Effect of testicle post-mortem storage on goat epididymal sperm quality: the first step towards cryobank for local Algeria breeds

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SUMMARY

The present study was designed to evaluate the effect of both temperature and storage time interval on the cauda epididymal sperm quality in bucks. This investigation addresses the pre-freeze/post-thaw quality of goat epididymal sperm as a function of testicle storage temperature (environment or +4°C) and time elapsed between animal's death and sperm recovery (0, 24, 48, 72 h) to establish the optimal protocols for the recovery and cryopreservation of epididymal sperm in this species. Testicles of 40 mature bucks collected at the abattoir were divided in two groups: half of the testicles (n = 40) were transported to the laboratory at environment temperature (E), whereas the remaining half (n = 40) at a refrigeration temperature (R) of +4°C. In the two groups (E) and (R), one testicle from each pair was processed after slaughter forming the time 0 groups (0E and 0R). The contralateral testicle was processed after 24, 48 or 72 h of storage, at the corresponding temperature. Sperm motility and kinetic parameters, viability, intact acrosom, HOStest and morphology were assessed in pre-freeze and post-thaw samples. Until 48 h post-mortem, both E and R temperatures are able to maintain very good pre-freeze epididymal sperm quality. After 48 h post-mortem, refrigeration temperature is fundamental to reduce epididymal sperm quality decay in pre-freeze samples. Therefore, when sperm cryopreservation is not immediately practicable, goat testicles should be transported and stored at 4°C up to a maximum of 48 h post-mortem to ensure an acceptable sperm quality.

Keywords: cryopreservation, epididymal sperm, goat, cryobank, temperature

RÉSUME

Effet de la température de conservation des testicules de bouc, sur la qualité du sperme épididymaire : Première étape vers la cryobanque de races locales algériennes

Cette étude permet d'évaluer la qualité en précongélation et en post-congélation du sperme epididymaire du bouc local en fonction de la température de stockage des testicules et du temps écoulé entre la mort de l'animal et la récupération du sperme (0, 24, 48, 72 h), pour établir les protocoles optimaux de la cryoconservation du sperme épididymaire dans cette espèce. Les testicules de 40 mâles pubères collectés à l'abattoir ont été divisés en deux groupes: la moitié des testicules (n = 40) ont été transportés au laboratoire à la température ambiante (E), tandis que l'autre moitié (n = 40) à la température de réfrigération (R) de + 4 °C. Dans les deux groupes (E) et (R), un testicule de chaque paire a été traité après l'abattage, formant les groupes de temps 0 (0E et 0R). Le testicule controlatéral a été traité après 24, 48 ou 72 h de stockage, à la température correspondante. La mobilité, la viabilité, l'intégrité acrosomial, la réaction à l'hypoosmose et la morphologie des spermatozoïdes ont été évaluées avant et après congélation. Jusqu'à 48 heures après la mort, les températures E et R permettent de maintenir une très bonne qualité de sperme épididymaire avant la congélation. Après abattage, les testicules du bouc peuvent être conservés 48 h à 4°c sans altérer les spermatozoïdes.

Mots-clés : cryoconservation, sperme épididymaire, bouc, banque de gènes, température

Introduction

Nowadays, examples of an application of post-mortem sperm collection from the epididymis for the conservation of endangered species as in ibex [43] and in constructing semen banks [15, 5, 31] are reported. Preservation of epididymal sperm from dead or slaughtered animals has been reported in several domestic and wild species: deer [22, 45, 34-35, 43], cattle [38], fox [52], horse [11], camelin [1], black and white tegu [54], rhesus monkeys [14], saltwater crocodiles [24], goat [9], ibex [44], and sheep [25,15,51]. However, sometimes, epididymal sperm extraction is not easily practicable in marginal regions, owing to the lack of facilities and expertise near the farming areas of local breeds.Several studies have been conducted to determine the entity of the decay of epididymal spermatozoa recovered post-mortem depending on some conditions (temperature and post-mortem time) in different species (cattle [37]; marmosets, baboons and chimpanzees [40]; red deer [36,45]; sheep [25]; wild ruminant [29, 8,18]. Overall, it has been observed that the resistance over the time of the spermatozoa collected from the testicles is species specific [29, 45]. In goat species, it was demonstrated that epididymal sperm can be successfully cryopreserved and used for in vitro fertilization and artificial insemination if they were immediately processed after animal death [9].

In this study, to establish an optimal protocol for the recovery and cryopreservation of goat epididymal sperm, the pre-freeze and post-thaw quality of epididymal spermatozoa as a function of post-mortem storage, constituted by the time elapsed between animal's death and sperm recovery (0, 24,

48, 72 h) and testicle storage temperature (environment or +4°C), was investigated. The investigation was carried out within a programme addressed to establish protocols for the recovery and the cryopreservation of viable sperm from the epididymis of slaughtered animals, as a tool in creating semen banks for endangered breeds.

Materials and Methods

EXPERIMENTAL DESIGN

Testes were collected at the abattoir from local Algerian goat breeds (mean age: 3.5±0.5 years), 1.30 h on average after slaughtering transported to the laboratory according to the experimental conditions that underwent: half (n = 40) at environment temperature of 21.5 ± 2.2°C (E) in a Styrofoam box, half (n = 40) at refrigeration temperature of +4°C (R) (during transportation were refrigerated at 4°C (R) with a fridge). From each goat, one testicle was processed at this time (control group T0), whereas the other testicle was stored at 4°C in a plastic bag (TR) and processed after. In the two groups (E, R) one testicle from each pair was processed within the first 4 hours after slaughter, forming the control group (T0E, T0R).Then, processed after 24h (T24E, n=6; T24R, n=6), 48h (T48E, n=6; T48R, n=7) and 72h (T72E, n=7; T72R, n=8) hours.

POST MORTEM SPERM RECOVERY

The sperm was collected by retrograde flushing method as previously reported [6, 35]. Briefly, the epididymis and vas deferens were dissected and separated from the testis and both cauda epididymis and vas deferens were isolated from the whole epididymis. Superficial blood vessels were cut and their contents removed by rinsing and wiping. The sperm was recovered in a glass tube by making a cut near the junction of the corpus and the proximal cauda. Then, the vas deferens was catheterized with a blunted 22 G needle and flushed by air injection to recover a maximum amount of sperm. Immediately after extraction, volume, sperm concentration, motility, viability, morphology, intact acrosome, and hypo-osmotic swelling test (HOST) reacted spermatozoa were recorded were evaluated.

