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EFFECT OF THREE COLLECTION METHODS ON THE CONCENTRATION AND KINEMATIC PARAMETERS OF STALLION EPIDIDYMIS SPERMATOZOA

EFECTUL A TREI METODE DE COLECTARE ASUPRA CONCENTRAȚIEI ȘI PARAMETRILOR CINEMATICI AI SPERMATOZOIZILOR DE EPIDIDIM DE ARMĂSAR

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ABSTRACT | REZUMAT

The semen stored in the tail of the epididymis is the last source of genetic material in case of sudden death or emergency castration of a valuable stallion. The aim of this study was to evaluate the efficiency of three harvesting protocols for epididymis stallion semen described in the literature from the point of view of concentration and kinematic parameters. In this study, 134 epididymis semen samples were processed with one of the three methods: retrograde air or extender lavage, flotation. Kinematic parameters and concentration were assessed using an electronic system CASA (Computer-Assisted Sperm Analysis, SCA® 103 Production, MICROPTIC). The results of this study revealed statistically significant differences between the three collection methods for the parameters concentration, volume, progressive motility, total motility, and velocity. The best method for harvesting stallion epididymis semen was the retrograde air lavage technique.

Keywords: stallion, epididymis semen, post-mortem, orchiectomy

Materialul seminal depozitat la nivelul cozii epididimului reprezintă ultima sursă de material genetic în caz de moarte subită sau castrare de urgență a unui armăsar valoros. Scopul acestui studiu a fost de a evalua eficiența celor trei metode de recoltare a materialului seminal epididimal de armăsar descrise în literatura de specialitate, din punctul de vedere al concentrației și parametrilor kinematici. În cadrul acestui studiu au fost procesate un număr de 134 de probe de material seminal epididimal cu una dintre cele trei metode : lavaj retrograd cu aer, diluant sau flotație. Parametri kinematici și concentrația au fost evaluate utilizând un sistem electronic CASA (Computer-Assisted Sperm Analysis, SCA® 103 Production, MICROPTIC). Rezultatele studiului au relevat diferențe semnificative statistic între cele trei metode de recoltare pentru parametri concentrație, volum,mobilitate progresivă,totală,velocitate.Cea mai bună metodă de recoltare a materialului seminal epididimal de armăsar a fost metoda lavajului retrograd cu aer.

Cuvinte cheie: armăsar, material seminal epididimal, post-mortem, castrare

Semen from the tail of the epididymis is the last source of genetic material in the event of emergency castration or the sudden death of a valuable stallion. The first gestations from frozen semen were obtained in this species with semen from the epididymis (2). However, the success rate remains lower, and the ideal method of harvesting, processing, and preservation is not fully elucidated. At the level of the tail of the epididymis and the vas deferens, there are up to 50 x 10.9 sperm (1). Therefore, when the purpose of castration is to collect semen from this level, it is necessary to collect as much as possible from the vas deferens and tail of the epididymis and to obliterate both ends to avoid loss of seminal material. There are two methods of harvesting: one by retrograde flush (Bruemmer, 2006) and the second by flotation method (4).

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For retrograde, flush the vas deferens is catheterized and instilled with extenders or buffered, balanced saline solutions such as Triode solution. Catheterization is performed into the vas deferens and the sample obtained is collected in a sterile recipient. Using this method eliminates the need to centrifuge samples. Retrograde flushing can be achieved solely by insufflation of air. The flotation technique refers to suspending the tail of the epididymis and vas deferens in a vessel prefilled with dilution medium for 10 minutes and shaking the vessel gently. The vas deferens and tail of the epididymis are incised in 10-15 locations. The need to add seminal plasma at the end is controversial (5).

The purpose of this study was to compare the concentration and kinematic parameters of epididymis semen using three different collection methods.

MATERIALS AND METHODS

Biological material

In this study, we included 142 epididymis semen

samples from stallions presented for elective orchiectomy. The batch of horses was heterogeneous, aged 2 to 12 years, of different breeds, light (± 500 kg) or heavy (± 800 kg).

