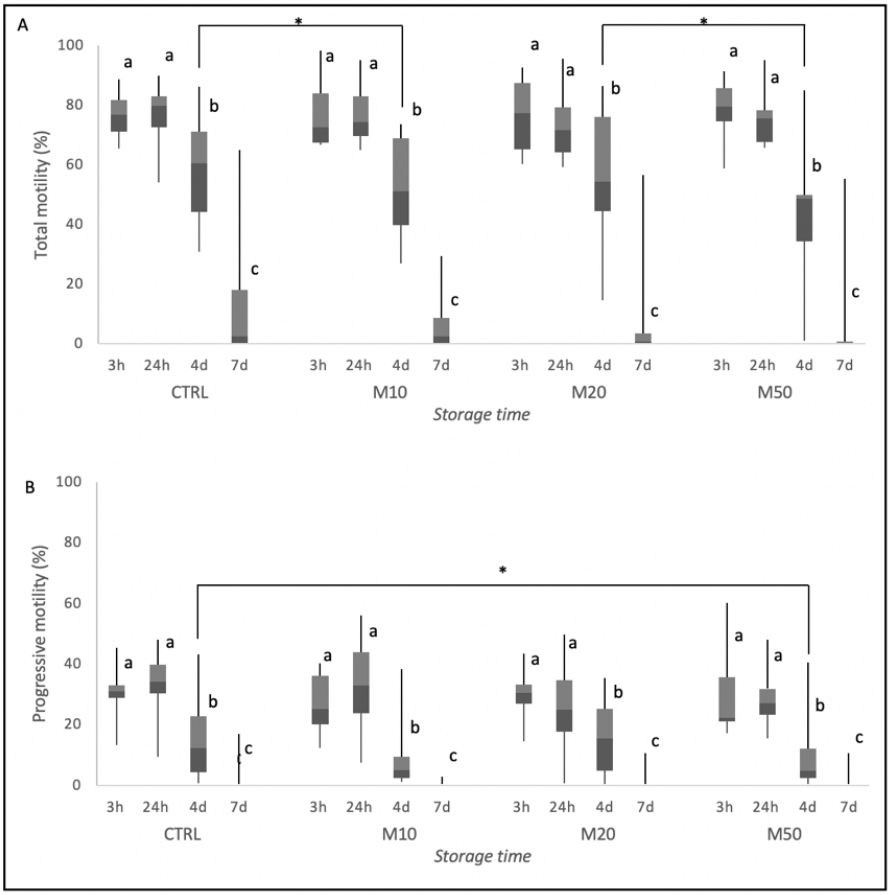

Figure 4.3 Total (A) and progressive motility (B) of dog semen (n=9) diluted with only egg-yolk tris-citrate glucose (YET-G; CTRL) or with EYT-G with the addition of three different concentrations (10, 20 and 50 µL/mL) of aqueous extract of Maca (M10, M20 and M50) stored at refrigeration temperature (4°C) and evaluated at 3h, 24h, 4 and 7 days (4d and 7d) of storage. Asterisk above the bar indicates statistical difference between groups at P ≤ 0.05. The letters a, b and c indicate statistically significant difference at P≤ 0.05 between time points within each group.

Sperm hyperactivation during cooling preservation was different in all groups, as illustrated in Table 4.3.1. Treatment with 20 and 50 µL/mL of Maca increased the percentage of hyperactivate sperm compared to CTRL after 3 h of storage ($p \leq 0.05$). On the contrary, after 4 days of storage at 4 °C, the percentage of hyperactivated sperm decreased in the M50-treated group compared to the CTRL group ($p \leq 0.05$). As shown in Table 4.3.2, the

temporal decrease of kinetic parameters showed a similar trend in the different groups, except for VCL. This parameter increased after 24h of storage (P≤ 0.05), only in M10 treated group. For kinetic parameters, after 3h at 4°C the CTRL group had higher VSL, VAP, LIN and STR than the M50 treated group (P≤ 0.05) and higher STR than the M20 treated group (P≤ 0.05). Moreover, after 24h of cooling, semen treated with 50 µL/mL of Maca (M50 group) had reduced (P≤ 0.05) VCL than semen diluted with the lowest concentration of Maca (M10 group).

Table 4.3.1. Sperm subpopulations (% of rapid, medium and hyperativated spermatozoa) of canine (n=9) semen of control (CTRL) and semen extender Maca treatment groups (M10, M20, M50) during storage at 4°C for 7 days. All values are expressed as median and interquartile range (IQR).

	Storage time			
Rapid progressive (%)	**3h**	**24h**	**4d**	**7d**
CTRL	14.4 (4.5-20.7) [a]	17.8 (8.1-29.7) [a]	6.5 (1.9-16) [a,x]	0 (0-1.3) [b]
M10	10.6 (7.4-17.6) [a]	24.2(6.6-29.1) [a]	2.1 (1-4) [b,xy]	0 (0-0) [c]
M20	12.2 (5.6-21.8) [a]	11.2 (5.3-20.2) [a]	5 (1.5-11) [b,x]	0 (0-1.5) [c]
M50	13.3 (9.4-20.8) [a]	12.6 (6-20.1) [a]	1.8 (0.5-2.4) [b,y]	0 (0-0) [c]
Medium progressive (%)				
CTRL	14.3(13.4-21.7) [a,xy]	12.3 (7.3-23.2) [a]	2.4 (1.7-13.8) [b]	0(0-1.5) [c]
M10	13.4 (6-20.7) [a,xy]	12 (6.2-21.5) [a]	2.1 (1-7.5) [b]	0 (0-0.1) [c]
M20	12 (11.9-21.1) [a,x]	15.2 (8.1-21) [a]	10.5 (0.9-15.5) [b]	0 (0-0.5) [c]
M50	14.5 (9-20.1) [a,y]	13 (7.7-22.4) [a]	2.5 (0.3-12.9) [b]	0 (0-0) [c]
Hyperactive sperm (%)				
CTRL	1.6 (0.9-3.7) [a,x]	1.3 (0.9-4.6) [a]	1.1 (0.6-4.4) [a,x]	0 (0-0.14) [b]
M10	3.5 (2.2-8.1) [a,xy]	2.88 (1.8-4.1) [a]	0.5 (0-1.2) [b,xy]	0 (0-0) [c]
M20	3.1 (1.4-5.9) [a,y]	2.2 (0.7-3.8) [a]	0.25 (0-1.6) [b,xy]	0 (0-0.2) [b]
M50	2.1 (1.1-6.7) [a,y]	1.7 (1.2-4) [a]	0 (0-0.8) [b,y]	0 (0-0) [b]

The letters a, b and c indicate statistically significant difference at P≤ 0.05 between time points within each row (group; letters x and y represent statistical differences among groups within each column (time point).

Table 4.3.2. Trends of semen kinetic parameters of canine (n=9) semen of control (CTRL) and semen extender Maca treatment groups (M10, M20, M50) during storage at 4°C for 7 days. All values are expressed as median and interquartile range (IQR).

	Storage time			
VCL	**3h**	**24h**	**4d**	**7d**
CTRL	85.4 (82.2-86.3) ^{a}	86.1 (84.6-86.6) ^{a,xy}	86.7 (74.8-88.2) ^{a}	0 (0-76.8) ^{b}
M10	85.7 (81.3-87.8) ^{a}	87.8 (86.1.-89.4) ^{b,x}	82.4 (78.2-84) ^{a}	0 (0-33.8) ^{c}
M20	84.2 (82.2-87.7) ^{a}	86.2 (82-87.6) ^{a,xy}	84.7 (40.5-85.8) ^{a}	0 (0-41) ^{b}
M50	85 (81.7-87.1) ^{a}	83.8 (80.2-86.2) ^{a,y}	78.9 (38.3-84.1) ^{a}	0 (0-0) ^{b}
VSL				
CTRL	61 (56.3-66.4) ^{a,x}	55.4 (48.5-62.2) ^{a}	44.7 (35.7-50.3) ^{b}	0(0-27.5) ^{c}
M10	60.6 (50-64.5) ^{a,xy}	56 (49.7-63.6) ^{a}	40.1 (34.2-46) ^{b}	0 (0-19.3) ^{c}
M20	59 (55.4-61.7) ^{a,xy}	57.3 (46.6-61.8) ^{a}	42.9 (19.5-51.5) ^{b}	0 (0-21.7) ^{c}
M50	57.9 (50.8-64.4) ^{a,y}	49.9 (44.5-59.7) ^{a}	43.2 (16.3- 51.3) ^{b}	0 (0-0) ^{c}
VAP				
CTRL	66.4 (61.8-70.7) ^{a,x}	61.2 (55.2-67.1) ^{a}	51.5 (42.8-57) ^{b}	0(0-34.4) ^{c}
M10	66.5 (56.2-69.6) ^{a,xy}	62.3 (57.2-68.7) ^{a}	47.6 (42-52.9) ^{b}	0 (0-22.6) ^{c}
M20	65 (63-66.6) ^{a,xy}	63.4 (53.4-68.1) ^{a}	50.5 (23.6-57.6) ^{b}	0 (0-24) ^{c}
M50	64.1 (57.2-69.5) ^{a,y}	58.8 (51.7-64.8) ^{a}	50.6 (19.8-57.9) ^{b}	0 (0-0) ^{c}
LIN				
CTRL	71.6 (66.2-79.1) ^{a,x}	67.4 (56.9-72.1) ^{a}	51.6 (40.3-62.4) ^{b}	0 (0-35.4) ^{c}
M10	73 (59.8-75.5) ^{a,xy}	65.4 (55.5-72.2) ^{a}	48.9 (41.5-54.1) ^{b}	0 (0-25) ^{c}
M20	70.1 (65.3-73.6) ^{a,xy}	66.3 (57.3-72.5) ^{a}	50.9 (2.9-60.1) ^{b}	0 (0-24.2) ^{c}
M50	66.7 (61.2-73.8) ^{a,y}	60.2 (55-71.9) ^{a}	50.3 (20.9- 63.6) ^{b}	0 (0-0) ^{c}
STR				
CTRL	91.4 (90.4-93.1) ^{a,x}	90 (87.5-92) ^{b}	86.3 (82.5-87.8) ^{c}	0 (0-80.4) ^{d}
M10	90.9 (88-92.3) ^{a,xy}	89.7 (85.6-92.1) ^{a}	83.9 (80.6-86.5) ^{b}	0 (0-41.9) ^{c}
M20	90.6 (87.5-92.4) ^{a,y}	89.9 (88.8 -92.8) ^{a}	84.9 (40.5-88.2) ^{b}	0 (0-41.8) ^{c}
M50	89.7 (87.4- 92.2) ^{a,y}	86.3 (85-91.4) ^{b}	85.2 (41-92.2) ^{b}	0 (0-0) ^{c}

The letters a, b and c indicate statistically significant difference at P≤0.05 between time points within each row (group); letters x and y represent statistical differences among groups within each column (time point).

