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Viability of ovine spermatozoa collected from epididymides stored at 18°-25°C for 48 hours post mortem

[*Viabilidade dos espermatozoides ovinos coletados de epidídimos armazenados a 18°-25°C até 48 horas após a morte*]

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ABSTRACT

The objectives of this study were to verify the time during which viable ovine spermatozoa could be recovered from the cauda epididymis kept at ambient temperature (18-25°C). Sperm collected in an artificial vagina (AV) were used as control. Spermatozoa samples were collected with an AV and from epididymis at 0 (G0), 6 (G6), 12 (G12), 24 (G24), and 48 (G48) hours *post mortem*. Total motility (TM), progressive motility (PM), hypo-osmotic membrane integrity test (HOST) and morphological changes were assessed. TM decreased (P<0.05) from 24 hours *post mortem (*70.0±1.9%) compared to AV (86.4±1.0%). PM decreased (P<0.05) from 12 hours after death (31.3±4.0%) compared to AV group (73.2±1.4%). The percentage of viable cells in HOST decreased (P<0.05) in the G48 (60.0±8.9%). Spermatozoa recovery was lower (P<0.05) 48 hours after death (2064.2±230.7 x 10^6 spermatozoa) compared to G0(2623.6±288.4 x 10^6 spermatozoa). In conclusion, under the conditions of this study, it would be possible to use epididymal spermatozoa recovered up to 24 hours after death for artificial insemination or *in vitro* fertilization; however, fertility trials are necessary to prove this hypothesis.

Keywords: ram, epididymis, room temperature

RESUMO

Os objetivos deste estudo foram avaliar o período pelo qual era possível recuperar espermatozoides ovinos viáveis da cauda de epidídimos mantidos em temperatura ambiente (18-25°C). O sêmen coletado em vagina artificial (AV) foi utilizado como controle. Os espermatozoides foram coletados dos epidídimos à zero hora (G0), às seis (G6), 12 (G12), 24 (G24) e 48 (G48) horas post mortem. *A motilidade total (TM), a motilidade progressiva (PM), a integridade de membrana plasmática em solução hiposmótica (HOST) e a morfologia espermática foram avaliadas. A TM diminuiu (P<0,05) a partir de 24 horas após a morte (70,0±1,9%) comparado ao sêmen coletado em AV (86,4±1,0%). A PM diminuiu (P<0,05) a partir de 12 horas após a morte (31,3±4,0%) comparado ao grupo AV (73,2±1,4%). A porcentagem de espermatozoides viáveis no HOST diminuiu (P<0,05) no G48 (60,0±8,9%). A recuperação espermática foi menor (P<0,05) 48 horas após a morte (2064,2±498,1 x 10^6 espermatozoides) comparado ao G0 (2298,4±288,4 x 10^6 espermatozoides). Em conclusão, nas condições deste estudo, é possível utilizar espermatozoides epididimários recuperados até 24 horas após a morte para inseminação artificial ou fertilização* in vitro, *porém testes de fertilidade são necessários para comprovar essa hipótese.*

Palavras-chave: carneiro, epidídimo, temperatura ambiente

INTRODUCTION

In livestock breeding, the preservation of gametes after the death of a genetically valuable animal can be important. Recovery of spermatozoa after death may offer the last chance to preserve genetic material from breeding animals which have died unexpectedly

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(García-Álvarez *et al*., 2009) and is important in formation of germplasm banks for endangered species (Martinez-Pastor *et al*., 2005a).

The epithelium of the epididymal tubules has absorptive and secretory activities that promote changes in sperm cells, culminating in maturation, during transit of sperm cells through epididymus (Vernet *et al*., 2004). It has been shown that viable sperm cells can be recovered from the cauda epididymis in bulls (Alapati *et al*., 2009; Ardon e Suarez, 2013), stallions (Weiss *et al*., 2008; Olaciregui *et al*., 2014), dogs (Hishinuma e Sekine, 2004) and rams (Kaabi *et al*., 2003). The use of genetic material obtained from the epididymis is particularly important following the sudden death of breeding livestock or in the preservation of endangered species. There is a consensus among the authors that when epididymis is refrigerated at 5°C, before spermatozoa recovery, the viability period increases (Fernández-Santos *et al*., 2009; Maroto-Morales *et al*., 2010; O'Hara *et al*., 2010; Nichi *et al*., 2016). In sheep, when the epididymis is refrigerated, the recovered spermatozoa do not present significant changes up to 24 hours *post mortem* (Kaabi *et al*., 2003; Tamayo-Canul *et al*., 2011). Few studies have reported the effects on spermatozoa quality when the epididymis is kept at ambient temperature after death, as occurs in real life sudden death scenarios. We hypothesized that spermatozoa viability would decrease as death time increased.

