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Characteristics of Epididymal Sperm Recovered from Slaughterhouse Derived Testes of Nondescript/Native Goats in the Philippines

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Abstract Nondescript/ native goats are a common sight in the country side because of their ability to thrive well in harsh environmental conditions and their ability to reproduce easily despite their small size (20-30kg). In order to supply the food chain, they usually end up in the slaughterhouse shortly before or after they have reached sexual maturity. With the intensification of live importation of foreign goat breeds, there is a pressing need to conserve local native goats in the country. However native bucks are not usually trained for ejaculated semen collection therefore epididymal sperm collection from post mortem is one option. In this study, scrotal intact testes (n=6) were collected within 1-2 hour after slaughter from matured native bucks. The objective of this study is to optimize a method of sperm collection from post mortem testes and determine the sperm characteristics using basic parameters such as motility, viability, sperm concentration, and sperm volume. The slice + swim-up (Method 1) or Mince +flushing (Method 2) were performed to isolate epididymal sperm. In both methods, the obtained percentage sperm motility ranged between 60-75% by conventional method of assessment. The average sperm concentration per mL was higher in Method 2 with 1.89 ± 0.36 $x10^9$ sperm compared to Method 1 with 1.23 ± 0.29 $x10^9$ but did not differ significantly by Student's T- test ($p>0.05$). In terms of sperm volume, an average of 770±200 µl was recovered in Method 2 compared with 500±110 µl in Method 1 and are not significantly different ($p>0.05$). In terms of percentage live sperm at the time of collection, Method 1 registered a higher mean percentage of 88.9±2.65 as compared with Method 2 with a mean percentage of 81.67±2.81 (Student's T-test $p>0.05$). The proportion of normal sperm was 76.9±8.48 and 69.4±5.34 in Methods 1 and 2, respectively. The presence of proximal (16-18%) and distal droplet (69-71%) was a common find which is indicative of a sub population of maturing sperms in the epididymis. We therefore conclude that using the two recovery methods a considerable population of viable epididymal sperm can be isolated from post mortem gonads of matured nondescript bucks.

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Keywords: goat epididymal sperm viability and motility characteristics, post mortem testes

Introduction

Goats are known as 'kambing' in the Philippines and their population is largely contributed by small scale goat producers in rural farming communities. The most numbered in the country are the native goats (Bondoc, 2005) raised under backyard conditions which comprise to about 98.35% of the total goat inventory of 3.67M head (Philippine Statistics Authority, 2014). Although they perform poorly in terms of production, they are mostly preferred by farmers due to their innate adaptability to the warm environment, perceived resistance to diseases and their ability to breed easily inspite of their small size ranging between 20-30 kg. More often that not they are slaughtered shortly before or after they have reached sexual maturity to supply the food chain. With the introduction of foreign goat breeds by artificial insemination (AI) using processed ejaculated semen to improve their production efficiencies, there is a compelling need to conserve native goat germplasm for future sperm banking. However, native goats are not trained for semen collection unlike the imported goat breeds. Post mortem male gonads or testicles collected from slaughtered matured native bucks therefore, is a viable option for sperm recovery for basic research in preparation for native goat germplasm conservation.

The epididymis is a specialized structure close to the exterior of the testis which serves as site for sperm maturation. It is where the sperm is provided with the microenvironment necessary for sperm maturation and acts as a storage unit for sperm before they are finally released upon ejaculation (Saenz, 2007).

The interest in preserving valuable genetic material has resulted in increased attention for possible recovery of viable sperm from the epididymis of dead animals (Foote, 2000). In fact sperm from the epididymis have been productively used for artificial insemination and for *in vitro* production of embryos in several species (Barati *et al*., 2009). Previous studies had already proven that epididymal sperm for instance ram epdidymal sperm, showed better resistance than ejaculated sperm in terms of stressors related to cryopreservation such as chilling, osmotic stress, crpyoprotectant agent addition and removal (Varisli *et al.*, 2009).

In the Philippines, there is lack of basic information on epididymal sperm from native goats even though research on other ruminants and non ruminant species is replete with literature (Kaabi *et al*., 2003, Karja *et al.*, 2010, Lima *et al.*, 2013, Guimaraes *et al*., 2012). It is therefore the interest of this study to firstly, optimize a method of epididymal sperm collection from post mortem native buck gonads and determine sperm characteristics using basic parameters

such as sperm volume, sperm concentration and sperm motility, sperm viability (live and dead sperm).

