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Progesterone does not potentiate the acrosome reaction in human spermatozoa: flow cytometric analysis using CD46 antibody

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The study was designed in order to investigate the action of progesterone on the spontaneous and ionophore-induced human spermatozoa acrosome reaction *in vitro*. The principle of the assay system is flow cytometric analysis of CD46 antibody binding to the inner acrosomal membrane. The technique is a simple and objective method of analysis, allowing fluorescent analysis of a large segment (5000 spermatozoa) of the spermatozoa population under investigation, with concomitant isolation of the live fraction of the spermatozoa population. Four concentrations of progesterone (1, 25, 50, and 100 μg/ml) were examined for their effects on spermatozoa capacitated for 4 and 24 h. In addition, motility parameters were examined by the CellSoft 2000 automated semen analyser system. Analysis of variance revealed that progesterone had no effect on either the spontaneous acrosome reaction or the ionophore-induced acrosome reaction at both 4 h and 24 h of spermatozoa capacitation times. Further, no effects on sperm motility parameters or on spermatozoa viability could be attributed to progesterone. We therefore conclude that progesterone has no objectively measurable effects on either the sperm acrosome reaction or sperm motility parameters, as measured in normal sperm populations.

Key words: acrosome reaction/CD46/flow cytometry/progesterone

Introduction

The ability of human spermatozoa to undergo capacitation and the acrosome reaction is a prerequisite for the preliminary phase of fertilization (Yanagimachi, 1988; Tesarik, 1989). The acrosome reaction of human spermatozoa has been well documented (Wassarman, 1987; Mortimer *et al.*, 1989) with regard to the physiological conditions required for its initiation and completion, the main trigger of the event being a rapid influx of calcium ions (Roldan and Harrison, 1989; Baldi *et al.*, 1991; Shimizu *et al.*, 1993). Several authors have attempted to induce this event by physiological and pharmacological techniques (Cross *et al.*, 1988; Florman *et al.*, 1989; Leyton and Saling, 1989; Harrison *et al.*, 1990), the most potent inducer, *in vitro* at least, being the family of calcium ionophores (Aitken *et al.*, 1984; Cummins *et al.*, 1991). However, the ionophores are capable of inducing the acrosome reaction in non-capacitated as well as capacitated spermatozoa (Russell *et al.*, 1979), thereby circumventing the normal physiological processes of the series of events comprising capacitation.

A variety of agents are reported to induce the acrosome reaction, ranging from solubilized zonae pellucidae, follicular fluid, peritoneal fluid, serum substitutes and platelet activating factor, to agents such as pentoxifylline, 2-deoxyadenosine, progesterone and other steroid hormones (Chan *et al.*, 1983; Margalioth *et al.*, 1988; Osman *et al.*, 1989; Palermo *et al.*, 1992; DasGupta *et al.*, 1994; Krausz *et al.*, 1994; Modotti *et al.*, 1994; Oehninger *et al.*, 1994a,b). A large portion of work has focused on the actions of progesterone on human sperm capacitation, including calcium influx (Blackmore *et al.*, 1990; Baldi *et al.*, 1991), hyperactivation (Uhler *et al.*, 1992), and the acrosome reaction (Parinaud *et al.*, 1992) *in vitro*. The most frequently used techniques for the detection of the acrosome reaction centre around fluorescence microscopy, i.e. labelling of spermatozoa with fluorescein isothiocyanate (FITC)-conjugated lectins (e.g. Liu *et al.*, 1988; Grunert *et al.*, 1990; Cummins *et al.*, 1991; Holden and Trounson, 1991). However, these techniques are quite labour intensive and subjective unless the assay is carried out in a double-blinded fashion, with a maximum observation of 100–200 spermatozoa from the total population in some studies (Parinaud *et al.*, 1992; Aitken *et al.*, 1993). Previously we reported (Carver-Ward *et al.*, 1994) a flow cytometric assay for the rapid, objective assessment of the acrosome reaction in human spermatozoa. In the assay, CD46 antibody (Ballard *et al.*, 1987, 1988; Liszewski *et al.*, 1991) is used to detect spermatozoa which have completed the acrosome reaction, as CD46 binds only to the inner acrosomal membrane (D'Cruz and Haas, 1992), and, when the flow cytometric detection is performed in conjunction with a supravital fluorescent stain, has been proven to be a reliable, rapid assay for the routine assessment of the acrosome reaction (D'Cruz and Haas, 1992; Carver-Ward *et al.*, 1994).

