DOI: 10.1002/vms3.681

ORIGINAL ARTICLE

WILEY

Epididymis incision as a method to collect epididymal sperm cells in alpacas

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Funding information
Alpaca Breeding Association Germany (AZVD)

Abstract

Background: Epididymal sperm can be collected post-castration or post-mortem. This method has already been described for alpaca (*Vicugna pacos*), but little data are available on success rate and the influence of diluent on it.

Objective: The aim was to investigate the effectiveness of epididymis incision as an extraction method.

Method: Post-castration epididymides (n = 78) were incised and semen was collected from cut surfaces. Further sperm were flushed with two semen extenders. For the left epididymis, a diluent without animal proteins and for the right, a diluent with egg yolk was utilised. Collected sperms were immediately spermatologically examined.

Results: Due to incorrect measurements, the samples of seven epididymides were not analysed. An evaluation was possible in 58 samples. Average density was 108.80 ± 83.28 million/mL and motility was 53.30 ± 18.17%. On average, 76.70 ± 11.60% of the sperm were vital in eosin-stained specimens. In the hypoosmotic swelling test, an average of 69.50 ± 10.48% of the sperm had an intact plasma membrane. Semen extender had no effect on spermatological parameters ($p > 0.05$). Overall success rate of sperm recovery was 83%. Extraction of epididymal sperm was possible in 68.80% of the 2-year-old males. The 13 epididymides from which no sperm recovery was possible were histologically examined and for 10, there was no histological evidence of sperm. The corrected success rate was 95.60%.

Conclusion: Testicular volume ($p = 0.0453$), but not age ($p = 0.62$), had an effect on the probability of obtaining sperm.

KEYWORDS
alpaca, epididymis, extraction method, sperm recovery, *Vicugna pacos*

1 | INTRODUCTION

The collection of epididymal sperm makes it possible to archive valuable genetic material from sires that are not suitable for mating or recently deceased. Epididymides from castrations or slaughtering can be used as a model.

Various methods have been described in the literature for obtaining epididymal sperm. In alpaca, the prepared and cleaned epididymis is usually divided into small pieces and placed in liquid to allow the sperm to float out. The volumes (1–4 mL) and temperatures (35–37°C) of the media used, as well as the incubation time vary (Abraham et al., 2016; Kershaw-Young & Maxwell, 2011; Mamani-Mango et al., 2019;

Morton et al., 2010b; Morton et al., 2007; Vasquéz et al., 2012). With this method of extraction, Morton et al. (2010b; 2007) achieved a success rate of 100% (n = 10 each) in three experiments and 90.90% (n = 11) success rate in one experiment. Abraham et al. (2016) used 44 epididymides after castration and 12 epididymides from deceased animals. They described success rates of 17–100% for different groups of subjects. The groups were divided according to testicular length in combination with age. Kershaw-Young & Maxwell (2011) conducted two experiments using 12 and 8 epididymides, respectively. No information was provided on the success of the extraction. A study by Vasquéz et al. (2012) used 28 epididymides, but the success rate was not stated. Mamani-Mango et al. (2019) compared sperm extraction by crushing the epididymis with retrograde flushing. For each method, the epididymides of 19 stallions were used and no information on the success rate was provided.

Alternatively, sperm collection at the isolated epididymides could be performed by retrograde flushing of the epididymis tail through the ductus deferens. To date, the use of this method has been reported in bulls (P. M. Papa et al., 2015), male horses (F. O. Papa et al., 2008), male dogs (Hori et al., 2015), Spix yellow-toothed guinea pigs (*Galea spixii Wagler*) (da Silva et al., 2017) and Iberian ibex (*Capra pyrenaica*) (Santiago-Moreno et al., 2009). For the camelid family, the method has been described for the dromedary camel (*Camelus dromedarius*) (Monaco et al., 2016) and alpaca (*Vicugna pacos*) (Mamani-Mango et al., 2019). Using this method of sperm collection, Santiago-Moreno et al. (2009) reported problems with catheterisation of the vas deferens in three of 23 epididymides in the Iberian capricorn. The same problem was observed in male dogs (Hori et al., 2015) and in dromedaries (Monaco et al., 2016). In addition, the ductus deferens of the dromedary ruptures in some cases and there is buffer leakage at the incision sites. Therefore, Monaco et al. (2016) could not obtain sufficient sperm from 4 of 18 epididymides in the dromedary, with only a few sperm collected from these four. Mamani-Mango et al. (2019) do not mention any problems with the performance of retrograde flushing. However, the total sperm yield with the retrograde flush with an average of 25.20 million sperm was significantly lower than with the crushing technique, having an average of 51.70 million sperm ($p = 0.005$) (Mamani-Mango et al., 2019).

