PhD THESIS

Collection, evaluation and conservation of equine extragonadal sperm reserves

(SUMMARY OF THE PhD THESIS)

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The sudden death or emergency castration of a valuable stallion meant in the past the definitive loss of its genetic characteristics. We now have the opportunity to harvest viable sperm from the post-mortem stallion and use the semen in order to obtain offspring and thus perpetuate superior genetic lines that would otherwise be lost. Moreover, the first gestation by artificial insemination in this species was obtained from epididymal semen in 1957.

The harvesting of epididymal semen after castration or sudden death is successfully practiced in many species of domestic and wild animals, thus contributing to the fight to protect endangered species. In horses, the importance also results from the economic impact that the loss of a valuable stallion can have by thwarting the genetic selection efforts sometimes extended over tens of generations. Also, the emotional value of horses and the inclusion of this species in the category of companion animals are additional arguments.

The functions of the epididymis are not fully elucidated in the stallion, but they can be deduced from several studies as well as from correlations with other species. The epididymis can be divided into three parts: the initial portion of the epididymal duct, two to three areas of maturation in the head and body of the epididymis, and a storage area in the tail of the epididymis. Fluids from the testicle are resorbed in the first part of the efferent ducts and the proximal part of the epididymal head is replaced by the secretion of the epididymal epithelium. These secretions differ along the epididymis. The sperm that leave the testicle are infertile, but those in the tail of the epididymis have the ability to fertilize. Sperm maturation is dependent on exposure to epididymal secretions, with different composition depending on the region. The presence of testosterone is necessary for the synthesis of epididymal proteins with a role in the maturation of sperm, especially in the head and body of the epididymis. Sperm maturation refers to the ability to fertilize, the acquisition of progressive mobility as well as some characteristics of membrane structure and metabolism. The mere stagnation of spermatozoa in different segments of the epididymis is not sufficient for maturation (Sostaric, 2008). The sperm in the head and body of the epididymis are immotile, while those in the tail of the epididymis are motile. The mobility characteristics of the sperm in the tail of the epididymis are similar to those of the sperm in the ejaculate. However, sperm in the tail of the epididymis are resistant to heat shock, unlike those in the ejaculate (Contri, 2012).This is most likely due to the exposure of the sperm to the secretion of the adnexal glands of the reproductive tract. This means that the sperm in the tail of the epididymis could be used for insemination. The storage of mature sperm is done at the level of the tail of the epididymis, but also at the level of the vas deferens and the ampoules of the vas deferens. In total, there are approximately 54 billion mature sperm in the tail of the epididymis of a mature stallion,

about 61% of the total number of sperm. The number of sperm in the epididymis is influenced by age (Bruemmer, 2006). The movement of sperm through the head and body of the epididymis takes place through the peristaltic movements of the smooth muscles. There are no peristaltic contractions in the tail of the epididymis, except when the muscles are stimulated to contract. Therefore, the time required for sperm to travel through the head and body of the epididymis is not influenced by ejaculation and lasts an average of 4.1 days. Therefore, fertility is not influenced by frequent ejaculations in terms of sperm maturation but is due to their concentration. The period of time spent by the sperm at the level of the tail of the epididymis is influenced by ejaculation and is maximal in stallions during sexual rest. Sperm removal is done regularly in the absence of ejaculation, around the urethra. In the case of some stallions, large amounts of sperm are stored in the tail of the epididymis, as well as in the efferent ducts and ampoules. In this case, sperm kept at this level for more than 7-10 days undergo significant changes with an impact on fertility (Sullivan, 2005).

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Mobility is one of the most important parameters for determining the viability of semen, but it does not directly correlate with the ability to fertilize (Amann, 1993). When leaving the seminiferous epithelium, the sperm are immobile and move through the peristaltic contractions of the seminiferous ducts. During the passage through the

epididymis, maturation processes take place, including obtaining mobility (Johnson, 1978).