4.3.4 DNA fragmentation

Results of DNA fragmentation are reported as median (IQR) and range (min–max) in Figure 4.3.4a. For all groups, storage time has an effect on DNA fragmentation ($p \leq 0.05$), especially after 7 days of cooling. Differences in DNA fragmentation between groups were found at 3 h and at 7 days of refrigeration. After 3 h of cooling, sperm DNA fragmentation was lower in the M10-treated group than in CTRL ($p \leq 0.05$) and M50 groups ($p \leq 0.05$). After 7 days of refrigeration of sperm, DNA fragmentation

increased ($p \leq 0.05$) in M50-treated group compared to the other two treated groups (M10 and M20).

4.3.5. Lipid Peroxidation

As displayed in Figure 4.3.4b, MDA concentrations increased in all treated groups after 7 d ($p \leq 0.05$), However, after 24 h of cooling, lipid peroxidation was lower in all Maca- treated groups (M10, M20 and M50) with respect to CTRL, although the difference was only significant between M20-treated group and CTRL group ($p \leq 0.05$).

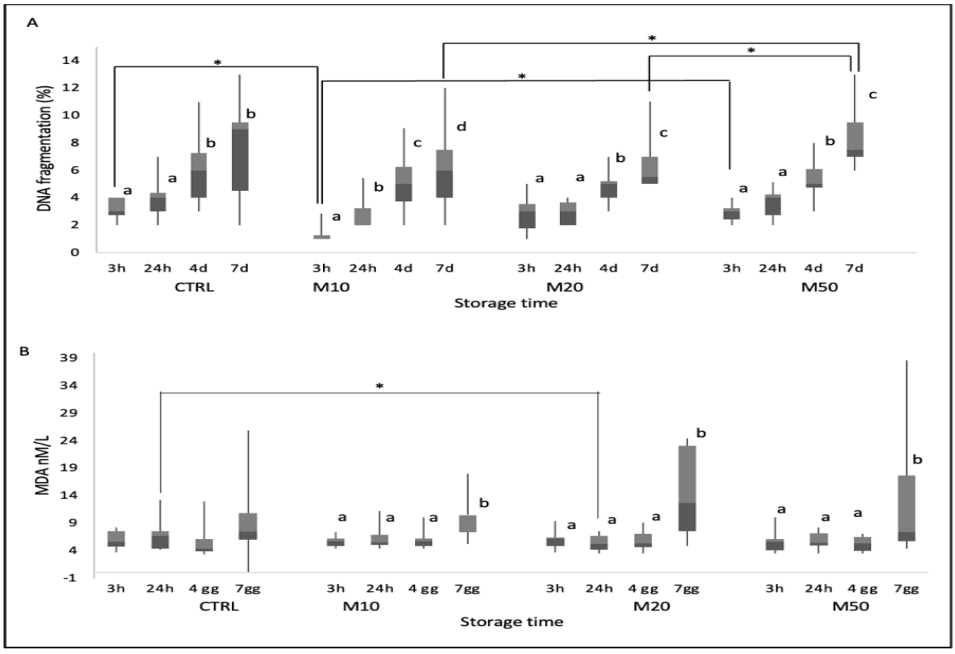

*Figure 4.3.4. Percentage of DNA fragmentation (**A**) and lipid peroxidation (**B**) of dog semen (n=9) diluted with only egg-yolk tris-citrate glucose (EYT-G; CTRL) or with EYT-G with the addition of three different concentrations (10, 20 and 50 µL/mL) of aqueous extract of Maca (M10, M20 and M50) stored at refrigeration temperature (4°C) and evaluated at 3h, 24h, 4 and 7 days (4d and 7d) of storage. Asterisk above the bar indicates statistical difference between groups at $P \leq 0.05$. The letters a, b and c indicate statistically significant difference at $P \leq 0.05$ between time points within each group.*

4.4 Discussion

This study shows for the first time the in vitro effects of Maca on canine spermatozoa preserved for 7 days at 4 ◦C. Cytoprotective effects of Maca have been demonstrated to be associated with its antioxidant activity by increasing the enzyme activity of superoxide dismutase and by direct free radical scavenging (Sandoval et al.2002; Vecera et al. 2007). As expected, during refrigeration in both control and Maca-treated groups, there was a progressive and significant reduction in semen quality. One of the factors that cause this impairment is the imbalance between oxidants and antioxidants (Dutta et al.2019). The protective action of the addition of antioxidants in canine semen extender for chilling has been already tested, suggesting that Lycopene or Vitamin E and B16 are able to preserve semen quality of chilled dog spermatozoa (Sheikholesami et al.2020; Michael et al.2009). Antioxidants counteract oxidative stress, improving membrane integrity and motility and preventing lipid peroxidation and DNA fragmentation of spermatozoa (Greco et al.2005).The results of this study indicate that Maca had a protective effect on canine chilled semen until 24 h of storage. Particularly, the semen extender treatment with 10 µL/mL preserved DNA and plasma membrane integrity of spermatozoa at 3 h of storage and after 24 h of storage, significantly increased VCL of spermatozoa and improved the percentage of rapidly progressive and hyperactivated sperm cells, albeit not in a significant manner. A previous study that investigated the in vitro effect of Maca on human spermatozoa reported an increase in total motility but not in VCL or in other kinetic parameters (Aoki et al.2019). The color of Maca hypocotyls or the use of different methods of cultivation, processing, and extraction (methanol, chloroform, DMSO and water) of Maca can change concentrations of Maca bioactive metabolites (Tafuri et al.2019; Gonzales et al.2006). The content of secondary metabolites (Macamides, Macaenes and Lepilidines) improves the cold shock resistance of spermatozoa (Clément et al.2012) For this reason, we decided to investigate the quantities of some constituents of the aqueous extract of Maca used in this study and to test the supplementation of the semen extender with three different concentrations.

Spermatozoa are highly prone to peroxidative damage due to the higher polyunsaturated fatty acid contents. MDA concentration is indicative of lipid peroxidation as a marker of oxidative stress, and it is an accepted diagnostic tool for humane infertility workup (Tavilani et al.2005; Collodel

et al.2015). Previous studies reported the high susceptibility of canine epididymal sperm to the deleterious effect of hydrogen peroxide, MDA and hydroxyl radical (Da Rosa Filho et al.2021). The MDA can exert a damaging effect by combining with other molecules such as proteins, DNA and RNA and provoking a reduction in sperm viability, motility, and DNA integrity (Das et al2009; Benedetti et al.2012). This study observed that an increase in the main by product of lipid peroxidation after 7 days of cooling determined a meaningful reduction of total and progressive motility and all kinetic parameters. Instead, DNA fragmentation and membrane integrity were not correlated with high MDA concentrations. Moreover, the semen with the semen extender addition of Maca had lower levels of MDA at 3 and 24 h of cooling storage, the only statistical difference compared at 24 h between control and semen extender with the addition of 20 µL/mL. From these results, we can only speculate that the addition of Maca had a protective role against oxidation, preventing the formation of lipid peroxidation. More cases and further studies on the effect of Maca during canine semen freezing are needed to clarify whether Maca could prevent the cold shock of spermatozoa.

In this study, contrasting and opposite effects at different Maca concentrations demon- strated dose-related effects of Maca. High concentrations of Maca affected sperm parameters by increasing DNA fragmentation and damaged membranes and also reducing some kinetic parameters (VCL, VSL, VAP, STR and LIN) between 3 and 24 h of storage at refrigerating temperatures. It was reported that Macamides and Macaenes have cytotoxicity It was reported that Macamides and Macaenes have cytotoxicity against different cancer cell lines by inducing apoptosis (Gonzales et al. 2006; Kuang et al. 2004; Fu et al. 2021). Cells undergoing apoptosis usually show several cellular changes, including DNA fragmentation and membrane disruption. Probably the toxicity of Maca on spermatozoa is expressed not only with increased apoptosis but also by reducing sperm velocities.

The addition of all concentrations of Maca to semen extender increased the hyperactivation of sperm cells at 3 h, but significance was apparent only in the semen with the addition of 20 and 50 µL/mL of Maca. A previous study reported that the interaction of human and mouse sperm with Maca tends to increase the amplitude of lateral head displacement, which is strictly correlated with hyperactivation (Aoki et al.2019; Chan et al.1990). Alkaloid components of Maca could be responsible for the hyperactivation of the

spermatozoa, as already described after the addition of caffeine alkaloids to the semen (Tesarik et al. 1992; Funahashi et al.2001). It has been described that the alkaloids contained in caffeine that are similar to those of Maca provoke sperm hyperactivation by increasing intracellular cyclic adenosine monophosphate (cAMP) or by promoting activation of calcium ion-permeable cation channels in the plasma membrane of sperm (Tesarik et al.1992; Ho et al.2001). Further analysis should be conducted to clarify the responsibilities and the mechanism underlying this effect of Maca. When hyperactivated, spermatozoa are able to swim through viscoelastic substances and successfully penetrate the zona pellucida; indeed, it is associated with increased sperm fertilizing capacity (Suarez et al.2008; Suarez et al.2008). For these reasons the hyperactivation is required when sperm enter the uterus and not during storage because this event determines an overall energy consumption reducing sperm lifespan.

