IONOPHORE-INDUCED ACROSOME REACTION IN RAM SPERMATOZOA: A POSSIBLE TEST IN THE DIAGNOSIS OF MALE SUBFERTILITY

EMPLEO DE LA REACCIÓN ACROSÓMICA INDUCIDA POR EL IONÓFORO DE CALCIO COMO POSIBLE MÉTODO DE DIAGNÓSTICO DE LA SUBFERTILIDAD EN EL MORUECO

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ADDITIONAL KEYWORDS

A23187. Acrosome reaction. Fertility rates. Semen evaluation. Ram.

PALABRAS CLAVE ADICIONALES

Ionóforo de calcio. Reacción acrosómica. Tasas de fertilidad. Contrastación seminal. Morueco.

SUMMARY

In order to compare the ionophore-induced acrosome reaction (AR) between spermatozoa obtained from subfertile and normal rams, a study with 8 rams of Manchega breed has been performed. Three subfertile rams with normal routine semen values were selected on the basis of low fertility rates (15.7 ± 1.6 p.100). The control animals consisted of 5 rams with good fertility (44.2 ± 1.6 p.100). All rams were examined clinically, including palpation of the testicles and epididymes to detect possible abnormalities. Semen was obtained twice weekly by artificial vagina. Five ejaculates were collected from each ram and evaluated for routine semen parameters. Additionally, semen samples were incubated in saline medium with or without A23187 and aliquots were taken at 0, 15, 30, 45 and 60 min for assessment acrosome reaction (AR) by a triple-stain tecnique. No statistical differences were found between subfertile and normal rams regarding routine semen evaluation. However, great variations were observed on the ocurrence of the ionophore-induced AR between subfertile and normal rams. Sperm obtained from subfertile males underwent the AR at a much lower rate ($p<0.001$) than spermatozoa collected from normal males. These results suggest that ionophore A23187 can be considered as a possible test for the studied of defective ram sperm function.

RESUMEN

El objetivo del presente trabajo ha sido estudiar las tasas de reacción acrosómica (RA) inducidas por el ionóforo de calcio (A23187) en espermatozoides procedentes de moruecos subfértiles o normales, con objeto de analizar las posibilidades de esta

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prueba como método para discernir entre ambos grupos de sementales. Para ello, se han empleado 3 moruecos de raza Manchega con bajas fertilidades (15,7 ± 1,6 p.100), pero con valores seminales rutinarios normales. Además, como grupo control, se han utilizado 5 machos con porcentajes de fertilidad normales (44,2 ± 1,6 p.100). Previamente, todos los animales fueron examinados clínicamente para la detección de posibles anomalías en su aparato genital. Se recogieron cinco eyaculados (ritmo sexual de 2 eyaculaciones semanales) de cada morueco mediante el empleo de una vagina artificial. Posteriormente, se procedió a la contrastación rutinaria de los mismos. Además, se incubaron muestras de dichos eyaculados en una solución salina con y sin A23187, evaluándose el porcentaje de RA, por medio de una técnica de triple tinción, a los 0, 15, 30, 45 y 60 minutos del inicio de la incubación. No se encontraron diferencias estadísticamente significativas entre moruecos subfértiles y normales en los parámetros rutinarios de evaluación seminal analizados. Sin embargo, se evidenciaron grandes variaciones entre los eyaculados pertenecientes a ambos grupos en los porcentajes de RA inducidos por el A23187. Los espermatozoides obtenidos de machos subfértiles presentaron unas tasas de RA estadísticamente inferiores ($p<0,001$) a las procedentes de los moruecos normales. Estos resultados sugieren que el A23187 puede ser considerado como una prueba eficaz para el estudio de algunas disfunciones espermáticas en el morueco.

