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IN VITRO INDUCTION OF THE ACROSOME REACTION IN OVINE SPERMATOZOA BY CALCIUM IONOPHORE A23187

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The relationship between concentration of calcium ionophore A23187 and incubation time upon the proportion of spermatozoa undergoing acrosome reaction (AR) *in vitro* was investigated in rams from a commercial artificial insemination (AI) program. Two ejaculates were collected by artificial vagina from each of nine rams of three breeds (Finn Dorset, Charolais and Suffolk) aged 8–36 months. Each ejaculate was diluted in a skimmed milk extender. Spermatozoa were thereafter incubated for 45 or 60 min in modified Tyrode's medium (TALP) which contained either zero, 0.1 or 1.0 µM/l A23187. After fixing in 10% formaldehyde, the number of spermatozoa that had undergone AR was determined by phase contrast microscopy. In pre-incubation samples, 21.3 ± 3.3% of spermatozoa had undergone AR. Percentages of acrosome reacted spermatozoa were significantly ($P < 0.001$) increased after incubation with A23187. After incubation with 0.1 µM/l A23187 for 45 and 60 min there were 22.4 ± 3.0% and 31.7 ± 4.3% acrosome reacted spermatozoa, respectively. After incubation with 1.0 µM/l A23187 for 45 and 60 min there were 46.2 ± 6.5% and 53.8 ± 5.9% acrosome reacted spermatozoa, whilst corresponding numbers in control samples were 17.0 ± 2.7% and 22.3 ± 4.2%. There was also a significant ($P < 0.001$) effect of individual animals upon the responses to different concentrations of A23187. These findings indicate that (i) A23187 can be used to assess the AR of ovine spermatozoa *in vitro* and (ii) there are effects of individual animals upon the proportion of spermatozoa undergoing AR.

Key words: Ram, semen, acrosome reaction, calcium ionophore, A23187

The only reliable test of the functional integrity of a semen sample is its capacity to fertilise the ovum and to sustain embryogenesis (Watson, 1979). However, the assessment of fertilising capacity *in vivo* is costly, time-consuming and laborious. Likewise, the value of routine semen analysis in the prediction of fertility is both subjective and only poorly predicts fertility (Rodriguez-Mártinez

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et al., 1997). By contrast, the induction of AR *in vitro*, by calcium ionophore A23187, has been found to be predictive of fertility both *in vitro* (Liu and Baker, 1998) and *in vivo* (Whitfield and Parkinson, 1995). Therefore, alternative methods of assessing semen have been sought, particularly *in vitro* methods of analysing the functionality of spermatozoa, namely *in vitro* induction of AR by calcium ionophore A23187. The assessment of the ability of spermatozoa to undergo the AR *in vitro* is thus increasingly widely used as a means of determining the fertility of sires (Parkinson, 1996).

The spermatozoa must undergo AR to fertilise the ovum via penetrating the zona pellucida and fusing with the plasma membrane of the ovum (Yanagimachi, 1994). Freshly ejaculated spermatozoa need to be capacitated before undergoing AR (Harrison, 1996). In mammalian spermatozoa, both capacitation and AR can be achieved in the absence of the oocytes under *in vitro* conditions, which mimic the physiological environment of the female reproductive tract (Yanagimachi, 1994).

Various compounds (e.g. heparin or A23187) have been used for the induction of AR *in vitro* in bull (Ax et al., 1985; Whitfield and Parkinson, 1992, 1995), stallion (Christensen, 1995) and dog (Uçar, 2000). A23187-induced ARs were also studied in rams (Shams-Borhan and Harrison, 1981; Watson et al., 1991; Garde et al., 1992). The conditions of *in vitro* induction of the AR by A23187, however, have not been fully established for the ram. Hence, the relationship between concentration of A23187 and incubation time upon the proportion of spermatozoa undergoing AR *in vitro* was studied in rams from a commercial AI program.

Materials and methods

Animals

A total of nine rams of three breeds (3 rams for each of Finn Dorset, Charolais and Suffolk) aged between 8 and 36 months from a commercial AI program was used in this study. Rams were allowed to graze freely in the pasture and fed approximately 250 g standard concentrated meal/ram daily with water *ad libitum*. All rams had previously been trained to serve an artificial vagina.

