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Quality assessment of murine spermatozoa obtained by percutaneous epididymal sperm aspiration (PESA)

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submitted by
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1. Introduction

Sperm collection in mice is an important technique in laboratory animal science and it is commonly applied in assisted reproduction. Spermatozoa are collected to cryopreserve specific mouse strains (Nakagata 2000; Wilson and Sheardown 2010), which is a fast, safe and cost-effective way to store genetically engineered mouse colonies for an indefinite amount of time. However, the prime field of use is the generation of new genetic strains via *in vitro* fertilization (IVF). IVF is furthermore an important routine practice to overcome fertility problems, for rederivation of a mutant strain and to accelerate the expansion of a line (Taft 2017; Nagy 2003). In order to determine the heritability of a genetic mutation through the germline into the next generation, sperm can be genotyped by PCR (Wilson and Sheardown 2010).

The standard procedure for sperm collection in mice is the surgical extraction of the *cauda epididymis* (SECE). This procedure can be done fairly quickly and results in a high number of mature spermatozoa (Nakagata 2000; Nagy 2003). However, the drawback of this method is, that it is conducted post mortem and therefore the mouse has to be euthanized. Alternatively, a one-sided excision of the epididymis under anesthesia can be attempted, although this procedure can only be performed twice in each mouse. And yet for a number of experiments a sperm collection method is needed, which allows repeated sperm collections from a single male. This can be due to a specific study design or to preserve a valuable individual, for instance a male with a specific genotype. Besides practical issues, there are also ethical concerns that demand refining sperm collection techniques and to pursuing different non-terminal sperm retrieval procedures, which allows keeping animals alive and furthermore preserve their fertility. Commercializing an *in vivo* sperm collection procedure would contribute to animal reduction and improve refinement in animal experimenting, as postulated by Russell and Burch (Russell and Burch 1959).

There are a number of different non-terminal sperm retrieval procedures in mice described in literature: electroejaculation, injection of drugs and flushing a uterus of previously mated females. However, none of the procedures above have arrived in routine practice, since the outcome is not proportional to the performed effort or is simply not adequate.

There have been multiple studies about electroejaculation in mice reporting lower fertilization rates, lower motility and lower sperm number in comparison with epididymal sperm, which was also found to be true for other species like horses (Cary et al 2004). Further drawbacks include a relatively high mortality rate and the requirement of specialized equipment (Scott and Dziuk 1959; Snyder 1966; Anderson et al. 1983; Tecirlioglu et al. 2002). Main concerns about the drug injection method are the inconsistency regarding success rate and possible negative side effects (Loewe 1937; Loewe 1938). The third method relies on the copulation of a male sperm donor with a female, which has to be euthanized afterwards for harvesting the ejaculation sample. This requires a relatively high animal number and a male with a functional sex drive, causing a problem in some strains. Further studies have demonstrated lower developmental potentials of embryos, presumably due to increased nuclease activity in capacitated sperm, which represents the majority of an ejaculation retrieved from the uterus (Yamauchi and Ward 2007).

A silver lining in the area of the non-terminal sperm collection techniques seems to be the microsurgical epididymal sperm aspiration (MESA) and the percutaneous epididymal sperm aspiration (PESA). These minimal invasive procedures were originally developed in human medicine to overcome infertility problems in cases of obstructive and non-obstructive azoospermia (Collins et al. 1995; Craft et al. 1995; Meniru et al. 1998; Glina et al. 2003; Bromage et al. 2008; Esteves et al. 2011; Jensen et al. 2016; Hao et al. 2017).

For MESA a small scrotal incision is made to puncture the epididymis under eyesight, whereas in PESA the epididymis is punctured through the scrotum. Thus, PESA is a refined and less invasive procedure, but with similar IVF fertilization rates and cryopreservation success (Boersma et al. 2015). Besides the numerous studies that have been conducted in humans, there is also literature that states the efficiency of PESA as an acceptable sperm retrieval technique in other mammals such as dogs (Varesi et al. 2013).

It has been shown that *in vivo* serial sampling of mice via PESA and MESA is possible and that the obtained sperm can be successfully used for IVF. In addition, these studies further revealed that the experimental procedures do not impair male fecundity (Del Val and Robledano 2013; Boersma et al. 2015), there is however no knowledge on possible long-term effects concerning repeated sperm collections in mice. Zhang et al. (2014) and Saade et al. (2008) reported that repeated PESA caused significant epididymal inflammatory changes as

well as a reduced sperm concentration and motility in rat studies, although the number of conducted PESA's did not correlate with the severity of inflammation or reduced motility. Nevertheless, the sperm quality of these samples have never been examined objectively via computer assisted sperm analysis (CASA).

