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Effect of Epididymis Handling Conditions on the Quality of Ram Spermatozoa Recovered Post-Mortem

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Abstract: The present research studied the effect of the interval between animal's death and sperm recovery (0, 24 or 48 h) on the qnality and freezability of ram spermatozoa from cauda epididymidis. Storage temperature of epididymes (room temperature or 5°C) was also analyzed. Spermatozoa were diluted with Tes-Tris-Fructose solution supplemented with egg yolk (10%) and glycerol (4%) and frozen using a programmable bio-freezer (-20°C min^{-1}). Pre-freeze and post-thaw sperm samples showed viable spermatozoa up to 48 h after the animal's death, although their quality declined significantly as post-mortem storage time increased. Epididymes sperm stored at 5°C showed better motility and a lower percentage of abnormal forms than epididymes stored at room temperature after 24 and 48 h. The fertilizing ability of cauda epididymis ram spermatozoa obtained at 0 and 24 h after the animal's death is similar to that of ejaculated spermatozoa. Therefore, a good protocol for post-mortem semen collection in rams when epididymal spermatozoa cannot be collected immediately is to preserve the epididymes at 5°C and process the samples in the first 24 h after the animal's death.

Key words: Mehraban, post-mortem, ram, spermatozoa recovered

INTRODUCTION

The unexpected death of animals of high genetic value or zoological interest, as well as the difficulty in collecting semen from wild species, is a handicap to the application of assisted reproduction techniques for the preservation of biodiversity. The recovery and freezing of viable sperm from the epididymes of dead animals (post-mortem recovery) is an interesting option for preserving male gametes and thus for maintaining germplasm banks. There are many studies on semen collection from the cauda epididymis of several species (Fournier-Delpech *et al*., 2001; Lambrechts *et al*., 2006), but there are no data available on the effects of different storing conditions on ram epididymal spermatozoa. The conditions (time and temperature) under which epididymis is handled could cause important changes in the viability of sperm samples. It should be kept in mind that animals die unexpectedly and far away from the lab where the sperm sample could be properly processed and stored. In breeding rams, Aguado *et al*. (2005) reported the preservation of ram sperm stored at room temperature for 0, 3, 6, 9, 12 and 24 h. These authors found semen of better quality, both before and after freezing, when it was collected in the first 3 h after death. Under similar conditions, Garde *et al*. (2004) found no remarkable variations in the fertilizing ability of ram epididymal samples processed in the first 24 h after death, noticing a marked diminution in sperm viability for longer periods of time. In red deer (*Cervus elaphus*) and moufflon (*Ovis musimon*), Garde *et al*. (2005) concluded that viability and *in vitro* fertility (percentage of penetration in hamster oocytes) of sperm decreased when

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the time between the animal's death and the moment of semen collection increased (up to 40 h at ambient temperature). They pointed out that there were appreciable differences between species with regard to the hamster oocyte penetration test, where a particularly marked decrease was observed in the case of the red deer.

These studies did not analyze the effect of storage temperature on epididymal semen quality. However, Kikuchi *et al*. (2004) in pig and Kishikawa *et al*. (2001) in mice suggested that when valuable male animals die unexpectedly and sperm cryopreservation is not possible immediately, temporal storage of epididymides at 40°C may help to preserve the genome of individuals. In order to establish a model for post-mortem sperm recovery in ram, we studied the effect of the interval between death and sperm collection (0, 24 or 48 h) as well as the storage temperature of epididymes (room temperature or 5°C) on the quality and fertilizing ability (evaluated by *in vitro* fertilization) of pre-freezing and post-thawed sperm from the cauda epididymidis.

MATERIALS AND METHODS

Experimental Design

This experiment was conducted in spring, 2007 at Ziaran Slaughter House and Physiology Laboratory of Animal Science Department, College of Agriculture, Karaj, Tehran University in Iran. Testicles from 50 Mehraban breed rams were collected at an abattoir. One testicle from each pair was transported at room temperature (22°C) and semen collection was carried out in the first 2 h after the slaughter of the ram (control group, CG). The other testicle was transported either at room temperature (22°C, group R) or at 5°C (group C). Testicles were stored prior to sperm recovery for 24 h (groups R24 and C24) or 48 h (groups R48 and C48).

