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Collection, storage and freezability of equine epididymal spermatozoa

Maria Elena Falomo[a], Melissa Rossi[a] and Roberto Mantovani[b]

[a]Dipartimento di Medicina Animale Produzioni e Salute, University of Padova, Padova, Italy; [b]Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente, University of Padova, Padova, Italy

ABSTRACT

The recovery of spermatozoa from the cauda epididymis may be the last chance to obtain genetic material from stallions undergone to castration. The aim of the current study was to evaluate the efficiency of cryopreservation and the use of two different extenders for equine epididymal spermatozoa. Testicles obtained from castration were divided into two groups: cauda epididymis processed immediately after orchiectomy and cauda epididymis processed after 24 h storage in saline solution at 4 °C of the testis. The epididymal spermatozoa were collected through manual slicing of the cauda epididymis of each testicle. In addition, spermatozoa obtained from different processed testes were diluted alternatively with either modified Palmer or EGG TECH® extenders to produce frozen straws. Motility parameters in fresh and frozen-thawed material were analysed by means of the computer-aided sperm analysis (CASA). The recorded CASA data were analysed with a mixed linear model. Motility parameters in fresh semen yielded better results than in frozen semen ($p = 0.008$), but no difference ($p > 0.05$) was observed between spermatozoa collected immediately after castration or after 24 h of storage; in frozen-thawed samples, EGG TECH® tended to improve the percentage of progressively motile spermatozoa in epididymal frozen-thawed semen ($p = 0.08$) compared with modified Palmer. We conclude that the processing of epididymal spermatozoa can occur up to 24 h after stallion castration and both common extenders used are suitable for preserving this material.

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Castration; cryopreservation; epididymis; horse; spermatozoa

Introduction

Traumatic injuries or severe diseases can prematurely end the breeding career of stallions with high genetic value. However, in spite of the high genetic value, some owners require voluntarily stallion castration for management reasons. In this case, to avoid the acquisition of behavioural problems due to the training to dummy and artificial vagina, the collection of semen result compromises. Thus, to preserve the genetic value of injured or bad tempered stallions, owners may request the recovery of epididymal spermatozoa after orchiectomy. Epididymal spermatozoa collection has been reported in different species (Cary et al. 2004; Bruemmer 2006; Martinez-Pastor et al. 2006; Toyonaga et al. 2011) and the first foaling using frozen epididymal spermatozoa was obtained in 1957 (Barker & Gandier 1957). Moreover, the motility of epididymal and ejaculated sperm in stallions have been found comparable (Monteiro et al. 2011), and sperm collected from the cauda epididymis have shown to possess an appropriate degree of maturity and fertility (Martinez-Pastor et al. 2006).

Epididymal spermatozoa can be collected through percutaneous epididymal sperm aspiration (PESA) in anesthetised and standing stallions, electro ejaculation in live horses under general anaesthesia or after castration (Cary et al. 2004; Bruemmer 2006). After surgical removal of the testes, the harvesting of spermatozoa from epididymis and ductus deferens can occur by three techniques: the flotation method, the retrograde flush technique or the standard flush technique of epididymis and ductus deferens (Cary et al. 2004; Bruemmer 2006). Depending on the method of extraction, it is possible to collect different sperm subpopulations (Martinez-Pastor et al. 2006). In the flotation method, the presence of erythrocytes does not appear to be detrimental to the spermatozoa (Cary et al. 2004). Moreover, no differences were found comparing spermatozoa coming from left or right epididymis (Contri et al. 2012). Results from previous research suggest that sperm can be harvested immediately after

CONTACT Prof. Roberto Mantovani roberto.mantovani@unipd.it DAFNAE, Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente, Università di Padova, Viale dell'Università 16, 35020 Legnaro (PD), Italy

castration or after 24 h of storage at 4–5 °C without any difference in terms of viability (Bruemmer 2006; Monteiro et al. 2011; Toyonaga et al. 2011). Furthermore, the cooling of epididymis wrapped in foil can last up to 96 h at 4 °C (Vieira et al. 2013). After cooling, diluted epididymal spermatozoa can be successfully frozen (Papa et al. 2008).

The aim of the current study was to evaluate the efficiency of cryopreservation of epididymal spermatozoa comparing spermatozoa harvested from the cauda epididymis immediately after the procedure of orchiectomy or after a 24-h storage at 4 °C of the testes. Furthermore, we investigated the effects of two different extenders, modified Palmer and EGG TECH®, on motility parameters (percentages of motile and progressively motile spermatozoa) before and after cryopreservation.

Materials and methods

Animals

The study included six privately owned stallions of mixed breeds and age subjected to castration under general anaesthesia, without the use of local anaesthetic. Indeed, in the preliminary phase of the study, intra-testicular administration of lidocaine revealed a spermicidal effect.

Experimental design

The testes were divided into two groups: testes in which cauda epididymis was immediately processed after orchiectomy (A testicle) and testes stored in saline solution at 4 °C for 24 h after orchiectomy and subsequently processed (B testicle). The 24 h storage at 4 °C was aimed to simulate the shipment of testes to a reference centre for the harvesting of epididymal spermatozoa.