EPIDIDYMAL SPERM RECOVERY

Immediately after collection, semen volume was determined by direct reading the graduations on collection tubes; sperm concentration was determined using a photometer (Spermacue, Spermacue Minitüb, Tiefenbach, Germany). The number of semen doses producible was calculated by multiplication of the sperm concentration by the semen volume and then dividing the total number of sperm per 200 × 106 sperm/straw.

MOTILITY

The motility was assessed using a Computer Assisted Sperm Analyzer (CASA; Spermclass analyzer, SCA Microptic, S.L., Version3.2.0, Barcelona, Spain). To facilitate the image capture, the samples were diluted (10–20 × 106Spz/ml) using Tris-extender. Subsequently, 5µl of each sample was placed onto a warmed (37°C) 20 µm Leja® 3 chamber slide (LejaProducts B.V., Nieuw-Vennep, The Netherlands). Leja slide was placed under a phase contrast microscope (NikonE200-LED microscope) on a warmed stage (37°C) and images were captured using a video camera (Camera Digital Basler A312 fc Germany) at magnification x10. Four sequences were scanned and at least 200 spermatozoa were analyzed. The standard settings were set at 25 frames/s, 20–90 µm2 for the head area and VCL>10 µm/s to classify a spermatozoa as motile [48]. Kinetic variables that were assessed were: total motility (TM, %), progressive motility (PM, %), movement linearity(LIN, %); straightness (STR, %); wobble (WOB, %); curvilinear velocity (VCL, µm/s); straight linear velocity (VSL, µm/s); average path velocity (VAP, µm/s); amplitude of later all movements of the head (ALH, µm); beat cross frequency (BCF, Hertz). With respect to the setting parameters, the system has a specific setup for goat sperm evaluation. It was set up as follows: VCL (m/s): 10 < slow < 40 < medium < 75 < rapid; VAP (m/s): 10 < slow < 40 < medium < 75 < rapid; LIN: circular < 50%; the progressivity: 80% of STR [50].

VIABILITY

Sperm viability was assessed in smears by eosin-nigrosin staining. The proportions of live and abnormality spermatozoa were then evaluated under oil immersion (magnification 1250x) using a phase-contrast microscope; 200 cells were counted [16].

INTEGRITY OF THE ACROSOME

Acrosome integrity (intact acrosomes) was assessed using Coomassie blue G-250 staining as described by HERRICK et al. [23]. Briefly, 100 µl of fixative solution (7.5% formaldehyde in phosphate-buffered saline) was added to 100 µl of thawed semen. After 10 min, the sample was centrifuged for 2 min at 10,000 g and the supernatant was discarded. The sperm pellet was washed, twice, by resuspending in 1 ml of ammonium acetate (0.1 M, pH 9) and re-centrifuging. Sperm was then resuspended in 0.5 ml of the same buffer. The sperm suspension (20 µl) was spread on a slide, air dried and stained with 0.04% (w/v) blue G-250 in 3.5% (v/v) perchloric acid for 10 min, and then gently rinsed with ultrapure water until it appeared blue. To calculate the percentage of spermatozoa with intact acrosomes, at least 200 sperm cells were assessed. Acrosomes with dark blue staining were considered to be intact, whereas, acrosome that did not stain with Coomassie blue was considered to be damaged.

HOS TEST: HYPO-OSMOTIC

Functional integrity of sperm membrane was evaluated using the hypo-osmotic swelling test (HOST). Briefly, 20 µl of sperm was incubated with 200 l of 100 mOsM hypo-osmotic solution for 60 min. Hypo-osmotic solution was prepared by mixing 9 g of fructose and 4.9 g of sodium citrate diluted in 1 l of distilled water at 37°C. The spermatozoa possessing a swollen and coiled tail were assumed to have the functional membrane. At least 150 spermatozoa were taken into account to calculate the percentage of reacted spermatozoa [10].

FREEZING AND THAWING OF EPIDIDYMAL SPERM

After collection, epididymal semen samples were diluted with Tris egg yolk extender, consisting of 20% v/v egg yolk, 10% wt/vol glycerol, 3.06% wt/vol Tris buffer, 1.25% wt/vol fructose,1.7% wt/vol citric acid, 27.5 mg gentamycin, per 100 ml (37°C) necessary to achieve a final concentration of 200 × 106 sperms/ml. Then were cooled to 4°C for 2h, packaged into 0.25 straws (Cryo Bio System, Group IMV Technologies, l'Aigle, France) and frozen horizontally suspending straws 4 cm above the liquid nitrogen for 12 min, and then completely immersed into nitrogen liquid. Two straws of each aliquot were thawed in a water bath (37°C. After cryostorage of 5 months, two straws per animal were thawed at 37°C for 2 min to determine the post-thaw epididymal semen quality.

STATISTICAL ANALYSIS

To analyze the effects of temperature and post-mortem time on buck epididymal spermatozoa quality, in the model fixed effects of the farm, the experimental group (temperature of conservation and post-mortem time) and sperm status (prefreeze, post-thaw) were included. Results are expressed means ± standard deviation.

Results

Epididymal sperm was successfully collected from all 80 testicles. The effects of post-mortem time (PMT) on the variable indexes of sperm production are shown in Table I. The effects of the testicle storage temperature (TEMP) and post-mortem time (PMT) on total and progressive motility and kinetic parameters, sperm viability, intact acrosome, hypo-osmotic swelling test (HOST), and abnormalities, on pre-freeze and post-thaw quality of epididymal sperm are shown, respectively, in Table II, Table III and Table IV.

Variable	Testicle storage time (h)			
	0	24	48	72
Testicle (n)	40	12	13	15
Volume (ml)	2.4±0.5	2.6±0.7	2.1±0.7	1.5±0.3
Concentration (10^9/ml)	4.4^{a}±0.6	4.9^{a}±0.8	3.9^{a}±0.4	2.2^{b}±0.6
Dose	21.7^{ab}±7.5	34.3^{a}±14.3	17.1^{b}±6.1	7.5^{b}±3.9

N: Number of testicle,Doses = semen doses; Different superscripts within the same column indicate differences (P<0.05).