Materials and methods

Matured bucks (n=6) as determined by oral dentition were selected as experimental animals prior to testicle collection from the slaughterhouse. Scrotal intact goat testicles (Figure 1) were cut shortly after the animal was bled, placed in a styropore box and brought to the laboratory immediately within 1-2 hours. The weight (grams) of the scrotal intact testicles were measured and recorded. After the removal of the scrotal sac the testicles were washed in Physiologic Buffered Saline (PBS) with 2-3 drops of povidone iodine. One testicle from each pair was measured for its weight before it was used in one of the two methods of epididymal sperm collection. The weight (grams) of the cauda epididymis was also taken for recording purposes. The right testicle was processed using slicing and swim-up technique (Method 1) while the left testicle was processed using mincing and flushing technique (Method 2).

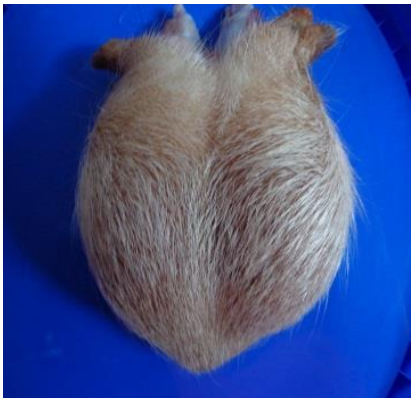

Figure 1. *Scrotal sac-intact goat testicle*

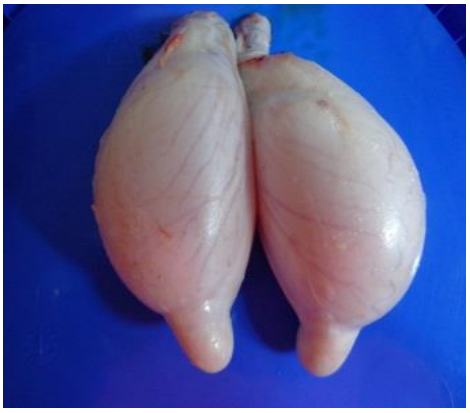

Figure 2. *Left and right testicle*

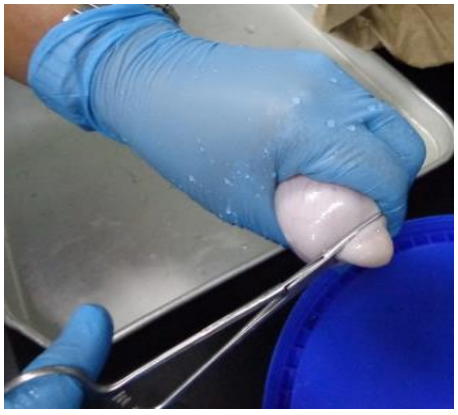

Figure 3. ***Cutting of the cauda epididymis***

Swim-up Technique (Method 1)

The parietal tunic was removed and the tail of the epididymis was sliced longitudinally halfway using sterile scalpel blade and immediately dropped into a 50 mL volume conical tube containing 15 ml of TRIS buffer solution. The solution was allowed to stand for 5-10 minutes to allow epididymal sperm to swim up to the upper part of the solution. The upper two thirds (2/3) of the Tris buffer solution was then pipetted out and transferred to a 10 ml volume conical tube for centrifugation set at 10,000 rpm for five minutes in order to form a sperm pellet.

Mincing and Flushing Technique (Method 2)

The cauda epididymis was minced into 4-8 pieces in a sterile petri dish and the exposed surface areas were flushed with TRIS buffer solution. Shortly thereafter, the flushed solution was pipette out and transferred in a 10 ml conical tube for centrifugation as described in Method 1 to form a sperm pellet.

Sperm Volume

After the centrifugation process the volume of sperm pellet at the conical end of the test tube was measured against the graduated lines in microliter units. The volume in microliters (µl) was recorded from each of the two methods.

Microscopic Sperm Evaluation

Microscopic evaluation was performed to determine percentage sperm motility by subjective method of assessment and the sperm concentration was determined using a hemocytomer. The percentage live and dead sperm, normal and abnormal sperm were also observed after differential staining with eosin nigrosin dye of representative samples from each methods of recovery.