INTRODUCTION

In the diagnosis of male subfertility a number of sperm characteristics are evaluated (volume ejaculated, sperm concentration, sperm motility, sperm morphology and acrosome status). These assays cannot be relied on to accurately predict ram fertility. An essential step for fertilization is the acrosome reaction (AR). The poor ability of mammals sperm of agricultural interest to undergo a spontaneous acrosome reaction in the absence of a zona pellucida poses difficult questions in terms of the development of diagnostic tests. The most common solution to this problem is to use the A23187 to induce AR (Harrison *et al.*, 1990). This metodology has been widely used to bypass the need for capacitation, and the AR so obtained in several species has been demonstrated to be equivalent to the natural AR (Green, 1978; Schams-Borhan and Harrison, 1981; Whitfield and Parkinson, 1995). The aim of our paper was to compare the ionophore-induced acrosome reaction among spermatozoa obtained from subfertile or normal rams in order to use this metodology as a test in the diagnosis of ram subfertility. Three subfertile rams selected on the basis of low fertility rates and five normal control rams of the same age were studied.

MATERIALS AND METHODS

Ram ejaculates selected for Artificial Insemination (AI) programs are subjected to several analysis each time they are collected. Ram semen with low initial sperm parameters is not used for AI. Animals producing semen with good or normal sperm characteristics are used for AI. Approximately 500-700 inseminations per ram are performed each year. The fertility of ewes is evaluated for each male to detect subfertile rams.

Three subfertile rams with normal routine semen values were selected on the basis of low fertility rates. The average fertility rate for these rams was 15.7±1.6 p.100. The control animals consisted of 5 rams with good fertility, randomly selected from the same age group as the subfertile rams. The average fertility rate

for the control rams was 44.2±1.6 p.100.

All rams were examined clinically, including palpation of the testicles and epididymes to detect possible abnormalities. Semen was obtained twice weekly by artificial vagina. Five ejaculates were collected from each ram and evaluated. Total number of spermatozoa, sperm motility, sperm morphology and percentage of intact acrosomes were calculated for each time semen was collected.

The total number of spermatozoa per ejaculate was calculated by measuring the volume and the sperm concentration of the ejaculate. The ejaculated volume was read immediately after collection from a graduated test tube and the sperm concentration was measured by a spectrophotometer after diluting a sample of semen with 0.05 p.100 glutaraldehyde solution (1:500 dilution rate) (Vijil, 1986).

The percentage of spermatozoa displaying forward progressive motility was estimated by diluting a drop of semen with 0.1 M sodium citrate, transferring it to a warm slide, mounting it with a coverslip and examining it under the microscope. Sperm morphology and percentage of intact acrosomes (PIA) were assessed through observation of semen fixed in buffered 2 p.100 glutaraldehyde solution and counting a total of 200 cells under a phase-contrast microscope (Pursel and Jonhson, 1974).

Ionophore-induced acrosome reaction was performed using conventional procedures for ram spermatozoa (Schams-Borhan and Harrison, 1981; Harrison *et al.*, 1990). The HEPES diluent used consisted of 142 mM-NaCl, 2.5 mM-KOH, 10 mM-glucose and 20 mM-Hepes, adjusted to 7.55 at 20°C with NaOH; a medium containing 222 mM-Sucrose in place of the NaCl was used for washing spermatozoa. Both media also contained 1 mg of PVA/ml and 1 mg of PVP/ml and had a final osmolality of 305 mOsm/kg. Ejaculated ram spermatozoa were separated from seminal plasma by dilution and washing through sucrose medium (Harrison *et al.*, 1982). They were then resuspended to approximately 0.5-1x10^8 cells/ml in 500 µl of saline medium, and incubated for 60 min in a 37°C water bath with Ca^{2+} (3mM) and 5 µl of 100 µM calcium ionophore A23187 in dimethylsulfoxide (DMSO) (final concentration of ionophore=1µM). Aliquots were removed and stained with the TST every 0, 15, 30, 45 and 60 minutes for evaluation of acrosome status. Control sperm suspensions did not receive ionophore or DMSO. All experiments were replicated on five independent occasions.