Table I: Sperm production as a function of post-mortem time of goat epididymal sperm.

Variable	Post mortem time (h) and storage temperature (°C)							
	0h		24h		48h		72h	
	21.5°C	4°C	21.5°C	4°C	21.5°C	4°C	21.5°C	4°C
Testicle (n)	20	20	6	6	6	7	7	8
TM (%)	98.3^{a}±0.6	98.7^{a}±0.1	80.5^{b}±0.7	98.2^{a}±0.1	78.3^{c}±0.8	95.1^{b}±1.0	35.3^{d}±0.6	62.1^{c}±0.7
PM (%)	29.5^{a}±0.5	29.8^{a}±0.70	29.0^{a}±0.5	29.2^{b}±0.7	30.1^{a}±0.8	39.1^{a}±1.2	22.4^{b}±1.2	27.2^{c}±0.6
VCL (µm/s)	138.4^{a}±0.8	139.4^{a}±0.9	99.4^{b}±0.5	116.1^{a}±1.2	77.2^{c}±0.8	86.5^{b}±0.5	66.3^{d}±0.6	67.2^{c}±0.7
VSL (µm/s)	69.4^{a}±0.5	69.7^{a}±0.6	71.8^{b}±1.2	47.1^{b}±0.7	61.3^{c}±1.2	71.2^{a}±0.6	47.3^{d}±1.4	44.2^{c}±0.7
VAP (µm/s)	89.6^{a}±0.3	90.4^{a}±0.3	60.5^{b}±0.8	67.1^{b}±2	67.3^{c}±1	77.2^{a}±0.9	36.3^{d}±0.6	30.2^{c}±0.7
LIN (µm/s)	48.6^{d}±0.6	49.6^{d}±0.6	66.2^{b}±1.1	41.23^{c}±0.9	78.3^{a}±0.7	81.5^{a}±1.1	55.3^{c}±0.6	47.1^{b}±0.8
STR (µm/s)	76.2^{c}±0.7	76.5^{c}±0.7	78.2^{b}±0.7	68.3^{b}±0.9	91.2^{a}±0.7	92.2^{a}±1.1	70.3^{d}±1.4	63.5^{c}±0.5
WOB (µm/s)	65^{d}±1	65.1^{d}±1	76.5^{b}±0.6	59.2^{c}±0.7	86.4^{a}±1	88.3^{a}±1.3	72.3^{c}±1.4	66.2^{b}±0.7
ALH (µm/s)	2^{a}±0.3	2.1^{a}±0.2	2.1^{a}±0.2	1.9±0.2	1^{b}±0.1	1.2±0.3	2.5^{a}±0.5	2.2±0.7
BCF(Hz)	2.8^{b}±0.4	2.9^{b}±0.4	3.1^{b}±0.1	3±0.5	2.2^{b}±0.8	3.2±0.6	4.3^{a}±0.6	4.2±0.7

TM = total motility; PM= progressive motility; VCL = curvilinear velocity; VSL = straight-line velocity; VAP = average path velocity; LIN = linearity; STR = straightness; ALH = amplitude of lateral head displacement BCF = beat cross-frequency;. Different superscripts within the same column indicate differences (P<0.05).

Table II: Effects of post mortem time and temperature on total motility, progressive motility and kinetic parameters of pre-freeze goat epididymal sperm.

Generally, all the parameter indexes of sperm production decrease over time (Table I). Post-mortem time (PMT) induced a significant reduction in most of these variables at 72 h of testicle conservation (0E v. 72E), with the exception of collected volume.

SPERM PRODUCTION

Post-mortem time did not have significant effect on semen volume, but a significant difference in sperm concentration ($p<0.01$), and number of doses ($p<0.05$) as shown in Table I. In particular at 72 hours of testicles storage, the lower concentration was observed and consequently a lower number of doses were produced for each epididymis. The same pattern occurred for volume, but after 48 hours of testicles storage (T48 vs T72).

EFFECTS OF POST-MORTEM STORAGE ON PRE-FREEZE EPIDIDYMAL SPERMATOZOA

Concerning the effects of post-mortem time (PMT) and testicle storage temperature (TEMP) on motility assessment on pre-freeze samples are presented in Table II, PMT and TEMP induced a significant reduction in total motility and progressive motility , at 72 h of testicle conservation (0E v. 72E; 0R v. 72R; $P < 0.001$; respectively). The percentage of motile spermatozoa decreased about 10% to 12% during the 3 days of storage in two groups (E, R). Except for progressive motility ($p>0.05$) no significative difference was observed between the environment temperature (E) and refrigeration temperature (R).

The kinetic parameter decrease significative ($P < 0.001$) from the most parameter with the post-mortem time, but

Post mortem time (h)	Storage temperature (°C)	Testicles (n)	Viability (%)	Host (%)	Acrosom integrity (%)	Abnormality (%)
0	21.5	20	$90.6^{a}\pm2.4$	$87^{a}\pm0.8$	$97.1^{a}\pm1.8$	2.3±0.5
	4	20	$91.5^{a}\pm2.4$	$87.5^{a}\pm0.7$	$97.2^{a}\pm1.8$	2.4±0.5
24	21.5	6	$76.1^{b}\pm1.2$	$67.1^{b}\pm0.9$	$73.5^{b}\pm1.2$	2.8±0.7
	4	6	$83.2^{a}\pm0.7$	$74.2^{a}\pm0.7$	$95.2^{a}\pm0.7$	2.2±0.7
48	21.5	6	$74.3^{b}\pm1.5$	$62.3^{c}\pm1.5$	$70.1^{c}\pm0.8$	3.3±0.6
	4	7	$82.4^{a}\pm1$	$68.1^{b}\pm0.8$	$84.2^{b}\pm0.7$	2.6±0.5
72	21.5	7	$40.2^{c}\pm0.7$	$47.2^{d}\pm0.7$	$52.^{d}\pm0.9$	3.4±0.5
	4	8	$43.2^{b}\pm0.7$	$55.2^{c}\pm0.7$	$69.2^{c}\pm0.7$	3.2±0.7

Different superscripts within the same column indicate differences ($P<0.05$).