In the study presented here we concentrate on the objective analysis of the actions of progesterone — at physiological and supra-physiological concentrations defined as those found in follicular fluid (Margalioth *et al.*, 1988; Uhler *et al.*, 1992; Shimizu *et al.*, 1993; Modotti *et al.*, 1994) — on the major events of capacitation, namely, actions on sperm motility

parameters (reversible event), and the acrosome reaction (irreversible event). Previously, it has been indicated that progesterone demonstrates marked effects on sperm motility (Uhler *et al.*, 1992; Modotti *et al.*, 1994) as detected by automated semen analysers. However, reports on the effects of progesterone on the acrosome reaction are conflicting. Some authors claim an enhancement of the acrosomal response (Aitken *et al.*, 1993; DasGupta *et al.*, 1994; Oehninger *et al.*, 1994) while others report no effect (Uhler *et al.*, 1992). All of the conflicting data have been obtained by the use of fluorescent microscopy techniques, either using variants of the lectin binding protocols (Tesarik *et al.*, 1992; Gearon *et al.*, 1994) or the 'triple stain' technique (Talbot and Chacon, 1981; De Jonge *et al.*, 1989; Baldi *et al.*, 1991). Also, there is much diversity with respect to capacitating conditions for spermatozoa prior to testing: some authors utilize routine in-vitro fertilization (IVF) media and culture conditions with concentrations of crystalline bovine/human serum albumin of 0.3–1% (Cross *et al.*, 1986; Suarez *et al.*, 1986; Tesarik *et al.*, 1988; Calvo *et al.*, 1993; Tao *et al.*, 1993; Brucker *et al.*, 1994; Gearon *et al.*, 1994; Oehninger *et al.*, 1994a,b), others use their alternative IVF conditions of 7.5–10% maternal serum (Cummins *et al.*, 1991; Takahashi *et al.*, 1992; Uhler *et al.*, 1993; Wang *et al.*, 1993), whilst others utilize concentrations more closely related to the serum content of oviductal fluid, i.e. 25–30 mg/ml (Yudin *et al.*, 1988; Aitken *et al.*, 1994). Further, two important studies regarding the effects of capacitation time on the acrosome reaction both utilized routine IVF media with 7.5–10% maternal serum and found a peak of acrosomal loss after 4 h of incubation, and indicated that extended capacitation times were, in fact, detrimental to the spontaneous acrosome reaction (Takahashi *et al.*, 1992; Wang *et al.*, 1993). Initial studies by ourselves indicated no differences in acrosomal response with respect to incubation time or protein concentration, even after 24 h of capacitation in media with high (35 mg/ml) BSA content (unpublished data). In the study presented here we attempt to repeat the basic concepts of the previously reported studies, using our routine laboratory techniques evolved for IVF and of flow cytometry, in an attempt to provide objective data from an in-vitro assay system upon which to base our conclusions.

Materials and methods

Reagents and stock solutions

Calcium ionophore (A23187) was obtained from Sigma Chemical Co., St Louis, MO, USA. A stock solution of 5 mmol/l in dimethylsulphoxide (DMSO; Sigma) was stored at −40°C in 0.25 ml aliquots. A working stock solution was obtained by diluting 1:10 in unsupplemented human tubal fluid media (HTF; Quinn *et al.*, 1985) and equilibrated at 37°C in 5% CO_2 in air for 3 h prior to use; 10 µl was added to each allocated sperm suspension, to give a final concentration of 10 µmol (Cummins *et al.*, 1991). The final concentration of DMSO was therefore <1%; this has been shown (Carver-Ward *et al.*, 1994) to have no significant deleterious effect upon spermatozoa.