Here we aimed to test whether PESA can be applied as a reliable method to assess sperm quality in mice. For this purpose, we compared sperm quality traits after collection with the PESA method versus the standard sperm retrieval method, i.e., surgical epididymis extraction (SECE). We hypothesized that there are no significant differences in sperm quality parameters between the two sperm retrieval techniques mentioned above.

The obtained sperm samples were analyzed by CASA and various sperm parameters, like sperm motility, swimming velocity and morphology were assessed. If PESA would provide reliable estimate of male sperm quality, this procedure can find broad applicability in various research areas.

2. Materials and Methods

2.1. Animals and Husbandry

For the purpose of the present study, we used 10 male outbred SWISS mice. All animals belonged to the institute's breeding colony and were 15 weeks of age when used for the experiment. Founders were purchased from Janvier Labs, France. Two weeks before the experiment started breeding activity was stopped (animals were sorted out from the breeder's list).

Mice were housed individually in an open top Makrolon type II cage (252 x 167 mm, Tecniplast, Buguggiate, Italy) equipped with soiled bedding (Lignocel®, heat treated, Rettenmaier KG, Vienna, Austria) and enriched with cellulose swabs (Pur-Zellin 4x5cm; Paul Hartmann GmbH, Wiener Neudorf, Austria) as nesting material. As mice were kept singly, the establishment of dominance relationships between males was avoided. Previous studies in mice have shown that males can differ in their sperm quality depending on their social status (Koyama & Kamimura 1999). All mice were kept in a barrier-free facility with a light/dark photoperiod of 12h (lights on at 3:00am). The room temperature was maintained at 20°C ± 1°C and relative humidity was constantly held at 55% ± 10%. Water and Food (ssniff® M-Z Extrudate, Spezialdiäten GmbH, Soest, Germany) were provided *ad libitum*.

2.2. Study Design

The aim of this study was to test whether percutaneous epididymal sperm aspiration (PESA) is a suitable method to reliably collect and assess sperm quality in mice. Conventional methods to collect and assess sperm quality apply a surgical extraction of the *caudae epididymides* (SECE). Here, we collected and assessed individual sperm samples applying both PESA and SECE for comparison.

We applied a within subject design and collected four samples from each male. First, we collected sperm by performing PESA from both, the right and the left epididymis. To rule out any side bias, the side of first collection was randomized. Immediately afterwards, we performed SECE, again collecting separate samples from each side and randomizing the side of first collection. All obtained samples were transferred into a specifically prepared medium

(TYH) and incubated at 37°C under 5% CO_2 to ensure optimal conditions for the specimen for the following sperm analyses.

2.3. Sample collection

Experimental mice were euthanized by cervical dislocation immediately before the sample collection started. Each mouse was then placed in dorsal recumbency and the abdomen was gently massaged downward to ensure that the testes were in the scrotum. The abdomen and scrotum were then rinsed with distilled water to improve visualization of the cauda epididymis and to avoid contamination with loose hair.
The experiment was carried out on two consecutive days and we tested five animals per day. Individual body mass was recorded from all mice one day prior to sample collection.

2.3.1. Percutaneous epididymal sperm aspiration (PESA)

The *caudae epididymes* were individually located at the base of the scrotum by visual inspection. The *cauda epididymis* normally appears to be right next to the scrotal raphe on the medial apical side of the scrotum. It presents itself as a pinhead-sized circular light yellow-greyish tissue with a visible curly structure, which has to be distinguished from the similar-looking circumjacent fatty tissue. Once located, the *cauda epididymis* was fixed using curved forceps (Figure 1 B). Therefore, the scrotal skin between testis and epididymis was carefully squeezed by the forceps to carefully separate the epididymal tissue from the testis and to restrain it. We then used a 30-gauge (0,3 mm x 8 mm) needle on an insulin syringe (Omnican 20, B Braun, Meslungen, Germany), containing 0,05 ml of pre-warmed TYH culture medium to puncture the epididymis (Figure 1 C). A gentle resistance was perceived once the epididymal tissue was punctured. Negative suction pressure was applied before the needle was retrieved. The content of the syringe was then transferred onto a culture dish and examined for the presence of sperm under a stereomicroscope (Figure 1 D). The procedure may involve multiple punctures at different sites in the epididymal tissue to locate sperm.

Two successful sperm samples were then transferred into a 35-mm culture dish containing a drop of 100 µl TYH covered with oil and mixed together before incubated at 37°C under 5% CO_2 for 30 minutes until sperm analysis was performed. The contralateral *cauda epididymis*

was subsequently sampled in exactly the same manner. The number of performed punctures and the incubation times were recorded.