Table III: Effects of post mortem time and temperature on percentage of viable sperm, HOST, acrosom integrity and abnormal sperm of pre-freeze goat epididymal sperm

Variable	Post mortem time (h) and storage temperature (°C)							
	0h		24h		48h		72h	
	21.5°C	4°C	21.5°C	4°C	21.5°C	4°C	21.5°C	4°C
Testicles (n)	20	20	6	6	6	7	7	8
TM (%)	$45.2^{b}\pm0.7$	$45.5^{b}\pm0.3$	$42.3^{c}\pm0.6$	$46.3^{b}\pm0.6$	$50.3^{a}\pm0.7$	$52.1^{a}\pm1$	$35.1^{d}\pm1.3$	$37.6^{c}\pm0.5$
PM(%)	$22.2^{b}\pm0.68$	$23.23^{b}\pm0.60$	$20.23^{c}\pm0.68$	$20.15^{c}\pm0.78$	$24.17^{a}\pm0.76$	$27.20^{a}\pm1.31$	$22.07^{b}\pm1.30$	$23.37^{b}\pm1.45$
VCL(µm/s))	$99.3^{a}\pm0.6$	$10.1^{a}\pm0.6$	$87.2^{b}\pm0.7$	$98.2^{a}\pm0.7$	$73.3^{c}\pm0.8$	$87.3^{b}\pm0.6$	$66.2^{d}\pm0.7$	$67.3^{c}\pm1.3$
VSL(µm/s)	$72.2^{a}\pm0.7$	$73.2^{a}\pm0.7$	$62.2^{b}\pm0.7$	$57.9^{b}\pm0.1$	$60.3^{c}\pm0.7$	$71.4^{a}\pm0.8$	$47.3^{d}\pm0.9$	$44.3^{c}\pm0.9$
VAP (µm/s)	$60.2^{b}\pm0.7$	$60.5^{b}\pm0.6$	$58.2^{c}\pm0.7$	$60.3^{b}\pm0.6$	$64.2^{a}\pm0.7$	$77.2^{a}\pm0.6$	$36.2^{d}\pm0.7$	$29.3^{c}\pm0.6$
LIN (µm/s)	$66.3^{c}\pm0.6$	$66.8^{c}\pm0.7$	$68.2^{b}\pm0.7$	$41.3^{c}\pm0.6$	$70.3^{a}\pm0.7$	$81.1^{a}\pm0.8$	$55.3^{d}\pm0.7$	$47.1^{b}\pm1.8$
STR (µm/s)	$79.2^{b}\pm0.7$	$80.2^{b}\pm0.6$	$79.2^{b}\pm0.7$	$67.3^{b}\pm0.6$	$82.3^{a}\pm0.7$	$92^{a}\pm2$	$70.2^{c}\pm0.9$	$64.3^{c}\pm0.6$
WOB (µm/s)	$77.2^{b}\pm0.7$	$78.2^{b}\pm0.8$	$73.3^{c}\pm0.6$	$64.3^{c}\pm0.6$	$79.3^{a}\pm0.6$	$88^{a}\pm2$	$72.3^{c}\pm0.6$	$66.3^{b}\pm0.7$
ALH (µm/s)	2.3±0.6	2.4±0.7	2.2±0.7	2.3±0.6	1±0.1	1.3±0.4	2.3± 0.8	2.3 ±1.2
BCF(Hz)	$4.3^{a}\pm0.6$	$4.2^{a}\pm0.4$	$3.5^{a}\pm0.5$	3.1±0.9	$2.1^{b}\pm0.8$	3.2±0.7	$4.3^{a}\pm0.9$	3.3±0.8

TM = total motility; PM= progressive motility; VCL = curvilinear velocity; VSL = straight-line velocity; VAP = average path velocity; LIN = linearity; STR = straightness; ALH = amplitude of lateral head displacement; BCF = beat cross-frequency; Different superscripts within the same column indicate differences ($P<0.05$).

Table IV: Effects of post mortem time and temperature on total motility, progressive motility and kinetic parameters of post-thaw goat epididymal sperm.

there was no difference in some parameter like: ALH and BCF. Testicle storage temperature (TEMP) affect significative (P < 0.001), almost all the parameters except: VCL at 72 hr (72E v. 72R; P>0.05), STR and WOB at 48 hr (48E v. 48R; P>0.005), ALH and BCF(0E v. 72E; 0R v. 72R; P>0.005; respectively).

Results relating to viability, host, and acrosome integrity assessment are shown in Table III. At 72 h of testicle storage, a significant decrease in the percentage of viable epididymal sperm acrosomal cells, Host and acrosome integrity, occurred at both temperatures of testicle storage (0E v. 72E; 0R v. 72R; P < 0.001 respectively) compared with the respective time 0 group (E, R). Considering sperm abnormalities, no significant differences were observed among different times in the environment temperature (21.5°C). Even in the refrigeration temperature (4°C) samples until the 72 h of testicle conservation, no difference was detected.

In the case of testicle conservation temperature (TEMP), refrigeration temperature, at the same post mortem temperature (PMT), corresponded on average to significant better epididymal sperm quality considering total motility, sperm kinetic parameters, viability, HOST and acrosome integrity parameters. Allowing to obtain higher values of these variables in samples stored under refrigeration temperature (4°C). For the main kinetic parameters (Table II), refrigeration temperature preserved the epididymal spermatozoa quality, allowing to obtain significantly higher values (24R, P < 0.001) from the 24 h up to 72 h of testicle storage.

EFFECTS OF POST-MORTEM STORAGE ON POST-THAW EPIDIDYMAL SPERMATOZOA

The effects of post-mortem temperature (PMT) and testicles storage temperature (TEMP) on total motility, progressive motility, kinetic parameters, sperm viability, Host, acrosome integrity and abnormalities on post-thaw epididymal sperm are shown, respectively, in Table IV and V. In the post-thaw samples, PMT had an effect(P <0.001) on the percentage of motile cells, most of the kinetic parameters, sperm viability, HOST, acrosome integrity (Table IV), unless abnormalities no difference significative was observed (p>0.05).Comparing testicles storage temperature (TEMP) effects, Refrigeration temperature (R) showed a significant effect (P<0.001) over time on the percentage of total motility, and all kinetic parameters (Table IV) . Moreover, no difference significative affected total motility (48hR ; p>0.05), progressive motility (24hR ,72hR ; p>0.05), VCL, ALH and BCF.