4.5. Conclusions

The main findings emerging from the results of this study are that the addition of 10 or 20 µL/mL of aqueous extract of Maca to the chilled extender had positive effects until 24 h of storage, while the highest concentration of Maca tested in this study (50 µL/mL) had an immediately deleterious effect on quality-related semen parameters. Maca cannot sufficiently protect canine semen for extending refrigeration storage time; however, low concentrations of Maca could be proposed before insemination in fresh, cooled or freeze- thawed semen to increase hyperactivation of sperm cells and to preserve DNA integrity of spermatozoa until fertilization.

4.6 References

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Chapter 5

Effect of aqueous extract of crocin addition to an extender for chilled canine semen

5.1 Introduction

In the last decades there has been an increasing interest in artificial insemination (AI) in dogs, both to overcome mating inability and to improve breeding programs. With regard to the latter, the shipment of chilled or frozen semen allows the movement of semen from genetically superior dogs across countries, avoiding the risks of animal transportation and improving genetic variability. Both processing and shipping procedures are easier and cheaper with chilled compared to frozen semen whose transport requires expensive equipment (Verstegen, et al. 2005). Moreover, vaginal insemination with chilled semen results in higher pregnancy rates and larger litter sizes compared to frozen semen, that requires intrauterine deposition (Linde-Forsberg et al. 2001; Pinto et al.1999; Rota et al.1995.)

A major limiting factor of chilled semen is the limited lifespan of spermatozoa after prolonged storage, as quality deteriorates at increasing times, imposing the use of chilled semen within 4.9 days (England et al. 1996). It follows the importance to develop strategies to extend the lifespan of sperm during storage at 4°C.

It is known that the reduced sperm longevity is due to oxidative stress, resulting from increased production of reactive oxygen species (ROS) and reduced antioxidants (Aitken et al. 2017; Silvestre et al. 2021; Henkel et al.2005). Oxidative stress (OS) affects sperm motility, membrane fluidity and DNA integrity, due to ROS-induced lipid peroxidation (Verstegen et al.2005). Therefore, enrichment of the semen extender with antioxidants has been proposed to prevent OS during prolonged storage at 4 °C.

The spice saffron (Crocus sativum) has anti-inflammatory, anti-proliferative, and anti-apoptotic properties (Hashemzaei et al. 2020), mainly due to its known antioxidant function (Assimopoulou et al. 2005). Crocin, one of the carotenoids responsible for the antioxidant capacity of saffron, is known to protect cells from oxidative damages by scavenging ROS (Rahaiee, et al. 2015). It has been reported that crocin protects spermatozoa from oxidative stress and consequent DNA damages in the deer, ram and goat (Domínguez-Rebolledo et al. 2010; Mata-Campuzano, et al. 2015; Longobardi et al.2020). Furthermore, sperm incubation with crocin improved motility, viability, membrane integrity, as well as blastocyst yields in cattle (Sapanidou et al. 2015).

To the best of our knowledge, the effects of crocin on dog semen have not yet been investigated. We hypothesized that the enrichment of semen

extender with crocin would improve dog sperm quality during storage at 4 °C, through its antioxidant action Therefore, the aim of this study was to evaluate the effects of different concentrations of crocin on sperm qualitative parameters, such as motility, viability and membrane integrity, as well as on sperm lipid peroxidation, during prolonged storage at 4°C.

5.2 Materials and Methods

5.2.1 Animals

The experiment was conducted in accordance with the code of ethics (D.lgs. 26—04/03/2014), and it was approved by the Ethics Committee of the Department of Veterinary Medicine and Animal Productions at the University of Naples Federico II, Italy (prot. no. PG/2021/0057934 of 07/06/2021).
Semen samples were collected from 10 dogs of the FOOF breeder center through their routine practice in the framework of breeding programs. The study included small breed (3 French bulldogs, 2 Jack Russel, 1 Pug, 1 Shih Tzu, 1 Poodle and 1 Cavalier King Charles Spaniel) and 1 large breed (Golden Retriever), with ages ranging from 1.5 to 8 years (median age was 6). Dogs received a standard commercial dog food twice daily and water ad libitum. All dogs received routine deworming treatments and vaccinations and shared the same environment for at least 6 months before the study.

5.2.2 Semen Collection and Processing

Semen collection was performed with an artificial vagina. Raw semen was evaluated for volume, color and concentration using a Burker's counting chamber. Each ejaculate was split into 4 aliquots that were diluted to reach a final concentration of 100 × 10s sperm/mL respectively in egg-yolk TRIS-citrate glucose (EYT-G: Tris 2.4 g, Citric Acid 1.4 g, Glucose 0.8 g, Penicillin G Sodium Salt 0.06 g, Streptomycin 0.1 g, 20 mL of egg yolk and distilled water to 100 mL), i.e., the control group and in YET-G supplemented with 0.5, 1, 2 mM crocin (C0.5, C1 and C2 groups). All aliquots were placed in a syringe without air, transported to the laboratory at 4 °C within 3 h and then stored in the fridge at 4 °C for 7 days. Control and treated semen samples were evaluated for sperm viability, membrane integrity, sperm motility, and lipid peroxidation after 3 h, 24 h, 4 days and 7 days (4 d and 7 d) of storage.

5.2.3 Membrane Integrity (Hypo-Osmotic Swelling Test)

The hyposmotic swelling (HOS) test was carried out at each time point for the assessment of the functional integrity of the sperm plasma membrane in control and treated groups. Twenty microliters of semen were incubated at 37 °C for 45 min with 80 µL of pre-warmed HOS solution (0.73 g sodium citrate and 1.35 g fructose in 100 mL of distilled water, 150 mOsm). After incubation time, a volume of 10 µL was placed on a glass slide and covered with a cover slip. Evaluations were conducted under phase-contrast microscopy (40×; Eclipse E200, Nikon, Tokyo, Japan) by operators unaware of the experimental design. The cells were classified as positive (damaged membrane) or negative (intact membrane) according to the presence or absence of coiled tails, respectively. A total of 200 spermatozoa were counted.

5.2.4 Motility Assessment

Sperm motility parameters (total and progressive motility, sperm subpopulations and semen kinetic parameters) were assessed by Sperm Class Analyzer (SCA) system (Microptic SL, Veterinary Edition, Barcelona, Spain) installed on a camera-equipped light microscope system (Eclipse E200, Nikon, Tokyo, Japan). The following parameters were considered for the assessment: total motility (%), progressive motility (%), the percentage of sperm subpopulations (rapid and medium progressive), average path velocity (VAP; µm/s), straight-line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), straightness (STR; %) and linearity (LIN; %).

SCA system settings for dog semen classified as spermatozoa all the particles sized between 10 and 80 µm2 and as progressively motile spermatozoa with 75% STR. The minimum velocity (VCL) values considered for slow-medium and rapid spermatozoa subpopulations were 50 and 100 µm/s; spermatozoa with VCL below 10 µm/s were considered static and spermatozoa with VCL > 150 µm/s and ALH >3.5 µm as hyperactive. Sixty frames per second with minimum contrast of 35 were acquired.

For the evaluation, an aliquot of control or treated (C0.5, C1 and C2) semen at each time point (3 h, 24 h, 4 d and 7 d) was diluted 1:3 with TRIS-glucose-citrate in order to reach a concentration of 30×10^6 sperm/mL as required by SCA system and incubated at 37 °C for 10 min before evaluation. Then, 5 µL were spotted onto a pre-warmed glass

microscope slide, covered with a glass coverslip (22 mm × 22 mm). At least 500 sperm cells in five randomly selected fields were evaluated.

5.2.5 Lipid Peroxidation

Sperm lipid peroxidation of control and treated samples at each time point was determined by assaying the Malondialdheyde (MDA) concentration by means of the thiobarbituric acid (TBA) test . In order to precipitate proteins, 100 µL of each sample was treated with 0.5 mL of cold 30% (w/v) trichloroacetic acid and centrifugate. One millimeter of supernatant was reacted with 1.3 mL of 0.5% (w/v) TBA at 85 °C for 40 min. In the TBA test reaction, each molecule of MDA reacts with two molecules of TBA with the production of a pink pigment having maximal absorbance at 532–535 nm. After cooling, the fluorescence was read at wavelengths of 536 nm for excitation and 557 nm for emission using a SPEX Fluoromax spectrophotofluorimeter (GloMax®-Multi Detection System, Promega, Madison, WI, USA). Concentrations of MDA calculated using a calibration curve ranged between 0.5–2 pmoles/mL and were expressed as nmol/L of proteins.

5.2.6 Statistical Analysis

Data were first recorded using a computerized spreadsheet (Microsoft® Excel® 2021, Redmond, WA, USA) and then imported into Statistical Package for Social Sciences (SPSS IBM® Statistics version 27.0, IBM Corporation, Armonk, NY, USA) for statistical analysis. The Kolmogorov–Smirnov test was utilized for normality analysis of the parameters. All data were expressed in median and interquartile ranges (IQR) for the violation of normality. Non-parametric tests were used for evaluation. The effect of storage time on sperm analysis data was evaluated in each group (control, C0.5, C1 and C2) using a Friedman test, and in case of significance, post hoc analysis with Wilcoxon's signed-rank test was conducted to compare individual storage times; differences between groups at each time point were also evaluated with Wilcoxon's signed-rank test. Differences were considered statistically significant when $p \leq 0.05$.

5.3 Results

5.3.1 Fresh semen

All ejaculates collected were white and milky in consistency. The of the volume of sperm-rich fraction was 2.5 (1.5-5.0) (median (IQR)) ml with a sperm concentration of 230 (174- 396) × 10^6 sperm/ml.