Sperm Concentration

A sample of epididymal sperm was sucked up to the 0.5 mark of the RBC dilution pipette. Then, the diluting fluid which consisted or 3% NaCl with tinge of eosin stain was sucked up to the 101 mark avoiding air bubble formation. The diluted sample was then shaken in a figure of eight motion to mix the sperm with the diluting fluid. Shortly thereafter about 4-5 drops of the diluted sperm was discarded. Then the hemocytometer was loaded with 10 µL of the sperm suspension in each of the upper and lower grid chambers. The spermatozoa were allowed to settle for 5 to 6 minutes before placing the hemocytometer on the stage of the microscope. The spermatozoa in five large squares were counted with the head of the spermatozoa considered as the reference in counting. As some spermatozoa transcended the lines at the edge of the squares, only spermatozoa on the top right lines were included in the count. The spermatozoa in any five of the 25 squares were counted and multiplied by the dilution factor and by 10,000 to yield a sperm count of n x10^7 sperm per mL.

Sperm Motility

A microdroplet of about 10µl of the sperm suspension was placed in a clean pre-warmed (37°C) microscope slide using a micropipette. A clean pre-warmed cover slip was later placed gently to avoid air bubble formation. At least five microscopic fields were examined in order to evaluate the percentage of motile sperm which was performed by a highly skilled technican with a trained eye for sperm motility evaluation. Sperm motility was demonstrated by the progressive wavy movement of sperm cells.

The evaluation of the movement of sperm was adapted from the scoring system devised by Mamuad *et al.* (2005). Reference in rating sperm motility in the experiment is shown in Table 1.

Table 1. Scoring system for the motility of sperm cells. (Mamuad *et al.,* 2005)

Motility (%)	Grade	Characteristics
91-100	Excellent Motility	90% or more of the spermatozoa is very rigorous in motion. Swirls caused by the movement of the sperm are extremely rapid and constantly going forward progressively.
76-90	Very Good Motility	Approximately 75-90% of the spermatozoa is in vigorous rapid motion. Waves and eddies form and rapidly but not so rapid as in excellent motility.
60-75	Good Motility	About 60-75% of the spermatozoa is in motion. Motion is vigorous but waves and eddies formed move slowly across the field of vision.
40-59	Fair Motility	From 40-55% of the sperm is in motion. The movements are largely vigorous or eddies are formed.
Less than 40	Poor Motility	Less than 40% of the sperm is in motion. The motion is not progressive but mostly weak and ocsillary.
0	Zero Motility	No motility is discernable.

Microscopic evaluation for live and dead and sperm morphology

A droplet (10µL) of epididymal sperm sample was stained with a droplet (10µL) stained with Eosin-Negrosin which is composed of one percent (1% w/v) eosin and five percent (5% w/v) nigrosin dissolved in three percent (3% w/v) sodium citrate dihydrate solution. After mixing the two droplets, a thin smear of the stained sperm was run across the surface of the glass slide and was air dried. When the slide is viewed microspically, sperms that are alive will not absorb the stain because of the intact plasma membrane and the sperms appear white as shown in Figure 4. On the other hand dead

sperm will absorb the color of eosin dye because there is loss of plasma membrane integrity and sperm will appear pinkish against the nigrosin background (Fig 4). A minimum of 200 spermatozoa are examined from each slide and counting is done in random fields over the slide to obtain representative figures. The number of live sperms are counted and the percentages of live sperms are calculated over the total number of sperm observed multiplied by 100. The shape of the head, neck or mid piece and the tail can be also examined. The presence of cytoplasmic droplet in the tail are indicative of immature sperm. Sperm cells with abnormal morphology are also counted over the total number of sperms counted and expressed as percentage abnormal sperm.