In mammals, two physiological types of mobility are described (Turner, 2006). Active mobility is characterized by low-amplitude symmetrical flagellar movements and results in a straight forward motion. This is characteristic of sperm after ejaculation and is needed to advance them to the level of the oviduct. It is an important parameter of fertility because without it the sperm cannot reach the oviductal level naturally. Hyperactive mobility impresses the flagellum with large amplitude asymmetric movements in a liquid medium. This gives the sperm a circular or eight-way motion. Its role is to detach from the tubal epithelium, advance to the oocyte and penetrate the cumulus and pellucid area (Mc Kinnon et al. Colab., Pp. 1292).

Harvesting semen from the tail of the epididymis is the last source of genetic material in case of emergency castration or sudden death of a stallion with high genetic value. The first gestations of frozen semen were obtained in this species with semen from the epididymis (Barker, 1957). However, the success rate remains lower, and the ideal method of harvesting, processing and preservation is not fully elucidated. Up to 50 x 109 sperm are located at the tail of the epididymis and the vas deferens (Amann, 1979). Therefore, when the purpose of castration is to harvest semen at this level, it is necessary to harvest as much of the vas deferens and tail of the epididymis as possible and to obliterate both ends to avoid loss of semen. There are two methods of harvesting, one by retrograde washing (Bruemmer, 2006) and the other by the flotation method (Cary, 2004). In order to wash the vas deferens, the vas deferens is catheterized and instilled at this average level of freezing or buffered saline, balanced as the Tirode solution. It is injected into the vas deferens and the sample obtained is collected in a sterile beaker. Using this method eliminates the need to centrifuge the samples. The flotation technique refers to maintaining the tail of the epididymis and the vas deferens in a vessel pre-filled with dilution medium, stirring the vessel lightly for 10 minutes. The vas deferens and the tail of the epididymis are incised in 10-15 locations. The need to add seminal plasma at the end is controversial (Mc Kinnon, 1273).

Historically, semen has been subjectively evaluated under an optical microscope, which allows the identification of morphological features and the assessment of mobility - the percentage of motile sperm, progressive mobility, speed and longevity during storage. It also assesses the concentration, volume, presence of urine or blood, bacteria. More recently, the evaluation of stallion semen is done objectively, with specific equipment and trained staff (Morar et. Al., 2005; Morar et. Al., 2006).

However, classical tests of mobility and morphology remain the basis for the evaluation of semen. Semen can be evaluated and diluted as long as environmentally friendly thinners are used and environmental factors are considered to have an impact on mobility parameters - excessive heat or cold, eco-gel, light, antiseptics, osmolarity, pH - ul. Subjective assessment of mobility can be done by an experienced user, but objective methods are preferable. These include spectrophotometry, videomicrography, photomicrography, etc. By far, however, the most commonly used are computer systems (CASA), much more accessible and easy to use. However, they have the disadvantage that they cannot assess the fertilization capacity, which depends on many parameters. Morphology is evaluated under an optical microscope at 1000x magnification. The morphology as well as the characteristics of the germ and somatic cells can be evaluated on Wright, Giemsa or eosin stained smears. It is recommended to evaluate at least 100 sperm and note the defects encountered. They are classified as primary, secondary or tertiary. The primary ones are considered to be genetic defects and have a testicular source. The secondary ones originate in the excretory ducts and the tertiary ones are due to faulty handling and / or storage after ejaculation. Morphological defects correlate positively with sperm motility, both positively and negatively. Therefore, a small number of sperm with morphological defects together with low mobility are indicators of poor post-ejaculation manipulation (Morar et. Al., 2006). The semen can also be evaluated using some specific or non-specific biochemical markers.

In the context of an increase in the material and sentimental value of horses, technological developments and limited research on post-mortem semen harvesting, the aims of this paper were as follows:

- Evaluation of the effects of some factors on the concentration and kinematic parameters of the stallion epididymal spermatozoa:
  - total anesthesia and local anesthesia;
  - harvesting method;
  - post-harvest processing method: centrifugation and refrigeration;
  - external factors: age, seasonality, thinners for semen.

• Study of vas deferens and ampullae of vas deferens (post-mortem) in the stallion.