Anaesthetic and surgical protocol

The orchiectomy was performed with the testicle covered. Anaesthesia involved a premedication step with xylazine 1.1 mg/kg (Xylazin Bio 2%, Bioveta) and butorphanol tartrate 0.01 mg/kg (Butomidor 10 mg/ml, Richter Pharma) administered intravenously as a bolus. Induction was performed with ketamine 2.2 mg/kg (Ketamidor 100 mg/ml, Richter Pharma) and diazepam 0.05 mg/kg (Diazepam 10 mg, Therapy), and maintenance was performed with an additional bolus of ketamine 0.2 - 0.5 mg/kg if needed.

After induction, the patient was directed to dorsal recumbence by manipulation of the head and tail, assisted by two people. Antisepsis of the groin area and penis was performed using chlorhexidine soap and lukewarm water, followed by dressing with betadine solution, and finally medical alcohol. The orchiectomy was performed by incision of the scrotum, one cm lateral to the scrotal median raffia, on either side. The incision was advanced all the way to the dartos, avoiding the incision of the vaginal tunic. The testicles were exposed by ventral pressure and dilacerations with fingers or swabs, followed by the application of a transfixed suture with resorbable, braided polyfilamentary suture (PGA Surgycril, SMI) 3/4 thick. Crushing of the testicular cord was achieved by applying a Reimers forceps approximately 2 cm distal to the transfixed ligature and crushing for about 10 minutes, followed by detachment of the testicle.

The detached testicle was ligated at the level of the vas deferens to avoid leakage of semen and packed in an individual sterile bag for identification. Each testicle was immediately stored in the carrying bag on ice bags. The testicles were kept at a constant temperature of about 5 degrees C all throughout the transport.

Recovery from anaesthesia was permitted after surpassing of the threshold of superficial anaesthesia, clinically expressed by cessation of nystagmus, with the horse assisted from the head and tail. There were no complications associated with anaesthesia or recovery from anaesthesia using this protocol.

Postoperatively, anti-infectious prophylaxis was performed by intramuscular injection of a combination of procaine - penicillin and dihydrostreptomycin at a dose of 8 respectively 10 mg/kg (Pen-Strep, Norbrook) as well as anti-tetanus gamma globulin with 6000 U.I immunoserum tetanicum equinum nativum / per animal, subcutaneously (Clostetan, Bioveta).

Analgesic and anti-inflammatory therapy was performed with flunixin meglumine 1.1 mg/kg intravenously (Niglumine 50 mg/ml, Dopharma). Analgesic therapy was continued by daily administration of phenylbutazone oral powder at a dose of 2.2 mg/kg/day for 5 days (Butagran Equi 200 mg/g, Dopharma) together with anti-bacterial therapy at the aforementioned dose and route of administration. The therapy was complemented by three-time-daily walks to rapid allures followed by local cold showers.

Retrograde flush with air or the semen extender method

The harvesting of semen was carried out in two stages. The first stage is the preparation of the tail of the epididymis for harvesting. First, the incision of the vaginal tunic was performed to easily visualize the epididymis. A haemostatic forceps was placed at the base of the tail of the epididymis, and the epididymis ligament and the body of the epididymis was incised under the haemostatic forceps, thus freeing the epididymis' tail. The tail of the epididymis has a sinuous trajectory, and the diameter increases from the body of the epididymis towards the vas deferens. To make retrograde lavage possible – from the vas deferens – careful preparation of the tail of the epididymis is necessary. For this, the connective tissue and blood vessels were gently dilacerated using Metzenbaum scissors. This led to the expansion and relaxation of the tail of the epididymis, making the lavage possible.

In the second stage, after preparation, the portion of the tail of the epididymis with a larger diameter was preserved, and the rest was detached by incision with scissors, above the collection tube. After detachment, an 18G gauge needle with a blunt tip was inserted into the vas deferens, to which a pre-filled syringe with a known volume of semen extender (Ghent, Minitube) was attached. The vas deferens was firmly squeezed to the needle, and the diluent was injected through the lumen of the vas deferens until it was completely emptied into the centrifuge tube. The procedure was completed with the injection of 10 ml of air, and the final volume was noted. When harvesting was carried out without an extender, 20-40 ml of air were injected using the technique described above with constant pressure to avoid rupture of the epididymal wall.