Collection and Quality Evaluation of Sperm

After isolation, the epididymis-testicle complexes were dissected into three parts: testicle, epididymis and cauda epididymis. Sperm was obtained by slicing the tissue of the cauda epididymis with a scalpel; the fluid was collected and its volume was estimated. To limit contamination, epididymis samples were carefully dissected free of blood clots and extraneous tissues. Care was taken not to cut blood vessels. Sperm concentration was determined using a haemocytometer. The percentage of Total Motility (TM) and Progressive Motility (PM) was determined microscopically, observing eight random fields in a flat drop (4 μL) of semen diluted in freezing extender (200x phase contrast; heating plate at 37.5°C). Acrosome integrity was determined using 5 μL of a semen sample fixed in 0.5 mL of glutaraldehyde fixative solution (GS glutaraldehyde at 2% in 100 mL aqueous solution with 2.9 g glucose monohydrate, 1 g sodium citrate tribasic dihydrate and 0.2 g sodium bicarbonate; Sigma, USA). A flat drop of fixed sample was placed on a microscope slide. For each group, two slides were observed by phase-contrast microscopy (600x), 200 spermatozoa were counted for each slide and percentage of spermatozoa with normal acrosome was noted.

The functioual integrity of the sperm plasma membrane was evaluated using the hypo osmotic swelling test (HOS test): 5 μL of semen were diluted in 0.5 μL of 100 mOsm kg^{-1} aqueous sodium citrate solution; after 18 min of incubation (at room temperature) samples were fixed with a drop of GS solution. Response to the test was quantified (percentage of spermatozoa swollen = percentage of positive endosmosis or % E+) with a phase-contrast microscope (400x).

Sperm morphology (percentage of Cytoplasmic Droplets (CD) and percentage of Total Abnormal Spermatozoa (TAS)) were determined from a sample of 5 μL of semen fixed in 0.5 mL of GS solution, using phase-contrast microscopy (600x). Sperm abnormalities were grouped according to the location

of the morphological anomaly (head, middle piece or flagellum) using criteria of sperm morphology established by WHO (2004). All the above mentioned parameters were calculated for pre-freezing and post-thawed semen. Recovery Rates (RR) for total motility, progressive motility, percentage of normal acrosomes and percentage of positive endosmosis were calculated, using the following formula:

$$RR = \frac{\text{Value after thawing}}{\text{Value before freezing}} \times 100$$

Freezing and Thawing of Sperm

Ten minutes after collection, semen was diluted 1:1 with Tes-Tris-Fructose extender (Sigma) containing 10% egg yolk and 4% glycerol. The diluted sample was chilled to 5°C (at a rate of -0.2°C min^{-1}) and further dilution with the same diluent's was carried out to yield a final concentration of 200×10^6 spermatozoa mL^{-1}. Diluted sperm was allowed to equilibrate for 2 h and was loaded into 0.25 mL straws. Finally, it was frozen in a programmable bio-freezer (Planner MRIII®) at a rate of -20°C min^{-1} to -100°C. Frozen samples were stored in liquid nitrogen until further use. Thawing was carried out in a water bath at 65°C for 6 sec.

***In vitro* Fertilization**

Briefly, visible follicles (3-7 mm in diameter) of slaughterhouse ovaries were aspirated into TCM-19 medium (Sigma) and complemented with gentamycine (0.4%; Sigma) and heparin (5 UI mL^{-1}; Sigma). Ouly oocytes with homogenous cytoplasm and a complete and compact cumulus cell investment were used. Oocyte maturation took place in TCM-199 medium supplemented with ovine 5 μg mL^{-1} FSH (Ovagen, ICP), sheep follicular fluid (10%) and gentamycine (0.4%), for 24 h at 38.5°C in a controlled atmosphere (5% CO_2) and humidity at saturation level. Six hundred selected oocytes were divided into six groups (four sets of 25 oocytes for each experimental group) for the IVF. These groups were used to compare the fertility of the five types of frozen epididymal semen corresponding to the experimental design and a control of frozen ejaculated ovine semen. Thawed sperm was rinsed and selected on a Percoll gradient (Percoll 45/90; Sigma) and the highly motile fraction was selected for IVF at a concentration of 10^6 cells mL^{-1}. Fertilization was carried out in a modified Brackett's defined medium (DM-H) buffered with 10 mM HEPES (Huneau and Crozet, 2001) and containing 20% (v/v) estrus sheep serum for 18 h at 38.5°C in a controlled atmosphere (5% CO_2) and humidity at saturation level. Embryos were then cultured in modified synthetic oviduct fluid (mSOF) according to Takahashi and First (2000) for 24 h at 38.5°C (5% CO_2, 5% O_2 and 90% N_2) and then the rate of embryo cleavage was determined.