• Description of the technique of aspiration of sperm located at the level of the ampullae of the vas deferens intraoperatively (developed within the team).

• Reporting the characteristics of ampullary sperm compared to epididymal sperm.

The first part (I) is structured in two chapters. Chapter I.1 contains data on the anatomy, physiology, endocrinology and pathology of the stallion's genitals as well as an extensive study of the characteristics of stallion sperm. Chapter I.2 describes the methods of harvesting, evaluating and preserving the stallion semen.

The second part (II) consists of six chapters, the first of which includes working hypotheses and purpose, the second includes the presentation of materials and working methods, followed by two chapters focused on: harvesting, preservation and evaluation of semen from the epididymis of the stallion (Chapter II.5) and the collection, evaluation and preservation of semen from the ampullae of the vas deferens (Chapter II.6). At the end, we are presenting the general conclusions of the study, the recommendations, the elements of originality and the bibliography containing 350 titles.

Chapter II.5 had as main objectives the evaluation of the factors that influence the concentration and kinematic parameters of the stallion epididymal spermatozoa as follows:

- local and general anesthesia
- harvesting method
- centrifugation, refrigeration
- age, seasonality, dilution media.

In order to achieve the first objective, we formed two batches of stallions presented for orchidectomy, of which the first batch was anesthetized using a total intravenous protocol (TIVA n = 96) and the second batch was anesthetized using a partial intravenous protocol (PIVA n = 36). From the TIVA group, we extracted a number of 10 stallions to which we injected lidocaine intratesticularly before the orhidectomy. After castration, the testicles were transported in refrigerated conditions to the laboratory equipped for the extraction and evaluation of epididymal sperm.

Samples were evaluated for mobility parameters using a computerized CASA system (SCA® 103 Production, MICROPTIC). The following parameters were computerized for each test: Concentration, PM-progressive mobility; TM- total mobility; VCL- curvilinear speed; VSL - linear speed; VAP - average speed; LIN - linearity; STR - linear displacement.

The Tiva anesthetic protocol leads to significantly higher averages than the Piva anesthetic protocol for the variables Progressive mobility ($p = 0.001$), Total

mobility (p = 0.002), Curvilinear speed (p = 0.017), Linear speed (p = 0.007) and Average speed (p = 0.010). Regarding local anesthesia with lidocaine TM parameters, VCL and VAP did not show statistically significant differences while PM and LIN values recorded different values with statistical significance only when egg yolk diluent was used.

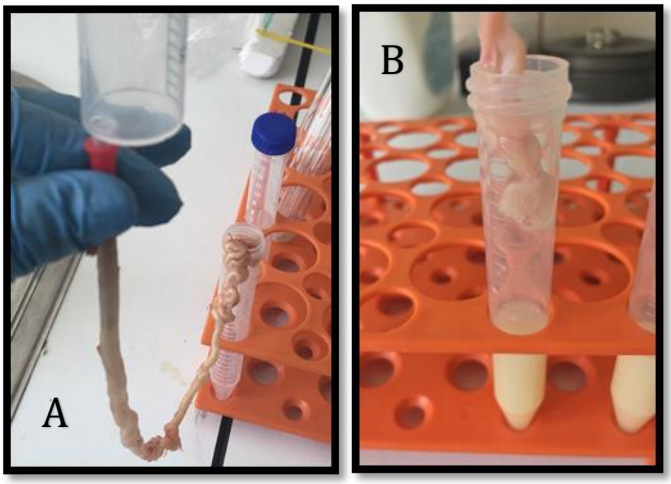

Fig. 1. A. The lumen of the vas deferens is catheterized with a blunt-tipped needle for retrograde lavage **B.** Appearance of the epididymal semen sample immediately after harvesting by retrograde lavage.

In order to achieve the second objective, we collected a number of 146 semen samples using either the retrograde air flush method (n=29), the retrograde flush with dilution medium (n = 88) or the flotation method (n = 17).

