IMPAIRMENT OF RAM SPERM-HYALURONAN BINDING ABILITY BY THE FREEZING EXTENDER

DIMINUIÇÃO DA CAPACIDADE DE LIGAÇÃO DOS ESPERMATOZÓIDES DE CARNEIRO AO ÁCIDO HIALURÓNICO INDUZIDA PELO DILUIDOR DE CONGELAÇÃO

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SUMMARY

The ability of ram sperm to bind to hyaluronan coated slides (HBS, %) was tested on fresh (FS) and chilled semen (CS) diluted with skimmed-milk based extender (CS-SM), chilled (CS-EY) and frozen-thawed (FTS-EY) semen diluted with tris-glycerol-egg-yolk based extender. The % of HBS (motile bound sperm/all motile sperm) was similar in FS and CS-SM groups (73,6 % *vs.* 71,2 %) both significantly higher than CS-EY (25 %) and FTS-EY (29,7 %) groups. Progressive individual motile sperm (IMS) of the FTS-EY group was the lowest (21,7 %), and no differences were found among FS (63,3 %), CS-SM (55 %) and CS-EY (48,3 %). IMS was positively correlated with HBS (r= 0,62; p<0,03). Although needing confirmation on a larger number of animals, the freezing extender used in the present work disrupted the mechanism of sperm hyaluronan binding prior to the freezing-thawing, suggesting a close relationship between this finding and lower fertility rates following artificial insemination with frozen-thawed semen.

RESUMO

Avaliou-se a capacidade de ligação dos espermatozóides de carneiro ao ácido hialurónico (% HBS), no sémen fresco (FS) e refrigerado (CS) diluído com leite de vaca desnatado (CS-SM) e sémen refrigerado (CS-EY) ou e congelado (FTS-EY) diluído com um diluidor de tris-glicerol-gema de ovo. A % de HBS (espermatozóides móveis ligados/total de espermatozóides móveis) nos grupos FS e CS-SM foram semelhantes (73,6 % *vs.* 71,2 %), ambas significativamente superiores aos grupos CS-EY (25 %) e FTS-EY (29,7 %). A diferença entre os grupos CS-EY e FTS-EY não foi significativa. A percentagem de motilidade individual progressiva (IMS) mais baixa foi obtida no grupo FTS-EY (21,7 %), não havendo diferenças entre os grupos FS (63,3 %), CS-SM (55 %) e CS-EY (48,3 %). Verificou-se uma correlação positiva entre a IMS e a HBS (r= 0,62; p<0,03). Embora necessitando confirmação num maior número de animais, o diluidor de congelação utilizado perturbou, previamente à congelação, a ligação dos espermatozóides ao ácido hialurónico, sugerindo a existência de uma relação com as baixas taxas de fertilidade obtidas na ovelha após a IA com sémen congelado.

INTRODUCTION

Misoprostol administration to synchronized ewes, 48 h after sponge removal, increased the content of hyaluronan in the vagina and cervix epithelial layer, advancing in required cervical transformations from 72 to 54 h (AI timing) after sponge removal (Leethongdee *et al.,* 2007). Although misoprostol administration during synchronized oestrus failed to increase the proportion of intracervical inseminations, fertility increased significantly when using CS, but non-significantly when FTS was used (Horta *et al.,* 2010; Barbas *et al.,* 2013). When frozen-thawed semen was

used in Saloia ewes, the fertility increase was of smaller magnitude and non-significant (Barbas *et al.,* 2013).

Those findings suggest that misoprostol treatment could be associated to a higher hyaluronan concentration in the cervix and vagina, increasing sperm adhesion to vaginal and cervical wall and so the number of sperm available for fertilization. Lower magnitude in fertility increase when using FTS suggests that the freezing-thawing process or the extender may affect the misoprostol mediated sperm hyaluronan binding mechanism.

Sperm-hyaluronan binding assays identify motile sperm in human, through binding of HABP1 (Ghosh *et al.,* 2002) and CD44 (Bains *et al.,* 2002) membrane proteins to hyaluronan in the epithelial extracellular matrix of genital tract, and have been used for characterization and selection of viable sperm to fertilize *in vitro* mature oocytes.

The aim of this work was to evaluate the effect of different extenders for chilling and freezing upon the capacity of ram sperm to bind hyaluronan.

MATERIAL AND METHODS

Weekly collected semen ejaculates (n= 3), from one fertile Portuguese Saloia ram were *in vitro* tested for hyaluronan bound sperm (HBS). Four experimental groups were considered: fresh semen (FS); chilled semen diluted with a skimmed milk based extender (CS-SM); chilled (CSEY) and frozen-thawed semen (FTEY) diluted with an egg yolk + glycerol based extender. Semen collected by artificial vagina was immediately incubated at 28 ºC and scored for volume, mass motility, sperm concentration by spectrophotometry, and percentage of progressive individual motile sperm (IMS) under microscope observation (400x). IMS was reevaluated in samples of FS, CS-SM and CS-EY after chilling, and of FTS-EY after thawing (Barbas *et al.,* 2013). In the FS group, 10 µL of semen were diluted $(24x10^3 \text{ spz mL}^{-1})$ in synthetic oviductal fluid medium (SOF, Tervit *et al.,* 1972) supple-