Spermatozoa were obtained by flotation method with manual slicing of the cauda epididymis of each testis. Incisions were made every 2 mm and then the cauda epididymis was allowed to float into a Petri dish with 10 ml of Equipro™ (minitube) at 37 °C for 10 min. A sample of 5 ml was centrifuged at 4 °C for 1400*g*/min for 8 min to facilitate the removal of most part of the supernatant. Thus, straws of frozen semen were produced diluting 2 ml of sample, obtained from the cauda epididymis of A or B testicle, with 2 ml of either modified Palmer (containing milk, 4% glycerol and 4% egg yolk; X extender) or EGG TECH® (Technologies International Ltd, Richmond, VA; Y extender), obtaining four aliquots for each stallion: AX, AY, BX and BY. Straws were tagged and loaded with 0.25 ml of diluted semen, each. Straws were then sealed by ultrasound and equilibrated for 30 min at 4 °C. Subsequently, straws were placed on a grid above liquid nitrogen for 15 min and, finally, were completely plunged in it. Samples were thawed after 24 h in a water bath at 37 °C for 30 s and then kept in Eppendorf tube.

Data recording

Semen analysis comprised assessment of sperm motility with computer aided sperm analysis (CASA, IVOS Animal Version 12.3D Bild 002, Hamilton Thorne Research, Bedford, MA). The CASA analysis was carried out in fresh (i.e. immediately before freezing) and in frozen-thawed samples. At each assessment, 15 µl of semen sample were placed on a pre-warmed Cell-Vu® slide with two counting chambers. Ten fields per sample were chosen manually at 40×. The settings for the CASA analysis were the same as used in the previous studies (Mantovani et al. 2002). A preliminary measure of repeatability within sample was assessed and analyses started when motility parameters differed $\leq$3% between subsequent measurements. The parameters examined were the following: percentages of motile (% MOT) and progressively motile spermatozoa (% PMS).

Statistical analysis

Data were analysed via a mixed model analysis using a hierarchical linear model for repeated measures (PROC MIXED, SAS user manual, V9.2. Cary, NC). The model accounted for the fixed effects of the extender (X or Y), the testicle (A or B) and their interaction. The stallion within extender–testis interaction was used as an error term for the previously described effects. Further factors accounted in the sub-plot of the hierarchical model were the type of semen analysed (fresh or frozen-thawed epididymal semen) and all the possible interaction between the type of semen analysed with the extender and testes. The degrees of freedom of the interaction extender*testes were used to test the effect of the extender within testicle (A or B, i.e. time 0 or 24 h). Further comparisons were carried out by decomposing the degrees of freedom of the interaction extender*testicle*type of semen analysed in order to compare the following:

- Extender X versus Y for testicle A analysed before freezing (i.e. fresh semen).
- Extender X versus Y for testicle B analysed before freezing (i.e. fresh semen).
- Extender X versus Y for testicle A analysed after freezing and thawing.

- Extender X versus Y for testicle B analysed after freezing and thawing.

Results

The % MOT and % PMS obtained resulted greater ($p = 0.008$ and $p < 0.001$, respectively) in fresh epididymal spermatozoa when compared with frozen-thawed epididymal spermatozoa (Table 1). The storage of testes at 4 °C for 24 h did not alter significantly the motility parameters (A versus B; $p > 0.05$). Indeed, we observed 68.5% versus 64.3% ($p = 0.63$) for % MOT and 12.9% versus 10.3% ($p = 0.38$) for % PMS, in A and B, respectively (Table 1). No differences ($p > 0.05$) were noticed in the comparison between the extenders used (X versus Y; Table 1). However, the storage at 4 °C for 24 h slightly modified the response of spermatozoa to different extenders. Indeed, in X extender, the % PMS in B tended to be lower than in Y ($p = 0.08$; Table 2). No differences were noticed for X or Y extenders in % MOT (Table 2). Furthermore, in fresh epididymal spermatozoa in Y extender, the % PMS in A was lower than in B testes (7.7% versus 18.8% in A and B, respectively; $p = 0.04$; Table 3), although no differences were noticed for both the extenders in % MOT (Table 3). No changes were detected in frozen-thawed semen both within A and B testicles when compared with different extenders ($p > 0.05$; Table 3).

Table 1. Different effects of analysis, extender and testicles in motility parameters. Values are least square means ± SE.

Item	MOT, %	PMS, %
Analysis:		
Fresh	74.6	15.1
Frozen-thawed	58.2	8.0
SE	5.1	1.6
P	0.008	<0.001
Extenders:		
- X	64.5	8.9
- Y	68.3	14.2
SE	6.1	2.1
P	0.66	0.08
Testicles:		
- A	68.5	12.9
- B	64.3	10.3
SE	6.1	2.1
P	0.63	0.38

MOT: total motility; PMS: progressively motile spermatozoa; X: modified Palmer; Y: EGG TECH®; A: cauda epididymis processed immediately after orchiectomy; B: cauda epididymis processed after 24 h storage at 4 °C.