Acrosome reactions were monitored using a Triple-Stain Technique (TST) (Garde *et al.*, 1992). Briefly, 50 µl of the sperm suspension were diluted with an equal volume of BWW medium (Biggers *et al.*, 1971), containing 1 p.100 trypan blue and were incubated at 37°C for 10 min. Afterwards, the samples were smeared on prewarmed glass slides and air dried. The slides were rinsed in water and blotted. The smears were then fixed in 3 p.100 glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 30 min, and then were rinsed with water and air dried. The smears were stained in 0.8 p.100 Bismark brown in 30 p.100 ethyl alcohol, pH 2.8, at 40°C for 10 min; rinsed in water; and air dried. Finally, smears were stained with 0.8 p.100 rose Bengal in 0.1 p.100 M Tris buffer, pH 5.3, at 24°C for 20 min. thoroughly rinsed in water and air

Table I. *Fresh semen characteristics in normal and subfertile rams used in Artificial Insemination programs*[*]. (Características seminales de moruecos normales y subfértiles empleados en programas de Inseminación Artificial).

	Vol[2]	Conc[3]	MM[4]	MI[5]	PIA[6]
Normal	1.01 ± 0.03	4.89 ± 049	4.26 ± 0.50	86.84 ± 2.23	88.54 ± 4.70
Subfertile	0.98 ± 0.03	5.02 ±0.34	4.38 ± 0.10	82.56 ± 4.51	90.45 ± 3.45

*Values are means ± SEM;[1]No difference was observed between the two groups in the parameters studied; [2]Ejaculated volume (ml); [3]Sperm concentration (x10^9/ml); [4]Massal motility (0-5); [5]Individual sperm motility (0-100 p.100); [6]Percentage of sperm with normal acrosome (0-100 p.100).

dried. After mounting, the slides were examined at x400 with a light microscope. Spermatozoa were classified into the following 4 categories: 1) live spermatozoa with normal acrosomes, 2) live spermatozoa without normal acrosomes (true acrosome reaction), 3) dead spermatozoa with normal acrosomes, and 4) dead spermatozoa without normal acrosomes (false acrosome reaction). The percentage of acrosome reaction (category 2) was estimated.

STATISTICAL METHODS

All statistical analyses were performed with the use of SAS statistical package (SAS Institute, Cary, NC).

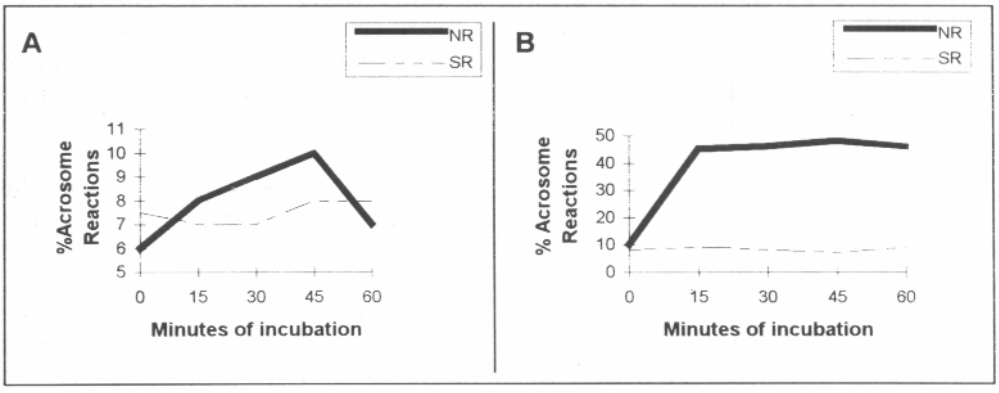

Figure 1. *Influence of exposure time on the ocurrence of the spontaneous (A) and ionophore-induced (B) acrosome reaction in spermatozoa appertaining to normal (NR) or subfertile rams (SR).* (Influencia del tiempo de incubación sobre las tasas de presentación de RA espontánea (A) o inducida por A23187 (B) en espermatozoides pertenecientes a moruecos de fertilidad normal (NR) o subfértiles (SR)).

Percentages of acrosome-reacted sperm were transformed by arc sine before analysis. All data were expresed as means ± SEM and compared by analysis of variance test. The results of fertility for the rams were compared with the Student's t-test.

RESULTS AND DISCUSSION

Preliminary studies demonstrate statistical differences between the two groups of rams in fertility rates (44.2±1.3 *versus* 15.7±1.6, $p<0.05$). However, no statistical differences were found between subfertile and normal rams regarding routine semen evaluation. The results are shown in **table I**. The palpation findings of all rams were normal.