Recent publications describe the collection of epididymal sperm samples in vivo. The vas deferens of male alpacas is deviated surgically and opened. This allows repeated collection of sperm directly from the vas deferens (Gomez-Quispe et al., 2016; Meza et al., 2018). The number of test individuals, and thus, the epididymides used was relatively small. Meza et al. (2018) used five stallions and achieved a success rate of 100%. Gomez-Quispe et al. (2016) used three stallions. After habituation of the animals, semen collection was carried out three times a week.

The goal of this study was to investigate the effectiveness of sperm extraction by incision of the epididymis. This method was chosen to reduce the necessary work steps and contamination of the samples with erythrocytes. The influence of the two diluents Andromed® and Triladyl® on sperm quality and collection rate was examined. In addition, the epididymides, from which no sperm could be obtained, were histologically examined to determine whether extraction should have been possible.

In addition, we wanted to investigate to what extent age affects sperm collection in our group of subjects.

2 | MATERIALS AND METHODS

2.1 | Animals and collection of the epididymis

Epididymides of 40 alpaca stallions of the Huacaya breed at an age of 2–8 years were used. The animals originated from different husbandry systems and were castrated at the request of the owner to facilitate husbandry. At the time of castration, the stallions were clinically healthy and there were no pathological deviations in terms of consistency of the testes detected.

2.2 | Measurement of testicles with the epididymides

The measurement of the testes, including the epididymides, was conducted in the processus vaginalis with the aid of a measuring tape, whereby length, width, height and circumference were recorded in centimetre. Using a formula to calculating the volume of an ellipsoid, the testicular volume was calculated as (length (l) × width (w) × height (h) × 0.5236).

2.3 | Castration and semen collection

Scrotal castrations were performed using standard procedures and under general anaesthesia. The surgical procedure is analogous to the description of scrotal castration by Baird et al. (1996) supplemented by subsequent wound closure.

After the castration, the epididymis was dissected and cleaned of blood. Clamps were applied at the level of the ductus deferens and the epididymis body. The removal of the epididymis was always conducted on the side of the clamp that was distant from the epididymis. The epididymis section in between was left in one piece until the incision was made. The opening at the level of the epididymis tail was performed with the aid of a new scalpel blade (Otto Rüttgers GmbH & Co. KG, Solingen, Germany).

The sperm emerging from the cut surfaces were aspirated with a syringe filled with diluent (Injekt, 2 mL volume, B. Braun Melsungen AG, Melsungen, Germany) with an attached intravenous catheter (Vasovet 22G × 1, 0.9 × 25 mm, B. Braun Melsungen AG. Melsungen, Germany) and transferred to a beaker (Schott Duran, 25 mL volume, Mainz, Germany). The remaining diluent contained in the syringe was then drizzled onto the cut surfaces, thereby flushing out further semen. The diluent used for flushing was maintained at 37°C temperature and a volume of 0.40 mL of the same was used. The samples from the left epididymis were rinsed with Andromed® (Minitube, Tiefenbach, Germany). The samples from the right epididymis were diluted with

TABLE 1 Composition of the diluents used

Diluent	Andromed®	Triladyl®
Composition	Bi-distilled water Fructose Glycerol Citric acid Buffer* Phospholipids Spectinomycin Lincomycin Tylosin Gentamycin	Bi-distilled water Glycerol TRIS Citric acid Fructose Spectinomycin Lincomycin Tylosin Gentamycin
		+ fresh egg yolk (1:4)

*No further information from the manufacturer.

Triladyl® (Minitube, Tiefenbach). The same procedure was adopted for samples from cryptorchid stallions. The composition of the semen extenders is shown in Table 1.

2.4 | Spermatological examination

Motility and density of the samples were determined using computer-assisted sperm analysis (CASA) (SpermVision® and AndroVision®, Minitube, Tiefenbach). The light microscopic vitality determination was conducted using supravital staining with eosin-y. The integrity of the plasma membrane was examined with the hypoosmotic swelling test (HOS test).

