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Ampullar and Ductus Deferens Aspiration Increases the Total Number of Viable Spermatozoa Recovered from Stallion Extragonadal Sperm Reserves

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ABSTRACT

Background: Equine spermatozoa acquire maturation and fertilization capacity during their passage from the testis towards the cauda epididymis and are concurrently stored in the cauda epididymis and ampullae of the ductus deferens. In case of sudden death or emergency castration, epididymal spermatozoa can be harvested and successfully preserved. Ampullar aspiration was not previously described, even though the ampullae of the ductus deferens and the ductus deferens are valuable sources of mature spermatozoa in equines. The aim of this study was to describe a technique to collect spermatozoa from the ampullae of the ductus deferens and the ductus deferens of stallions during routine castration and to compare kinematic parameters of these spermatozoa to spermatozoa harvested from the cauda epididymis of the same stallion.

Methods: Fourteen ampullae of the ductus deferens were successfully aspirated during the routine castration of 10 stallions, followed by epididymal sperm harvest. Concentration and motility parameters were assessed, computer-assisted and were compared to epididymal spermatozoa.

Result: Ampullar and ductus deferens spermatozoa increased the total number of spermatozoa recovered by approximately 6%. Progressive motility (PM), velocity of the average path (VAP), velocity of the curved line (VCL) and linearity (LIN) did not differ significantly ($P \geq 0.05$) between the ampullar spermatozoa (AS) and the epididymal spermatozoa (ES). Total motility (TM) was significantly lower ($P=0.04$) in the AS group.

Key words: Ampullae, Ductus deferens, Epididymal, Spermatozoa, Stallion.

INTRODUCTION

Equine spermatozoa acquire maturation and fertilization capacity during their passage from the testis towards the cauda epididymis and are concurrently stored in the cauda epididymis and ampullae of the ductus deferens. Catastrophic injury or sudden death may prematurely terminate a stallion's reproductive life. However, epididymal sperm reserves have been harvested successfully as the last source for gamete rescue, according to the intense research of several authors." Several authors have intensely researched the subject. Preservation of equine epididymal spermatozoa is possible both cool stored (Monteiro *et al.*, 2013) and long term cryopreservation (Olaciregui *et al.*, 2014; Papa *et al.*, 2008) and viable pregnancies can be obtained (Guasti *et al.*, 2017; Monteiro *et al.*, 2011). Epididymal spermatozoa can be retrieved from several domestic and wild species, such as the goat (Turri *et al.*, 2013), dog (Luvoni and Morselli, 2017), human (Van Peperstraten *et al.*, 2006), donkey (Gloria *et al.*, 2011), red deer, roe deer and chamois (Martinez-Pastor *et al.*, 2005), cat (de Sousa Barbosa *et al.*, 2019), boar (Chang *et al.*, 2016), sheep (Abella *et al.*, 2015), alpaca (Mamani-Mango *et al.*, 2018), mouflon and fallow deer (Boveda *et al.*, 2018).

The ampullae represent a dilation of the ductus deferens, that plays an important role in both the maturation and storage of spermatozoa (Parillo and Verini Suplizi, 2008).

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Nonetheless, they have a secretory role and an enzymatic activity, secreting glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase and alkaline phosphatase (Baumber and Ballunger, 2005; Turner and McDonnell, 2003). Several species have ampullae of the ductus deferens, including equines, bovines, several reptiles, the human, rabbit and camels. The lumen of the ampulla of the ductus deferens is dilated by variable amounts of spermatozoa in stallions at rest (Pozor and McDonald, 2002), to a diameter up to 4 mm, measurable by ultrasonography (Schnobrich *et al.*, 2016). In contrast, in geldings, the lumen's diameter is less than 1 mm and is usually impossible to be measured. Before ejaculation, ampullae are filled with liquid, dilate several times and empty during ejaculation (Weber and Woods, 1993). The concentration of spermatozoa stored in the ampullae of the ductus deferens is similar to that found in a normal ejaculate (Gebauer *et al.*, 1974). Antegrade catheterization of ampullae near the cauda epididymis was previously described for ampullar spermiostasis treatment that was refractory to conservative treatment (Mc Kinnon *et al.*, 2011). To the authors' best knowledge, aspiration of ampullar spermatozoa has not been previously described.