Table 2. Correlation between the effects of extender in the different testicles, in motility parameters. Values are least square means ± SE.

	A				B			
Variable	X	Y	SE	P	X	Y	SE	P
MOT, %	66.0	71.1	8.6	0.68	63.0	65.6	8.6	0.83
PMS, %	11.3	14.4	2.9	0.46	6.5	14.0	2.9	0.08

MOT: total motility; PMS: progressively motile spermatozoa; X: modified Palmer; Y: EGG TECH®; A: cauda epididymis processed immediately after orchiectomy; B: cauda epididymis processed after 24 h storage at 4 °C.

Discussion

The flotation technique results a reliable and rapid technique to obtain equine epididymal spermatozoa. Indeed, we obtained samples with general good motility parameters. The differences between samples of fresh and frozen-thawed epididymal spermatozoa are undoubtedly due to the damage induced by cryopreservation at the spermatozoa membrane and metabolism (Medeiros et al. 2002). Comparing the results with another study where the semen was obtained through flotation technique and suspended with a milk-based extender (E-Z Mixin with amikacin and penicillin), in fresh semen, we obtained approximately the same % MOT, but lower % PMS (74.6% versus 74% and 15.1% versus 35.5%, respectively); however, in frozen-thawed semen, we observed greater values of % MOT (58.2% versus 32.6%, respectively), and still lower values of % PMS (8.0% versus 18.4%, respectively; Cary et al. 2004). These differences can be due to the individual variability that exists between stallions (Aurich 2005) or to different set ups of the CASA systems among studies. No differences were noticed in the comparison between epididymal spermatozoa collected immediately or after 24 h of storage at 4 °C of the testis, supporting the hypothesis that it is possible to store the cauda epididymis before harvesting of spermatozoa. These results are in agreement with other studies that support the cooling at 5 °C which is the best way to stop the deterioration of

Table 3. Different interaction between extender and testicles in fresh and frozen-thawed spermatozoa. Values are least square means ± SE.

Item	MOT, %	PMS, %
Fresh		
-XA	76.5	14.8
-YA	85.0	19.8
P	0.56	0.29
-XB	59.7	7.7
-YB	77.3	18.0
P	0.24	0.04
Frozen-thawed		
-XA	55.5	7.8
-YA	57.2	9.0
P	0.91	0.80
-XB	66.3	5.3
-YB	53.8	10.0
P	0.40	0.32
SE	10.2	3.25

MOT: total motility; PMS: progressively motile spermatozoa; XA: modified Palmer used in cauda epididymis processed immediately after orchiectomy; YA: EGG TECH® used in cauda epididymis processed immediately after orchiectomy; XB: modified Palmer used in cauda epididymis processed after 24 h storage at 4 °C; YB: EGG TECH® used in cauda epididymis processed after 24 h storage at 4 °C.

epididymal spermatozoa that seems to occur even within a few hours from collection (Martinez-Pastor et al. 2005; Bruemmer 2006; Monteiro et al. 2011; Toyonaga et al. 2011; Vieira et al. 2013). The kind of extender used appears as an important factor. Comparing our post-thaw values, obtained with the use of EGG TECH® or modified Palmer in A or B testes, and results obtained in other studies where different extenders were used, we can come to the conclusion that the use of both modified Palmer and EGG TECH® result ameliorative for % MOT, but not for % PMS. For example, for the extender Botucrio® (Papa et al. 2008), on one hand, it has been reported a 19.6% PMS, although with a variability that resulted three times greater than that observed in the present study (i.e. 11.43% versus 3.25% of standard deviation, respectively). On the other hand, INRA-82 or EDTA-lactose (Papa et al. 2008) produced lower % PMS than that obtained with the extenders used in this study (4.0 ± 7.37% and 1.1 ±3.19%, respectively). This study demonstrates that EGG TECH® and modified Palmer give good results in the dilution of equine epididymal spermatozoa. Moreover, EGG TECH® tended to improve the percentage of progressively motile spermatozoa compared with modified Palmer. The reasons for different values in post-thaw motility and progressive motility of epididymal spermatozoa may be due to the substances contained in these extenders (Papa et al. 2008), and their different abilities in protecting equine spermatozoa from cold shock and cryopreservation damages.

Conclusions

The results of this study confirm that equine epididymal spermatozoa, obtained after 24 h storage at 4 °C of the testis, maintain acceptable motility and progressive motility, making it possible the use in the preparation of fresh or frozen semen doses. Moreover, modified Palmer and EGG TECH® are both suitable for the production of frozen straws of equine epididymal spermatozoa.

Ethical animal research

The study was carried out on material collected *ex vivo* after castration requested by the animal owner. Explicit owner informed consent for participation in the study was stated, and general permission for *ex vivo* examination was obtained by authors.

Acknowledgements

M. E. Falomo was the main contributor to study design, did the majority of the study execution and provided guidance on manuscript preparation; R. Mantovani and M. Rossi gave the main contribution to data analysis, data interpretation and manuscript preparation.

Disclosure statement

The authors deny any financial interest or benefit arising from the direct applications of their research.

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