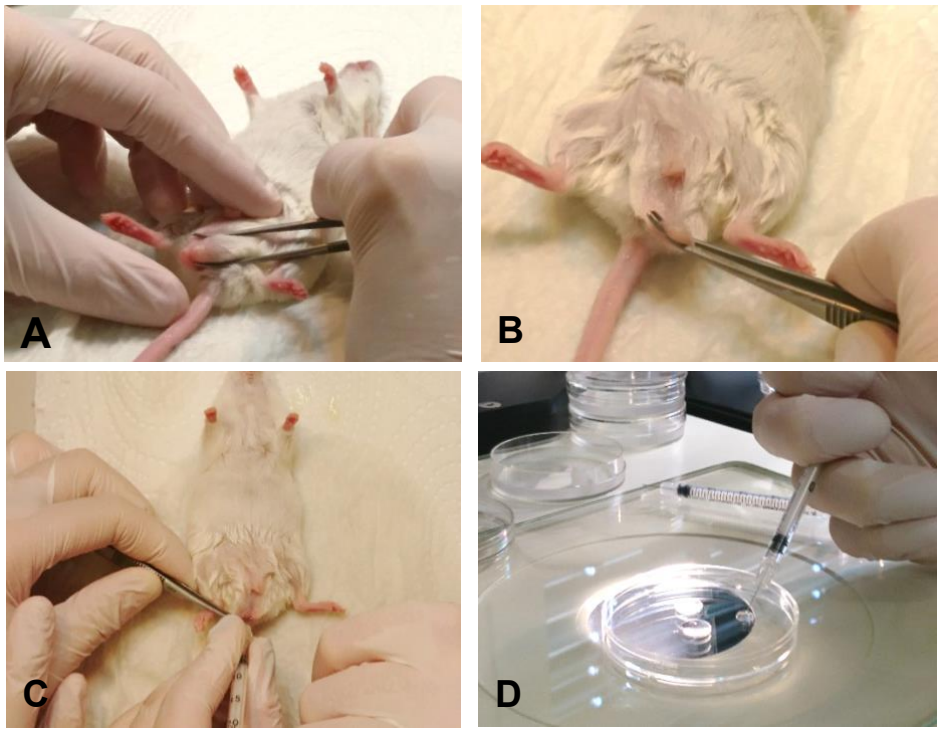

Figure 1. PESA (Percutaneous epidydimal sperm aspiration). (A) The abdomen is gently massaged downward to ensure that both testes are located in the scrotum. (B) The left *cauda epididymis* is identified and fixated with curved forceps by applying gentle pressure. (C) Sperm aspiration is performed by puncturing the *cauda epididymis* with an insulin syringe. (D) Immediately after retrieval the samples were examined for motile sperm under a stereomicroscope.

2.3.2. Surgical extraction of cauda epididymis (SECE)

After performing a bilateral PESA, we dissected each mouse and extracted both *caudae epididymides*. Hence, we had to gain access to the abdominal cavity and isolate the reproductive organs. For this purpose, we made a horizontal cut on the lower third of the abdomen with surgical scissors. The testes were then pulled out of the scrotum in a cranial direction. The tissue was always handled carefully, to ensure not to damage any tissue or accidentally squeeze the epididymis. The cauda epididymis, which is generally located right below the testis, was extracted using scissors and forceps. To avoid sample contamination, we removed any surrounding fat tissue.

The isolated *cauda epididymis* was transferred into a pre-warmed 200 µl drop of TYH in a 35 mm culture dish covered with mineral oil. The tissue was then cut four to six times with micro scissors to allow the spermatozoa to swim out for 10 minutes. During this time the samples were incubated at 37°C under 5% CO_2. Subsequently, the epididymal tissue was removed and 10 µl of the sperm suspension were transferred into a 190 µl drop of TYH covered with mineral oil. This 1:20 dilution was performed to achieve an appropriate sperm density for the computer assisted sperm analysis (CASA). The diluted sperm sample was then incubated for another 20 minutes at 37°C and 5% CO_2 before CASA was performed.

2.4. Sperm analysis

2.4.1. Sperm motility and swimming velocity

We used computer-assisted sperm analysis to assess sperm quality traits.
The Microptic® Automatic Diagnostic System requires a high-resolution camera connected to a phase-contrast microscope (Nikon Eclipse E200) using a 10x objective to track and record individual sperm. Mouse standard was set as default configuration in the SCA® Motility main module (Microptic S.L. 2021).

After gently shaking the incubated samples on a heating plate (37 °C) for one minute we transferred 3 µl into a standard count 4 chamber slide (Leja Products B.V., Nieuw-Vennep, Netherlands) for imaging. Ten videos were then recorded at different sites evenly distributed throughout the slide to ensure an objective sample estimate.
We used CASA to determine sperm motility (%) and sperm swimming velocity (curvilinear and straight-line velocity).

