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EFFECTS OF LIDOCAINE ON EQUINE EPIDIDYMAL SPERMATOZOA, AFTER 24 HOURS INTRAEPIDIDYMAL EXPOSURE, USING TWO COMMERCIAL EXTENDERS

EFECTELE LIDOCAINEI ASUPRA SPERMATOZOIZILOR EPIDIDIMALI ECVINI, DUPĂ 24 DE ORE DE EXPUNERE INTRAEPIDIDIMALĂ LA LIDOCAINĂ, UTILIZÂND DOI DILUANȚI COMERCIALI

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ABSTRACT | REZUMAT

Epididymal spermatozoa is the last source for gamete rescue in case of emergency castration or sudden death of a valuable stallion, thus an ideal harvesting and preservation technique should be employed. Routinely, 2% lidocaine intratesticular administration was used to provide analgesia prior to castration, but studies on the effect of lidocaine on epididymal spermatozoa motility parameters are limited. The purpose of this study was to determine the effects of lidocaine on equine epididymal spermatozoa, after 24 hours intraepididymal cool storage using two commercial extenders. We hypothesized that intraepididymal prolonged exposure to lidocaine, might affect motility parameters of epididymal stallion spermatozoa and different extenders might have an impact. Sperm was collected from 20 epididymides of routinely castrated 3-year-old KWPN stallions. Testicles were transported to an equipped facility and cooled stored for 24 hours. From each sample an aliquot was diluted in a commercial egg yolk-based extender, and another in a commercial extender containing defined milk proteins. Motility parameters were registered 30 minutes after dilution, computer assisted. There were no statistical differences between motility parameters of spermatozoa exposed to lidocaine and spermatozoa not exposed, however progressive motility and linearity significantly differed among the two extenders.

Keywords: lidocaine, equine epididymal spermatozoa, motility

Spermatozoizii epididimali reprezintă ultima sursă de material genetic în caz de castrare de urgență sau moarte subită a unui armăsar valoros, deci este important să se utilizeze cea mai bună tehnică de recoltare și conservare. Administrarea intraparenchimatoasă de lidocaină 2% este utilizată de rutină la castrarea armăsarilor pentru a asigura analgezia locală, însă studiile asupra influenței acesteia asupra parametrilor de motilitate ai spermatozoizilor sunt extrem de limitate. Scopul acestui studiu a fost evaluarea parametrilor de motilitatea a spermatozoizilor de armăsar expuși la lidocaină, după 24 de ore de conservare prin refrigerare, intratesticular. Am pornit de la ipoteza că expunerea prelungită a spermatozoizilor la lidocaină poate afecta parametri de motilitate și diluanți diferiți pot avea un impact semnificativ. Materialul seminal a fost recoltat din 20 de epididime după castrarea a 10 armăsari KWP N în vârstă de 10 ani. Testicolele au fost transportate la un laborator echipat pentru prelevare de la nivelul epididimului și stocate la temperatură de refrigerare timp de 24 de ore. Din fiecare probă o mostră a fost diluată într-un diluant pentru material seminal pe bază de gălbenuș de ou iar o altă mostră într-un diluant pe bază de lapte. Parametrii de motilitate au fost evaluați după 30 de inute utilizând un sistem computerizat. Nu au existat diferențe statistic semnificative între parametri de motilitate ai spermatozoizilor tratați cu lidocaină versus cei netratați cu excepția motilității progresive și a linearității.

Cuvinte cheie: lidocaină, spermatozoizi epididimali, motilitate

Regional analgesia by means of intratesticular 2% lidocaine administration during surgical procedures such as orchidectomy has proven to be effective in reducing pain during routine castration (7,16) or laparoscopic cryptorchidectomy in horses (8).

Lidocaine is frequently used for stallion castration due to its relative low cost, reduced analgesia onset time and because castration is a short surgical procedure, requiring only a short duration of anaesthesia and analgesia. Lidocaine acts by directly binding to the neuronal voltage-gated sodium channels (NVGS) thus inhibiting them. The presence of NVGS in human testes and spermatozoa is well known (5,14). This mechanism of action refers to both local and general intravenous administration. Administration of lidocaine subcutaneously on the incision line, intratesticularly and/or funicularly significantly reduces pain during castration, regardless of the anaesthesia protocol (7,

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14). In human medicine, lidocaine is the anaesthetic of choice during testicular biopsy, testicular sperm extraction, and testicular sperm aspiration which are routine techniques used to obtain spermatozoa for intra-cytoplasmic sperm injection in patients suffering from azoospermia (9,15). However, intravenous administration of lidocaine during stallion castration does not seem to have an analgesic effect (16).