Flotation method

For this method, the epididymis prepared according to the technique described above - dilaceration and removal of blood vessels was placed in a sterile container - plastic petri dish and incised 10-15 times using a 12-scalpel blade. Extender (Ghent) preheated to physiological temperature – 37 ° C was added and kept in contact for 30 min., constantly homogenizing. This allows active sperm extraction. After 15 minutes of incubation at 37°C, the sample was filtered through a nylon filter, and the evaluation was carried out identically, regardless of the extraction method used.

Table 1

Motility parameters of stallion epididymal spermatozoa depending on harvesting technique

	CONC	VOL mL	PM %	TM %	VCL	VSL	VAP mm/s	LIN %	STR
FLOTATION									
nr	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00	17.00
min	61.82	3.50	7.85	38.56	35.90	9.97	17.92	22.67	47.98
max	375.00	15.00	71.52	100.00	105.10	30.93	55.51	44.01	66.62
mean	222.90	6.50	26.15	90.99	58.29	17.61	30.57	29.26	54.14
Standard	98.83	4.90	18.75	15.2	19.57	6.00	10.34	5.90	5.51
FLUSH AIR									
nr	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00
min	123.86	0.10	0.00	8.81	18.55	5.88	11.05	26.43	48.92
max	8451.4	10.00	71	100	101.75	39.95	62.81	46.80	69.11
mean	1707.35	6.63	12.71	64.37	37.21	12.59	20.75	32.84	55.70
Standard	2024.16	3.61	16.85	29.88	18.24	7.08	10.87	6.25	5.39
FLUSH									
nr	95.00	95.00	95.00	95.00	95.00	95.00	95.00	95.00	95.00
min	20.80	0.01	0.00	4.86	16.10	4.37	7.29	9.33	37.39
max	7687.83	10.00	64.74	100.00	103.13	26.18	49.55	54.33	70.11
mean	1333.80	2.13	10.05	56.43	37.70	11.16	19.61	29.88	53.44
Standard	1621.62	2.46	13.77	29.66	18.89	5.10	9.29	7.31	7.02

CONC - concentration (M/ml), VOL - volume (mL), PM - progressive mobility (%), TM - total mobility (%), VCL - curvilinear velocity (mm/s), VSL - linear velocity (mm/s), VAP - medium speed (emm/s), LIN – linearity (%), STR - linearity (%)

Table 2

Comparison of mean variables between three different harvest techniques using ANOVA method

Variable	Harvest protocol	*N*	*m*	*σ*	The F test	
					F(2, 131)	*p*
Concentration (M/ml)	Flotation	17	222.90	98.83	4.604	**0.012**
	Air Flush	29	1706.21	2059.98		
	Extender Flush	88	1293.29	1605.85		
Volume (mL)	Flotation	17	6.50	4.90	29.690	**<0.001**
	Air Flush	29	6.51	3.61		
	Extender Flush	88	2.14	2.46		
Progressive mobility (%)	Flotation	17	26.15	18.75	8.013	**0.001**
	Air Flush	29	12.98	17.08		
	Extender Flush	88	9.97	13.85		
Total Mobility (%)	Flotation	17	90.99	15.22	10.799	**<0.001**
	Air Flush	29	64.70	30.35		
	Extender Flush	88	56.32	29.45		
Curvilinear speed (mm/s)	Flotation	17	58.29	19.57	8.694	**<0.001**
	Air Flush	29	37.38	18.54		
	Extender Flush	88	37.70	19.18		
Linear speed (mm/s)	Flotation	17	17.61	6.00	9.286	**<0.001**
	Air Flush	29	12.70	7.17		
	Extender Flush	88	11.08	5.17		
Mean speed (mm/s)	Flotation	17	30.57	10.34	8.623	**<0.001**
	Air Flush	29	20.92	11.02		
	Extender Flush	88	19.63	9.52		
Linearity (%)	Flotation	17	29.26	5.90	2.369	0.098
	Air Flush	29	32.90	6.36		
	Extender Flush	88	29.80	7.45		
Trajectory (%)	Flotation	17	54.14	5.51	1.528	0.221
	Air Flush	29	55.62	5.47		
	Extender Flush	88	53.21	6.94		