Statistical Analysis

Sperm quality data were evaluated using General Linear Model (GLM) procedures of the SAS™ program to analyze variance components. Factors of variation were the type of post-mortem storage of epididymes and spermatozoa status (pre-freeze or post-thaw). Comparison of means was performed with Duncan's test ($p<0.05$). The rates of embryo cleavage were compared using the Chi-square test.

RESULTS

Table 1 shows the mean values of general parameters for the 100 testicles and epididymes processed in this study.

Table 1: General parameters of epididymal sperm recovery in this study

Parameters	Mean±SEM
Age (years)	4.06±0.12
Testicle weight (g)	191.11±4.9
Epididymis weight (g)	34.36±0.07
Cauda epididymis weight (g)	18.14±0.4
Spermatozoa/cauda epididymis ($\times 10^6$)	1439.00±85.6
Concentration ($\times 10^6$ cells mL^{-1})	3678.00±102.0
Sperm straws (50×10^6 spermatozoa/straw)/cauda epididymis	29.00±3.2

Table 2: Effect of post-mortem storage on epididymal rams spermatozoa motility (Mean±SEM)

	Percentage of total motility		Percentage of progressive motility	
Group	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
CG	$80.3\pm0.7^{a,\alpha}$	$69.8\pm1.0^{a,\beta}$	$68.4\pm1.0^{a,\alpha}$	$55.8\pm1.0^{a,\beta}$
C24	$78.4\pm1.3^{a,\alpha}$	$64.6\pm1.6^{a,\beta}$	$64.7\pm2.7^{a,\alpha}$	$48.8\pm2.3^{a,\beta}$
R24	$69.1\pm4.1^{b,\alpha}$	$52.3\pm5.1^{b,\beta}$	$46.5\pm6.7^{b,\alpha}$	$33.6\pm5.5^{b,\beta}$
C48	$64.4\pm2.7^{b,\alpha}$	$48.7\pm3.2^{b,\beta}$	$52.4\pm3.8^{b,\alpha}$	$30.7\pm2.3^{b,\beta}$
R48	$58.1\pm1.9^{c,\alpha}$	$38.1\pm3.6^{c,\beta}$	$21.4\pm1.1^{c,\alpha}$	$13.2\pm0.6^{c,\beta}$

Different superscripts (a, b, c) indicate a difference ($p<0.05$) among groups. Different superscripts (α, β) indicate a difference ($p<0.05$) among pre-freeze and post-thaw spermatozoa

Table 3: Effect of post-mortem storage on acrosomal status and HOS-test reactivity of epididymal ram spermatozoa (Mean±SEM)

	Percentage of normal acrosomes		Percentage of swollen spermatozoa (E+)	
Group	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
CG	$84.3\pm0.9^{a,\alpha}$	$62.7\pm1.2^{a,\beta}$	$84.2\pm0.6^{a,\alpha}$	$57.5\pm0.9^{a,\beta}$
C24	$80.2\pm1.1^{a,\alpha}$	$52.6\pm1.3^{b,\beta}$	$81.9\pm1.6^{a,\alpha}$	$54.4\pm2.7^{a,b,\beta}$
R24	$78.4\pm1.7^{a,\alpha}$	$55.4\pm3.5^{b,\beta}$	$83.3\pm1.1^{a,\alpha}$	$53.7\pm3.4^{a,b,\beta}$
C48	$68.5\pm5.5^{b,\alpha}$	$42.9\pm2.6^{c,\beta}$	$77.9\pm3.8^{b,\alpha}$	$51.4\pm3.0^{a,b,\beta}$
R48	$67.2\pm2.0^{b,\alpha}$	$38.7\pm3.9^{c,\beta}$	$73.0\pm2.9^{c,\alpha}$	$47.9\pm3.1^{b,\beta}$

Different superscripts (a, b, c) indicate a difference ($p<0.05$) among groups. Different superscripts (α, β) indicate a difference ($p<0.05$) among pre-freeze and post-thaw spermatozoa

Motility

No significant decrease was observed after 24 h at 5°C in comparison to the control values. Pre-freeze motility shows a significant decrease after 48 h (at both 5°C and room temperature) and 24 h at room temperature with regard to control and C24 groups (Table 2). The pre-freezing values of total and progressive motility of all experimental groups decrease significantly ($p<0.05$) after thawing.