5.3.2Membrane integrity (HOS)

The results of sperm membrane integrity during storage time, with the differences among time points within each group and the differences among groups within time point are shown in Figure 5.3A. A decrease ($P \leq 0.05$) of membrane integrity was observed at 7 days in all groups. Moreover, in the semen treated with 2 mM crocin (C2) a significant decrease ($P<0.05$) was recorded already at 4 days of storage at 4°C Furthermore, a higher ($P<0.05$) proportion sperm with intact membrane was recorded in C0.5 and C1 groups compared to the control group also after 7 days of storage.

5.3.3 Motility

Results of total and progressive motility during storage time, as well as the differences among time points within group and among groups within time point are reported in Figure 5.3B and 5.3C. No differences were observed in total and progressive motility among groups at any time point (Figure 5.3 B and C). During preservation at 4 °C, total and progressive sperm motility decreased ($P \leq 0.05$) only at 7 days in the control group and in 0.5 and 1 mM crocin groups. Instead, the treatment with 2 mM crocin determined a reduction of total and progressive motility already at 4 days of storage. Moreover, in C1 group progressive motility increased after 24 hours of storage at 4°C to return to original values at 4 d and then decrease at the latest time.

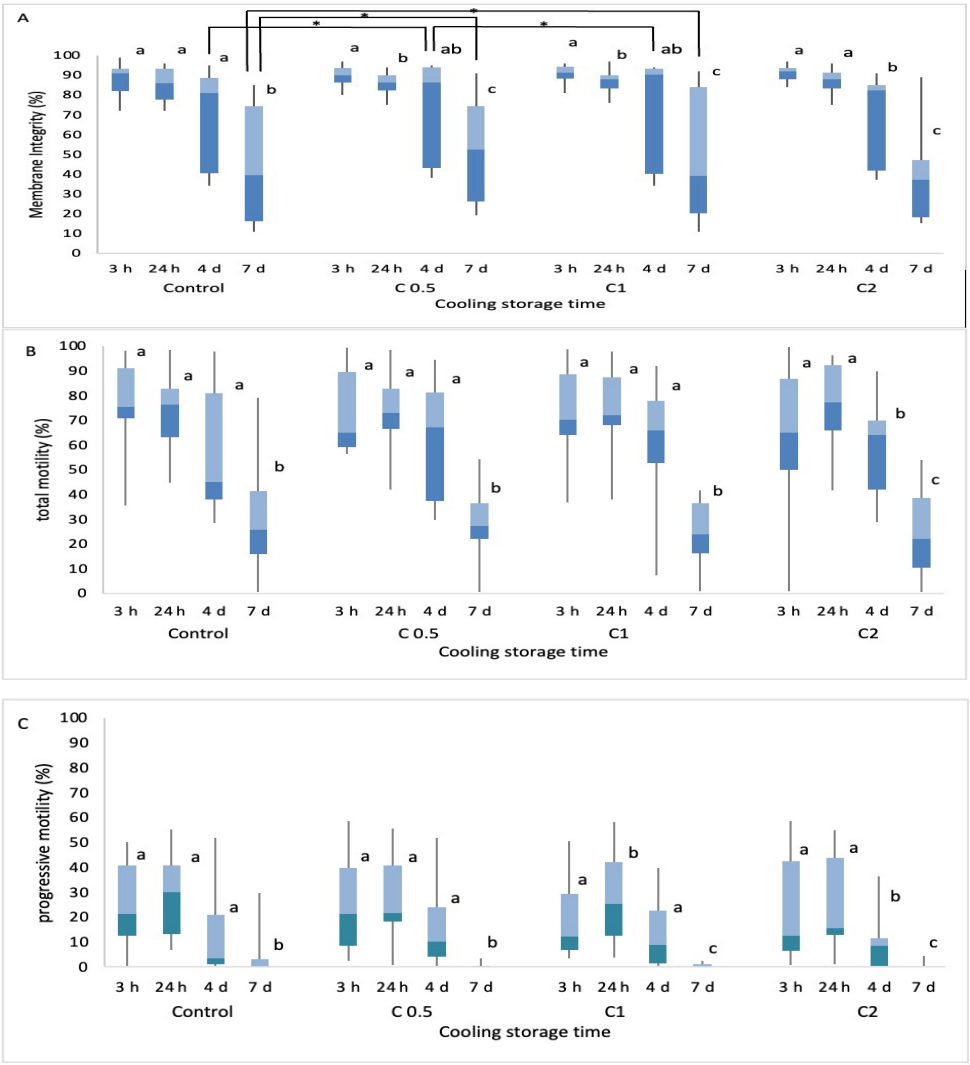

Figure 5.3 Membrane integrity (A), total (B) and progressive motility (C) of dog semen (n=10) diluted with only egg-yolk tris-citrate glucose (EYT-G; control) or with EYT-G 99supplemented with three different concentrations (0.5, 1, 2 mM) of crocin (C0.5, C1 and C2) after storage at refrigeration temperature (4°C) for 3h, 24h, 4 and 7 days (4d and 7d). Asterisks indicate significant difference at P≤0.05 among groups within each time point. [a,b,c] *Different letters indicate significant differences among time points within each group (P<0.05)*

5.3.4 Kinetic parameters

The temporal decrease of kinetic parameters and the pattern observed within time points and groups are showed in Table 5.3.
The percentage of hyperactivated spermatozoa was similar among groups at all time points (Table 5.3). This parameter decreased at 7 d of storage in all groups even if the difference was not statistically different in the control group.
With regard to treatment, no differences in VCL were detected between the control and treated groups. However, in C0.5 group VCL was higher ($P \leq 0.05$) than in C1 group at 3h and 4 days of semen storage ($P \leq 0.05$). In all groups VCL dropped ($P < 0.05$) only after 7 days of storage. The semen treated with 0.5 mM crocin (C0.5) exhibited higher ($P < 0.05$) VSL and VAP than the control group ($P \leq 0.05$) at 4 d of storage at 4°C. Extending storage to 7 d resulted in a decrease of VAP in all groups; however, all treated groups had lower ($P < 0.05$) values than the control.
No differences in LIN and STR were observed among groups at 3 h, 24 h and 4 d of storage. Instead, at the latest time in the control group LIN was higher ($P < 0.05$) compared to most groups and STR was higher ($P < 0.05$) than in C2 group. However, in relation to storage time, both parameters decreased ($P < 0.05$) in the control group at 4 h compared to earlier times, whereas this decrease ($P < 0.05$) was only detected in the C0.5 group at 7 d.
Likewise, Finally, BCF was higher ($P < 0.05$) in C0.5 group compared to the control at 4 d of cooling storage, with intermediate values in the other treated groups. The temporal pattern of BCF was also similar to that of LIN and STR, with high values preserved up to 4 d and 7 d, respectively in the C0.5 and the control groups.

Table 5.3. Trends of hyperactivated spermatozoa (%) and semen kinetic parameters of canine (n=10) semen of control (CTRL) and Crocina groups (C0.5, C1 and C2) during storage at 4°C for 7 days.

	Storage time			
Hyper (%)	*3h*	*24h*	*4d*	*7d*
CTRL	2.9 (0-8) [a]	2.5 (0-8.2) [a]	0.3 (0-4.1) [a]	0 (0-1.1) [a]
C0.5	1.6 (0.2-11.4) [a]	1.9 (0.6-4.1) [a]	0.5 (0-4.3) [a]	0 (0-0) [b]
C1	2.5 (0.2-7.8) [a]	3.5 (0-7.3) [a]	0.9 (0-3.4) [ab]	0 (0-0) [b]
C2	2.2 (0.7-7.8) [a]	2.6 (1.2-9.8) [a]	0 (0-2.7) [a]	0 (0-0.4) [b]
VCL				
CTRL	73 (56.4-91.4) [a,xy]	77.7 (55.4-128.6) [a,xyz]	34.2 (19.3-91.2) [ab,xy]	24.1 (17.3-73.2) [b]
C0.5	84.2 (54.3-104.3) [a,x]	70.5 (49.5-107.1) [a,x]	65.5 (27.1-91.6) [a,x]	19.8 (16.2-30.3) [b]
C1	64.2 (40.7-95.2) [a,y]	83 (63.3-122.7) [b,y]	48.1 (26.9-71.8) [a,y]	19.8 (17.4-42.2) [c]
C2	68.7 (37.7-118.6) [a,xy]	81.6 (48.3-116.8) [b,xz]	44.4 (19.4-73.7) [a,xy]	17.5 (10.3-21.6) [c]
VSL				
CTRL	37.1 (26.4-49) [a]	39.3 (34-73.8) [a,xy]	13.7 (2.3-40.5) [b,x]	6.4 (3.2-24.3) [b,x]
C0.5	38 (20.2-57.5) [a]	39.2 (24.8-57.3) [a,xy]	26.8 (7.3-52.8) [a,y]	2.2 (1.3-7.4) [b,y]
C1	27.4 (21.5-43-1) [a]	39.9 (29.3-57.6) [b,x]	20.7 (8.4-29.3) [a,xy]	3.3 (1.5-13.6) [c,xy]
C2	34 (14.8-46) [ab]	35.5 (24.7-46.2) [a,y]	19.7 (3.4-29.7) [b,xy]	1.7 (0.7-4.8) [c,y]
VAP				
CTRL	49.2 (42.2-63.2) [a]	50.9 (40-92.7) [a,xy]	19.8 (5.7-52) [b,x]	11.2 (6.9-41.2) [b,x]
C0.5	53.2 (40.4-74.4) [a]	48 (32-75.7) [a,x]	36.6 (12.5-61.4) [a,y]	6.2 (5.1-14.2) [b,y]
C1	44.3 (28.2-61.2) [a]	56.7 (43-84.5) [b,y]	31.7 (13.8-41.8) [a,xy]	6.8 (3.4-23.7) [c,y]
C2	44.8 (24-81.2) [a]	53 (33.2-75.9) [a,x]	28.4 (6.7-44.9) [b,xy]	6.3 (3.4-10.3) [c,y]
LIN				
CTRL	47.6 (40-54.7) [a]	46.7 (41.4-55.4) [a]	29.8 (14.9-41.5) [b]	26.4 (17.3-33.2) [b,x]