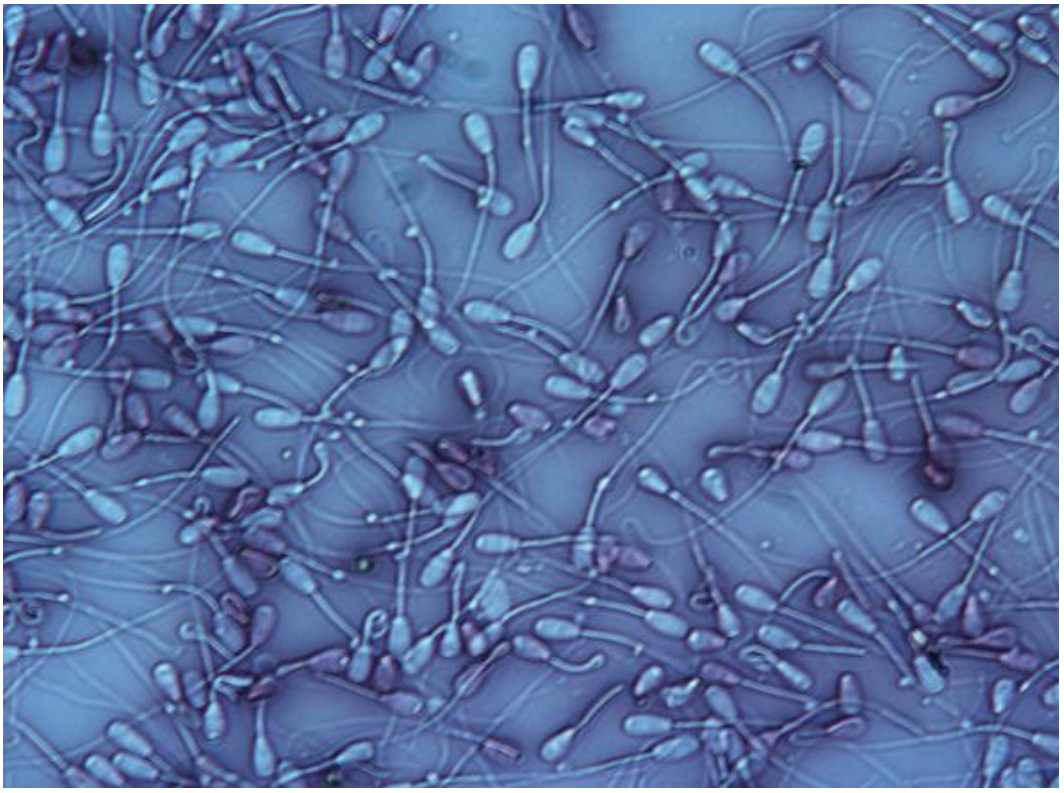

Figure 4. Eosin nigrosin stained goat epididymal sperm (live sperms appear white while dead sperms appear pink and other visible morphological abnormalities such as coiled tail, bent tail and detached head). Some cytoplasmic droplets i.e. white droplet in the middle part of the sperm tail can also be seen.

Statistical Analysis

All data gathered from the measured sperm parameters in the two methods of sperm recovery were reported as mean ± s.e.m and significant differences were statistically analyzed by Student's T-test at $P<0.05$.

Results

In this study, testicles (n=6 pairs) from matured native goats were immediately excised from the body after the animal was bled. Scrotal intact native goat testicles initially measured for their weights were found to weigh between 135-160 grams. After the scrotal sac was removed, the weight of one testicle was between 30-40 grams averaging 37.5grams. The average weight of the epididymis was 1.39 grams.

The volume (µL) of epididymal sperm obtained by Method 2, which is mincing+flushing registered an average of 770 ±200 µL which was higher but not significantly different ($P>0.05$) when compared with the swim-up method (Method 2) with a mean value of 500 ±110 µL shown in table 2. The average sperm concentration per mL (n x10^7± s.e.m) obtained in method 2 was higher (189 ±36.8x10^7) compared with the swim up method (123 ±29.7 x10^7). However, statistical analysis revealed no significant difference ($P>0.05$) between two methods in terms sperm concentration.