Semen collection and initial assessment

Two ejaculates were collected by an artificial vagina (using an oestrogen-treated teaser ewe) from each of nine rams over at intervals of 2 to 21 days during July (i.e. in the non-breeding season). Each ejaculate was examined for mass activity (0–5) and motility (% spermatozoa exhibiting progressive motility) at a total magnification of 200×. The volume of each ejaculate was recorded and the concentration of spermatozoa was determined with an improved Neubauer

haemocytometer. Smears were prepared using nigrosin/eosin staining for the assessment of sperm morphology (Barth and Oko, 1989). Only samples with > 50% progressively motile and < 30% morphologically abnormal spermatozoa were included (Boundy, 1993) in this study.

Experimental design

In order to ascertain the conditions under which A23187 could be used to induce ARs in ram spermatozoa, a 2 × 2 factorial experiment was designed to investigate the effects of concentration of ionophore and time on the proportion of spermatozoa undergoing AR.

Semen processing

Samples were diluted at 1:2 or 1:3 (semen: diluent) in UHT skimmed milk (Evans and Maxwell, 1987) at 30–32 °C depending on their initial volumes. Once the samples were transported to the laboratory at around the same temperature (in a shirt's pocket) within 3 h, the seminal plasma and diluent was removed by layering suspension onto an equal volume of Percoll solution, containing 45% Percoll (Sigma®) and 55% TALP (see below). After centrifuging at 275 *g* for 5 min, supernatant was separated and pellet was resuspended to pre-centrifugation volume in TALP. The suspension was then further diluted in TALP to a final concentration of 40×10^6 sperm/ml.

Induction of ARs

The methods used to induce ARs by calcium ionophore (A23187; Sigma) were based on those previously described for the bull (Christensen et al., 1994). The incubation medium (TALP) was based on a modified Tyrode's medium, previously described for incubation of spermatozoa *in vitro* (Bavister and Yanagimachi, 1977) and further modified by Christensen et al. (1994). Washed spermatozoa were incubated for 45 or 60 min with 0.1 and 1 μM/l A23187. Control samples were incubated in TALP medium alone. After fixing in 10% formaldehyde, the number of spermatozoa that had undergone AR was determined by phase contrast (PC) microscopy.

Evaluation of ARs

All spermatozoa which exhibited the acrosomal region fully, in a single microscopic field, were evaluated as described by Cardullo and Florman (1993). The criteria (Uçar, 2000) used for the assessment of the stage of AR were as follows: (i) The acrosome was considered to be 'intact' where the entire acrosomal cap (particularly the apical ridge) had uniform, dark background with a smooth, clear outline (Cross and Meizel, 1989). (ii) All other spermatozoa were

considered to be acrosome 'reacted', as determined by the degree of acrosomal changes (i.e. decondensation, swelling, fuzzy appearance, irregular outlines or complete loss). One hundred spermatozoa were counted from each slide to evaluate the AR by their direct visualisation under PC microscopy at a total of 7000× magnification.

Statistical analysis

Data, which were normally distributed, were subjected to analysis of variance (MINITAB for Windows, 1996) with respect to treatment (i.e. concentration of A23187) and time, and with respect to individual rams. Where statistically significant effects were noted, comparisons between individual pairs of means were quantified by calculating least significant differences (Snedecor and Cochran, 1967).

Results

The initial characteristics of ejaculates were ranged from 65 to 85% (75%, as average) progressively motile and 15–30% (20%, as average) morphologically abnormal/dead spermatozoa.

There was a statistically significant ($P < 0.001$) interaction between concentration of A23187 and incubation time upon the number of spermatozoa having undergone AR (Table 1).

Table 1

Analysis of variance table

Term	df	F-ratio	Significance
Ram	8	3.79	$P < 0.001$
A23187 concentration	2	15.54	$P < 0.001$
Incubation time	2	6.75	$P < 0.01$
A23187 concentration × Incubation time	2	24.02	$P < 0.001$

In pre-incubation samples, 21.3 ± 3.3% of acrosome reacted spermatozoa were present (Table 2). In control samples (no A23187), there was no significant increase in the numbers of reacted spermatozoa after incubation for 45 and 60 min (17.0 ± 2.7% and 22.3 ± 4.2%, respectively). Likewise, following incubation with 0.1 µM/l A23187, there was no increase (22.4 ± 3.0%) in the number of reacted spermatozoa at 45 min, however it was significantly ($P < 0.05$) higher (31.7 ± 4.3%) at 60 min of incubation. After incubation with 1.0 µM/l A23187, there was significant ($P < 0.05$) increase by 45 min (46.2 ± 6.5%) and further by 60 min (53.8 ± 5.9%).