There are few studies looking at the effect of local anaesthetics on sperm motility parameters. In humans, results are conflicting. In one in vivo study, lidocaine induced hyperactivation of sperm cells (4) whereas *in vitro*, there seemed to be no negative effect at therapeutic concentrations (2). In stallions, administration of 10 mL intraparenchymal 2% lidocaine did not decrease total motility (TM), progressive motility (PM), velocity of the average path (VAP), velocity of the curved line (VCL), linearity (LIN), normal morphology (M), and membrane integrity (MI) of the spermatozoa *in vivo*, even though relevant concentrations of lidocaine can be detected in epididymal flush, regardless of the blood barrier (3). This is important in case of emergency castration of valuable stallions, when cauda epididymis spermatozoa are the last source of genetic material.

Even though viability of spermatozoa significantly decreases after 72 hours, they can be successfully cryopreserved and maintain their fertilization capacity *in vitro*, after 96 hours of intra-epididymal storage at 4 °C (6). Choosing the best extender for each stallion is important, due to the extremely limited quantity, and extenders can influence the motility parameters of refrigerated epididymal spermatozoa (17). To the author's best knowledge, there is no other study on the effect of different commercial semen extenders on epididymal spermatozoa at 24 hours after intratesticular injection of lidocaine.

The purpose of this study was 1. to determine the effects of lidocaine on the motility parameters of equine epididymal spermatozoa after 24 hours intra-epididymal cool storage and 2. to compare the effect of two commercial extenders on the motility parameters of equine epididymal spermatozoa.

Because lidocaine does cross the blood barrier into the epididymis of the stallion, as showed by Boye (2019), and facilities equipped with the means to extract equine epididymal spermatozoa might be at a considerable distance, we conducted this study increasing to 24 hours the time of exposure of spermatozoa to lidocaine, which is generally the minimum amount of time required to ship the testicles.

We hypothesized that intra-epididymal prolonged exposure to lidocaine, might affect kinematic parameters of epididymal stallion spermatozoa and that different extenders might have an impact.

MATERIALS AND METHODS

Animals and castration method

Ten 3-year-old KWPN client owned stallions were routinely castrated using a closed technique with the stallions in dorsal recumbency. After a thorough examination of the testicles to detect any gross modifications, each stallion was sedated using xylazine hydrochloride (1.1 mg/kg, IV) and butorphanol tartrate (0.03 mg/kg, IV). Anaesthesia was induced using ketamine hydrochloride (2.2 mg/kg, IV) and diazepam (0.07 mg/kg, IV). Following aseptic preparation of the scrotal area 10 mL 2% lidocaine hydrochloride (Lido Bel, Bela-Pharm, Germany) was randomly administered to 4 stallions in the parenchyma of the testes using an 18 g 1.5-inch needle. Each testicle was removed using a transfixic ligature (PGA USP 2, SMI, Surgicryl) and a Reimer's emasculator placed approximately 2 cm above the transfixic ligature. Immediately after the removal of the testicle, a haemostatic forceps was placed over the vas deferens in order to prevent leakage and contamination of spermatozoa. Following removal, each testicle was individually packed in a sterile bag, identified and stored at 5 °C for transportation, avoiding direct contact of the epididymis with the ice packs. 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 sulphate (procaine penicillin 4.000 UI/kg and streptomycin sulphate 15 mg/kg IM).

Sperm collection and analysis

After 24 hours intra-epididymal cooled storage at 5°C, 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 was recovered using a retrograde flush technique as previously described (14).

From each stallion, an aliquot was extended to 20 x 10^6 sperm/ml in an extender for chilled semen containing defined milk proteins (EquiPlus, Minitüb, Tiefenbach, Germany; pH 6.8 ± 0.2, 320 ± 20 mOsm/L), and another aliquot was diluted to 20 x 10^6 sperm/mL in an extender for chilled semen containing egg yolk (Gent, Minitüb; pH 6.6-6.8, 310-330 mOsm/L), randomly. Each sample was assessed after 30 minutes maintenance at room temperature for 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