Acrosomal Status and Cell Membrane Integrity

Epididymal spermatozoa stored for 24 h (C24 and R24) show no significant differences in pre-frozen percentages of normal acrosomes (NA) in comparison with the control group (Table 3). In contrast, this percentage decreases significantly ($p<0.05$) after 48 h of storage (C48 and R48). However, NA percentages of post-thaw samples show significant differences ($p<0.05$) amongst the three groups defined by storage time (0, 24 and 48 h). In these groups, acrosome status of post-thaw samples is not affected by the temperature at which the epididymes are stored.

Before freezing, the percentages of swollen spermatozoa in 48 h groups (R48 and C48) are significantly different ($p<0.05$) to those in the control and 24 h groups (R24 and C24). After thawing, these differences ($p<0.05$) are only detected between the control group and R48.

All groups show a significant decrease in the percentage of spermatozoa with normal acrosome after thawing. This effect of the freezing procedure is also observed in the percentage of swollen spermatozoa.

Abnormal Spermatozoa and Cytoplasmic Droplets

In pre-freezing sample, epididymes stored at room temperature show the percentage of total abnormal spermatozoa than the control group (Table 4). This increase is not observed when epididymides are stored at 5°C. After thawing, the TAS percentage shows significant differences only between group R48 and all other groups. The pre-frozen percentage of spermatozoa with CD is not affected by post-mortem handling of epididymes (Table 5). In post-thaw samples, this percentage decreases significantly in group R48 in comparison with groups C24 and R24.

Post-Thaw Recovery Rate

Analysis of the recovery rates for total motility and progressive motility after storage (Table 6) shows that the freezing procedure produces a significant reduction in the 48 h storage groups (C48 and R48). Rates of normal acrosomal spermatozoa are affected by freezing at both 24 and 48 h but sample R24 does not differ from the control group. Recovery rates of E+ do not change significantly amongst groups (Table 6).

Different superscripts $^{(\alpha, \beta)}$ indicate a difference ($p<0.05$) among pre-freeze and morphological defects in the flagellum are the most frequent abnormality in pre-frozen samples (64.5%, Fig. 1) and these undergo a significant increase in post-thaw samples (74.8%).

***In vitro* Fertility**

In IVF experiments, cleaved oocytes using epididymal sperm from groups CG, R24 and C24 do not differ significantly from values observed with post-thaw ejaculated ram semen (Fig. 2). When epididymal semen is recovered 48 h after the animal's death, the embryo cleavage rate reduces significantly ($p<0.05$) for both storage temperatures (C48 and R48) with regard to the other groups.

Table 4: Effect of post-mortem storage on percentage of abnormalities in epididymal rams spermatozoa (Mean±SEM)

Group	Pre-freeze	Post-thaw
CG	$6.4\pm0.9^{a,\alpha}$	$11.0\pm0.7^{a,\beta}$
C24	$7.2\pm0.9^{a,b,\alpha}$	$12.3\pm0.7^{a,\beta}$
R24	$14.6\pm5.3^{b,\alpha}$	$16.4\pm2.3^{a,\alpha}$
C48	$11.5\pm4.3^{a,b,\alpha}$	$16.2\pm3.9^{a,\alpha}$
R48	$25.0\pm3.7^{c,\alpha}$	$35.1\pm3.3^{b,\alpha}$

Different superscripts (a, b, c) indicate a difference ($p<0.05$) among groups. Different superscripts (α, β) indicate a difference ($p<0.05$) among pre-freeze and post-thaw spermatozoa

Table 5: Effect of post-mortem storage on percentage of cytoplasmic droplets in epididymal rams spermatozoa

Group	Pre-freeze	Post-thaw
CG	$65.2\pm1.9^{a,\alpha}$	$47.8\pm3.0^{a,b,\beta}$
C24	$68.6\pm3.4^{a,\alpha}$	$55.1\pm5.3^{a,\beta}$
R24	$69.9\pm3.5^{a,\alpha}$	$54.5\pm5.4^{a,\beta}$
C48	$67.5\pm4.1^{a,\alpha}$	$42.5\pm6.3^{a,b,\beta}$
R48	$67.5\pm3.8^{a,\alpha}$	$40.0\pm5.8^{b,\beta}$

Different superscripts (a, b) indicate a difference ($p<0.05$) among groups. Different superscripts (α, β) indicate a difference ($p<0.05$) among pre-freeze and post-thaw spermatozoa

Table 6: Post-thaw recovery rate (%) of qualitative parameters in epididymal rams spermatozoa according to storage method (Mean±SEM)