2.5 | Histology

The 13 epididymides, from which no sperm extraction was possible, were histologically examined. The epididymides pieces were fixed in buffered 10% formalin and then embedded in paraffin. Then, 4-μm-thick sections were made and stained with haematoxylin–eosin. The evaluation was performed using a microscope with positive phase contrast (Helmut Hund GmbH, Wetzlar, Germany) at 1000× magnification (objective 100×/1.25 oil). The presence or absence of sperm in the preparation was assessed.

2.6 | Groups

Depending on the success of sperm recovery and the results of the histological examination, the epididymides were divided into different groups. The groups were defined as follows:

Group 1: Semen was obtained from the epididymides of this group, and therefore, no histological examination was carried out.

Group 2: From these epididymides, no semen was extracted and no sperm was detected histologically.

Group 3: The sperm collection was not successful in these epididymides, but sperm was found in the histological examination.

TABLE 2 Classification of epididymides into groups 1 (n = 65), 2 (n = 10) and 3 (n = 3)

Group	Semen gained	Histology performed	Sperm detected (histology)
1	x	–	–
2	–	x	–
3	–	x	x

x: yes.
–: no.

The classification of groups is shown in Table 2.

The median age of the stallions in group 1 was 42 months (min. 24 months; max. 96 months). In group 2, minimum and median age of the alpacas was 24 months and maximum age was 96 months. The median age of the stallions in group 3 was 24 months (min. 24 months; max. 48 months). Figure 3 shows the age distribution of the subjects (groups 1–3).

2.7 | Statistics

The following programmes from the statistical programme package BMDP/Dynamic, Release 8.1 (Dixon, 1993), were used: BMDP1D (simple data description), BMDP3D (*t*-test for dependent samples), BMDP2V (one-way analysis of covariance with repeated measurements), BMDPLR (multiple logistic regression) and BMDP6D (correlation analysis). Motility, vitality and plasma membrane integrity were provided as a percentage of all sperm counts of the respective sample (= 100%).

Following the experimental design, the influence of the diluent on semen density was determined using the *t*-test for dependent samples. The effect of the diluent on motility and on the results of the vitality determination are determined by a one-way analysis of covariance with repeated measurements with respect to the diluent, taking into account the log(testicular volume) as a covariate. Using multiple logistic regression, the correlation between sperm gain and age, as well as testicular volume was analysed. Because the testicle volume has a right-skewed distribution, it was logarithmically transformed in this analysis to achieve an approximate normal distribution. Correspondingly, the data description of this characteristic was conducted by specifying the geometric mean value and the dispersion factor also known as geometric standard deviation. The correlation analysis examines the relationship of individual testicular parameters to each other and to the testicular volume. The significance level in the evaluation of the statistical analyses is $\alpha = 0.05$.

3 | RESULTS

3.1 | Testicular dimensions

The testicles of the stallions had an average length of 5.20 ± 0.98 cm, a width of 3.50 ± 0.79 cm and a height of 3.10 ± 0.65 cm. The testicle

TABLE 3 Epididymal sperm parameters (mean ± SD) in samples treated with Andromed® (n = 29) and Triladyl® (n = 29)

Semen extender	Concentration (mio./ mL)	Motility (%)	Vital sperm (eosin y) (%)	Curled tail in HOS-test (%)
Andromed®	103.49 ± 69.91	54.30 ± 18.17	74.50 ± 11.62	69.71 ± 10.31
Triladyl®	114.22 ± 97.05	52.25 ± 18.43	78.88 ± 10.55	69.33 ± 10.84

volumes calculated from the values collected were skewed to the right. The geometric mean of the testicular volume calculated by logarithmic transformation was 26.92 cm^3 and the dispersion factor was 1.857.

After performing the multiple logistic regressions, there was no evidence of a statistically significant influence of age on sperm collection. However, the testicular volume showed a statistically significant positive correlation with the probability of obtaining sperm ($p = 0.0453$). The correlation of the individual testicular parameters with each other (at least $r \geq 0.684$) and with the testicular volume (at least $r \geq 0.858$) was high.

3.2 | Success rate

Sperm was obtained from 65 of the 78 epididymides. Thus, the success rate of sperm collection in relation to all epididymides was 83%.

3.3 | Spermatological examination

The CASA system used did not have any preferences for the species alpaca at the beginning of this work. Therefore, the settings for the optimal detection of alpaca sperm were defined together with Minitube when the first samples were examined. For this reason, incorrect measurements with insufficient detection rates occurred at the beginning and the samples of seven epididymides could not be evaluated. With samples of 58 epididymides, an analysis was possible. The average density of the samples was 108.80 ± 83.28 million/mL (min. 12.30 million/mL; max. 475.90 million/mL). The diluent had no statistically verifiable influence on the density of the extracted sperm cells ($p = 0.44$).