All samples were evaluated twice, the first analysis was performed 30 minutes after collection (T0) to assess fresh sperm quality traits, the second analysis was performed 90 minutes after collection (T1) to assess the decline in sperm quality traits and to estimate sperm longevity.
In addition, we further visually inspected each sperm sample under a light microscope to compare our subjective assessment with the CASA system. Manual and CASA measurements were performed immediately after each other to reduce any time influence on sperm performance.

2.4.2. Sperm morphology

We further assessed sperm morphology to determine whether and how PESA affects sperm morphology traits. Therefore, an aliquot of each sperm sample was fixed on an object slide and stained using SpermBlue (Microptic S.L., Barcelona, Spain) following the exact instructions of the manufacturer. Mouse standard was chosen as the default configuration setting in the SCA® Morphology main module (Microptic S.L. 2021).

Stained samples were then analyzed using a 60x objective (phase-contrast microscope, Nikon Eclipse E200). Per sample, 50 spermatozoa were assessed and we determined the frequencies of morphological malformations. Only abnormalities that are defined in Table 1 were taken into account. Multiple abnormalities per spermatozoa were rare but taken into consideration.

Table 1. Different types of morphological abnormalities that were taken into account in our evaluations.

head and neck abnormalities	detached head
	head deformities (pear-shaped/narrow at the base)
	cytoplasmic droplet
	bent neck
midpiece abnormalities	bent midpiece
tail abnormalities	bent tail
	coiled tail
	broken tail

2.5 Ethical statement

For our experiment we used mice that had been used as breeders in the in-house breeding colony. Before we used the animals for our experiment, the mice had been sorted out as breeders and were euthanized. We collected our samples post mortem. All tasks were performed in accordance with the Good Scientific Practice guidelines (1.7.2019) of the University of Veterinary medicine, Vienna.

2.6. Statistical analysis

The statistical tests were performed with SPSS for Windows (SPSS, Version 24.0 Inc., Chicago, IL, USA).

To test whether there are differences in sperm quality parameters between PESA and SECE samples, we performed linear mixed models (LMMs) with mean sperm motility (T0, T1), swimming velocity (VCL, VSL at T0 and T1), the decline in motility and swimming velocity and frequency of morphological abnormalities as dependent variables. Sample collection procedure, side of collection (left or right epididymis) and the interaction of both were specified as fixed factors. Animal ID was included as a random factor to control for the non-independence of multiple samples from one individual.

3. Results

3.1 Sperm motility and swimming velocity

The statistical details of all results mentioned in this paragraph are summarized in Table 2.

In this study we were able to retrieve motile sperm from all mice by using both PESA and SECE techniques.
Not surprisingly, SECE proofed to supply a higher sperm number than PESA (Table 3). Interestingly, SECE also revealed a higher proportion of motile sperm compared to PESA (Figure 2A) and sperm motility was highly variable between individuals within the PESA method (as evidenced by the wide variation ranges observed). Similarly to sperm motility, sperm swimming velocity was also significantly affected by the sample collection procedure. Both, sperm curvilinear velocity (Figure 2B) and straight line velocity (Table 3) were significantly lower in PESA compared to SECE samples. We did not find any significant differences in sperm motility (Figure 2A), curvilinear velocity (Figure 2B) or straight line velocity (Table 3) depending on the sampling side and there were no significant interactions between sampling procedure and side on sperm traits.

After 90 minutes, sperm motility (Table 3), curvilinear velocity (Table 3) and straight line velocity (Table 3) were significantly lower in PESA compared to SECE samples. No side difference in motility (Table 4), curvilinear velocity (Table 4), or straight line velocity after 90 minutes of incubation (Table 4) was detected and all interactions between sampling procedure and side were non-significant.

The decline in motility over 90 minutes of incubation was lower in PESA compared to SECE samples (Figure 3A), whereas the decline in curvilinear velocity (Figure 3B) and straight line velocity (Table 3) were both higher in PESA compared to SECE samples. Again, no side effects on the decline in sperm motility, curvilinear velocity or straight line velocity were detected and there were no significant interactions between sampling procedure and side on the decline in sperm traits.

Table 2. Statistical results (F-values and p-values) are given for each measured sperm quality parameter and the associated fixed factors. Sample collection procedure, side of collection (left or right epididymis) and the interaction of both were defined as fixed factors in our linear mixed model (LMM). Results are considered statistically significant with $p<0,05$.