Group	Total motility	Progressive motility	Normal acrosomes	Swollen spermatozoa (E+)
CG	87.1 ± 2.6^{a}	81.8 ± 3.5^{a}	74.4 ± 1.7^{a}	68.4 ± 1.5^{a}
C24	82.4 ± 4.6^{a}	76.3 ± 6.2^{a}	65.7 ± 3.1^{b}	66.1 ± 2.7^{a}
R24	78.8 ± 4.7^{a}	82.2 ± 6.3^{a}	$70.5\pm3.1^{a,b}$	64.4 ± 2.7^{a}
C48	$75.9\pm5.5^{a,b}$	60.7 ± 7.4^{b}	$64.1\pm3.6^{b,c}$	66.1 ± 3.2^{a}
R48	66.6 ± 5.1^{b}	57.9 ± 6.9^{b}	57.1 ± 3.1^{c}	65.8 ± 2.9^{a}

Different superscripts (a, b, c) indicate a difference ($p<0.05$) among groups in each parameter

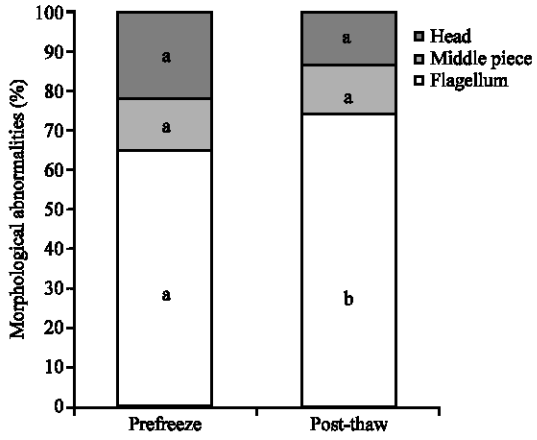

Fig. 1: Comparison of pre-freeze and post-thaw proportion of different kind of abnormalities on ram epididymal spermatozoa. Different letters (a, b) indicate differences among columns ($p<0.05$)

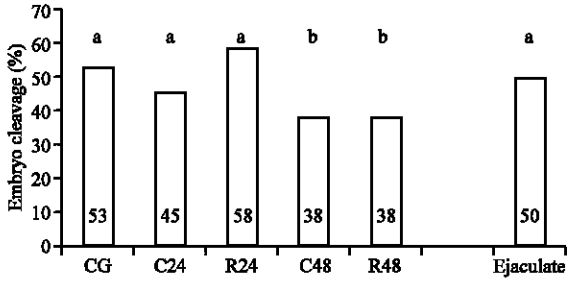

Fig. 2: Percentage of cleavage (24 h) by different storage types of frozen-thawed epididymal rams spermatozoa. Different letters [a, b] indicate differences among groups ($p<0.05$)

DISCUSSION

The qualitative parameters of ram epididymal spermatozoa observed in the control group (80.3% TM, 84.3% NA, 84.2% E+ and 65.2% CD) are similar to those published by Aguado *et al.* (2005) and Cognié *et al.* (1999). Total motility of epididymal sperm from breeding rams has been estimated at between 70 and 80% by other authors. According to Amann *et al.* (2000), in ram cauda epididymidis, about 88% of the sperm had an intact plasma membrane. This result is similar to the one obtained for ovine ejaculate, with 85% of swollen spermatozoa (Nehring, 2003). The percentages of CD described in the present study are similar to those reported by Amann *et al.* (2000). In contrast, Blash *et al.* (2001) observed that pre-freeze motility and viability of goat epididymal sperm were higher than those of ejaculated semen.

The quality parameters, total motility, progressive motility and normal spermatozoa, which do not show significant variations during the first 24 h post-mortem at 5°C, drop significantly when the epididymis is stored for 48 h. Aguado *et al.* (2005) in ram and Garde *et al.* (2005) in red deer and moufflon, find that viability of sperm collected from the cauda epididymis decreases progressively as the time between the animal's death and sperm collection increases.