C0.5	47 (40-48.7) [a]	41.8 (35.3-52.2) [a]	35.8 (27.4-44.8) [a]	14.2 (8.3 -24) [b,y]
C1	42.2 (40.1-52.5) [a]	46.3 (40.5-51.7) [a]	33 (31.1-40.5) [b]	17.3 (10-24.9) [c,xy]
C2	39.8 (35.1-45.1) [a]	41.5 (35.7-46.4) [a]	26.4 (17.3-33.2) [a]	9.9 (4.5-22.2) [b,y]
STR				
CTRL	66.4 (60-74) [a]	70.1 (65.2-73.1) [a]	53.8 (44.2-70.9) [b]	48.6 (42.3-62.5) [b,x]
C0.5	66.9 (61.2-71.2) [a]	64.1 (55.5-72.1) [a]	62.2 (49.8 -71.4) [a]	48.6 (36.4-54.5) [b,xy]
C1	65.4 (61-69.4) [a]	68 (60-71) [a]	60.2 (53.2-68.5) [ab]	59.3 (44.3-57.1) [b,x]
C2	63.4 (55.2-69.4) [a]	65.9 (57.7-71.3) [a]	60 (46.4-68.2) [a]	33.4 (11.8-50.7) [b,y]
BCF				
CTRL	8.8 (5.5-13.4) [a,x]	10.8 (6.4-13.7) [a]	3.8 (0.7-10.6) [b,x]	2 (1-8.2) [b,x]
C0.5	9.1 (4.9-12.9) [a,xy]	7.5 (5.6-13.2) [a]	6.9 (2.2-11.2) [a,y]	0.9 (0.6-2.5) [b,xy]
C1	6.8 (4.2-12) [a,y]	9.9 (6.8-14) [b]	5.5 (2.6-10.4) [a,xy]	0.9 (0.4-5) [c,xy]
C2	7.8 (4-14.2) [a,xy]	7.5 (4.7-13.6) [a]	6 (0.8-11) [b,xy]	0.8 (0.4-1.5) [c,y]

5.3.5 Lipid peroxidation

As shown in Figure 5.3.5, no differences in lipid peroxidation, indicated by the MDA concentrations, were detected among groups at any time points. A slight similar decrease was recorded at 24 h compared to 3 h in the control and the C0.5 groups, with intermediate values at 4 and 7 d. Unexpectedly, no increase in lipid peroxidation was observed at prolonged storage times.

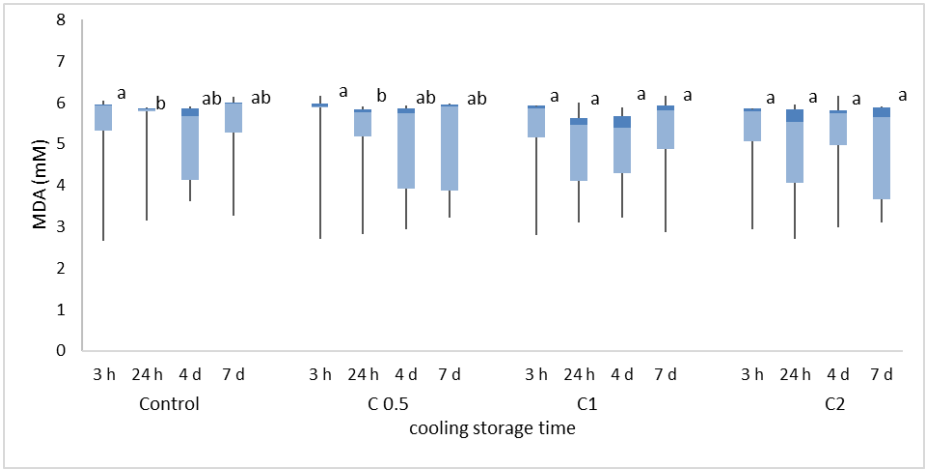

Figure 5.3.5. Lipid peroxidation Membrane integrity (A), total (B) and progressive motility (C) of dog semen (n=10) diluted with only egg-yolk tris-citrate glucose (EYT-G; control) or with EYT-G 103supplemented with three different concentrations (0.5, 1, 2 mM) of crocin (C0.5, C1 and C2) after storage at refrigeration temperature (4°C) for 3h, 24h, 4 and 7 days (4d and 7d). Asterisks indicate significant difference at P≤0.05 among groups within each time point. [a,b] Different letters indicate significant differences among time points within each group (P<0.05).

5.4 Discussion

The hypothesis of this study was that supplementation of the extender with crocin, an active constituent of saffron with antioxidant properties, would improve the quality of canine semen stored at 4°C. The rationale behind this work originates from the evidence of oxidative stress occurring after prolonged refrigeration and of beneficial effects of crocin on sperm quality parameters reported in other species. To the best of our knowledge, this is the first study to assess the effects of crocin on chilled canine semen. The results of the study demonstrated that the enrichment of the extender with 0.5 mM crocin improves sperm membrane integrity and sperm kinetics after 4 days storage at 4°C, without affecting though lipid peroxidation.

In order to evaluate the effects of crocin on sperm quality a dose-response trial was carried out, using concentrations (0, 0.5, 1 and 2 mM) previously tested in bovine, goat and buffalo species (Sapanidou et al. 2022; Longobardi et al. 2020 and 2021) showing that the most effective concentration for canine chilled semen was the lowest tested (0.5 mM), while the highest (2 mM) exerted in part a deleterious effect. The effects of

the treatment were assessed at different times, such as 3 h, 24 h, 4 days and 7 days. Regardless of the treatment, a deterioration of semen quality was recorded after 7 days of storage, time at which sperm membrane integrity, total and progressive motility, as well as kinetic parameters, were significantly reduced. When the extender was supplemented with the highest concentration of crocin (2 mM) a decrease of membrane integrity, total motility, and progressive motility was observed earlier, i.e., at 4 days storage, indicating a potential toxic effect. However, in any case the decline in semen quality was unexpectedly not associated to increased lipid peroxidation. The worsening of semen quality at prolonged storage time at 4°C is in agreement with a previous study suggesting that chilled canine semen should be used for AI within 4.9 days (England, et al. 1996). It has been previously shown that over extended storage at 4° C spermatozoa switch from aerobic to anaerobic metabolism, due to oxygen consumption, and that the activation of glycolysis results in lactate production and hence reduced pH of the medium, leading in turn to decreased metabolism, ATP production and motility. (Mann et al.1964)

The most interesting findings of the present study regard the improvement of semen quality obtained with 0.5 mM crocin. Indeed, the addition of 0.5 mM crocin in the extender significantly increased the proportion of spermatozoa with intact membrane at both 4 and 7 days compared to the control group. Moreover, despite similar values of total motility and progressive motility, after 4 days of storage most of the sperm kinetic parameters improved in C0.5 group, compared to the control. In fact, average path velocity (VAP), straight-line velocity (VSL) and beat cross frequency (BCF) were significantly higher when semen was stored for 4 days in the presence of 0.5 mM crocin compared to the control. The curvilinear velocity (VCL) was also higher although the difference was not significant. Furthermore, in the C0.5 group straightness (STR) and linearity (LIN) were preserved up to 4 days, whereas a decrease was observed at this storage time compared to earlier times in the control group. It is known that motility is one of the most important indicators of the potential fertilizing ability of spermatozoa (Vijayaraghavanet al. 2003) and sperm kinetics has been associated to fertility in various species (Marshburn et al 1992; Broekhuijse et al. 2012)

A correlation between oxidative stress and semen quality parameters was demonstrated in dogs (Del Prete et al.2018). It is known that sperm quality deteriorates at increasing storage times due to the occurrence of oxidative

stress, resulting from an unbalance between ROS production and antioxidant systems (Silvestre et al. 2021). For this reason, the focus of researchers has been recently addressed to strategies based on the utilization of antioxidants to counteract the chilling-related oxidative stress. An improved semen quality of chilled canine semen has been previously obtained by supplementing extender with antioxidants such as Lycopene or Vitamin E and B16, Maca extracts, antioxidant enzymes (Sheikholeslami et al.2020; Michael et al. 2009; Del Prete et al.2022)

The beneficial effect of crocin here reported is likely related to its antioxidant properties. However, the improved semen quality was not associated to decreased lipid peroxidation, which was unexpectedly not affected by either treatment or storage time. A slight decrease of lipid peroxidation was only recorded in the control group and C0.5 groups at 24 h, returning to initial values at 4 and 7 days. This contrasts with our previous experiment with maca extracts, in which after 7 days storage chilled semen showed an increase of lipid peroxidation (Del Prete et al. 2022).

There are several reports on the beneficial effects of crocin on semen quality in different domestic species, in most of which, though, crocin was added to the semen extender before freezing rather than chilling and hence, results cannot be compared. In the goat supplementation of 1 mM crocin in the extender decreased oxidative stress, improving sperm motility and the DNA integrity of frozen-thawed sperm (Longobardi et al. 2020). In cattle crocin improved sperm viability, motility and kinetic parameters (VCL, VSL, VAP, ALH) after thawing but a reduction of lipid peroxidation was only observed after 2 h post-thawing incubation (Sapanidou et al. 2022). In another study the incubation of frozen-thawed semen with crocin improved sperm membrane integrity and decreased DNA fragmentation and ROS levels (Longobardi et al. 2021).