Table 1

Motility parameters for Equi Plus group

	TM %	PM %	VCL mm/s	VAP mm/s	LIN %
EP 1	99.86	30.52	64.06	35.59	36.68
EP 2	99.74	44.06	74.10	39.67	32.52
EP 3	98.59	13.47	50.43	22.11	29.52
EP 4	97.78	19.70	52.16	23.91	22.67
EP 5	97.67	11.20	42.96	22.07	30.93
EP 6	73.56	16.09	43.20	26.43	41.81
AV	**94.53 ± 9.41**	**22.51 ± 11.45**	**54.49 ± 11.24**	**28.30 ± 6.85**	**32.36 ± 5.94**
EPL 1	82.03	16.09	46.81	25.39	29.86
EPL 2	72.36	16.91	49.50	27.23	27.82
EPL 3	99.31	34.52	63.49	37.70	39.84
EPL 4	97.71	12.61	45.61	24.34	29.34
AV	**87.85 ± 11.20**	**20.03 ± 8.51**	**51.35 ± 7.14**	**28.67 ± 5.31**	**31.72 ± 4.75**

EP: Equi Plus without lidocaine, EPL: Equi Plus with lidocaine, AV: Average results
Motility parameters TM: total motility, PM: progressive motility,
VCL: Velocity of the average curve, VAP: Velocity of the average path, LIN: Linearity

(Microoptic, Barcelona, Spain) using the following settings: 10x Nikon, negative phase contrast (PC-) optics, calibrate value 0.82µm /pixel, gird distance: 10 µm, box size: 200pixels, VCL/VAP area 4 μm^2/min, area:75 μm^2/max, 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 four fields were assessed using 20 µm heated Leja slides.

Statistical method

An unpaired t-test was used to compare kinematic parameters of lidocaine exposed and non-exposed samples using two different semen extenders (Graph Pad Prism 6.0). Significance was assessed at $p < 0.05$.

RESULTS AND DISCUSSION

Effect of lidocaine on motility parameters, using Equi Plus semen extender

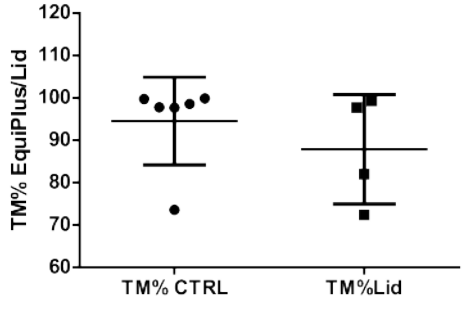

Fig. 1A. Total motility for the samples diluted in Equi Plus extender. Lidocaine did not significantly affect TM

A number of ten pellets were suspended in Equi Plus Semen extender and distributed in one of the two groups: Equi Plus without lidocaine (EP) (n=6) and Equi Plus with lidocaine (EPL) (n=4). Table 1 provides detailed information about the motility parameters analysed in both EP and EPL groups and the average results ± standard deviation. There were no statistical differences between the two groups (Fig. 1 A-E).

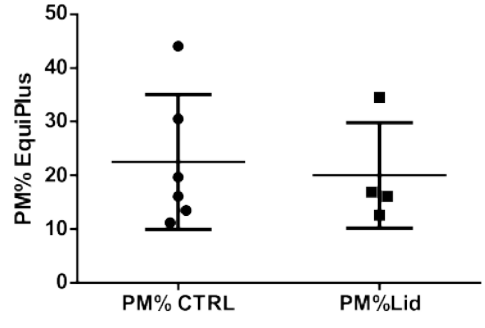

Fig. 1B. Progressive motility for the samples diluted in Equi Plus extender. Lidocaine didn't significantly affect PM

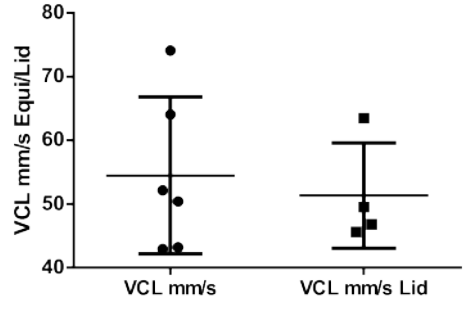

Fig. 1C. Velocity of the average curve for the samples diluted in Equi Plus extender. Lidocaine did not significantly affect VCL

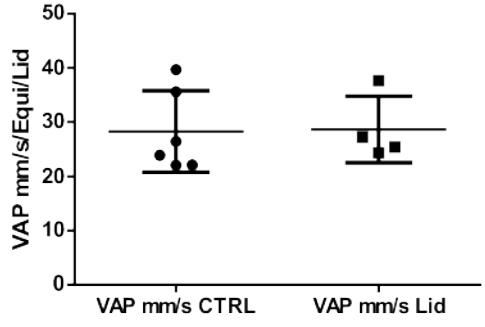

Fig. 1D Velocity of the average path for the samples diluted in Equi Plus extender. Lidocaine did not significantly affect VAP