The vitality of the sperm, determined using eosin staining, averaged 76.70 ± 11.60% (min. 41.00%; max. 92.50%). In the hypoosmotic swelling test, 69.50 ± 10.48% (min. 41.00%; max. 89.00%) of the sperm cells had an intact plasma membrane. The samples had an average motility of 53.30 ± 18.17% (min. 19.30%; max. 92.00%). The diluent had no statistically significant effect on the results of the vitality determination ($p = 0.12$) or motility ($p = 0.54$). The spermatological parameters for both diluents are listed in Table 3.

3.4 | Histological examination

Sperm were found in the preparations of three of the 13 histologically examined epididymides. Sperm was thus detected in 23% of the epididymides from which no sperm could be obtained.

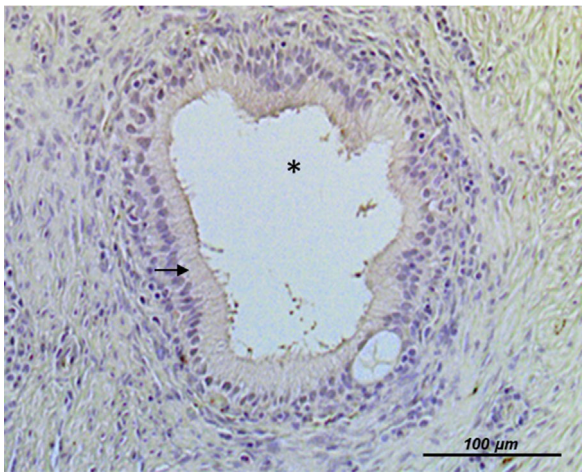

FIGURE 1 Formalin-fixed paraffin-embedded tissue, Epididymidis. Section of the epididymal duct of an alpaca male (24 months old). No sperm was detected in the lumen (*) of the ductus epididymidis (→). Hematoxylin Eosin Stain. Objective 200×

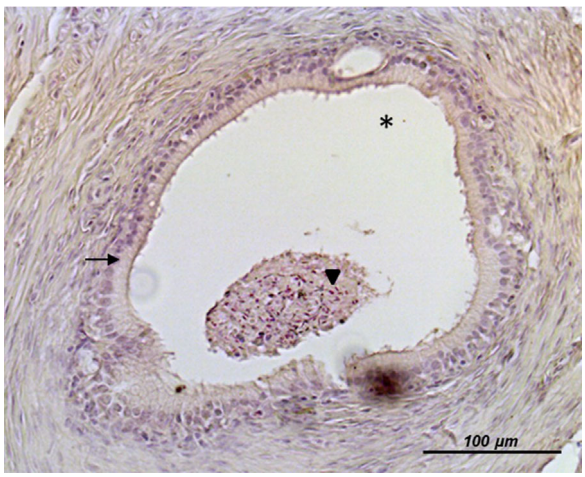

FIGURE 2 Formalin-fixed paraffin-embedded tissue, Epididymidis. Section of the epididymal duct of an alpaca male (24 months old). The lumen (*) of the epididymal duct (→) contained a conglomerate of cell debris and sperm (▼). Hematoxylin Eosin Stain. Objective 200×

The preparations with sperm were from two animals aged 24 months and one stallion aged 48 months (group 3). Figure 1 shows the epididymal duct of an alpaca male (24 months old) without sperm. Group 2 consisted of three stallions aged 24 months and one 8-year-old animal. The epdidymal duct with sperm of an alpaca male at the age of 24 month is shown in Figure 2.

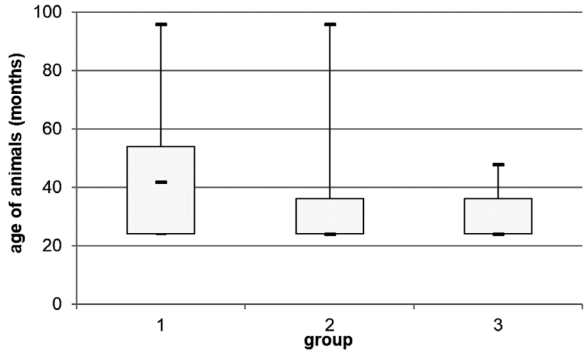

FIGURE 3 Boxplots showing age of animals in group 1 (n = 33), group 2 (n = 4) and group 3 (n = 3). Quartiles (top and bottom of boxes), medians (short horizontal lines within the boxes) and the minimum and maximum age (horizontal line at the end of vertical whiskers) are shown for each boxplot

3.5 | Age

The age distribution of the animals in group 1 had a wider range than that of groups 2 and 3. Statistically, no significant effects on sperm collection ($p = 0.62$) were supported for age (Figure 3).