Significant differences (at a p threshold <0.01) occurred between the averages recorded for the variables Concentration, Volume, Progressive Mobility, Total Mobility, Curvilinear Speed, Linear Speed and Average Speed. In the case of the Concentration variable, the average is much lower than the averages for the other two protocols. For the variable, the average volume for the average Flush protocol is much lower than the average for the other two protocols. Regarding the variable Progressive Mobility, the average for the Flotation protocol is higher than the averages for the other two protocols. Averages of the variable Total mobility differs significantly, the average for Flotation being higher than the other two averages. For the variable Speed the average curvature for Flotation is much higher than the other two averages. The average of the Linear Speed variable for the Flotation protocol is higher. In the case of the variable, the average speed for flotation is higher than the other two averages.

To meet the third objective, we first performed two batches of centrifuged samples (n = 47) for 10 minutes at 600G and non-centrifuged samples (n = 95), respectively. We also used two different refrigeration protocols: the first refers to the refrigeration of the testicles for 24, 48 or 72 hours before the collection of epididymal sperm and the second to the refrigeration for 24, 48 or 72 hours of the epididymal samples collected, followed by evaluation of the concentration and kinematic parameters. For none of the variables are there significant differences (at the threshold p = 0.05) between the average of the centrifuged samples and the average of the non-centrifuged samples.

Significant differences (at a p threshold <0.01) occur between the averages recorded at different harvest times for the variables Progressive Mobility, Total Mobility, Curvilinear Speed, Linear Speed and Average Speed. In the case of all these variables, the mean at time 24 is higher than the other two averages and for each pair of moments the averages differ significantly between the two moments (p <0.001). That is, the averages differ significantly between 24 and 48, 24 and 72, and 48 and 72. For all dependent variables where the averages differ significantly between refrigeration intervals and between harvest times, for each refrigeration interval (24, 48, and 72) , the highest average occurs at harvest at 24 hours and the lowest average at harvest at 72 hours.

Also, significant differences (at a threshold p <0.01) occur between the averages recorded at different refrigeration periods for the variables Progressive Mobility, Total Mobility, Curvilinear Speed, Linear Speed and Average Speed. For all these variables, the mean at time 24 is higher than the other two averages.

In order to meet the fourth objective, we formed two lots for each parameter evaluated: the effect of age - stallions under 5 years old (n = 84) and stallions over 5 years old (n = 58), the effect of seasonality - October - February (n = 73) - off-season and March-September (n = 42) - season, extender effect: Gent, Minitube (n = 73) and Equi Plus (n = 69). Between the ages of 0-5 years and 5+ years, the averages differ significantly only for the variable Concentration (p <0.001). Between the two seasons, the averages differ significantly only for the variables Total mobility (p = 0.005) and Linearity (p = 0.041) (Fig. 40). For none of the variables are there significant differences (at the threshold p = 0.05) between the mean of the samples for which the Gent diluent was used and the mean of the samples for which the Equi diluent was used.

The conclusions of this study are summarized in Table 1.

Table 1

General results – dependent variables whose mean significantly differ between them depending on the values of the independent variables

Dependent variable	Independent variable							
	Refrigeration	Anesthesia	Harvest protocol	Centrifugation	Extender	Age	Season	Harvest moment
Concentration (M/ml)			X			X		
Volume (mL)			X					
Progressive motility (%)	X	X	X					X
Total motility (%)	X	X	X				X	X
Curvilinear speed (mm/s)	X	X	X					X
Linear speed (mm/s)	X	X	X					X
Mean speed (mm/s)	X	X	X					X
Linearity (%)							X	
Straightness (%)								

Observation. For each independent variable, X was denoted when the averages of the dependent variable differ significantly depending on the values of the independent variable.

Chapter II.6 had as main objectives the following:

• measuring the deferential channels and the ampullae of the deferential channels on parts from deceased stallions;

• description of a surgical technique for harvesting the ampullary seminal material during the orhidectomy operation;

• evaluation of semen from ampullae and vas deferens;

• comparative evaluation of the ampullary seminal material with the epididymal one.