mented with 2,5 mg mL^{-1} glucose (SOF-g, the only medium allowing ram sperm to bind hyaluronan, 270 mOsmol L^{-1} , 7,2-7,4 pH) and incubated at 28 ºC (30 min). The remaining fresh semen was equally divided in two aliquots: CSSM group where semen was diluted to 1,2 spz mLx109 and chilled at 4 ºC for 60 minutes in a refrigerated chamber; and the CS-EY and FTS-EY groups, where semen was diluted to the same concentration (Marques *et al.,* 2006) and refrigerated at 4 ºC for 90 minutes. In FTSEY group, semen was frozen in liquid nitrogen vapours before immersion into LN2. After dilutions, semen was packed in 0,25 mL Cassou mini-straws (300x106 spz). In each group, samples from 3 ejaculates were tested for their ability to bind hyaluronan coated slides (HBA®, Sperm-Hyaluronan Binding Assay, Biocoat, Inc, supplied by ORIGIO). Prior to counting % HBS under contrast phase microscopy (400x), sperm samples (10 µL) from the chilled and thawed groups were further diluted (SOF-g) to a final concentration (24x103) and incubated at 28 ºC (30 minutes). Samples (10 µL) in slides covered with a coverslip with a grid were incubated at 28 ºC (10 min). At least 250 motile sperm were counted for 10 minutes and classified as bound motile sperm (BMS, adhered sperm head with vigorous tail movements) or not bound (FPMS, free progressive motile sperm). The effects of treatment on % HBS ([BMS / (FPMS+BMS)] x 100) and IMS were computed by ANOVA and differences among groups by post-hoc LSD. Correlation between IMS and HBS was calculated by the Pearson product-moment correlation coefficient method. The measure of variability of means is expressed by their standard errors. Significance was accepted at p≤0,05.

RESULTS

Fresh ejaculates (n= 3) presented a mean volume of 1,08±0,19 mL, a sperm concentration of 4,3±0,06 x 10^9 spz mL^{-1}, a mass motility of 4, an IMS of 63,3±3,3 %.

The % HBS of FS and CS-SM groups were similar (p= 0,77), and both were significantly higher than those of CS-EY (p= 0,0003 for FS and p= 0,0005 for CS-SM) and FTS-EY (p= 0,0006 for FS and p= 0,0009 for CS-SM) groups **(table I)**. Considering all groups, the % HBS was calculated from a total of 2156 motile sperm cells **(table I)**. In FTS-EY, IMS was significantly lower than in all other groups (p= 0,003; **table II**). IMS was positive and significantly correlated with the % of HBS (r= 0,62; p= 0,034) considering paired values from all groups (n= 12), and is explained by the following regression equation: HBS= 9,635 + 0,855 x IMS (F [1,10]= 6,07; p= 0,034).

DISCUSSION

To our knowledge, this is the first time that a sperm-hyaluronan binding assay was tested in ram semen, either fresh, chilled or frozen, and diluted with different extenders. The values of % HBS obtained in the FS (71,2 %) and the CS (73,6 %), are close to the human semen (around 80 %). Those obtained after adding the freezing extender (25 %), which were not reduced after freezing (29,7 %), show

Table I. *Hyaluronan binding assay: total motile sperm and percentages of hyaluronan bound sperm (HBS) from fresh (FS), chilled (CS-SM and CS-EY) and frozen-thawed (FTS-EY) semen from three ejaculates.* (Teste de ligação ao ácido hialurónico: total de espermatozóides móveis e percentagem de espermatozóides ligados ao ácido hialurónico (HBS) no sémen frasco (FS), refrigerado (CSSM e CSEY) e congelado (FTSEY) de três ejaculados).

Groups	HBA	
	Total motile sperm (n)	HBS %±SE
From fresh	779	73,62±3,34[a]
Chilled CS-SM	467	71,15±10,34[a]
Chilled CS-EY	638	25,00±3,30[b]
Frozen-thawed	272	29,73±1,44[b]
ANOVA		p= 0,0004

a≠b: p<0,05, LSD.

Table II. *Individual motile sperm (IMS) from fresh (FS), chilled (CS-SM and CS-EY) and frozen-thawed (FTS-EY) semen from three ejaculates, evaluated after dilutions and previously to HBA tests.* (Motilidade individual progressiva (IMS) no sémen fresco (FS), refrigerado (CSSM e CSEY) e congelado (FTSEY), de três ejaculados, após a diluição e antes do teste HBA).

Groups	IMS (%)±SE
Fresh	63,33±3,33[a]
Chilled CS-SM	55,00±2,89[a]
Chilled CS-EY	48,33±8,33[a]
Frozen-thawed	21,67±4,41[b]
ANOVA	p= 0,003

a≠b: p<0,05, LSD.

that the extender, but not the freezing process itself, reduced the ability of sperm to bind hyaluronan. It is reasonable to state that the freezing extender constituent(s) may impair the binding of sperm membrane protein(s) to hyaluronan, decreasing sperm adhesion to the extracellular matrix in cervix and vagina, explaining the lower fertility rates when FTS is used, and the moderate increase of fertility rate in misoprostol treated ewes inseminated with FTS compared to the significant increase when CS was used (Barbas *et al.,* 2013).

Our results show that, besides the ability of thawed sperm to swim across cervical mucus (Richardson *et al.,* 2011), sperm-hyaluronan binding ability might be an important pre-requisite for colonizing the genital tract with high numbers of viable sperm available for fertilization.

Differently from humans, where it is proposed the use of a protein enriched human tubal fluid medium for incubation of sperm prior and during the HBA, our results show that in the ram the only medium allowing sperm binding was SOF+glucose, denoting species differences.

An overall positive correlation of sperm motility with sperm-hyaluronan binding ability was observed in the present study, in agree-

ment with results on human semen (Ghosh *et al.,* 2002; Yagci *et al.,* 2010).

Although needing confirmation on a larger number of animals, the freezing extender used in the present work disrupted the mechanism of sperm hyaluronan binding prior to the freezing-thawing process. This may explain the lower fertility rates obtained after transvaginal artificial insemination with FTS in this species.

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