Sperm quality parameters	procedure	side	interaction
Motile sperm (%)	LMM: F=312,84; p<0,001	LMM: F=2,64; p=0,12	LMM: F=0,5; p=0,5
VCL (μm/sec)	LMM: F=17,27; p<0,001	LMM: F=0,13; p=0,72	LMM: F=0,000; p=0,99
VSL (μm/sec)	LMM: F=8,24; p=0,008	LMM: F=0,57; p=0,46	LMM: F=0,196; p=0,66
Motile sperm_T1 (%)	LMM: F=121,59; p<0,001	LMM: F=0,34; p=0,57	LMM: F=1,01; p=0,33
VCL_T1 (μm/sec)	LMM: F=58,73; p<0,001	LMM: F=0,03; p=0,86	LMM: F=0,15; p=0,71
VSL_T1 (μm/sec)	LMM: F=6,81; p=0,016	LMM: F=0,03; p=0,87	LMM: F=0,16; p=0,7
Motility decline (%)	LMM: F=49,72; p=0,001	LMM: F=1,32; p=0,26	LMM: F=0,14; p=0,71
VCL decline (μm/sec)	LMM: F=4,07; p=0,05	LMM: F=0,02; p=0,88	LMM: F=0,095; p=0,76
VSL decline (μm/sec)	LMM: F=1,69; p=0,20	LMM: F=0,05; p=0,82	LMM: F=0,08; p=0,78
Morphological deformities (%)	LMM: F=42,42; p<0,001	LMM: F=0,999; p=0,32	LMM: F=1,26; p=0,27

Table 3. Comparison of the measured motility parameters between PESA and SECE sampled epididymal sperm (mean values ± standard error of mean).

	PESA		SECE	
	mean	SEM	mean	SEM
sperm (M/sample)	0,54	0,11	8,39	0,31
estimated motility (%)	22,55	3,95	85,25	2,36
motile sperm (%)	18,95	2,76	71,02	1,73
motile sperm_T1 (%)	8,92	2,05	42,6	2,36
motility decline (%)	10,03	2,42	28,42	2,16
VCL (μm/sec)	143,43	14,33	203,65	4,23
VCL_T1 (μm/sec)	45,05	11,41	144,64	7,72
VCL decline (μm/sec)	98,37	17,48	59,02	7,49
VSL (μm/sec)	54,68	5,2	69,86	3,54
VSL_T1 (μm/sec)	13,80	4,01	37,56	3,08
VSL decline (μm/sec)	40,87	5,79	32,29	2,81

Table 4. Results from the sperm quality evaluation of PESA and SECE collected sperm samples, comparing the right and left sided epididymis. Results are given in mean values ± SEM.
No significant differences were found.

	right epididymis		left epididymis	
	mean	SEM	mean	SEM
sperm (M/sample)	4,65	0,97	4,27	0,89
estimated motility (%)	54,5	7,6	53,3	8,19
motile sperm (%)	47,37	6,58	42,59	6,17
motile sperm_T1 (%)	26,65	4,68	24,87	4,21
motility decline (%)	20,72	2,72	17,72	3,43
VCL (μm/sec)	170,93	13,52	176,15	11,63
VCL_T1 (μm/sec)	93,67	14,86	96,02	15,17
VCL decline (μm/sec)	77,26	13,55	80,13	14,79
VSL (μm/sec)	64,27	4,7	60,27	4,81
VSL_T1 (μm/sec)	26,93	4,72	24,44	4,23
VSL decline (μm/sec)	37,34	5,36	35,83	3,81

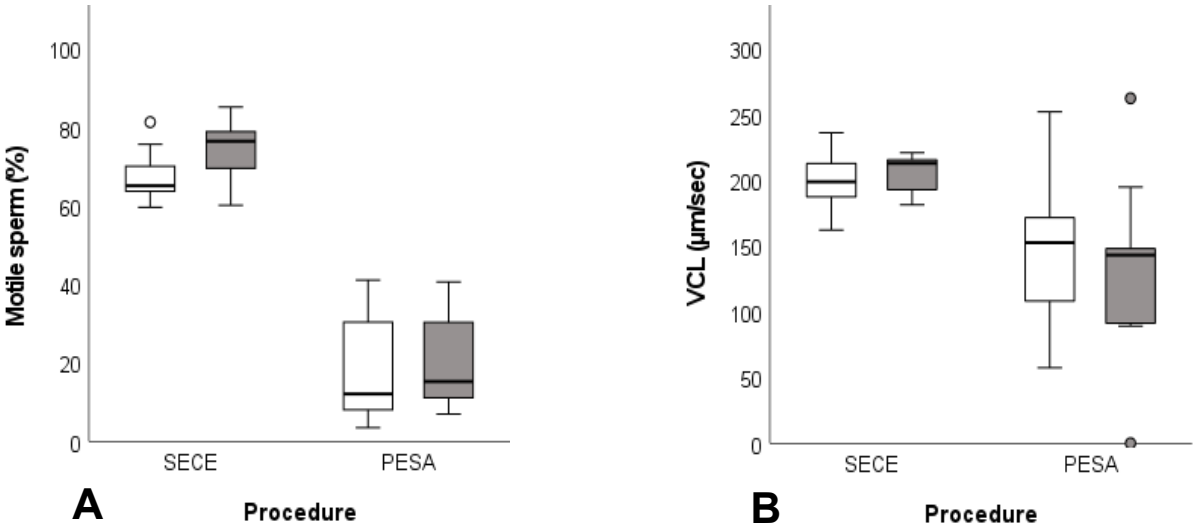

Figure 2. Comparison of sperm motility (A) and curvilinear velocity VCL (B) between sperm aliquots retrieved from the epididymis via SECE and PESA. White bars represent results from left-sided epididymal samples and grey bars from right-sided samples respectively. Values are given as quartiles. Sperm motility (A) and VCL (B) were both significantly lower in PESA sperm samples.
No significant differences in sperm motility (A) and VCL (B) were observed, comparing left and right sided sperm samples.