Table 2

The effect of concentration of A23187 and incubation time upon the percentage (mean ± SEM) of spermatozoa having undergone AR ($P < 0.001$)

A23187-treatment	Incubation time		
	0 min	45 min	60 min
0 μM/l	$21.3^{a} \pm 3.3$	$17.0^{a} \pm 2.7$	$22.3^{a} \pm 4.2$
0.1 μM/l	$21.3^{a} \pm 3.3$	$22.4^{a} \pm 3.0$	$31.7^{b} \pm 4.3$
1.0 μM/l	$21.3^{a} \pm 3.3$	$46.2^{c} \pm 6.5$	$53.8^{d} \pm 5.9$

[a–d]The values in each column with different superscripts are significantly different from each other ($P < 0.05$)

There was also a significant effect ($P < 0.001$) of individual animals upon the responses to different concentrations of A23187.

Discussion

The results of the present study showed that there was a marked interaction between concentration of A23187 and incubation time upon the number of spermatozoa having undergone AR. As the concentration of A23187 increased, the percentage of spermatozoa with intact (non-reacted) acrosomes decreased over the incubation time. Following incubation with 1.0 μM/l, the number of reacted spermatozoa was markedly increased both by 45 min and further by 60 min of incubation. These findings indicate that (i) the superior concentration of A23187 used in TALP medium (containing 3 mM/l $CaCl_2$) was 1.0 μM/l A23187 and (ii) the superior incubation time required for the induction of AR was 60 min in ram spermatozoa.

Relatively few studies upon the *in vitro* induction of AR by A23187 in ram spermatozoa exist in the literature (Shams-Borhan and Harrison, 1981; Watson et al., 1991; Garde et al., 1992). The present results are however, comparable with the findings of Shams-Borhan and Harrison (1981), who studied the effect of A23187 concentration and incubation time upon AR in rams, although the proportion of acrosome reacted spermatozoa in pre-incubation samples was considerably higher (21% *vs* 5%) in the present study. Furthermore, in the presence of 0.1 μM/l A23187 in HEPES medium, Shams-Borhan and Harrison (1981) observed a marked increase (from 5% to 30%) in the number of spermatozoa having undergone AR by 40 min. Differences between the two studies might be due to the fact that (i) the present study was conducted during the summer (non-breeding) season, which would increase the percentage of spermatozoal abnormalities including those of the acrosome (Öztürkler et al., 1997) and that (ii) all

the semen samples were processed after 3 h of collection (because of unavoidable transportation to the laboratory) which might decrease the number of spermatozoa with intact acrosomes because of ageing (or cell damage) leading to false AR (Uçar, 2000) prior to incubation (Sarhaddi et al., 1995).

There was also an apparent effect of individual animals upon the responses to different concentrations of A23187 during AR. Similar observations have also been made in other species (Whitfield and Parkinson, 1995; Christensen, 1995; Uçar, 2000). Watson et al. (1991), studying the effect of individual rams upon the AR, found a variation between individual ejaculates in the readiness of the spermatozoa to undergo an AR, but this could not be attributed consistently to any particular ram.

A23187-induced ARs have been shown to be predictive of *in vitro* fertilisation rates in subfertile men (Liu and Baker, 1998) and of *in vivo* fertility of frozen-thawed bull spermatozoa (Whitfield and Parkinson, 1995). These data imply that *in vitro* induction of the AR provides a sufficiently accurate model of the events during fertilisation *in vivo,* to allow estimates of fertility of spermatozoa to be made. However, there appears to be no study in the literature whether such a relationship exists between A23187-induced AR and *in vitro*/*in vivo* fertility in the ovine species. Thus, future studies are required in this area.

In conclusion, the overall results indicate that (i) there exists a significant relationship between A23187 concentration and incubation time on the basis of A23187-induced acrosome reaction *in vitro* and (ii) there are effects of individual animals upon the proportion of spermatozoa having undergone acrosome reaction.

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