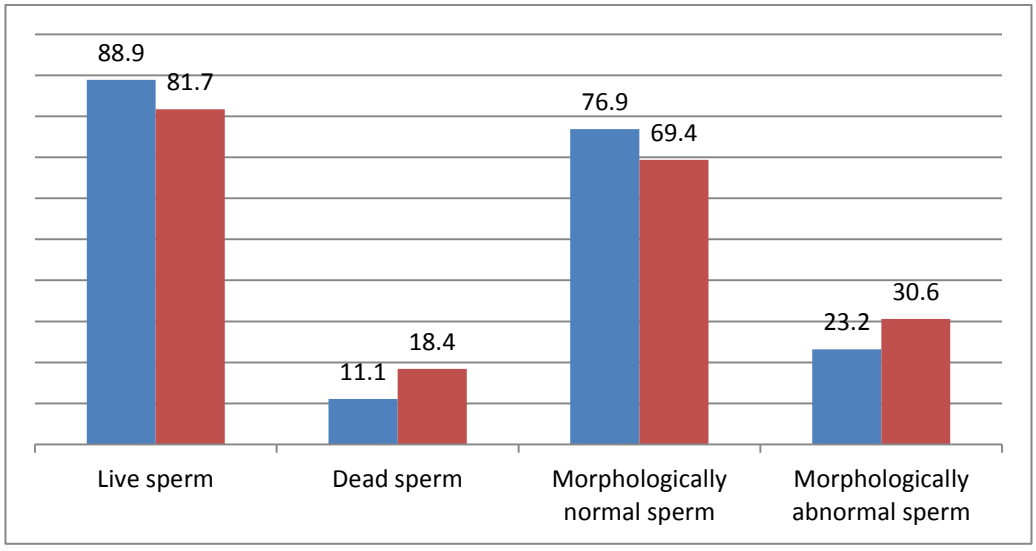

Figure 5. Percentage (%) live and dead sperm, morphologically normal and abnormal sperm in Method 1 (blue column) and method 2 (red column)

Conventional or subjective method of sperm motility evaluation revealed sperm motility scores between 60–75% suggesting that immediately recovered post mortem epididymal sperm remains to be alive and were still of good quality. As shown in figure 5, a greater proportion (%) of live sperm was obtained in Method 1 (88.9±2.76) compared with Method 2 (81.7±2.8) but the mean values were not statistically different by Student's T-test, (P>0.05). Similar to this finding is the higher percentage (% mean ± s.e.m.) of morphologically normal sperm in method 1 (76.9 ±8.5) compared with method 2 (69.4±5.3) but the values did not significantly differ (P>0.05). The proportion (%) of proximal cytoplasmic droplet (16-18%) and distal cytoplasmic droplet (69-71%) were a common find. This observation is indicative of the presence of a sub-population of immature and maturing sperms in the excised cauda epididymal tissue at the time of recovery. The occurrence of other sperm abnormalities from both methods such as coiled tail (2.2~7.6%), bent tail (4.3~5.3%) and detached head (0.4~3.1%) were also observed and the percentage values are presented in table 3.

Table 2. Epididymal sperm volume (µL) and sperm concentration (nx10^7 per mL) after recovery by Method 1 Slicing+swim-up and Method 2, Mincing+flushing method

Method of sperm recovery	Volume of sperm recovered (µL)	Sperm Concentration (n x10^7 sperm per ml)
Slicing+Swim-up	500 ± 110^b	123 ± 27.9^a
Mincing+flushing	770 ± 200^b	189 ± 36.8^a
p value	0.28	0.18

Values in column with the same superscript do not differ by student's T test (Statisitical significance P<0.05)

Table 3. Morphological characteristics of fresh epididymal sperm after using two methods of sperm recovery

Sperm abnormalities	Sperm Recovery Technique		Statistical significance by Student's T-test (P < 0.05)	
	Method 1 Slice+Swim-up	Method 2 Mince+Flushing	P value	
Proximal Droplet	16.7±2.5	18.8±1.5	0.38	ns
Distal droplet (%)	69.5±6.5	71.5±3.2	0.10	ns
Coiled tail (%)	7.6±4.5	2.24±0.7	0.07	ns
Bent tail (%)	5.38±1.8	4.3±1.4	0.32	ns
Detached head (%)	0.4±0.2	3.1±2.1	0.07	ns

ns= not significantly different

Discussion

The ability to successfully recover viable epididymal sperm from post mortem testes constitutes the initial step in germplasm conservation of genetically diverse native animals for future utilization using assisted reproductive biotechniques. Optimizing a procedure that will yield good quality epididymal spermatozoa from post mortem testes is a key element when one is to engage in basic and applied research relative to sperm cryopreservation of indigenous/native goats in the Philippines for future Animal Genetic Resources (AnGR's) cryobanking.