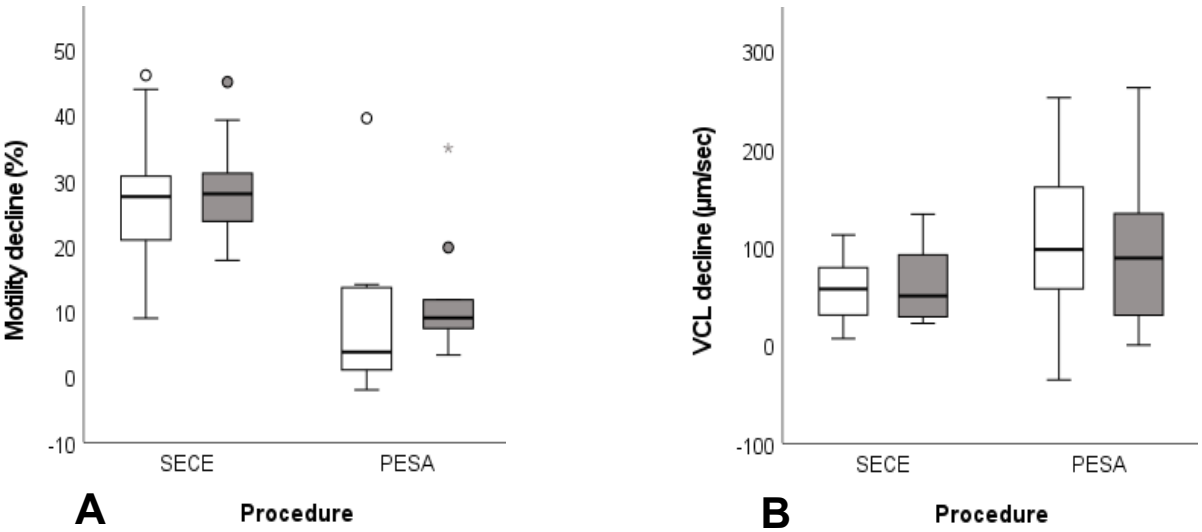

Figure 3. Graphic representation of the decline in sperm motility (A) and curvilinear velocity VCL (B) in PESA and SECE samples. White bars represent results from left-sided epididymal samples and grey from right-sided samples respectively. Values are given as quartiles. Left and right sided samples did not differ significantly from each other. The motility decline (A) was lower in PESA samples, whereas VCL decline (B) was higher in PESA sampled sperm.

3.2 Sperm morphology

We found significantly more morphological deformities in PESA samples than in sperm samples gained via SECE. No significant side difference was observed in either procedure technique, and no significant interaction between sampling collection procedure and side was found (Table 2).

Both in PESA and SECE samples tail abnormalities proofed to be the most significant fraction of morphological deformities (Figure 4).

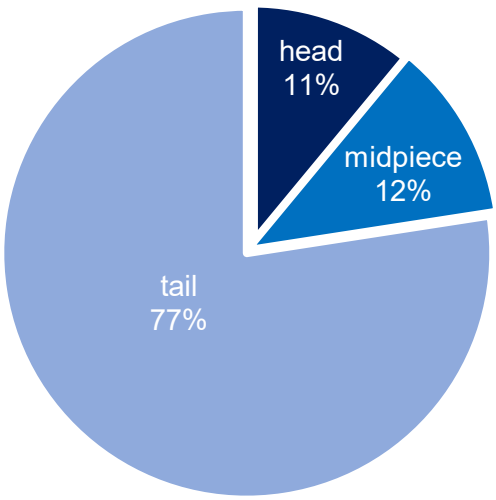

Figure 4. Percentage of morphological abnormalities in relation to the locations of occurrence in both PESA and SECE. Biggest fraction with 77% represents tail abnormalities, 11% make up head and neck abnormalities and 12% of the total number of abnormalities are midpiece deformities.

4. Discussion

The purpose of this study was to objectively evaluate the quality of sperm samples retrieved via percutaneous epididymal sperm aspiration (PESA) in laboratory mice and to compare the results to the routine collection procedure (SECE), which entails the surgical harvest of the cauda epididymis and subsequent preparation in a petri dish.