Testicles storage temperature (TEMP) had a significant effect on the percentage of sperm viability, HOST and acrosom integrity (table V) (P < 0.001), testicle storage temperature did not influence sperm abnormalities, with the exception of the 48E, where environment temperature (E) caused an increase of abnormal forms.

Discussion

Recovery and cryopreservation of viable sperm from the epididymidis can be an alternative tool to collect male gametes, especially in wild species or in situations where a traditional collection is difficult owing to the lack of expertise and/or facilities, such as in local farm breeds farmed in marginal areas. Considering that no data are available in the literature on the quality decay of goat epididymal sperm, the aim of this work was to evaluate the effects of the testicle post-mortem storage, as a function of time elapsed between animal's death and sperm recovery and testicle storage temperature, in pre-freeze and post-thaw samples, to understand how to maintain epididymal semen quality in field conditions before arriving at the laboratories.

Considering post-mortem time, our data reveal that time elapsed after death, in particular between 24 and 48 hours of testicles conservation, had relevant effects on goat epididymal sperm production, reducing the possibility of extracting epididymal sperm cells by the cauda epididymidis

Post mortem time (h)	Storage temperature (°C)	Testicles (n)	Viability (%)	Host (%)	Acrosom integrity (%)	Abnormality (%)
0	21.5	20	48.3^{a}±0.6	54.3^{a}±0.6	78.3^{a}±0.6	2.5±0.6
	4	20	49.2^{a}±0.6	54.5^{a}±0.6	79.3^{a}±0.6	2.3±0.6
24	21.5	6	41.2^{bc}±0.7	50.2^{b}±0.7	63.3^{c}±0.6	2.2±0.7
	4	6	43.3^{b}±0.7	54.3^{b}±0.6	65.3^{a}±0.7	2.3±0.6
48	21.5	6	42.3^{b}±0.7	54.3^{a}±1.3	65.3^{b}±0.7	3.3±0.7
	4	7	45.2^{a}±0.7	57.1^{a}±0.8	66.3^{a}±0.7	1.6±0.5
72	21.5	7	40.1^{c}±1.7	40.3^{c}±0.6	48.3^{d}±0.6	3.1±0.8
	4	8	43.3^{b}±0.6	45.1^{c}±1.1	50.4^{b}±0.8	3.4±1.2

Different superscripts within the same column indicate differences (P<0.05)

Table V: Effects of post mortem time and temperature on percentage of viable sperm, HOST, acrosome integrity and abnormal sperm of post-thaw goat epididymal sperm

and consequently the number of semen doses (from 21 at T0, to 7 at T72, for each epididymides). This can be explained by the fact that tissues of the vas deferens lumen and of the cauda epididymis, after death progressively undergo a deterioration process, decomposition, and dehydration. This process is more marked in samples stored at environment temperature that compromises epididymis handling condition during sperm extraction [51].

Pre-freeze spermatozoa quality parameters as total motility, progressive motility, kinetic parameters, viability, Host, and acrosome integrity, showed no significant variation until 48h of testicles conservation, both at environmental (E) and refrigerator temperature (4°C). At 72 hours of testicles storage a significant decrease in total motility, progressive motility, kinetic parameters, viability, Host and acrosome integrity, the probability was observed at both temperatures of conservation, with particular incident in samples stored at this environmental temperature. These results are in accordance with other researchers [46, 50, 51, 2], on the other hand, the values of this study were not in agreement with the findings of other researchers who recorded that only sperm motility and not live sperm cells were affected by storage temperature or post-mortem time [37, 7], probably because of the cold shock protective effect of factor, such as lecithin [41], contained in the epididymal fluid. These protective factors are effective within around 48 h after death [43]. Cauda part of the epididymis induces motility by the secretion from the epithelium. The carnitine along with the forward motility proteins of the epididymis regulates the sperm motility. With the passing of the time and the time of storage of the sperms, the content of carnitine got reduced which instead reduced the sperm motility [21]. The significant decrease in spermatic quality of epididymal spermatozoa recovered post-mortem and maintained under similar conditions have been reported in mice [30], ram [33, 26], boar [28], bull [19], stallion [53],camel [47], [39] in bucks and [20] in red deer confirm that the viability of sperm collected from the cauda epididymides post-mortem decreased progressively relative to the time of harvesting spermatozoa. In live animals, the cauda epididymides provide a suitable environment for the immature spermatozoa to become mature and acquire motility. Furthermore, studies have shown that spermatozoa recovered from cauda epididymides become motile only when they make contact with seminal fluid or media [3,32] and remain functional even hours after the death of an animal [14,13].

The main sperm kinetic parameters: ACT (%),VAP (µm/s), VCL (µm/s), VSL (µm/s), LIN(%), STR(%) and BCF (Hz) decreased significantly in advance with respect to total motility, already at 48 h post-mortem in the two temperatures (E, R), as confirmed by other authors [45,12]. In this study, the percent intact acrosome and Host decreased significantly ($p<0.05$) from 0 to 48 h. The results of this study were in agreement with the findings of other scientists [46] who recorded 88±0.4% intact acrosome of cauda epididymal spermatozoa collected post-mortem in goat and stored up to 48 h at 4°C. These researchers observed that this recorded percent intact acrosome in addition to sperm motility (50.6±0.5 %) and percent livability (60.5±0.7 %) of cauda epididymal spermatozoa was a good quality and so indicate their high fertilizing potential, because of the acrosome is the central regulator of fertilization which mediates the sperm oocyte fusion as well as exocytosis of its constituents to bring out the process of fertilization. That is why the intactness of the acrosome decides the fate of fertilization deterioration of sperm quality which instead raises the question about the probable outcome of fertilization. It can be assumed that with the passage of the storage time the free radical mediated oxidative stress was key player as previously reported [46, 49]. Post-mortem time did not influence the percentage of sperm abnormalities within 48 h of testicle conservation.