4 | DISCUSSION

The aim of this study was to establish a simple method for the collection and the dilution of epididymal alpaca sperm with as few work steps as possible. The samples obtained should be able to be examined with CASA, so no diluents with disruptive components were used.

In this study, a technique for obtaining epididymal sperm was used, which has not been described to date. This was done to reduce the contamination of the samples with erythrocytes because these cells prevent an examination of the sperm cells by CASA. When the epididymis is crushed, centrifugation and washing is necessary to eliminate the cell contamination. To reduce the amount of erythrocytes added during sperm collection, the epididymides were incised in the present study and not disintegrated. This procedure diverges from the techniques used by other groups working with epididymides after castration (Abraham et al., 2016; Kershaw-Young & Maxwell, 2011; Morton et al., 2007). In preliminary tests (not listed in the manuscript), the cutting and swim out method led to contamination with blood cells, so that an additional centrifugation step would have been necessary before CASA.

The only extraction method that appears to allow an even greater reduction of cell contamination is the retrograde flush of the epididymis. Retrograde flushing is more complex and requires more practice to perform. The most frequently mentioned problem when using this method is the faulty catheterisation of the ductus deferens (Hori et al., 2015; Monaco et al., 2016; Santiago-Moreno et al., 2009). In alpaca, retrogradely flushed samples had a significantly lower density than samples obtained by crushing the epididymis (Mamani-Mango et al., 2019). To ensure a uniform, efficient, and animal size-independent implementation, retrograde flushing was not used in the present study.

As a result, the incision of the epididymis appeared to be the best way to combine low contamination and practicality for semen collection. Furthermore, this procedure was independent of testicle size and could therefore always be performed uniformly.

In the present study, the success rate of sperm collection in relation to all epididymides was below the values of comparable studies (Morton et al., 2010b; Morton et al., 2007). However, if it is considered that in 10 epididymides, there were no sperm, and consequently, none could be obtained, the corrected success rate was 95.60%. The corrected success rate is within the range of variation stated in the literature (Morton et al., 2010b; Morton et al., 2007).

There are various reasons that could explain the azoospermia in these 10 epididymides. In the case of the 2-year-old stallions, it seems reasonable to assume that sperm production had not started yet. Genetics, diet, climate and the time of year of birth all affect the time of puberty in the alpaca stallion (Tibary & Vaughan, 2006).

A significant increase in body temperature, especially for longer than three days, can have fatal effects on spermatogenesis. These effects can be long lasting or irreversible. The hyperthermia can be generated by high ambient temperatures or high fevers due to general illnesses or infectious diseases (Pearson et al., 2014). Which factors were related to the azoospermia of the animal at the age of 8 years could not be determined on the basis of the general examination and the data collected.

Morton et al. (2010b, 2007) divided their investigations into two experiments in which different diluents, additives and protocols were used. For all experiments, epididymal sperm was obtained by dividing the epididymis and then allowing the sperm to float out for 30 min in 4 mL of Androhep at 37°C. Morton et al. (2010b) stated a success rate of 90.90% in experiment 1 and 100% in experiment 2. In the earlier study, a success rate of 100% was achieved in both experiments (Morton et al., 2007). The present study was the first histological examination of the epididymal tissue after sperm extraction. The results of the histology clearly showed that in some stallions, no sperm could be obtained because there were no sperm cells in the epididymis.

The average age of the alpaca stallions used in the present study was 41.25 ± 19.17 months. However, the wide age range of the animals, from 24 to 96 months, should be noted. Because 42.50% of the stallions had an age of 24 months, the arithmetic mean value does not adequately reflect the actual age distribution. This should be considered when comparing the present study with the data of Morton et al. (2010b).

The alpaca males in the study of Morton et al. (2010b) were, on average, almost 3 years old (experiment 1: 33.90 ± 3.90 months; experiment 2: 32.50 ± 1.80 months). Morton et al. (2007) mentioned a mean of the age of the animals of 32.90 ± 5.80 months in experiment 1 and 22.50 ± 0.90 months in experiment 2.