The objective of this study was to evaluate the duration of viability of ovine spermatozoa stored in the cauda epididymis at room temperature (18-25°C) up to 48 hours *post mortem* compared to semen collected in an artificial vagina.

MATERIAL AND METHODS

The project was approved by Animal Use Ethics Committee of Agricultural Science Campus of Federal University of the State of Paraná, protocol number 052/2016. 10 rams, aged between 1 and 2 years, were used. The animals were housed in an intensive system, with access to native grass pasture during the day, and were confined in the evening. They received food supplementation with corn, soybean meal, and mineral mix. Water was supplied *ad libitum.*

Semen was collected with an artificial vagina. A sheep was used as a dummy. The artificial vagina was filled with water at 42 to 45°C. Collections were performed twice a week for two months (January and February) in transition time for breeding season. After this period same rams were slaughtered. The testes were taken to the laboratory in Styrofoam box at room temperature (18-25°C). The transport time of the testicles to the laboratory did not exceed one hour.

The epididymides were randomly distributed into five groups corresponding to the time they had been kept at room temperature i.e. zero hours (G0), six hours (G6), twelve hours (G12), twenty-four hours (G24) and forty-eight hours (G48). Each group consisted of four anatomical specimens from four different rams.

After harvesting using an artificial vagina (AV) or from cauda epididymis spermatozoa were analyzed.

Testes and epididymides were washed with 0.09% sodium chloride solution warmed to 35°C. The tail of epididymis was divided into warmed petri dishes and maintained at 35°C. Using anatomical tweezers, scissors and a scalpel blade, superficial blood vessels were dissected to minimize blood contamination of spermatozoa. The cauda epididymis was sectioned and light pressure was applied to expel spermatozoa from the tubules (Kaabi *et al*., 2003). The cauda epididymis was then lavaged with 2mL of extender (200mL distilled water, 1.4g glycine, 2.97g sodium citrate, 3g fructose, 0.004g amikacin, 15mL skim milk, 5g egg yolk and 4.6 distilled water) warmed to 35°C. Petri plates were angled so that the extender washed the spermatozoa to accumulate at one end of the plate. After five minutes a pipette was used to collect the diluted spermatozoa from the Petri dish and the sample was placed in a conical tube (Falcon BD)(Martinez-Pastor *et al*., 2006).

The volume of ejaculate collected with the artificial vagina was measured in a graduated glass collecting cup. Diluted spermatozoa volume collected from the cauda epididymis was measured in a conical bottomed tube (BD Falcon).

Total motility (TM) and progressive motility (PM) were evaluated by optical microscopy (Coleman, N 107, Brazil). A drop of spermatozoa diluted to concentration of 400 x

10^6 sperm per mL was deposited between slide and cover slip warmed at 37°C and examined at 400x magnification. All the analysis was performed by the same person.

Spermatozoa concentration was determined by cell counting in a Neubauer chamber. The dilution of AV semen was 1: 400 and the spermatozoa collected from epididymis was 1: 200.

To perform the hypo-osmotic swelling test (HOST), 10µL of spermatozoa was diluted in 50µL water (Hishinuma e Sekine, 2004) heated to 37°C. After incubation for 60 minutes in a water bath at 37°C, 10µL of this suspension was placed on a slide and covered with a cover slip and evaluated in an optical microscope at 400x magnification. Two hundred cells per sample were evaluated, sperm with swollen or coiled tails were considered to have functional membranes and were classified as intact.

Sperm morphology was assessed by a differential count of 200 cells on slides prepared with swabs of diluted spermatozoa. Slides were stained by immersion in saturated Red Congo stain per one minute and Gentian Violet 0.5% per thirty seconds, according to the method of Cerovsky (1976).

Data were analyzed in a randomized block design with six treatments (AV, G0, G6, G12, G24 and G48) and ten (AV) or four (epididymis per group) blocks. The data used in the analyses were the average of the values obtained from the duplicate of each epidydimal or artificial vagina sample. The variables showed normal distribution and variance homogeneity according to the Kolmorogov-Smirnov method. T-test were used to compare AV and G0 groups. All groups were analyzed by ANOVA followed by the Tukey test. Statistical testes were implemented in the Action Stat software, version 3.1.43.724.694 for Windows. The level of significance was $P<0.05$.