We may speculate that the protective effects of crocin on canine chilled semen are due to its antioxidant functions, as it is known that crocin is a ROS scavenger (Singla et al. 2011; Sapanidou et al. 2005). Oxidative stress is indeed known to affect the fluidity, integrity, and flexibility of the sperm plasma membrane, interfering with fertilizing capacity (Said et al.2005). However, in this study we measured MDA concentration to assess lipid peroxidation, a reliable marker of oxidative stress and predictor of fertility in other species (Tavilani, et al. 2005; Tavilani et al. 2008; Collodel, et al. 2015) while ROS levels were not evaluated. Lipid peroxidation was not affected by either treatment or storage time; however, the MDA levels in

spermatozoa were relatively low in all groups and hence, due to the limitation of the assay and the high variability, small differences among groups could not be detected. In any case, based on our results, we cannot conclude that the improvement of membrane integrity and sperm kinetics observed in C0.5 group after 4 days storage is due to reduced oxidative stress. Therefore, the beneficial effects may be related to other functions of crocin, such as the capability to increase intracellular detoxifying enzymes and to modulate membrane fluidity, leading to changes in its permeability to oxygen and other molecules (Assimopoulou et al. 2005).

5.5 Conclusion

In conclusion, we demonstrated that the enrichment of extender with the crocin improves to a certain extend canine semen quality, particularly after 4 days of storage at 4 °C. At this time point crocin increased the percentage of sperm with intact membrane and most of the kinetic parameters measured by CASA. It is worth pointing out that the beneficial effect was lost at 7 days, at which time deterioration of semen quality occurred in all groups, regardless of the treatment. Therefore, the treatment improved semen quality parameters after 4 days storage but was not effective at further extending lifespan of spermatozoa under chilling conditions. Further studies are undoubtedly required to validate these results, by assessing other fertility-associated parameters including fertilizing ability, and to elucidate the mechanism of action of the compound, by evaluating ROS levels and DNA fragmentation. Finally, in future perspective it would be interesting to assess the effect of crocin on quality of frozen semen.

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Chapter 6

Effect of Maca aqueous extract addition to an extender for frozen canine semen

Effect of aqueous extract of Maca addition to an extender for frozen canine semen

6.1 Introduction

Cryopreservation of canine semen has become an increasingly popular technology (Peña et al., 2006). The use of frozen semen for artificial insemination allows to choose the best timing for insemination and the semen coming from anywhere in the world. Long-distance transportation of frozen semen reduced the movement of animals and its costs and preserve the genetic diversity of species (Watson and Holt, 2001; Comizzoli et al., 2009).

The outcome of artificial insemination with frozen semen is dictated by the quality of semen after thawing. The cold shock resistance of semen is different between individuals and ejaculates, and it can be further influenced by the contents of semen extender and the cryopreservation protocols (Szaz et al., 2000). During freezing process, the excess formation of reactive oxygen species (ROS) leads to a condition of oxidative stress. The large amount of polyunsaturated fatty acids and the low content of protective enzymes in sperm cytoplasm make sperm much more sensitive to the oxidative stress (Sharma and Agarwal, 1996; Neagu et al., 2011) . The major consequence of cryopreservation is an extensive oxidative damage to all cellular components, including proteins, lipids and DNA (Kim et al., 2010). This causes a decreased semen quality, such as viability, motility, DNA integrity, provoking acrosome damage, cell apoptosis, interfering with the fertilization capability (Sharma and Agarwal, 1996; Kim et al., 2010; Neagu et al., 2011).

In order to reduce reactive oxygen species generation due to osmotic stress during the cryopreservation procedure, different attempts have been made to protect spermatozoa during the freeze-thaw process by extender supplementation of antioxidants (Mahiddine and Kim, 2021).

Lepidium meyenii (Maca) is an Andean plant of the Brassicaceae family native of Peru. Maca composition depends on the ecotypes (hypocotyl colors) and the condition of cultivation (altitude, temperature), drying and extraction procedures (Korkmaz, 2018).Typical Maca elements include glucosinolates, alkaloids (macaines), alkamides (macamides) fatty acids, phenols (flavonoids, tannins), and several microelements (Korkmaz, 2018; Tafuri et al., 2019).

In this study for the first time the effect of the addition of aqueous extract of Maca on the sperm post-thaw quality of dog spermatozoa was examined.

6.2 Material and Methods

6.2.1 Animals

In this study were involved 10 dogs of different breeds (Rottweiler, English Setter, Shih Tzu, English Pointer, Miniature poodle) ranging between 2 and 8 (median of 5.5) years of age. All animals were housed in the FOOF breeder center and fed with standard commercial dog food twice daily and water *ad libitum*. The dogs were routinely vaccinated and dewormed according to national recommendations. All procedures were carried out in compliance with the code of ethics (D.lgs. 26 - 04/03/2014), and there were approved by the Ethics Committee of the Department of Veterinary Medicine and Animal Productions at the University of Naples Federico II, Italy (prot. no. PG/2021/0057934 of 07/06/2021). A written inform consent was signed by the legal representative of the breeding center.

6.2.2 Semen collection and processing

One ejaculate was collected by artificial vagina from each of 10 dogs as part of a routine reproductive examination of dogs. Immediately after, sperm rich fractions were examined for volume (mL) by aspiration into a 5-mL pipette, and motility by Sperm Class Analyzer (SCA) system (Microptic SL, Veterinary Edition, Barcelona, Spain). Where sperm concentration was too high, a 30 µl aliquot was first diluted 1:1 v/v in TRIS-citrate glucose buffer (TCG: Tris 2.4 g, Citric Acid 1.4 g, Glucose 0.8 g, Penicillin G Sodium Salt 0.06 g, Streptomycin 0.1 g and distilled water to 100 ml) at 37 °C.
Raw semen was centrifuged on a cushion (Glucose 59.95 g, Sodium citrate tribasic dihydrate 3.7 g, Disodium EDTA 3.7 g, Sodium bicarbonate 1.2 g, in 1 L of deionized water) at 600 x g for 10 minutes to partial eliminate the seminal plasma. After removal of supernatant, sperm concentration was measured using a Bürker counting chamber. A two-step freezing protocol was used to reach a final concentration of 200 x 10^6 sperm/mL. Semen was first diluted at room temperature (RT: 20-25°C) with TCG with 20% egg yolk and 6% glycerol to reach a concentration of 400 x 10^6 sperm/mL, placed in a RT water container and cooled over 30 minutes to 4°C in the fridge. Samples were then diluted 1:1 (v/v) with TCG with 20% egg yolk, 7% glycerol and 1% Equex STM without (Ctrl) or with the addition of 10 µl/mL of aqueous extract of Maca (Maca) and equilibrated for additiona1 5

minutes at 4°C. The preparation and the composition of the aqueous extract of Maca was the same as in our previous study (Del Prete et al., 2022). The choice of concentration was based on the results of previous experiments with the aqueous extract of Maca (Del Prete et al., 2022). Diluted semen was loaded into 0.5 mL straws, suspended 7 cm over liquid nitrogen vapor for 10 min, then plunged into and stored in liquid nitrogen. After at least 4 weeks two straws per sample were thawed in a 37°C water bath for 30 and emptied 1:3 in TCG to reach a concentration of 30 x 10^6 spz/ml.
Sperm levels of malondialdehyde (MDA), an indicator of lipid peroxidation, were evaluated before (fresh semen; pre) and immediately post-thaw (post-thaw). In order to verify the effect of Maca on post-thaw sperm longevity for up to 2 h of incubation of 37°C, thermal-resistance test was conducted, evaluating samples at 1 (T1) and 2 h (T2) of incubation for motility, sperm membrane integrity and mitochondrial membrane potential.

6.2.3 Motility and kinetic parameters

Motility was assessed using Sperm Class Analyzer (SCA) system (Microptic SL, Veterinary Edition, Barcelona, Spain) installed on a camera-equipped light microscope system (Eclipse E200, Nikon, Japan). The parameters included were: total motility (%), progressive motility (%), the percentage of sperm subpopulations (rapid, medium and slow), average path velocity (VAP; µm/s), straight-line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), straightness (STR; %) and linearity (LIN; %), amplitude of lateral head (ALH; µm), wobble (WOB= VAP/VCL; %), beat cross frequency (BCF ; beats/s). The same SCA preset for dog semen was used for all examinations. All particles sized between 10 and 80 μm^2 were considered sperm and classified as progressive motile in case of STR > 75 %. The SCA cut-off values for spermatozoa subpopulations were based on curvilinear velocity (VCL) = rapid > 100 > medium > 50> slow; spermatozoa with VCL > 150 µm/s and ALH >3,5 µm were considered as hyperactive and spermatozoa with VCL below 10 µm/s were considered as static. For the evaluation, five µl of control or Maca-treated semen at each time point (Fresh semen, Post-thaw, T1, T2) were placed in a Leja analysis chamber (Leja, Nieuw-Vannep, Netherlands) pre-warmed at 37 °C. At least 500 sperm cells in five randomly selected fields were evaluated.

6.2.4 Membrane integrity and mitochondrial activity

Triple fluorescent labelling with propidium iodide (PI; Invitrogen™, Eugene, Oregon, USA), SYBR green-14, and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi- dazolylcarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR, USA) was performed to detect changes in mitochondrial transmembrane potential and membrane integrity (Figure 6.2.3).
SYBR green-14 stains live sperm green and PI enters cells with compromised membrane integrity, staining them red (dead spermatozoa). JC-1 is a green-fluorescent monomer at low membrane potential and forms aggregates in mitochondria with high membrane potential, emitting a bright red- orange fluorescence. Therefore, three sperm populations were identified with the SYBR-14/PI/JC-1 stain: dead sperm (PI+), viable sperm with high mitochondrial membrane potential (HMMP; SYBR-14+/PI-/JC-1+) and viable sperm with low mitochondrial membrane potential (LMMP; SYBR-14+/PI-/JC-1-). For the evaluation, thirty µL of each sample were incubated with 3 µl of SYBR green solution (5 µl SYBR a10x + 15 µl PBS), 2 µl of PI solution (10 mg/ml) and 2 µl JC-1 solution (1 mg/ml) at 37 °C in the dark for 15 min. At least two hundred cells per sample were evaluated using confocal microscopy (*DM6 B, Leica Microsystems CMS, GmbH*).