In most animals the *cauda epididymis* was found to be on the medial apical side of the scrotum with one side generally more prominent in size compared to the contralateral organ. The epididymal tail can be described as a pinhead-sized circular light yellow-greyish organ with a visible curly structure embedded in fat tissue. In order to yield sufficient sperm via PESA we observed that training is key, since the *cauda epididymis* is not always easy to locate through the scrotal skin. Thus, inaccurate aspiration, meaning not restricted to the cauda epididymis exclusively, leads to sperm samples of decreased quality (contaminated with fat tissue, cells and immature spermatozoa). Boersma et al. (2015) reported a successful PESA sperm retrieval in B6D2F1 males, hence it also appears to be applicable in dark coated mice despite inferior visual orientation. However, they did not succeed in yielding sufficient sperm using PESA in each animal.

In order to gain an overall assessment, we retrieved the sperm aliquot for microscopic evaluation directly from the center of the sperm drop. By doing so we excluded a possible distortion of the measurement by only analyzing the most motile spermatozoa, which are found at the edge of the sample. As anticipated, we observed a big difference in the mean total number of spermatozoa between PESA (0,54 ± 0,1 x 10^6 spermatozoa/sample) and SECE (8,39 ± 0,3 x 10^6 spermatozoa/sample) specimen, due to the different volumes of suspension obtained with both procedures.

The overall mean motility in PESA sperm only reached 18,95% ± 2,8% compared to the standard retrieval procedure (SECE), which achieved a mean motility rate of 71% ± 1,7%. Also, all other measured velocity parameters were also strongly reduced in PESA compared to SECE sperm, suggesting that PESA has a damaging effect on sperm traits. Additionally, we observed a wider range of values in PESA samples compared to the control procedure. This dispersion in values is most likely related to the more or less accurate execution of the sperm aspiration (sampling in the right spot). However, there will always be anatomical and

physiological differences between individuals since we worked with outbred swiss mice, which do have a broader genetic background in contrast to inbred strains.

Regarding specimen longevity, we discovered the decline in motility was lower in PESA samples, while the decline in curvilinear velocity and straight line velocity both were higher in PESA sperm than in the SECE control group. A possible explanation for the reduced decline in motility in PESA sperm could be a mathematical distortion, since the motility in PESA samples was already very low at the beginning (T0) in comparison to SECE specimen. Alternatively, and not mutually exclusive, we hypothesize, that the higher decline in sperm swimming velocity in PESA were likely influenced by the negative suction pressure during the aspiration, causing a damaging effect on the spermatozoa. Damage related to suction pressure could also explain the overall worse sperm motility and swimming velocity in PESA compared to SECE sperm.

The hypothesis that the negative suction pressure during percutaneous sperm aspiration does have some effect on the sperm sample is further supported by the morphological differences between PESA and SECE samples in our experiment. PESA sperm samples displayed a significantly higher number of morphological abnormalities. According to Varesi et al. (2013) these results do not match the sperm quality results in dogs, where no morphological difference between PESA and the control group were found. We argue that this discrepancy in results could on one hand be due to the significantly greater average size of the murine spermatozoa compared to the canine specimen (Cummings and Woodall 1985). On the other hand, the researchers in the canine study used a 26G cannula for percutaneous puncture, which has a larger lumen in contrast to the 30G needle, we used for our experiment. Therefore, both sperm and cannula size could potentially have damaging effects on the spermatozoa morphology. No significant difference regarding the frequency of morphological deformities between left and right sided samples in either procedures were observed.

Our study further shows that sperm motility parameters and the prevalence of morphological deformities differ only slightly between the right and left sided cauda epididymis of the same animal regardless of the collection method (Table 4; Figure 3). These findings reflect with the outcome in dogs (Hori et al. 2004; Varesi et al. 2013). This leads us to the conclusion, that PESA represents a reliable sperm retrieval method with reproducible results.

Despite the seemingly lower quality of PESA specimen compared to the standard SECE technique, the overall aim of PESA is only to obtain few quality spermatozoa. Boersma et al. (2015) reported a mean fertilization rate of 84,4% with PESA subtracted sperm and no difference in litter size after IVF (in vitro fertilization) compared to standard SECE procedure. This just shows that only a small number of vital spermatozoa are enough for IVF, especially if intracytoplasmic sperm injection (ICSI) is applied. Further research is needed though to determine, if PESA samples with low spermatozoa concentration can decrease the success of cryopreservation techniques.