RESULTS

The total motility of spermatozoa collected from the cauda epididymis decreased ($P<0.05$) after 24 hours after death. PM of epidydimal spermatozoa decreased ($P<0.05$) after 12 hours *post mortem*. Percentage of intact sperm in HOST was only different ($P<0.05$) in the G48 when compared to sperm collected in the artificial vagina or any other group. There was no difference in the percentage of cells with acrosomal defects ($P>0.05$) in any groups, but the G24 had higher ($P<0.05$) concentrations of tail defects when compared to other groups. Detailed results of TM, PM, HOST and sperm morphology are shown in Table 1.

Table 1. Mean ± standard error of total motility (TM%), progressive motility (PM%), plasma membrane integrity (HOST% intact), acrosomal defects (%) and tail defects (%) in the semen collected with the artificial vagina (AV) and collected from the epididymides at 0(G0), 6(G6), 12(G12), 24(G24) and 48(G48) hours after death

Group	TM (%)	PM (%)	HOST (% intact)	Acrossomal defects (%)	Tail defects (%)
AV	86.4±1.0a	73.2±1.4a	90.4±1.4a	2.0±0.5a	13.8±1.9b
G0	79. 0±4.9ab	62.0±4.9a	82.0±0.7a	1.0±0.5a	10.0±1.8b
G6	81.3±4.0ab	65.0±3.3a	77.1±2.0a	1.8±0.6a	16.6±2.2ab
G12	72.5±1.6ab	31.3±4.0ab	79.1±5.2a	2.0±1.2a	23.5±5.9ab
G24	70.0±1.9b	26.3±3.2ab	77.4±1.2a	4.0±1.4a	34.4±7.4a
G48	39.0±8.6c	14.0±4.5b	60.0±8.9b	4.1±1.4a	27.6±7.7ab

Different letters in the same column are significantly different ($P<0.05$).

Mean ± standard error for concentration ($x10^6$ spermatozoa/mL), volume (mL) and total numbers of sperm cells ($x10^6$ total spermatozoa) of semen and spermatozoa collected from the epididymides at 0, 6, 12, 24 and 48 hours after death are shown in Table 2. Concentration, volume, and total numbers of sperm cells decreased ($P<0.05$) after 48 hours from death. The volume and total sperm number were higher in the groups where spermatozoa were collected from the epididymides in groups G0, G6, G12 and G24, as compared to collection in the artificial vagina.

Table 2. Mean ± standard error of concentration (x10^6 spermatozoa/ml), volume (mL) total number of sperm cells (total cells x 10^6) in semen collected in the artificial vagina (AV) and collected from the epididymides at 0(G0), 6(G6), 12(G12), 24(G24), and 48(G48) hours after death

Group	Concentration (x10^6 spermatozoa/mL)	Volume	Total sperm cells (total spermatozoa x 10^6)
AV	2571.8±163.37a	1.1±0.04b*	2623.6±237.9b*
G0	2298.4±288.4ab	3.1±0.2a*	6936.6±550.1a*
G6	1763.8±380.0ab	2.6±0.1a	4336.5±858.1ab
G12	2137.5±114.8ab	3.0±0.1a	6349.3±357.7a
G24	1437.5±247.8ab	2.8±0.2a	4239.8±883.4ab
G48	1036.0±230.7b	1.9±0.2b	2064.2±498.1b

Different letters in the same column are significantly different (P<0.05) *Differed in T-test (P<0.05).

DISCUSSION

Collection of viable spermatozoa after death has been described in several species (Kaabi *et al*., 2003; Tittarelli *et al*., 2006; Weiss *et al*., 2008) but few studies have evaluated the time during which sperm cells remain viable when exposed to environment temperature. Results found in this study are in line with results of other authors who evaluated epididymal spermatozoa in sheep (Kaabi *et al.*, 2003; Abella *et al.* 2015) and wild ruminants (Martinez-Pastor *et al.*, 2005a).

Spermatozoa recovered from epididymides have the same fertility and viability as ejaculated semen (Álvarez *et al*., 2012). In this work, we were able to confirm that ovine spermatozoa recovered from epididymis immediately after death have similar TM, PM, membrane integrity and morphological defects when compared to semen collected in an artificial vagina. Furthermore, total sperm cells recovered in G0 were higher than AV. This result was expected since epididymis cauda have the spermatozoa storage function. *Post mortem* spermatozoa recovery might be an ultimate source of spermatozoa for genetically valuable animals untrained for collection and wild animals when electroejaculation is not possible(Kaabi *et al*., 2003; Abella *et al*., 2015).