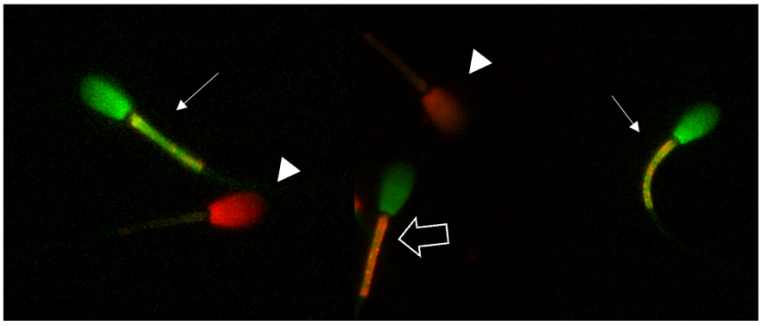

Figure 6.2.3. Example of canine sperm cells stained by SYBR-14/PI/JC-1: dead sperm (PI+; head arrow), viable sperm with high mitochondrial membrane potential (HMMP; SYBR-14+/PI-/JC-1+; big arrow) and viable sperm with low mitochondrial membrane potential (LMMP; SYBR-14+/PI-/JC-1-; small arrows).

6.2.5 Lipid peroxidation

Sperm concentration of malondialdehyde (MDA) was measured using the thiobarbituric acid (TBA) procedure as previously described (Esterbauer et al., 1990). In brief, 100 µL of each sample was mixed with 0.5 mL of cold 30% (w/v) trichloroacetic acid in order to precipitate proteins. One millimeter of supernatant was incubated with 1.3 mL of 0.5% (w/v) TBA at 85 ◦C for 40 min. In this test, one molecule of MDA reacts with two molecules of TBA yielding a pink chromogen having maximal absorbance at 532–535 nm. After cooling, the fluorescence was read immediately using a SPEX Fluoromax spectrophotofluorimeter (GloMax®-Multi Detection System, Promega Madison, WI, USA; excitation wavelengths, 536 nm; emission wavelengths, 557 nm). Concentrations of MDA were calculated using a calibration curve ranged between 0.5–2 pmoles/mL. The results expressed as nmol/L of proteins.

6.2.6 Statistical analysis

Data were analyzed with Statistical Package for Social Sciences (SPSS IBM® Statistics version 27.0, IBM Corporation, Armonk, NY, USA). Because of non-normal distribution (Shapiro-Wilk test) results were expressed as median and interquartile range (IQR) and non-parametric test were used. The statistical comparisons between the groups at each time point (Fresh semen, Post-thaw, T1, T2) were performed with Wilcoxon. The effect of storage time in each group (Ctrl or Maca) on semen parameters was evaluated using a Friedman test, and in case of significance, post hoc analysis with Wilcoxon's signed-rank test was used to compare individual storage times. Values were considered statistically significant when $p \leq 0.05$.

6.3 Results

6.3.1 Fresh semen

Fresh semen was of normal appearance, milky -white in color. The volume ranged between 0.4 and 12 mL (median of 2.4 mL), with a median concentration of 312.5 x 10^6 sperm/ml (IQR: 269,5- 462 sperm/mL). Fresh

sperm presented a viability (SYBR-14+/PI-) of 84 (79.3-85) % and HMMP (SYBR-14+/PI-/JC-1+) of 38.5 (36-44.8)% (Fig. 6.2.3).

6.3.2 Motility and kinetic parameters

Sperm motility and kinetic parameter assessment evaluated by SCA of fresh semen (pre) and of control and Maca-treated semen after frozen- thawed (post-thaw, T1 and T2) are shown in Table 6.3A and 6.3B. As expected, a significant reduction ($p \leq 0.05$) in total and progressive motility in both control and treated semen was observed after thawing (pre vs post; post vs T1; Table 6.3A). The percentage of hyperactivated spermatozoa remained constant during the four time points analyzed in control, while in the semen treated with Maca increased significantly at T1 ($p \leq 0.05$). As shown in Table 1, the temporal decreases of motile-sperm subpopulations showed a similar trend in both groups. Although this effect of cryopreservation on motility parameters, total motility and the percentage of sperm with medium velocity in Maca-treated semen were higher than control after 1 hour of incubation at 37°C (Table 6.3A). At the same time point, WOB was found remarkably high in Maca-treated group than control (Table 6.3B). There were no other differences between groups in semen kinetic parameters between the two groups (Table 6.3.2).

Effect of aqueous extract of Maca addition to an extender for frozen canine semen

Table 6.3A *Total and progressive motility and the percentages of hyperactivated, rapid, medium and slow-moving spermatozoa assessed using Sperm Class Analyzer (SCA) in Control and Maca-treated (10 µL/mL of aqueous extract of Maca) dog semen (n = 10) before freezing (Pre), immediately after thawing and after 1 (T1) and 2 h (T2) of incubation at 37°C. All values are expressed as median and interquartile range (IQR); asterisk indicates statistical difference between groups at p≤ 0.05; the letters indicate statistically differences at at p≤ 0.05 between time points within each group.*

	Total motility (%)		**Progressive motility (%)**		**Hyperactive (%)**	
	Control	***Maca***	***Control***	***Maca***	***Control***	***Maca***
Pre	86.4 (79.8-93)[a]	-	25.8 (20-39.7)[a]	-	2.8 (0.5-4.1)[a]	-
Post	31.2 (25.6-60.1)[b]	40.4 (28.5-58)[b]	7.4 (1.5-14.9)[a]	9.3 (0.6-23)[a]	0.5 (0-2.9)[a]	1.8 (0-6.7)[a]
T1	23.8 (15.9-36.3)[c]	33.1 (18-40.1)[*, c]	4.7 (0-7.9)[b]	5.5 (0.4-12.1)[a]	1.9 (0-11.1)[a]	6.8 (1.7-16.9)[b]
T2	25.6 (12.7-32.8)[c]	26.8 (10.5-42)[c]	0.5 (0-7.1)[b]	3.6 (0.4-9.6)[a]	4.3 (0-12.3)[a]	10 (0.7-17.9)[b]
	Rapid (%)		**Medium (%)**		**Slow (%)**	
	Control	***Maca***	***Control***	***Maca***	***Control***	***Maca***
Pre	16.4 (4.6-32)[a]	-	26.2 (23.2-29.8)[a]	-	35.8 (32.1-45.4)[a]	-
Post	5.7 (0-16.1)[a]	8.5 (1.7-20.7)[a]	2.1 (1.6-6.5)[b]	4.1 (2.6-6.9)[b]	25.5(19.4-32.2)[a]	26.9 (21.3-29.1)[a]
T1	4.7 (0-10.6)[a]	4.5 (1.4-16.1)[a]	1.4 (0.4- 2.8)[b]	2.3 (0.7-4)[*, b]	17.7 (12.2-22.1)[b]	19.2 (13-25.5)[b]
T2	1.1(0-10)[a]	4.6 (0.4-15.8)[a]	0.9 (0-2.1)[b]	2.1 (0.2-3.7)[b]	19.5 (11.9-22.3)[b]	21 (9.8-23.8)[b]

Table 6.3B *Sperm velocities in control and Maca-treated (10 µL/mL of aqueous extract of Maca) canine semen (n=10) at different time points, before freezing (Pre), immediately after thawing (Post) and after 1 (T1) and 2 h (T2) of incubation at 37°C. All values are expressed as median and interquartile range (IQR). Significant differences between groups are indicated by an asterisk with p ≤ 0.05; within each group (column), means with different letters (a–c) differed between time points (p ≤ 0.05).*

	VCL (µm/s)		VSL(µm/s)		VAP(µm/s)	
	Control	*Maca*	*Control*	*Maca*	*Control*	*Maca*
Pre	70.8 (57.5-82.9) [a]	-	40.1 (33.4-46.6)[a]	-	50.5 (45.8-60.9)[a]	-
Post	51.4 (28.7-71.8) [a]	59.9 (39.7-81.5) [a]	26.9 (15.8-33.7)[a]	34.7 (15.4-44.4) [a]	34.2(20.1-40.9)[b]	40.1 (24-52.7) [b]
T1	49.9 (25.2-77.6) [a]	57.7(37.1-80) [a]	21.1 (5.9-31)[ab]	23.2 (8.8-38.1) [a]	28 (10.5-42.1)[b]	31.7 (15.7-50.3) [b]
T2	33.5 (24-79.7) [a]	52.8 (25.9-88.9) [a]	9.3 (4.3-34.1) [b]	21.2 (7.1-29.3) [a]	15.3 (8.9-46.1)[b]	29.5 (11.7-48.6) [b]
	LIN (%)		STR(%)		WOB(%)	
	Control	*Maca*	*Control*	*Maca*	*Control*	*Maca*
Pre	51.5 (49.9-53.6) [a]	-	70.1 (66.9-73.7) [a]	-	70.7 (66.5-72.4)[a]	-
Post	37.2 (33.1-46.2) [b]	46 (23.1-51.7) [a]	64.1 (57-67) [a]	70.2 (52.5-71.9) [a]	55.2 (50.3-63.2) [b]	62.9 (41.8-66.9) [b]
T1	27.2 (17.7-32.4) [c]	30.8 (16.8-34.4) [a]	57.1 (48.1-59.8) [a]	59 (38.5-62.9) [a]	43.4 (36.9-51.4) [c]	48.4 (44.6 -53.1)[*b]
T2	24.8 (9.9-33.3) [c]	23.1 (15.1-34.4) [a]	55 (35.8-61.6) [a]	53.9 (47.8-64.3) [a]	46 (31.4-51.1) [c]	43.5 (31.3-51.5) [b]
	ALH (µm)		BCF (beat/s)			
	Control	*Maca*	*Control*	*Maca*		
Pre	1.7 (1.3-1.9) [a]	-	11.4 (9.1-12.7) [a]	-		
Post	1.3 (0.9-1.7) [a]	1.5 (1.1-1.8) [a]	6.1 (4.6-8.9) [a]	7.9 (4.6-11.5) [a]		
T1	1.4 (0.9-1.9) [a]	1.4 (1.2-2) [a]	5 (2-7.6) [a]	5.8 (3-8.7) [a]		
T2	1.1 (0.9-2) [a]	1.4 (0.9-2.3) [a]	3.4 (0.9-7.6) [a]	5.2 (2-7.7) [a]		

VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; ALH: amplitude of lateral head; WOB: wobble (VAP/VCL); BCF: beat cross frequency.