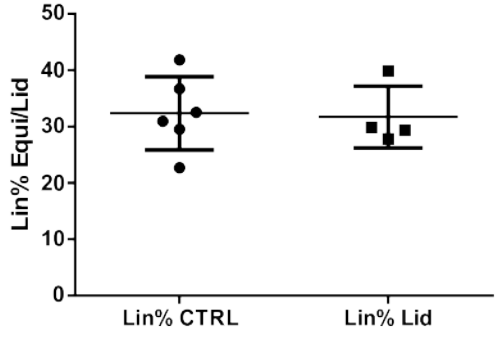

Fig. 1E Linearity for the samples diluted in Equi Plus extender. Lidocaine did not significantly affect Lin

Effect of lidocaine on motility parameters, using Gent semen extender

The remaining samples, extracted from the epididymides of the ipsilateral testicle of the same stallion were suspended in Gent extender and distributed in one of the two groups: Gent without lidocaine (Ge) (n=6) and Gent with lidocaine (GeL) (n=4). Table 2 provides de-tailed information about the motility parameters ana-lysed in both Ge and GeL groups and the average re-sults ± standard deviation. TM, VCL and VAP showed no statistically significant difference among the two groups (Fig. 2 A-C) whereas PM and LIN were significantly different among the two groups (Fig. 2 D, E).

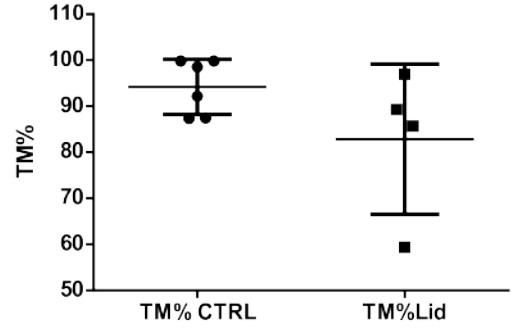

Fig. 2A. Total motility for the samples diluted in GENT extender. Lidocaine did not significantly affect TM

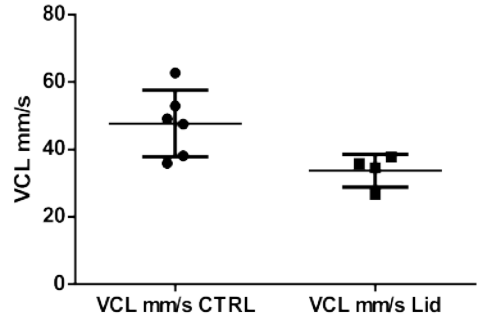

Fig. 2B. Velocity of the average curve for the samples diluted with Gent extender. Lidocaine did not significantly affect VCL

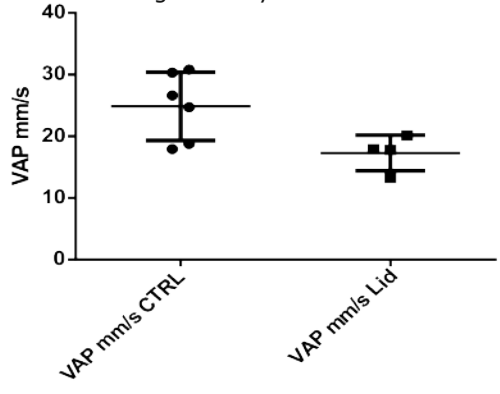

Fig. 2C. Velocity of the average path for the samples diluted in Equi Plus extender. Lidocaine did not significantly affect VAP

Table 2

Motility parameters for Gent group

	TM %	PM %	VCL mm/s	VAP mm/s	LIN %
Ge 1	98.54	22.13	52.93	26.60	26.79
Ge 2	99.83	25.36	62.73	30.82	24.63
Ge 3	87.42	7.98	35.90	17.92	25.74
Ge 4	87.35	19.09	49.11	24.71	26.29
Ge 5	99.82	13.82	47.51	30.31	44.01
Ge 6	92.16	8.34	38.11	18.75	26.09
AV	**94.19 ± 5.46**	**16.12 ± 6.61**	**47.72 ± 9**	**24.85 ± 5.06**	**28.93 ± 6.77**
GeL 1	85.65	6.61	34.57	17.92	28.00
GeL 2	59.39	15.16	26.68	13.26	26.76
GeL 3	89.29	9.45	35.78	17.80	27.62
GeL 4	96.99	51.81	37.75	20.12	29.90
AV	**82.83 ± 14.1**	**20.76 ± 18.19**	**33.70 ± 4.20**	**17.28 ± 2.49**	**28.07 ± 1.14**