Extra gonadal sperm reserves are reported to be between 60×10^9 and 89×10^9 spermatozoa in the stallion, the ampullae of the ductus deferens storing approximately 7% of these reserves (Amann *et al.*, 1979). Therefore, we hypothesized that the ductus deferens and ampullae might be a valuable source of viable spermatozoa in cases of emergency castration or sudden death of stallions, while ampullar spermatozoa might be the sole source in case of testicular compromise.

Kinematic parameters assessed by computer-assisted sperm analysis (CASA) is a widely used, efficient and cost-effective method to determine fertility in males of different species: stallion, human (Romero *et al.,* 2018) buffalo (Singh *et al.,* 2017), bull (Rai *et al.,* 2018), ram (Rajashri *et al.*, 2018).

MATERIALS AND METHODS

Castration and ampullar aspiration

Ten client-owned, 5 to 9 years, old mixed breed stallions have been routinely castrated using a closed technique with the stallions in dorsal recumbency. Castrations were performed at the Teaching Equine Hospital of the Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Cluj, Romania, between 2017 and 2021. After a thorough examination of the testicles to detect any gross modification, each stallion was sedated using xylazine hydrochloride (1 mg/kg IV) and butorphanol tartrate (0.03 mg/kg IV). Anesthesia was induced using ketamine hydrochloride (2 mg/kg IV) and diazepam (0.05 mg/kg IV). Following aseptic preparation of the scrotal area, 2 parallel incisions were made 1 cm on each side of the median raphe. The testicle was forced from the bottom and the incision was carried on through the dartos and scrotal fascia without incising the vaginal tunic. The ductus deferens was palpated through the vaginal tunic and a 1-2 cm incision was performed, using a scalpel. Each ductus deferens was isolated using the shanks of a hemostat (Fig 1). An approximately 5 mm incision was performed along the ductus deferens reaching the lumen, allowing the passage of a gastroscope flushing tube (Equivet) until the ampulla was reached (Fig 2). The tube was pre-cut at a 70 cm length and advanced towards the ampulla until resistance was encountered. A 20 ml syringe was attached at the outer end of the tube and aspiration was slowly performed along the ampullae and ductus deferens while retracting the tube. Aspiration was ceased 1 cm from the incision line, in order to avoid blood contamination. The obtained sample was mixed in 5 ml extender for chilled semen containing egg yolk (Gent, Minitüb; pH 6.6-6.8, 310-330 mOsm/L) by flushing the sampling tube and was then transferred to the laboratory. A hemostatic forceps was placed at the level of the ductus deferens in order to prevent leakage and contamination of spermatozoa. No post-surgical complications were reported, associated with the ampullar aspiration.

Following ampullar aspiration, each testicle was removed using a transfix ligature (PGA USP 2, SMI, Surgicryl) and a Reimer's emasculator placed approximately 2 cm above the ligature. Each testicle was individually packed in a sterile bag, identified and transported to the in-house laboratory with the aim to recover epididymal spermatozoa. After castration, the stallions were medicated with tetanus toxoid (6000 UI IM), flunixin meglumine (1.1 mg/kg IV) and a combination of procaine penicillin and streptomycin sulfate (procaine penicillin 4.000 UI/kg and streptomycin sulfate 15 mg/kg IM).

Epididymal sperm collection and sperm analysis

Each cauda epididymis and vas deferens were carefully removed from the testicles after placing a mosquito forceps at the palpable base of the cauda epididymis and dissected free of blood vessels and connective tissue, using an aseptic technique. Spermatozoa were recovered using a retrograde flush technique as previously described (Eichelberger *et al.*, 2007).