There were no significant differences found between the 2 groups of rams in spontaneous reactivity **(figure 1.A)**. However, great variations were observed on the ocurrence of the ionophore-induced acrosome reaction between subfertile and normal rams **(figure 1.B)**. Values of ionophore-induced AR are significantly different ($p<0.001$) between subfertile and normal rams at all time points after zero. Spermatozoa obtained from subfertile males underwent the acrosome reaction at a much lower rate ($p<0.001$) than spermatozoa collected from normal males.

There was significant difference ($p<0.001$) in ionophore-induced acrosome reaction among rams within the control group **(table II)**, but not among rams appertaining to subfertile group. These differences between individuals were observed after incubation for 15-30 minutes, but by 45-60 minutes all rams showed substancial percentages of spermatozoa acrosome-reacted. In contrast, no significant difference ($p>0.05$) was observed in spontaneous acrosome reaction among males from the good fertility group (data not shown).

In many previous studies, the percentage of acrosome-reacted ram spermatozoa has been assessed by staining with 10 p.100 Giemsa or by fixation in a glutaraldehyde solution (Goldman *et al.*, 1991). These techniques, however, can only differentiate between the absence or the presence of the acrosome; they cannot differentiate between spermatozoa with true acrosome reactions (live) and those with degenerative changes that are associated with cell death. With the TST, it is possible to differenciate clearly between four sperm categories and, in particular, to distinguish live cells that have undergone a true acrosome reaction. Therefore, we have used the TST to assess changes in acrosomal status when spermatozoa were incubated under different conditions.

The purpose of this study was to induce an acrosome reaction in ram spermatozoa with the aid of ionophore, and to compare the response obtained between subfertile and normal rams. The results of our study show that spermatozoa obtained from subfertile rams undergo the ionophore-induced acrosome reaction at a much lower rate ($p<0.001$) than sperm collected from normal males. These data implicate an unexplained incapacity to undergo the ionophore-induced acrosome reaction by the rams of low fertility. In a high proportion of such cases the spermatozoa exhibit a partial or complete refractoriness to the calcium influx induced by this ionophore. The possible significance

Table II. *The effect of Ionophore on the occurrence of the acrosome reaction in spermatozoa from five normal rams**. (Efecto del tratamiento con ionóforo de calcio (A23187) sobre las tasas de RA en espermatozoides de 5 moruecos fértiles).

	Time (minutes)				
	0	15	30	45	60
Ram A	6.5 ± 1.3	51.7 ± 6.2[1]	50.3 ± 6.8[1]	45.3 ± 6.2	47.3 ± 7.1
Ram B	8.4 ± 2.0	45.8 ± 3.9[1]	47.3 ± 7.2[1]	41.2 ± 5.8	45.2 ± 8.3
Ram C	7.5 ± 1.9	20.3 ± 6.4	31.2 ± 5.9	35.2 ± 8.3	39.4 ± 7.2
Ram D	4.5 ± 2.0	56.2 ± 5.9[1]	45.2 ± 5.4[1]	43.3 ± 7.2	42.3 ± 4.9
Ram E	8.7 ± 1.7	27.5 ± 1.2	28.5 ± 4.9	39.9 ± 4.5	41.2 ± 5.6

*Values are means ± SEM; values are percent reacted; [1]$p<0.01$ *versus* Ram C and E

of lipid peroxidation as a key factor in the development of defective sperm function has been reported (Aitken *et al.*, 1984). On the other hand, no significant difference was observed in spontaneous reactivity between the 2 groups of rams. This poor ability of mammalian sperm to undergo a spontaneous acrosome reaction in the absence of a zona pellucida poses difficult questions in terms of the development of diagnostic tests. However, our results indicate that the use of the divalent cation ionophore A23187 to induce the acrosome reaction *in vitro* could be considered as a possible test for the study of defective ram sperm function. Finally, we have obtained significant differences between spermatozoa from fertile rams in their ability to undergo the ionophore-induced acrosome reaction. This fact, reported firstly by Watson *et al.*, (1991) may be due to variations in seminal fluid constituents or perhaps due to differences in membrane lipids composition.

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