Assessment of concentration and mobility parameters

Epididymis samples were evaluated for mobility parameters using a computerized CASA system (SCA® 103 Production, MICROPTIC). For each sample, the following parameters were recorded: PM- progressive mobility; TM- total mobility; VCL- curvilinear velocity; VSL – linear speed; VAP – average speed; LIN – linearity; STR – linear displacement.

Concentration and mobility were analysed with Sperm Class Analyzer SCA software (Microoptic, Barcelona, Spain) using the following settings: 10x Nikon, negative contrast (PC-), calibration value 0.82 µm/pixel, grid size: 10 µm, box size: 200 pixels, VLC/VAP determination area 4 µm2/min: 75 µm2/max, static cell determination threshold <10 µm/s, mean for slow speed 45 µm/s, fast >90 µm/s, straight line advancement STR >75, VAP point 5 pixels, connectivity 12 pixels. A total of 500 sperm were evaluated for each sample in a minimum of 4 fields using constantly heated 20 µm Leja blades. The results were saved as analysis reports.

Statistical processing of results

To compare the averages of dependent variables between the three harvesting protocols, the ANOVA method was used. For all variables where significant differences between media were identified, the means between two harvesting protocols were compared. The Hochberg test was used for the variables Progressive Mobility, Curvilinear Velocity, Linear Speed and Average Speed (for which dispersions were equal), and the Games- Howell test for the other variables (for which dispersions were uneven). The data obtained was processed with MedCalc Statistical Software version 19.7.1 (MedCalc® Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2021).

RESULTS AND DISCUSSIONS

Table 1 shows the maximum and minimum values, mean ± standard deviation of the three batches. Table 2 presents the comparative results of the kinematic parameter averages according to the harvesting protocol using the ANOVA method.

Significant differences (at a $p<0.01$ threshold) appear between the averages recorded for the three harvesting protocols in the case of the variables Concentration,Volume, Progressive Mobility, Total Mobility, Curvilinear Velocity, Linear Velocity, and Average Velocity. In the case of the variable Concentration, the means differ significantly between the Flotation and Air Flush protocols ($p=0.002$) and between Flotation and Medium Flush ($p<0.001$). The average for Flotation is much lower than the averages for the other two protocols (Fig. 1).

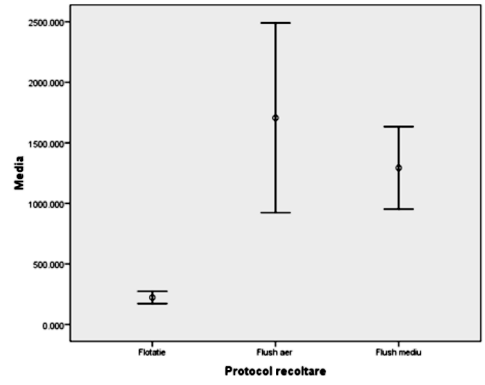

Fig. 1. Trust interval for the average of the variable Concentration (M / ml), corresponding to the probability P = 0.95, depending on the values of the variable Harvest protocol

In the case of the Average Volume variable for the Flush protocol the average is much lower than the averages for the other two protocols. Means differ significantly between Flotation and Medium Flush protocols ($p=0.006$) and between Air Flush and Medium Flush ($p<0.001$) (Fig. 2).