Spermatozoa recovered from cauda epididymides are affected by the time after death, especially when epididymides are kept at room temperature. This depreciation is a result of spermatozoa aging and epididymides tissue degeneration and decomposition process (Martinez-Pastor *et al*., 2005b). Studies in rats revealed that degenerative testicular changes start approximately six hours after death, but in the epididymis, these alterations only begin 12 hours after death (Songsasen *et al*., 1998). Under room temperature conditions, total motility and progressive motility decreases up to 24 hours post mortem in sheep (Kaabi *et al*., 2003; Bergstein-Galan *et al*., 2017). Total motility is the spermatic parameter most affected by time after death (Monteiro *et al*., 2013). In this work, the TM decreased 24 hours after death and HOST (% intact) decrease 48 hours after death. Sperm morphology was not different between groups. The findings of our study corroborate those of Kaabi *et al*. (2003) who also found decreased (P<0.05) TM in sheep, after 24 hours, and reduced percentage of viable cells in hypo-osmotic test after 48 hours, after the death of the animal, when the epididymides were kept at room temperature (22°C).

Some studies report that TM or viability of spermatozoa declined 48 or more hours after the death of the animal (ram: (Tamayo-Canul *et al*., 2011), bull: (Martins *et al*., 2009)) although, these authors worked with epididymides refrigerated at 4 to 5°C, demonstrating that lower storage temperature can extend sperm survival. However, a clinical scenario is that an animal of husbandry interest, or of an endangered species, may be found dead and refrigeration will not have occurred. For this reason, we studied the effect of storage at room temperature (18-25°C) on viability of ovine epididymal spermatozoa. Bergstein-Galan *et al*. (2017) reported that it is possible to cryopreserve spermatozoa from the cauda epididymis that have been exposed to room temperature for up to 24h post mortem without loss of viability or fertility. Based on our results we hypotheses that ovine epididymal spermatozoa exposed to ambient temperature could yield good fertility up to 24 hours *post mortem*, however efforts should be focused on

preservation (refrigeration or cryopreservation) of epididymal spermatozoa as soon as possible.

Martinez-Pastor *et al*. (2006) compared a slicing method with retrograde flushing for recovery of red deer spermatozoa, these authors found no difference between the methods in sperm recovery and motility immediately after collection but identified better motility, acrosome integrity and viability in sperm collected by retrograde flushing when spermatozoa were diluted and cooled for two hours. In our preliminary work, we identified limitations of the retrograde flushing method on ram cauda epididymis. The bore of the epididymal tubules was very narrow and dissection of the connective tissue sometimes resulted in rupture of the tubules making it impossible to flush the entire organ. The average number of spermatozoa recovered in this study was similar to other authors who used the same spermatozoa collection method from ovine cauda epididymis (Kaabi *et al*., 2003; Lone *et al*., 2011). Sperm recovery decreased beyond 48 hours following death in accordance with findings of other studies in sheep (Lone *et al*., 2011), horses (Neild *et al*., 2006) and deer (Martinez-Pastor *et al*., 2005). Over time, total motility and sperm viability reduces the effectiveness of sperm recovery by slicing method which relies upon sperm migration from the lumen of epididymal duct into the dilution medium.

The period after death has a negative effect on the gestation rate. According to Abella *et al.* (2015), the loss in gestation rate per day of storage corresponds to 7% in Corridale ewes and 12% in Ile de France ewes when epididymal spermatozoa were refrigerated to 4°C. Bergstein-Galan *et al.* (2017) did not found fertility loss when ovine epididymal spermatozoa were frozen up to 24 hours after death. Kaabi *et al.* (2003) reported a decrease ($P<0.05$) in the rate of oocytes cleavage fertilized by thawed spermatozoa recovered from ovine epididymis cauda 48 hours after death (38%), when compared to immediately after death epidydimal samples (53%). However, these authors used preserved (refrigeration or cryopreservation) epididymal spermatozoa, the injuries caused by preservation processes may have affected the results. Unfortunately, no fertility tests were performed in the present study.

CONCLUSION

It is possible to recover sperm with motility and viability from ovine cauda epididymis stored at room temperature for up to 48 hours after death, however 24 hours after death there is a decrease in spermatic quality and 48 hours after death there is a reduction in spermatozoa yield. In conclusion, we believe that spermatozoa recovered 24 hours after death presented parameters consistent with sperm samples with acceptable fertility however further studies on the fertilizing capacity and the effect of time and temperature of epididymal spermatozoa storage, must be carried out in order to confirm this possibility.

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