From each sample, including the ampullar aspiration samples, an aliquot was extended to 20×10^6 sperm/ml in an extender for chilled semen containing egg yolk (Gent, Minitüb; pH 6.6-6.8, 310-330 mOsm/L). Each sample was assessed after 30 minutes of maintenance at room temperature for concentration and motility parameters, using a computer-assisted sperm analysis system (SCA® Production, MICROPTIC). Total motility (TM), progressive motility (PM), velocity of the average path (VAP), velocity of the curved line (VCL) and linearity (LIN) were recorded for each sample. Sperm motility was assessed with Sperm Class Analyzer -SCA (Micro optic, Barcelona, Spain) using the following settings: 10x Nikon, negative phase contrast (PC-) optics, calibrate value 0.82 µm/pixel, gird distance: 10 µm, box size: 200 pixels, VCL/VAP area 4 μm^2/min, area: 75 μm^2/min, static cells threshold <10 µm/s, slow medium 45 µm/s, rapid >90 µm/s, progressive STR >75, VAP points

5 pixels, connectivity 12 pixels. A total of 500 spermatozoa in minimum of four fields were assessed on warmed 20 µm Leja slides.

Statistical analysis

The arithmetic mean and its standard deviation were calculated for each assessed parameter for ampullar (AS) and epididymal (ES) spermatozoa. After checking by the Shapiro-Wilk test, the data with normal distribution was analyzed by the ANOVA test (repeated measurements variables), while those without were analyzed by the Kruskal-Wallis test. Differences were considered statistically significant if the P value was ≤0.05. The obtained data were processed with MedCalc® Statistical Software version 19.7.1 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2021).

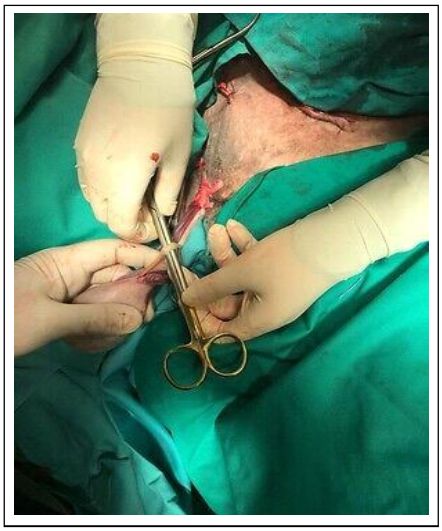

Fig 1: Ductus deferens, isolated on the shanks of a hemostat after the incision of the tunica vaginalis.

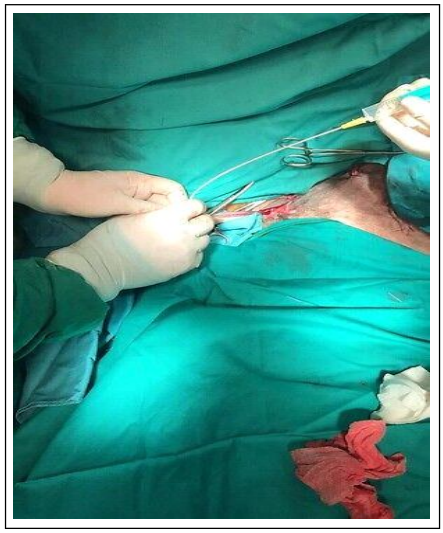

Fig 2: Aspiration tube, introduced in the lumen of the ductus deferens through a small incision, advanced toward the caudal part of the ampulla of the ductus deferens.

RESULTS AND DISCUSSION

Ampullar and ductus deferens content was successfully aspirated in 14 out of the 20 ampullae. In two stallions the content aspirated was urine, as demonstrated by the gross aspect and the creatinine concentration (Crea 207 mg/dL). In one stallion we successfully aspirated 3 ml content from both sides. The content was represented by a dense mass of mostly nonviable, decapitated spermatozoa warranting the clogged ampullae diagnosis. In the remaining samples, the mean number of ampullar spermatozoa (AS) harvested per stallion was 752.89±370.28×10^6 (from 353×10^6 to 1338.43×10^6), whereas the mean number of epididymal spermatozoa (ES) was 12695.28±5609.02×10^6 (from 5342 ×10^6 to 21245×10^6). Table 1 shows the mean values and standard deviation of kinematic parameters of both groups.