One big advantage of PESA is that it can be done repeatedly, if necessary, until motile sperm is obtained. In human reproductive medicine, PESA has established a reputation for itself of being a safe and fast technique with minimal trauma (Roselund et al. 1998; Ron-El et al. 1998). In mice no histopathologic alterations were found after a one-time PESA (Boersma et al. 2015). However, further research is necessary to determine the risk of trauma after repetitive PESA in mice. Studies in rats concluded that there were significant histological changes (hematoma, inflammation) after repetitive PESA procedures (Saade et al. 2008; Zhang et al. 2014).

Unlike the routine practice of killing male mice in order to harvest their sperm, PESA is a feasible alternative to yield sperm of sufficient quality for subsequent IVF (Boersma et al. 2015). On the downside however, PESA is not a recommendable procedure to measure sperm quality in laboratory mice, as the procedure itself seems to affect sperm quality. Nevertheless, repetitive PESA has the potential to be applied in various research fields to further investigate reproductive biology, pharmacokinetics, toxicology and mutagenesis in mice.

Based on all these advantages we propose to always start sperm collection under anesthesia and analgesia by performing percutaneous epididymal sperm aspiration, since it is less invasive than microsurgical epididymal sperm aspiration (MESA). For the best possible outcome, we suggest to carry out PESA in both bilateral epididymis to retrieve enough spermatozoa for ART. Should the number of gametes still not suffice, one can easily switch to perform MESA by cutting open the tunica vaginalis and directly puncturing the *cauda epididymis* under eyesight.

Being the most refined sperm sampling method there is today, PESA can make a valuable contribution to reduce animal numbers in biomedical research. Unfortunately, we cannot

recommend PESA though as a tool to assess male sperm quality (i.e., sperm swimming velocity and morphology). Further research is needed to determine the efficiency and possible side effects of repeated sperm collection in mice.

5. Abstract

To test whether percutaneous epididymal sperm aspiration (PESA) can be applied as a reliable method to assess sperm quality in laboratory mice, we compared sperm quality traits between PESA sperm samples and sperm aliquots obtained by the standard sperm retrieval method, surgical epididymis extraction (SECE). The collected sperm samples were analyzed using computer assisted sperm analysis (CASA) and various sperm parameters, including sperm motility, swimming velocity and morphology were assessed. In this study, we were able to retrieve motile sperm from all mice by using both PESA and SECE techniques. PESA sperm generally provided a lower total number of spermatozoa and showed significantly lower sperm motility and swimming velocity with a wide range of variation between individuals. Also, there were significantly more morphological deformities in PESA samples compared to sperm samples gained *via* SECE. Therefore, we cannot recommend PESA to measure sperm quality in laboratory mice.

Zusammenfassung

Diese Studie wurde durchgeführt um zu testen, ob die perkutane epididymale Spermienaspiration (PESA) als zuverlässliche Methode für Spermienqualitätsbestimmungen in Labormäusen herangezogen werden kann. Dafür haben wir Qualitätsmerkmale einerseits von Spermien, die *via* PESA gewonnen wurden und andererseits von Spermien, die *via* chirurgische Extraktion der Nebenhodenschwänze (SECE) erworben wurden, miteinander verglichen. Die erhaltenen Spermienproben wurden mittels Computerunterstützer Spermienanalyse (CASA) evaluiert, wobei verschiedene Parameter, unter anderem Spermienmotilität, Schwimmgeschwindigkeit und Morphologie bestimmt wurden.

Im Rahmen dieser Studie konnten wir von allen Mäusen sowohl mittels PESA als auch durch chirurgische Präparation der Nebenhoden motile Spermien gewinnen. PESA Proben zeigten grundsätzlich eine geringere Spermienkonzentration als auch eine geringere Motilität und Schwimmgeschwindigkeit. Eine hohe Variabilität zwischen den einzelnen Tieren konnten in PESA Proben beobachtet werden. Zusätzlich konnten in PESA gewonnen Proben signifikant mehr morphologische Veränderungen von der Norm beobachtet werden. Unter Anbetracht der Ergebnisse ist PESA keine zuverlässige Methode zur Spermien-Qualitätsbestimmung bei Labormäusen.

6. Abbreviations

ART	Assisted reproductive technology
C	Celsius
CASA	Computer assisted sperm analysis
CO_2	Carbon dioxide
G	Gauge
ICSI	Intracytoplasmic sperm injection
ID	Identification
i.e.	Id est (that is)
IVF	In vitro fertilization
LMM	Linear mixed model
MESA	Microsurgical epididymal sperm aspiration
ml	Mililiter
mm	Millimeter
µl	Microliter
PESA	Percutaneous epidiymal sperm aspiration
SECE	Surgical extraction of the cauda epididymis
SEM	Standard error of mean
SPSS	Statistical package for social sciences
T0	Time point zero (base value)
T1	Time point one
TYH	Toyoda, Yokoyama, Hoshi sperm medium
VCL	Curvilinear velocity
VSL	Straight line velocity

7. Tables and Figures

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8. References

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