However, during this time, an increase in abnormal sperm was manifested, in agreement with previous observations [46, 51]. In relation to the testicle storage temperature, samples of the refrigerator group in general showed a better sperm quality than those of the environment group. After 48 h of testicle conservation, refrigeration temperature was essential to prevent epididymal sperm quality decay over time. After 48 h of testicle conservation, refrigeration temperature was essential to prevent epididymal sperm quality decay over time. Considering our results, even for a short period, goat epididymides should be transported at +5°C. Over time (24, 48, 72 h), the beneficial effects of refrigeration temperature allowed us to obtain a significant better epididymal sperm quality considering the percentage of total motility, progressive motility, sperm kinetic parameters, percentage of live sperm cells, intact acrosom, Hostest and morphology of spermatozoa. In particular, refrigeration temperature induced an increase in total motility of the +62% in the 72R group than in the 72E group. The same condition was observed by other authors in goat [51], rams at 24 h [25] and bulls at 48 h of post-mortem time [37]. As stated in our study, refrigeration had a significant positive effect already after the 24 h of testicle conservation also on the most important sperm kinetic parameters. Considering that these parameters are reputed as fertility indicators [3, 17, 27], if cryopreservation is not immediately feasible, it becomes essential to prevent the loss of speed and linearity of epididymal sperm cells through the testicle refrigeration over time. The refrigeration temperature seems to prevent the decay of epididymal sperm quality over time, in particular in sperm motility, inducing a reduction in the metabolism of sperm cells, in terms of mitochondrial oxidative phosphorylation and glycolytic activities [42]. Considering the effects of PMT and TEMP on post-thaw samples, our data showed that samples obtained by testicles stored under environment temperature decrease their sperm quality in a significant way at thawing (total motility, progressive motility, the main kinetic parameters, viability, intact acrosome, Host, and sperm abnormalities) in pre-freeze samples, this situation occurred later, between the 48 and 72 h after death. Consequently, the detrimental effects of cryopreservation non samples of testicles conserved at environment temperature occurred in advance over time.

In rams, at least for total motility, this occurs situation occurred at 48 h of testicle conservation at the environment temperature [25]. In refrigerated goat samples instead, a significant decrease in these parameters occurred later, at 48 h of testicle conservation, which was also observed in boars [28]. Therefore, the effect of 4°C refrigeration also has a positive impact on post-thaw samples, allowing a lower decline in buck epididymal sperm quality through the different post-mortem time of storage.

In conclusion, despite the reduction in sperm characteristics during storage, epididymal sperm of goat species (Capra hircus) , recovered post-mortem and stored at refrigeration temperature shown very good motility, viability, HOStest, intact acrosome and abnormality spermatozoa even after 24-48 h post-mortem. By using this protocol, good epididymal semen quality, can be easily achieved at reduced costs, improving the use of this source of germ plasm in gene banking.

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References

1. - ABDEL KHALEK A.E., EL HARAIRY M.A., SHAMIAH SH.M., ABU EL HAMD M.A., KHALIL W.A.: Some physical characteristics and response to hypo-osmolarity levels of epididymal camel spermatozoa stored at 25° C or 5° C. *J. Agric. Sci. Mansoura Univ.,* 2009, **34**, 8657-8666.
2. - ABU A.H., KISANI A.I., AHEMEN T.: Evaluation of sperm recovered after slaughter from cauda epididymides of red Sokoto bucks. *Vet. World,* 2016, **9,** 1440-1444.
3. - AMANN R.P., SEIDEL GE J.R., MORTIMER R.G.: Fertilizing potential in vitro of semen from young beef bulls containing a high or low percentage of sperm with a proximal droplet. *Theriogenology*, 2000, **54**, 1499–1515.
4. - AMANN R.P., ALMQUIST. J.O.: Reproductive capacity of dairy bulls VIII. Direct and indirect measurement of testicular sperm production. *J. Dairy Sci*., 1962, **45**, 774-781.
5. - BAGIROV V., NASIBOV S.H., KLENOVITSKII P.: Animal gene pool preservation and conservation. *Russ. Agr. Sci*., 2009, **35**, 112–115.
6. - BENHENIA K., LAMARA A., FATMI S., IGUER-OUADA M.: Effect of cyclodextrins,cholesterol and vitamin E and their complexation on cryopreserved epididymal ram semen. *Small. Rumin. Res***.,** 2016, **141**, 29–35.
7. - BERTOL M.A.F., WEISS R.R., THOMAZ-SOCCOL V., KOZICKI L.E., FUJITA A.S., AZEVEDO DE ABREU R., GREEN K.T.: Viability of bull spermatozoa collected from the epididymis stored at 18-20°C. *Braz. Arch. Biol. Technol*., 2013, 56, 777-783.
8. - BISSET C., BERNARD R.T.F.: The effect of prolonged cold storage of eland (Taurotragus oryx) cauda epididymides on the spermatozoa: possible implications for the conservation of biodiversity. *Theriogenology*, 2005, **63**, 1592–1604.
9. - BLASH S., MELICAN D., GAVIN W.: Cryopreservation of epididymal sperm obtained at necroscopsy from goats. *Theriogenology*, 2000, **54**, 899–905.
10. - BUCAK M.N., TUNCER P.B., SARIÖZKAN S., ULUTAS P.A.: Comparison of the effects of glutamine and an amino acid solution on post-thawed ram sperm parameters, lipid peroxidation and anti-oxidant activities. *Small Rumin. Res*., 2009, **81**, 13–17.
11. - CARY JA., MADILL S., FARNSWORTH K., HAYNA J.T., DUOOS L., FAHNING ML. : A comparison of epididymal sperm and electrotechnical collection in stallions. *Can. Vet. J*., 2004, **45**, 35–41.
12. - CHATIZAA F.P., BARTELSB P., NEDAMBALEC T.L.,WAGENAARA G.M.: Computer assisted sperm analysis of motility patterns of postthawed epididymal spermatozoa of springbok (Antidorcas marsupialis), impala (Aepyceros melampus), and blesbok (Damaliscus dorcus phillipsi) incubated under conditions supporting domestic cattle in vitro fertilization. *Theriogenology*, 2012, **78**, 402–414.
13. - CHAVEIRO A., CERQUEIRA C., SILVA J., FRANCO J., MOREIRA DA SILVA F.: Evaluation of frozen thawed cauda epididymal sperms and *in vitro* fertilizing potential of bovine sperm collected from the cauda epididymal. *Iran. J. Vet. Res., 2015,* **16,**188-193.
14. - DONG Q., RODENBURG S.E., HUANG C., VANDEVOORD C.A.: Cryopreservation of Rhesus monkey (Macaca mulatta) epididymal spermatozoa before and after refrigerated storage. *J. Androl*., 2008, **29,** 283–293.
15. - EHLING C., RATH D., STRUCKMANN C., FRENZEL A., SCHINDLER L., NIEMANN H.: Utilization of frozen–thawed epididymal ram semen to preserve genetic diversity in Scrapie susceptible sheep breeds. *Theriogenology,* 2006, **66**, 2160–2164.
16. - EVANS G., MAXWELL W.M.C.: Handling and examination of semen. In: W.M.C. MAXWELL (éd.). Salamon's Artificial Insemination of Sheep and Goats. *Butterworts*, Sydney, 1987, pp. 93-106.
17. - FARRELL P.B., PRESICCE G.A., BROCKETT C.C., FOOTE R.H.: Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. *Theriogenology.,* 2000, **49**, 871–879.
18. - FERNÁNDEZ-SANTOS M.R., SOLER A.J., RAMÓN M., ROS-SANTAELLA J.L., MAROTO MORALES A.: Effect of post-mortem time on post-thaw characteristics of Spanish ibex (Capra pyrenaica) spermatozoa. *Anim. Reprod. Sci.,* 2011, **129**, 56– 66.