In this study, two methods of epididymal sperm recovery were compared to determine the characteristics of sperm that can be recovered. A larger volume of spermatozoa can be isolated in mincing+flushing (Method 2) because of the greater surface area to be exposed allowing greater number of spermatozoa to be flushed. However, the presence of epithelial cells and blood isolates in such procedure can not be avoided primarily due to the cut capillary vessels releasing hemolyzed blood cell contents. Furthermore, the likelihood of getting morphologically abnormal sperm eg. detached head and dead sperm is increased due the mechanical method of isolation causing a larger number of retrievable sperms.

In the slicing and swim-up or method 1, the volume of sperm was slightly lower because sperms gradually released themselves freely into the

Tris buffer solution only from the sliced portion of the cauda epididymis. Such procedure produced a pale white turbid appearance with no traceable tinge of hemolyzed blood. As a result, there is lesser chance of getting morphologically injured, abnormal or dead cells. The slice+ swim-up technique proved to be an advantage in separating potential spermatozoa for further semen processing (extended/liquid semen) prior to preservation below physiological temperature.

Ehling and co workers (2006), isolated ram epididymal sperm by slicing the cauda epididymis and suspending it it a one step freezing medium. Filtration of the sperm suspension with 50-75µm nylon grid was performed to remove the somatic cells. They reported an average pre-freeze sperm motility of 79.7% and 93.7% acrosome intact sperms. In a related experiment by Ocampo *et al*. (2015) native goat epididymal sperm isolated by the slice and swim-up method were stored at refrigerated temperature conditions of 4-5 °C after dilution with an extender optimized by Beltran and co-wokers (2013) with some minor modifications. Results showed that epididymal sperm in semen extender were able to survive the refrigerated condition for up to 48-72 hours based on the daily evaluation for percentage sperm motility and percentage live and dead sperm recorded. Lima and co workers (2013) reported that sustained sperm viability for goat epididymal sperm recovered by retrograde flushing of the vas deferens and cauda epididymis was achieved while at 4°C with the use of coconut water with egg yolk as the semen extender.

The proportion of recovered live sperms in our study is between 80-88% with acceptable sperm motility scores that ranges from 60-75% that qualify them for preservation at low temperature. Cryopreservation of these sperm yielded post thaw cryosurvability in terms of motility that ranged between 15-35% (Gautane *et al*., 2015). Epididymal sperm cryopreservation have been reported successful for goats (Blash *et al.*, 2000; Uttam Datta *et al*., 2011), the Indonesian spotted water buffalo (Yulnawati *et al.*, 2009), water buffalo (Barati *et al*., 2009) and European bison (Kozdrowski *et al*., 2011). The fertilizing potential of isolated epididymal sperm by Method 1 was performed by sperm penetration assay and yielded fertilization rates of 60.92% for freshly recovered and 41.36%, for processed epididymal sperm, respectively (Verdarero *et al.*, 2015).

Nonetheless, sperm yield in either recovery methods basically consisted of a mixture of matured and immature sperm. Sperms with proximal cytoplasmic droplet accounted to about 16-18% and was 69-71% for sperms with distal cytoplasmic droplet. The cytoplasmic droplet is a remnant of the cytoplasm of a maturing spermatid (Cooper, 2005) and the location of the droplet, determines the stage of maturity. Developmental changes in the

spermatozoa occurs as it travels the three segments of the epididymis namely the head (*caput*) , the body (*corpus*) and the tail (*cauda*). It is during this epididymal transit where sperm modifications that are morphological and functional in nature occur to complete the maturation process. The finality of these maturational events terminates in the development of a fully matured sperm that acquires the ability to interact with the oocyte after it is ejaculated from the caudal reserves of the epididymis.

Thus, the more distal the droplet is from the head, the more advanced the maturation process for the sperm is (Cooper, 2005). Harayama *et al.*, (1996) explained that the shedding of the cytoplasmic droplet occurs when the sperm are introduced to seminal fluid. The occurrence of cytoplasmic droplets from the cauda epididymis in our study is a clear indication of the readily available pool reserve each time the next ejaculation cycle commences.

Thus, with these recovery methods native/nondescript goat epididymal sperm of good quality can be isolated. The slicing and swim-up method allows a better population of more motile and live sperm without blood contaminants compared with the mincing and flushing method. Further utilization of epididymal sperm either through artificial insemination and/or IVF to produce viable offspring or quality embryos, respectively and most importantly, sperm cryopreservation is highly anticipated in the next experimental trials.

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