In order to fulfill the objectives of this study, we performed a first stage on parts from stallion carcasses on which we measured the vas deferens, the ampullae of the vas deferens and we performed the catheterization of the ampullae in order to aspirate the ampullary contents. In the second stage, we collected ampullary semen from 10 stallions during the orhidectomy surgery and we compared the kinematic parameters taking as a control group the epididymal samples from the same stallion.

The mean length of the vas deferens measured from the emergence of the tail of the epididymis and including the ampulla of the vas deferens was 69.9 ± 1.4 cm. The length and width of the vas deferens ampullae measured on the outside were on average 17.6 ± 1.8 cm and 3.9 ± 0.3 cm, respectively. The mean length and width of the ampullary lumen were 16.5 ± 1.7 cm and 3.4 ± 1.3 mm, respectively.

Fourteen of the 20 differential ampoules were successfully aspirated during elective orhidectomy. In two of the stallions, the aspirated content was urine, a fact demonstrated by the macroscopic appearance but also due to the creatinine concentration (Crea = 20.7 mg / dl). At one of the stallions, 3 ml of contents from both ampoules were aspirated. The harvested substance was represented by a dense mass of non-viable sperm, cellular debris, epithelial cells and the diagnosis of ampullary stasis was established.

The mean concentration of ampullary sperm (AS) per stallion was 752.8 ± 370.2 x 106 (353 x 106 to 1338.4 x 106) and the mean number of epididymal sperm (ES) was 12695.2 ± 5609 x 106 (from 5342 x 106 to 21245 x 106). There were no statistically significant differences between the parameters PM, VCL, VSL, VAP, LIN and STR between the two groups AS and ES ($p \leq 0.05$). TM was significantly reduced in the AS group ($p = 0.04$).

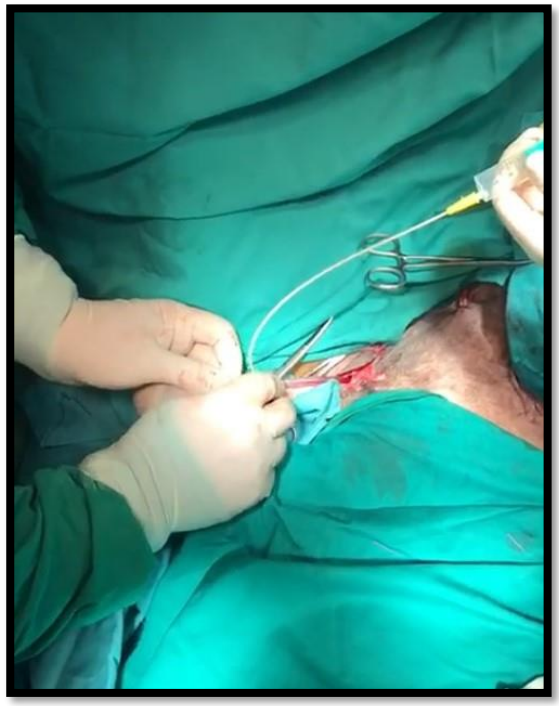

Fig. 2. Collection of semen from the ampulla of the vas deferens during orihyectectomy. The vas deferens is catheterized retrograde to the ampoule from which the stored semen is aspirated.

Chapter II.7 presents the conclusions and general recommendations of the study:

1. General anesthesia used during castration has a negative impact on the quality of epididymal stallion semen;

2. Local anesthesia with lidocaine does not have a negative impact on epididymal semen;

3. The best technique for harvesting semen from the tail of the epididymis is represented by retrograde air insufflation;

4. Centrifugation does not improve mobility parameters, as in the case of semen from ejaculate;

5. Harvesting semen in the first 24 hours maintains the quality of epididymal sperm;

6. Refrigeration is a viable technique for preserving epididymal semen;

7. The age of the stallion influences the concentration of epididymal semen but not mobility parameters;

8. Harvest season influences mobility parameters but not concentration;

9. Diluents for milk-based semen are similar to those for egg yolk in terms of mobility parameters;

10. The collection of semen from the ampoules of the vas deferens increases by about 6% the total volume of sperm harvested;

11. The mobility of ampullary sperm is similar to that of epididymal sperm;

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