Present data indicate that refrigeration enables us to obtain better quality semen samples than storage at room temperature. The beneficial effect of refrigeration on various parameters of sperm quality, especially motility, may be explained by the reduced metabolic rate of sperm cells when they are at 5°C (Salamon and Maxwell, 1983). In this sense, Sankai *et al*. (1968) found that motility of mouse epididymal spermatozoa decreases when the storage temperature is increased, suggesting that this effect is related to changes in spermatozoa metabolic activity. Nevertheless, Kikuchi *et al*. (2004) concluded that motility of boar spermatozoa collected at 4°C and stored for 1 or 2 days, decreased significantly in comparison with that of control spermatozoa from non-refrigerated epididymis. Also, motility of dog spermatozoa recovered from epididymides stored at 4°C decreased significantly within the first 48 h of refrigeration (Yu and Leibo, 1998). These results may evidence that there are differences between species relative to maintenance of spermatozoa viability from post-mortem stored epididymis.

In the present study, post-thaw total motility in both the control group (69.8%) and C24 group (64.6%) is similar to that of ovine ejaculate (70%) mentioned by other author (Garde, 1993). The good freezability of sperm from the cauda epididymidis was also observed by Rath and Niemann (1990) who found 72.2% post-thaw motility in boar spermatozoa. Also, Kikuchi *et al*. (2004) showed that post-thaw motility of boar spermatozoa from epididymides stored for 1, 2 or 3 days at 4°C does not differ from that of the controls. These authors proposed that although the reason for the survival of spermatozoa in epididymides at 4°C is unclear, epididymal fluid may contain an unknown cold shock protection factor. Nevertheless, the post-thawing/pre-freezing cell motility rate of goat epididymal sperm shows a higher decrease than that of ejaculated semen (Blash *et al*., 2001). This result may be explained taking into consideration that post-thawing motility appears to depend more on the initial quality of the semen than on the freezing method itself (Fernandes *et al*., 1990).

The results of this study show that acrosomes of ram epididymal spermatozoa might be sensitive to long storage periods (48 h). Our observations also indicate that the percentage of epididymal spermatozoa with intact acrosomes is affected by the freezing procedure. Kikuchi *et al*. (2004) suggested that pig acrosome integrity may be damaged during cryopreservation and this causes a decrease in sperm fertilizing ability. According to these authors, sperm motility and oocyte penetration ability (reflected by acrosome integrity) are affected by different mechanisms during cold storage of the epididymides. This interpretation may explain the differences between motility parameters (better when preserved at 5°C) and the percentage of normal acrosomes (better when preserved at room temperature) in post-thawing ram spermatozoa analyzed in this study. Yu and Leibo (1998) determined that there is no significant decrease in membrane integrity and acrosome integrity of dog spermatozoa recovered from epididymes and stored at 4°C within the first 48 h of refrigeration. The percentage of epididymal spermatozoa with intact acrosomes is very high and shows very little variation within the first day of refrigeration. In the present study, the plasma membrane integrity of post-thawing epididymal spermatozoa does not seem to be much affected by epididymis handling conditions.

The fertilizing ability of epididymal sperm is well known. In ewes inseminated with cauda epididymis spermatozoa, Fournier-Delpech *et al*. (2001) reported gestation rates of 78-80%; this rate is slightly higher than the one achieved with ovine ejaculates (72%). In this study, no significant difference in IVF was found between epididymal (control and storage for 24 h) and ejaculated spermatozoa. In contrast, the fertilizing ability of epididymal spermatozoa stored during 48 h decreased significantly. In addition, Garde *et al*. (2004) found that fertilizing ability evaluated by hamster oocyte *in vitro* penetration assay using red deer epididymal spermatozoa, gradually decreased as the storage period was prolonged. However, there were no significant differences when using mouflon spermatozoa. These results might reflect a difference amongst several ruminant species.

A reduction in the fertilizing ability of epididymal sperm is described by Kishikawa *et al*. (2001) when the time between the animal's death and the recovery of gametes increases (Kikuchi *et al*., 2004) in boar and in mouse. Such important differences in the fertilizing ability of frozen and thawed sperm from ovine and porcine epididymes might be explained by the different freezing ability of spermatozoa from the two species (Holt, 2000). Ikeda *et al*. (2002) suggested that the maintenance of acrosomal integrity in unreacted status, rather than the maintenance of sperm motility, is important for *in vitro* fertilization ability. This hypothesis coincides with the observation that both the cleavage rate and the acrosome status are similar in the R24 and control groups.

In conclusion, frozen-thawed spermatozoa collected from epididymides stored at 5°C for 24 h show a fertilizing ability similar to frozen-thawed ejaculated spermatozoa. These data suggest that ram epididymides must be stored at 5°C for 24 h when epididymal spermatozoa cannot be immediately collected and cry-preserved. We think that these storage conditions could also be used for epididymal sperm recovery in wild ruminants.

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