Analogous to the results obtained in the present study, the age distribution in Morton et al. (2007) shows that age is not the decisive factor for successful sperm collection.

For the dilution of samples, we used commercial semen extenders, which already contained glycerol as cryoprotectant to reduce work steps and enable an examination with CASA. The two diluents used had no significant effect on the density of the sperm cells or on the rate of extraction. The differences in the composition of the semen extenders (Table 1) do not seem to affect collection rate.

The use of the both extenders has been described for alpacas. McEvoy et al. (1992) used Triladyl® for cryopreservation of alpaca ejaculates. Abraham et al. (2016) used Andromed® to allow sperm to float out of the epididymis. This is the first time that the two diluents have been compared under identical conditions for epididymal alpaca sperm collection.

The mean density of sperm determined in the present study was 108.80 ± 83.28 million/mL. The density of the spermatozoa varied widely between the individuals (min.12.30 million/mL; max.475.90 million/mL). In order to be able to compare the achieved results adequately with the available literature, the average sperm count per animal was calculated. This value indicates the average of the total sperm yield achieved per animal and was 112.58 ± 139.46 million in the current work. The sperm count per animal was thus within the range of the values from crushed epididymides of 75.30 ± 22.50 to 143.10 ± 43.50 million/stallion stated in the literature (Morton et al., 2010b; Morton et al., 2007). So, the new method is therefore equivalent. Additionally, the values for the spermatological parameters were within the range of variation in the literature, including vitality (Gomez-Quispe et al., 2016; Vasquéz et al., 2012), plasma membrane integrity (Gomez-Quispe et al., 2016; Vasquéz et al., 2012) and motility (Gomez-Quispe et al., 2016; Kershaw-Young & Maxwell, 2011; Morton et al., 2010b; Morton et al., 2010a; Vasquéz et al., 2012).

Because of the high number of stallions used, the results of the present study were from a heterogeneous animal population, which reflects the situation in the field. Because the castration age of the alpaca stallions is on average 2 years, this age group was included in the present study. Furthermore, the question should be answered whether epididymal sperm can be successfully obtained from 2-year-old males.

In group 1, 54.60% of the stallions were 2 years old. This showed that sperm could be obtained from 68.80% of the 2-year-old stallions. However, some studies specify a minimum age of 3 years or more for alpaca stallions (Kershaw-Young & Maxwell, 2011; Mamani-Mango et al., 2019; Meza et al., 2018). The reason for this could be that the number of sperm produced increases during the development of the animals from sexual maturity to breeding maturity. In alpacas, sperm production begins at the age of 10–12 months (Smith et al., 1994) and breeding maturity occurs at approximately 2.50–3 years (Knauf et al., 2008).

5 | CONCLUSION

In this work, we were able to establish epididymis incision as a new method to collect epididymal sperm from alpacas after castration. The samples obtained with this method showed a low cell contamination, which is beneficial for CASA. In the future, CASA will play an increasingly important role in the spermatological examination of alpaca semen. This should be taken into consideration when the collection method is chosen.

The histological examination revealed that 76.92% of the epididymides, from which no sperm could be extracted, did not contain sperm at all. In the future, more histological investigations would supply further information about production and presence of sperm in individual animals.

As it was possible to obtain sperm from 68.80% of the 2-year-old males, more studies with animals at the age of 2 years could provide interesting information about the relationship between age and sperm production.

Furthermore, the sperm quality could be determined more precisely with additional morphological examinations, in particular, the percentage of normal sperm. The comparison of the sperm quality of different age groups could help to determine the optimal age for sperm recovery in the alpaca.

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

AW proposed and designed the experiment. MA and HW collected and analysed samples. KF performed statistical data analysis. AW, MA and HW drafted and edited the manuscript. All authors read and approved the final manuscript.

DATA AVAIBILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ACKNOWLEDGEMENTS

The authors would like to thank the Alpaca Breeding Association Germany (AZVD) for financial support. The funds provided were used for the procurement of necessary consumables. The grant had no impact on the design and implementation of the study, analysis or interpretation of the data and publication.

ETHICAL STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. The use of the testes, including the epididymides, for the examination was approved by the responsible Regierungspräsidium Gießen (kTV 7-2017).

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How to cite this article: Auer, M., Wagner, H., Failing, K., & Wehrend, A. (2022). Epididymis incision as a method to collect epididymal sperm cells in alpacas. *Veterinary Medicine and Science*, *8*, 157–163. https://doi.org/10.1002/vms3.681