6.3.3 Membrane integrity and mitochondrial activity

Figure 6.3.2 shows the modifications of the percentage of viable sperm with HMMP (A) or LMMP (B) at different time points in both groups (control and Maca). As shown in Figure 6.3.2A, the percentage of viable sperm with HMMP decreased significantly at thawing, and at T2 in both groups (P<0.05). The portion of viable sperm with LMMP remain constant between pre and post-thaw in both groups, while a decreasing was evident between post-thaw and T1 only for control group and between T1 and T2 for Maca group (Figure 6.3.2 B). No differences were found between groups regarding those parameters.

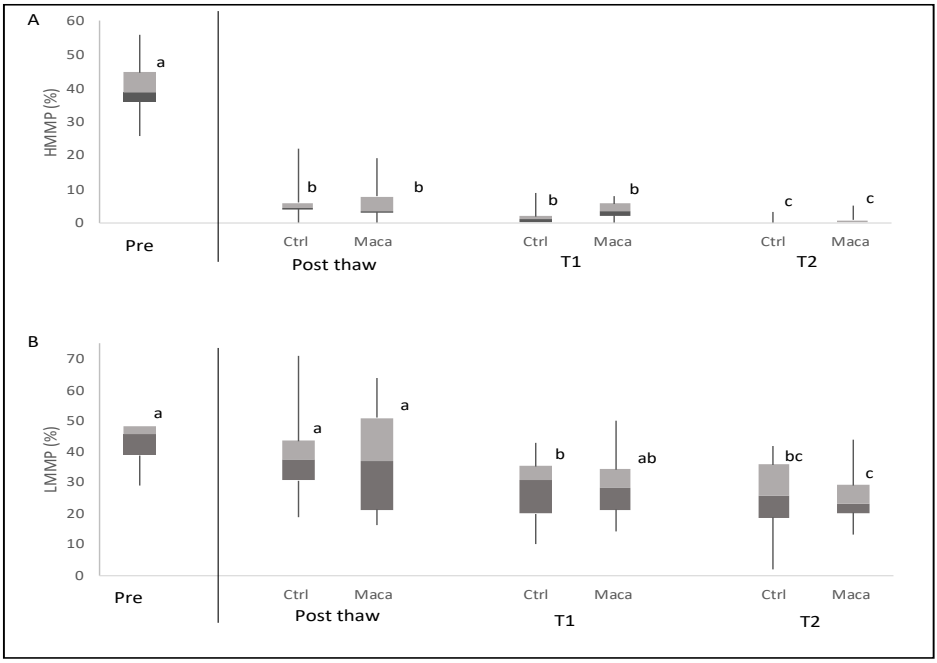

Figure 6.3.2 The percentage of viable sperm with high mitochondrial potential (HMMP: SYBR-14+/PI-/JC-1+) (A) and with low mitochondrial membrane potential (LMMP; SYBR-14+/PI-/JC-1-) (B) in Control and Maca-treated (10 µL/mL of aqueous extract of Maca) dog semen (n = 10) at different time points, before freezing (Pre), immediately after thawing (Post) and after 1 (T1) and 2 h (T2) of incubation at 37°C. For each box, the central line represents the median, the edges of the boxes represent the IQR (25th and 75th percentiles), the whiskers represent the extreme points; the letters (a-c) indicate statistically differences at p≤ 0.05 between time points and groups.

6.3.4 Lipid peroxidation

As shown in Figure 6.3.3, lipid peroxidation did not differ between pre-freezing and post-thawing semen in both groups, however the MDA concentration was lower in Maca-treated semen than in control (P<0.05).

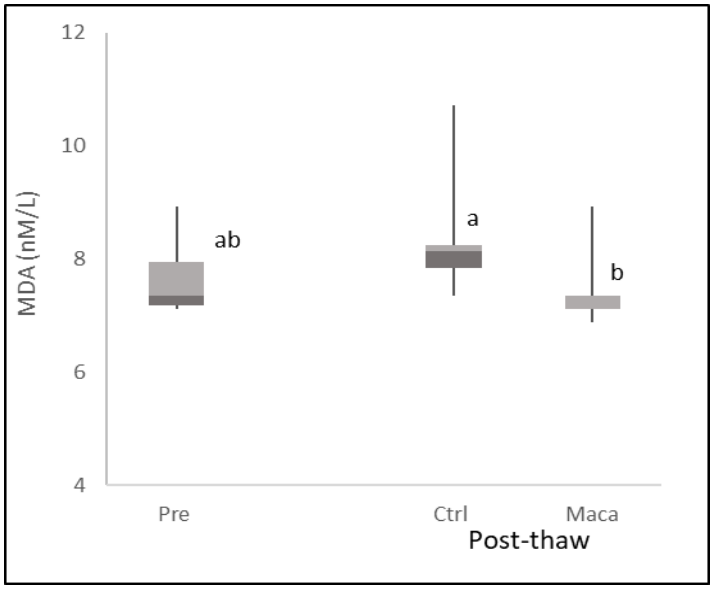

Figure 6.3.3 *The percentage of lipid peroxidation before freezing (Pre) and immediately after thawing (Post-thaw) in Control and Maca-treated (10 µL/mL of aqueous extract of Maca) dog semen (n = 10). For each box, the central line represents the median, the edges of the boxes represent the IQR (25th and 75th percentiles), the whiskers represent the extreme points; the letters (a-c) indicate statistically differences at p≤ 0.05 between time points and groups.*

6.4 Discussion

This study explored for the first time the effect of Maca addition on cryopreservation of canine semen, evaluating the qualitative semen parameters immediately after thawing and after 1 and 2 hours at 37°C.
Sperm motility is the most commonly used indicator of semen quality before and after freezing-thawing process (Martìnez, 2004). Motility is expression of structural and functional competence of spermatozoa, and it is necessary to reach and colonize the oviduct (Scott, 2000; Yanagimachi, 1994). Although fertilization have been obtained also with semen motility of 20 or 30 %, it has been proposed that a minimum of 40-50% of post-thaw sperm motility is required for fertility in dogs (Concannon and Battista, 1989; Linde-Forsberg and Forsberg, 1989). In this study, both control and treated semen had a total motility, immediately after thawing, ranged between approximately 26 and 60%. Canine sperm cells frozen with an extender supplemented with Maca exhibited higher total motility, especially the subpopulation of sperm with medium velocity 1 hours after thawing than control semen. Moreover, the percentage of cells with LMMP decrease slower in Maca-treated samples during thermal-stress time. The *in vitro* beneficial effect of Maca on motility is already reported in dog, human and canine spermatozoa (Aoki et al., 2019; Del Prete et al., 2022).
Canine frozen semen with the supplementation of Maca is responsible for a surge in hyperactivation and WOB of sperm cells after one hour at 37°C. Movements of hyperactivation are considered part of the capacitation process and it is an event crucial for acrosome reaction and fertilization (De Lamirande et al., 1997). In the literature, there is a suggestion that that hyperactivated bovine sperms with high WOB are able to efficiently progress in a fluid similar to the actual cervical and oviductal mucus to arrive to the oocyte more quickly (Hyakutake et al., 2018). The improving of sperm movement and hyperactivation are required when spermatozoa are placed in the uterus during natural or artificial insemination. The use of an extender with Maca for canine semen cryopreservation, that dramatically increase the percentage of hyperactivated spermatozoa could be useful to improve fertilization rates. The association of Maca with sperm hyperactivation was already reported 3 hours after the addition of the same aqueous extract of Maca in cooled semen (Del Prete et al., 2022). Our previous study discussed as a possible justification for this association the presence of alkaloids in Maca (Del Prete et al., 2022). Indeed, alkaloids are responsible for the

increasing of cyclic adenosine monophosphate and intracellular calcium, that is an important factor in the regulation of sperm movement (Tash and Means, 1983; Funahashi, et al., 2001; Wang et al., 2009).
What emerges from this study is a protective role of Maca against lipid membrane peroxidation of canine spermatozoa, that is a primary marker of oxidative stress. Lipid peroxidation is considered as sub lethal cryodamage that could cause DNA fragmentation, increasing phosphatidylserine translocation index and intracellular hydrogen peroxide levels (Lucio et al., Kim et al., 2010). Membrane composition of dog spermatozoa with high polyunsaturated fatty acids increases the sensitivity to lipid peroxidation and reduce the resistance to cooling (Drobnis et al., 1993; Bencharif et al., 2008). Lipid peroxidation is caused by an increased production of ROS during the cryopreservative procedure (Lucio et al., 2016). Our results confirm what was already suspected in a previous study on the beneficial effect of Maca on plasma membrane (Del Prete et al., 2022). This effect could be explained by the antioxidant activity of phenolic compounds and specific alkamides contained in Maca (Korkmaz , 2018; Tafuri et al., 2019). The yellow ecotypes (hypocotyl colors) of Maca used in this study have been reported to have highest phenols than most of the others (Korkmaz, 2018). Phenols can act as effective inhibitor of peroxidation, by chelating redox-active metal ions and inhibiting free-radical mediated events (Rice-Evans, 2001). Maca-specific alkamides called 'Macamides' scavenge free radicals and thus protect sperm cells from oxidative damage (Tafuri et al., 2019).

6.5 Conclusions

In conclusion, supplementation of the frozen extender with 10 µl/mL of aqueous extract of Maca improves the cold shock resistance of spermatozoa, protecting sperm against lipid peroxidation during frozen-thawed process, and activates canine sperm motility and hyperactivation after thawing, improving the fertility.

6. 6 References

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