19. - FOOTE R.H.: Fertilizing ability of epididymal sperm from dead animals. *J. Androl.,* 2002*,* **23,** 839-844.
20. - GARDE J., ORTIZ N., GARCIA A., GALLEGO L., LANDETE, C.T.,LOPEZ A.: Post-mortem assessment of sperm characteristics of the red deer during the breeding season. *Arch. Androl*., 1998, **41,** 195-202.
21. - GHOSH P.R., SANYAL S., BANDOPADHYA S., BASU S.: The distribution of carnitine and sialic acid in different parts of epididymis of Black Bengal goat (*Capra hircus*). *Indian J. Anim. Reprod., 1993,* **14,** 41.
22. - GUERIN Y., LOCATELLI Y. , COMIZOLLI P., MAUGET R .,MERMILLOD P., XAVIER X., LEGENDRE., GATTI JL., DACHEUX JL. : Conservation and use epididymal sperm sheep and deer in artificial insemination and in vitro fertilization. *Les actes du BRG*., 2003, **4**, 173-183.
23. - HERRICK S.B., SCHWEISSINGER D.L., KIM S.W., BAYAN K.R., MANN S., CARDULLO R.A.: The acrosomal vesicle of mouse sperm is a calcium store. *J. Cell. Physiol*., 2005, **202**, 663–671.
24. - JOHNSTON S.D., LEVER J., MCLEOD R., QUALISCHEFSKI E., BRABAZON S., WALTON S.,COLLINS S.N. :Extension, osmotic tolerance and cryopreservation of saltwater crocodile (Crocodylus porosus) spermatozoa. *Aquaculture,* 2014*,* **426–427**, 213–221.
25. - KAABI M., PAZ P., ALVAREZ M., ANEL E., BOIXO J.C., ROUISSI H., HERRAEZ P., ANEL.:Effect of epididymis handling conditions on the quality of ram spermatozoa recovered post-mortem. *Theriogenology, 2003,* **60**, 1249–1259.
26. - KARJA N.W.K., RESPATY E.M.A., NURAINI I., PRIHATNO S.A.,GUSTARI S.: Characteristic of frozen-thawed epididymal spermatozoa and refrigerated storage of ram spermatozoa. *J. Indonesian Trop. Anim. Agric*., 2010, **35,** 63-67.
27. - KATHIRAVAN P., KALATHARAN J., EDWIN M.J.,VEERAPANDIAN C.: Computer automated motion analysis of crossbred bull spermatozoa and its relationship with in vitro fertility in zona-free hamster oocytes. *Anim. Reprod. Sci*., 2008, **104**, 9–17.
28. - KIKUCHI K., NAGAI J., KASHIWAZAKI N., IKEDA H., NOGUCHI J.O., SHIMADA A.: Cryopreservation and ensuing *in vitro* fertilization ability of boar spermatozoa from epididymides stored at 5°C. *Theriogenology,* 1998*,* **50**, 615-623.
29. - KILLIAN I., LUBBE K., BARTELS P., FRIEDMANN Y., DENNISTON R.S.: Evaluating epididymal sperm of African wild ruminants: longevity when stored at 4°C and viability following cryopreservation. *Theriogenology*, 2000, **53**, 336.
30. - **30.** - KISHIKAWA H., KAWA H., TATENO H., YANAGIMACHI R.: Fertility of mouse spermatozoa retrieved from cadavers and maintained at 4°C. *J. Reprod. Fertil., 1999,* **116**, 217-222.
31. - LEON QUINTO T., SIMON MA., CADENAS R., JONES J., MARTINEZ HERNANDEZ F., MORENO JM., VARGAS A., MARTINEZ F., SORIA B.: Developing biological resource banks as a supporting tool for wildlife reproduction and conservation. *Anim Reprod Sci*., 2009, **112**, 347–361.
32. - LIMA I.C.S., ANDRADE I.R.A., AGUIAR G.V., SILVA M.M., CATUNDA A.G.V., MARTINS G.A., GADELHA C.R.F., CAMPOS A.C.N.:*In vitro* evaluation of goat cauda epididymal sperm, cooled in different extenders at 4°C. *Arch. Zootec.,* 2013*,* **62**, 429-437.
33. - LONE F.A., ISLAM R., KHAN M.Z., SOLI K.A.: Effect of transportation temperature on the quality of caudal epididymal spermatozoa of ram. *Anim. Reprod. Sci.,* 2011*,* **123,** 54-59.
34. - MALO AF., ROLDAN ERS GUARD J., SOLER AJ., GOMENDIO M.: Antlers honestly advertise sperm output and quality. *Proc. R. Soc. B*., 2005, **272**, 149-157.
35. - MARTINEZ PASTOR F., GARCIAMACIAS V., ALVAREZ M., CHAMORRO C., HERRAEZ P.,DE PAZ P., ANEL L.: Comparison of two methods for obtaining spermatozoa from the cauda epididymis of Iberian red deer. *Theriogenology.,* 2006*,* **65**, 471-485.
36. - MARTINEZ PASTORA F., GUERRAB C., KAABIB M., DIAZA A.R., ANEL E., HERRAEZ P.P.,ANEL DE PAZ L.: Decay of sperm obtained from epididymes of wild ruminants depending on postmortem time. *Theriogenology.,* 2005, **63**, 24-40.
37. - MARTINS C.F., DRIESSEN K., COSTA P.M., CARVALHONETO J.O., DE SOUSA R.V., RUMPF R., DODEC, M.N.: Recovery, cryopreservation and fertilization potential of bovine spermatozoa obtained from epididymides stored at 5°C by different periods of time. *Anim Reprod. Sci.,* 2009*,* **116**, 50-57.
38. - MARTINS C.F., RUMPF R., PEREIRA D.C., DODE M.N.: Cryopreservation of epididymal bovine spermatozoa from dead animals and its uses in vitro embryo production. Short communication. *Anim. Reprod. Sci.,* 2007*,* **101**, 326–331.
39. - NASIR M., NJIDDA A.A., HASSAN A.M.: Testicular histometry, gonadal and extra gonadal sperm reserve of red Sokoto bucks fed cotton seed cake. *J. Sci*., 2014, **4**, 227-232.
40. - O'BRIEN J.K., HOLLINSHEAD F.K., EVANS K.M., EVANS G., MAXWELL W.M.C. : Flow cytometric sorting of frozen–thawed spermatozoa in sheep and non-human primates. *Reprod. Fertil. Dev.,* 2003*,* **15**, 367–375.
41. - ROBAIRE B., HERMO L.: Efferent ducts, epididymis and vas deferens: structure, function and regulation *In* : E Knobil and JD Neill (éd.):The physiology of reproduction, Raven Press, New York, 1994, 999–1080.
42. - SALAMON S., MAXWEEL W.M.: Storage of ram semen. *Anim. Reprod. Sci*., 2000, **62**, 77–111.
43. - SANTIAGO MORENO J., ASTORGA RJ., LUQUE I., COLOMA AM., TOLEDANO DIAZ A., PULIDOPASTOR A., GOMEZ GUILLAMON F., SALAS VEGA R., LOPEZ SEBASTIAN A .: Influence of recovery method and microbial contamination on the response to freezing–thawing in ibex (Capra pyrenaica) epididymal spermatozoa. *Cryobiology,* 2009*, **59***, 357–362.