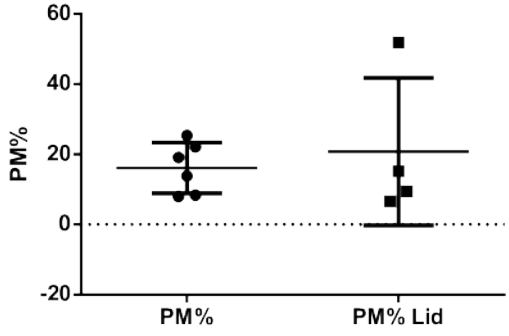

Fig. 2D Progressive motility for the samples diluted in Gent extender. Lidocaine significantly affected PM

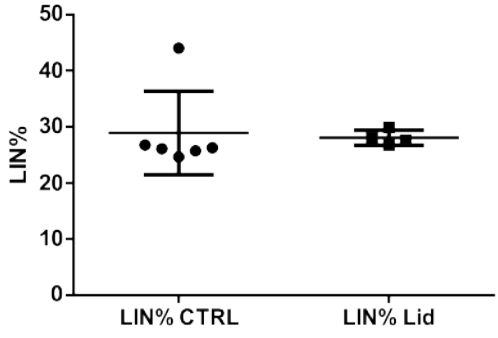

Fig. 2E. Linearity for the samples diluted in Gent extender. Lidocaine significantly affected Lin

The present study evaluated motion characteristics of chilled epididymal sperm, after 24 hours intra-epididymal exposure to lidocaine, using two commercial extenders. Testing different semen extenders is not feasible with epididymal sperm, due to quantity limitations. Applying a general protocol that works best for a significant number of stallions might be the better option. In the current study, the extender for chilled semen containing defined milk proteins produced better results for parameters PM and LIN, compared with the egg yolk extender, in epididymal spermatozoa exposed to lidocaine for 24 hours. These results are similar to other studies, where egg-yolk extenders were proved less effective for chilled storage of epididymal spermatozoa (12, 13) regardless of the lidocaine exposure.

In order to be able to preserve genetic material from deceased or emergency castrated stallions, a method has been described where intra-epididymal storage can keep spermatozoa viable for up to 96 hours post castration when epididymides are cooled stored, but motility and most importantly progressive motility decreases time-dependent (11. In the current study, generally, motility parameters were decreased in all four groups, probably due to the prolonged intra-epididymal cooled storage and because of the stallions young age, but lidocaine did not have a negative impact. In comparison to the Boye et. al. study (2019) where spermatozoa was harvested right after castration, we cooled stored the testicles 24 hours prior to collection, in order to assess the effect of lidocaine on stallion spermatozoa after longer exposure. This is important because facilities equipped for the procedure are commonly situated at a considerable distance.

Lidocaine is routinely used for local analgesia during castration, due to its proven positive effects. Similar to another study (3), lidocaine did not seem to affect motility parameters of epididymal spermatozoa even after 24 hours intra-epididymal exposure. The concerns about lidocaine local analgesia were based on the lack of studies, even though results published on human sperm motility showed no negative impact of lidocaine on human spermatozoa, at different concentrations, *in vitro* (2). Furthermore, studies on male humans and stallions prove that lidocaine does cross epididymal barrier after being administered in the testicular parenchyma (2, 14). Interestingly, in the Boye 2019 study, high concentrations of lidocaine did affect the progressive motility and linearity of spermatozoa. However, this only happened at doses exceedingly more than 100 times the dose used routinely for this procedure.

In the current study, progressive motility and linearity were lower for the samples diluted in Gent chilled extender, regardless of the exposure to lidocaine. Prediction of the fertility of equine sperm prior to cryoconservation remains challenging, and even though latest studies suggest the use of more than one technique in assessing fertility (1), progressive motility is still an important parameter, used to determine the minimum standard requirements for semen for artificial insemination (20).

CONCLUSIONS

The administration of 10 mL 2% lidocaine intra-parenchymatous during routine castration, did not negatively affect motility of epididymal spermatozoa after 24 hours intra-epididymal cooled storage, when milk protein chilled semen extender was used. However, when egg-yolk chilled semen extender was used, progressive motility and linearity significantly decreased.

Based on these findings, the injection of lidocaine during routine castration can be used even when epididymal sperm is to be retrieved and milk-based extenders seem to improve kinematic parameters of epididymal spermatozoa.

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Institutional Review Board Statement:

Ethical review and approval were waived for this study, due to the fact that animals were castrated in a clinical setting, at the request of the animal's owner, using generally approved anaesthesia and surgical protocols. Animals were not part of an experimental group.

Conflicts of Interest:

The authors declare no conflict of interest.

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