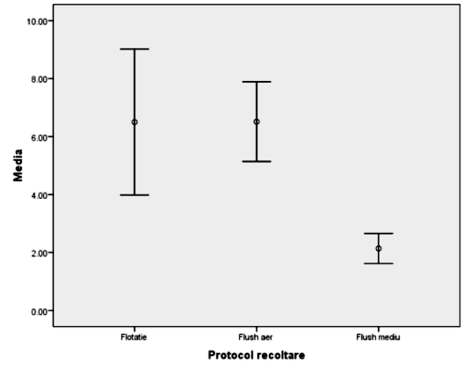

Fig. 2. Trust interval for the average of the variable Volume (mL), corresponding to the probability P = 0.95, depending on the values of the variable Harvest protocol

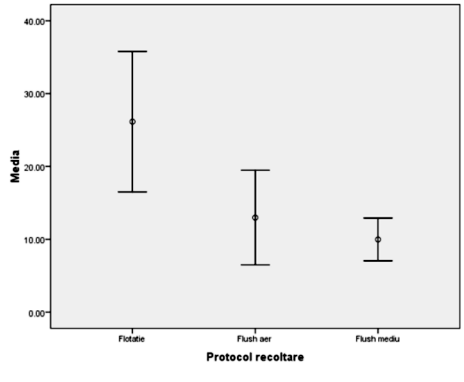

Fig. 3. Trust interval for the average of the variable Progressive motility (%), corresponding to the probability P = 0.95, depending on the values of the variable Harvest protocol

Regarding the Progressive Mobility variable, the average for the Flotation protocol is higher than the averages for the other two protocols. The differences in means are significant between Flotation and Air Flush protocols (p=0.016) and between Flotation and Medium Flush (p<0.001) (Fig. 3).

The means of the Total Mobility variable differ significantly between the Flotation and Air Flush protocols (p=0.001) and between Flotation and Medium Flush (p<0.001), the mean for Flotation being higher than the other two means (Fig. 4).

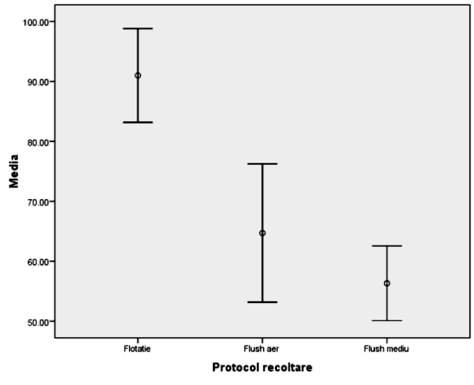

Fig. 4. Trust interval for the average of the variable Total mobility (%), corresponding to the probability P =0.95, depending on the values of the variable Harvest protocol

For the Curvilinear Velocity variable, the means differ significantly between the Flotation and Air Flush protocols (p=0.001) and between Flotation and Medium Flush (p<0.001). The average for Flotation is much higher than the other two averages (Fig. 5).

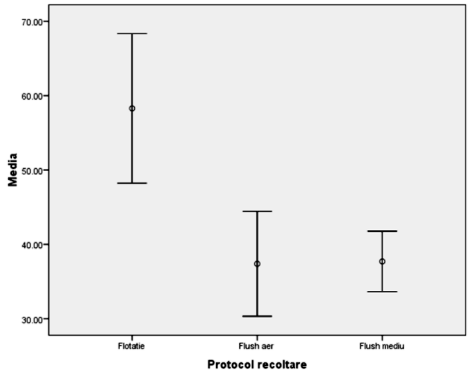

Fig. 5. Trust interval for the average of the variable Curve speed (mm/s), corresponding to the probability P = 0.95, depending on the values of the variable Harvest protocol

The mean of the Linear Velocity variable for the Flotation protocol differs significantly from the mean for the Air Flush protocol (p=0.018) and that for the Medium Flush protocol (p<0.001), being higher than them. In the case of the Average Speed variable, the means differ significantly between the Flotation and Air Flush protocols (p=0.006) and between Flotation and Medium Flush (p<0.001). The average for Flotation is higher than the other two averages.

CONCLUSIONS

The results of this study show that the best harvesting method is retrograde air flushing or air insufflation. The volume of semen obtained is higher in the case of air insufflation compared to the other two methods. The concentration of semen is much lower when using the flotation technique. Total mobility and progressive mobility are superior using the flotation technique, as this is an active extraction technique, but due to the very low concentration, this improvement in mobility parameters becomes irrelevant.

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