44. - SANTIAGO MORENO J., TOLEDANO DIAZ A., PULIDO PASTOR A., GOMEZ BRUNET A ., LOPEZ SEBASTIAN A.: Birth of live Spanish ibex (Capra pyrenaica hispanica) derived from artificial insemination with epididymal spermatozoa retrieved after death. *Theriogenology,* 2006*,* **66**, 283–291.

45. - SOLER A.J., PEREZ-GUZMAN M.D., GARDE J.J.: Storage of red deer epididymides for four days at 5°C: effects on sperm motility, viability, and morphological integrity. *J. Exp. Zoolog. Part A Comp. Exp. Biol*., 2003, **295**, 188–199.

46. - SWAIN D.K., TARAI A., MOHAPATRA A .P. K.,KUNDU A. K. **:** Evaluation of longevity of canine epididymal spermatozoa stored at 4°C. *Indian. J. Vet. Res.,* 2011*,* **20,** 16–20.

47. - TAJIK P., HASSAN-NEJAD LAMSOO M.R.: Assessment of epididymal sperm obtained from dromedary camel. *Iran. J. Vet. Res*., 2008, **9**, 46-50.

48. - TAMAYO-CANULA J.B., ALVAREZA M.B., LÓPEZ-URUENAA E.B., NICOLASA M., MARTINEZ-PASTORA B., ANELA F., ANELA C., PAZA C.: Undiluted or extended storage of ram epididymal spermatozoa as alternatives to refrigerating the whole epididymis. *J. Anim. Reprod. Sci.,* 2011*,* ***126***, 76–82.

49. - TARAI A., SWAIN D.K, MOHAPATRA A.P.K., KUNDU A.K.: *In vitro* study of longevity of canine epididymal sperm at 25°C. *Indian. Vet. J., 2010,* **87,** 1198–1200.

50. - TURRI F., MADEDDU M., GLIOZZI T.M., GANDINI G.,PIZZI F.: Influence of recovery methods and extenders on bull epididymal spermatozoa quality. *Reprod. Domest. Anim.*, 2012, **47**, 712–717.

51. - TURRI F., MADEDDU M., GLIOZZI T.M, GANDINI G., PIZZI F.: Effect of testicle post mortem storage on goat frozen-thawed epididymal sperm quality as a tool to improve genebanking in local breeds. *Animal*, 2013, **8**, 440–447.

52. - VERDIER Y **.:** Selection, identification and partial characterization of sperm antigens fox(Vulpes vulpes) for use in a vaccine contractive , Nancy1 University Henri Poincaré medecine. These for the degree of Doctor of the University Nancy 1; Discipline: Biological and medical engineering, 2002, 64.

53. - WEISS R.R., MURADÁS P.R., GRANEMAN L.C., MEIRA C.: Freezing sperm from cauda epididymis of castrated stallions. *Anim. Reprod. Sci*., 2008, **107**, 302-360.

54. - YOUNG C., CURTIS M., RAVIDA N., MAZOTTI F., DURRANT B.: Development of a sperm cryopreservation protocol for the Argentine black and white tegu (Tupinambis merianae). *Reprod. Fertil. Dev*., 2013, **26**, 168–169.