Collection of viable epididymal spermatozoa from a deceased or injured stallion is possible and widely used (Bruemmer, 2006). The first pregnancy using frozen semen was obtained from epididymal spermatozoa (Barker and Gandier, 1957). Epididymal spermatozoa can be successfully retrieved after 48 hours and viable pregananices can be obtained (Stawicki *et al.*, 2016). Motility parameters of epididymal spermatozoa are similar between routinely castrated healthy stallions and those that died because of acute illness (Gloria *et al.*, 2016).

In humans, irrigation of the ampullae and ductus deferens with or without spermicides is routinely performed during vasectomies, in order to obtain early azoospermia (Oliveira *et al.*, 2018). Catheterization and lavage were used in stallions as well, in order to treat ampullar spermiostasis refractory to conservative treatment (McKinnon *et al.*, 2011). However, aspiration of stallion ampullae was not previously described. In the current study, the aspiration of spermatozoa from the ampullae of the ductus deferens and along the ductus deferens increased the total number of viable spermatozoa by an average of 5.93%. This is important in case of sudden death or emergency castration

Table 1: Motility parameters for ampullar spermatozoa (AS) compared with testicular spermatozoa (TS).

	PM %	TM %	VCL mm/s	VSL mm/s	VAP mm/s	LIN %	STR %
TS n=14	25.23±19.06	92.97±6.84	54.15±21.99	16.66±8.49	28.80±13.10	29.68±5.87	53.92±4.64
AS n=14	25.46±18.67	76.85±22.09	42.25±23.18	13.76±6.85	22.79±12	33.94±7.27	57.81±6.64
p value	P=0.96	P=0.043	P=0.10	P=0.31	P=0.15	P=0.14	P=0.14

PM: Progressive motility; TM: Total motility; VCL: Curve speed; VSL: Linear speed; VAP: Average value; LIN: Linearity index; STR: Straightness index. Values are expressed in mean±standard deviation.

of a valuable stallion, in order to maximize the quantity of genetic material that can be retrieved and conserved, as well as in the case of testicular compromise. Epididymal spermatozoa can be succesfully cryopreserved even after 96 hours intra epididymal storage (Vieira *et al.,* 2013) and ampullar spermatozoa have similar characteristics as we have shown in this study.

According to data presented in Table 1, there were no significant differences of PM, VCL, VSL, VAP, LIN or STR between the epididymal and ampullar sperm. However, TM was significantly lower in the AS group. Ampullar spermatozoa were extended in an egg yolk-based semen extender (Gent, MiniTube) and maintained at room temperature for 2 hours before kinematic analysis. Egg yolk-based extenders may negatively impact motility in equine epididymal spermatozoa (Neuhauser *et al*., 2018) and probably caused the decrease of TM in ampullar and ductus deferens spermatozoa. Epididymal spermatozoa were mixed with the same extender, but maintained for a shorter time, approximately 30 minutes.

In two stallions the aspirated content aspirated was urine, we, therefore, hypothesized that the flushing tube passed the colliculus seminalis and entered the urethra.

Equine sperm prediction remains challenging and even though latest studies suggest the use of more than one technique in assessing fertility (Battut *et al*., 2017), progressive motility is still an important parameter, used to determine the minimum standard requirements for semen for artificial insemination (http://www.wbfsh.org/files Semen%20 standards.pdf). In the current study, progressive motility of ampullar spermatozoa was similar to epididymal spermatozoa. Despite these findings, there are still limitations with regard to the ampullar aspiration of deceased stallions, where ampullar spermatozoa might suffer more degeneration. However, ampullar and ductus deferens aspiration can successfully be used for both sperm retrieval and therapeutically.

CONCLUSION

The technique described is easy to perform and safe for the stallion, increasing the number of viable spermatozoa harvested during castration. This is important in case of sudden death or emergency castration of a valuable stallion, but also in that of sudden death when the retrieved extragonadal sperm reserves represents the last source of genetic material.

Conflict of interest: None.

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