Progesterone (4-pregnene-3,20-dione) was obtained from Calbiochem Corporation, La Jolla, CA, USA. A stock solution of 2 mg/ml in DMSO was stored at −40°C in 0.25 ml aliquots. A fresh weekly working stock (200 µg/ml) solution was obtained by diluting 1:10 in HTF media supplemented with 3 mg/ml bovine serum albumin (BSA; Irvine Scientific, Santa Ana, CA, USA). Progesterone content of each daily stock solution was assessed in triplicate by a chemiluminescence/fluorescence assay (Kodak Clinical Diagnostics Ltd, Amersham, UK). The progesterone working stock was not used unless the actual assayed progesterone content was 200 µg/ml ± 20 µg/ml (100 µg/ml = 166 µmol/l).

Percoll gradients (Sigma) were made according to the protocol of Dravland and Mortimer (1985) using HTF-BSA as the diluent.

Anti-human CD46 monoclonal antibody (AMAC Inc., Westbrook, ME, USA) was made up as a stock solution of 10 µg/ml in PBS (phosphate-buffered saline, Sigma). A stock solution of fluorescein-conjugated goat anti-mouse Ig (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) of 50 µg/ml, and a stock solution of ethidium bromide (Sigma), also of 50 µg/ml, were both made up in PBS.

Sperm preparation

A total of 24 semen specimens were obtained from the andrology section of the IVF clinical laboratory. All specimens were verified as having previous fertilization success, i.e. by natural conception or by intrauterine insemination (IUI)/IVF. All specimens were collected by masturbation into sterile, non-toxic containers after 48–72 h of sexual abstinence. All specimens were processed within 1 h of collection and after completion of liquefaction at room temperature for 30 min.

Routine semen analysis was carried out using the Makler chamber (Sefi Medical Instruments, Haifa, Israel) containing 5 µl liquefied semen linked to a CellSoft automated semen analyser system (CellSoft 2000, Cryo Resources Ltd, NY, USA). The set-up for each parameter measurement was according to the guidelines of Mortimer and Mortimer (1988). Sampling was performed in 12 random fields with a minimum of 200 sperm analysed for each parameter. Semen was consistently diluted to 30×10^6 sperm/ml with homologous seminal plasma prior to counting. Only specimens exhibiting $>50\times10^6$ sperm/ejaculate with >70% motility and >50% forward progressive motility were allocated to the study (i.e. highly normal sperm parameters). Morphology was assessed for each specimen, pre-Percoll, post-Percoll, and post-treatment, by staining with Diff-Quik (Baxter Scientific Products, McGaw Park, IL, USA) and according to strict criteria (Kruger *et al.*, 1986). Spermatozoa with <30% normal forms were also excluded from the study.

Specimen allocation

A total of 24 specimens were selected for inclusion in the study based on the criteria outlined above. The flow chart in Figure 1 details the allocation and treatment of each individual specimen. Specimens were subjected to Percoll density gradient centrifugation as previously described (Carver-Ward *et al.*, 1994), after which a post-Percoll analysis was carried out. Processed specimens were diluted to contain 50×10^6 motile spermatozoa/ml. This gives a final concentration for progesterone incubation of 9×10^6 /ml in each aliquot. A 0.5 ml aliquot was incubated for 4 h at 37°C/5% CO_2. A second 0.5 ml aliquot was incubated for 24 h. After termination of incubation a further semen analysis was carried out and the specimen diluted to 2.5 ml with HTF-BSA. This was further divided into five 0.5 ml aliquots, to each of which was added progesterone to give final concentrations of 0(A), 1(C), 25(E), 50(G), and 100(I) µg/ml. These dilutions were based on previously reported studies (Margalioth *et al.*, 1988; Uhler *et al.*, 1992; Shimizu *et al.*, 1993; Modotti *et al.*, 1994). Aliquots were incubated as above for 1 h, after which a further semen analysis was performed. Aliquots were made up to 5 ml with HTF-BSA and centrifuged at 300 *g* for 10 min to remove the progesterone. Pellets were resuspended up to 1 ml with HTF-BSA

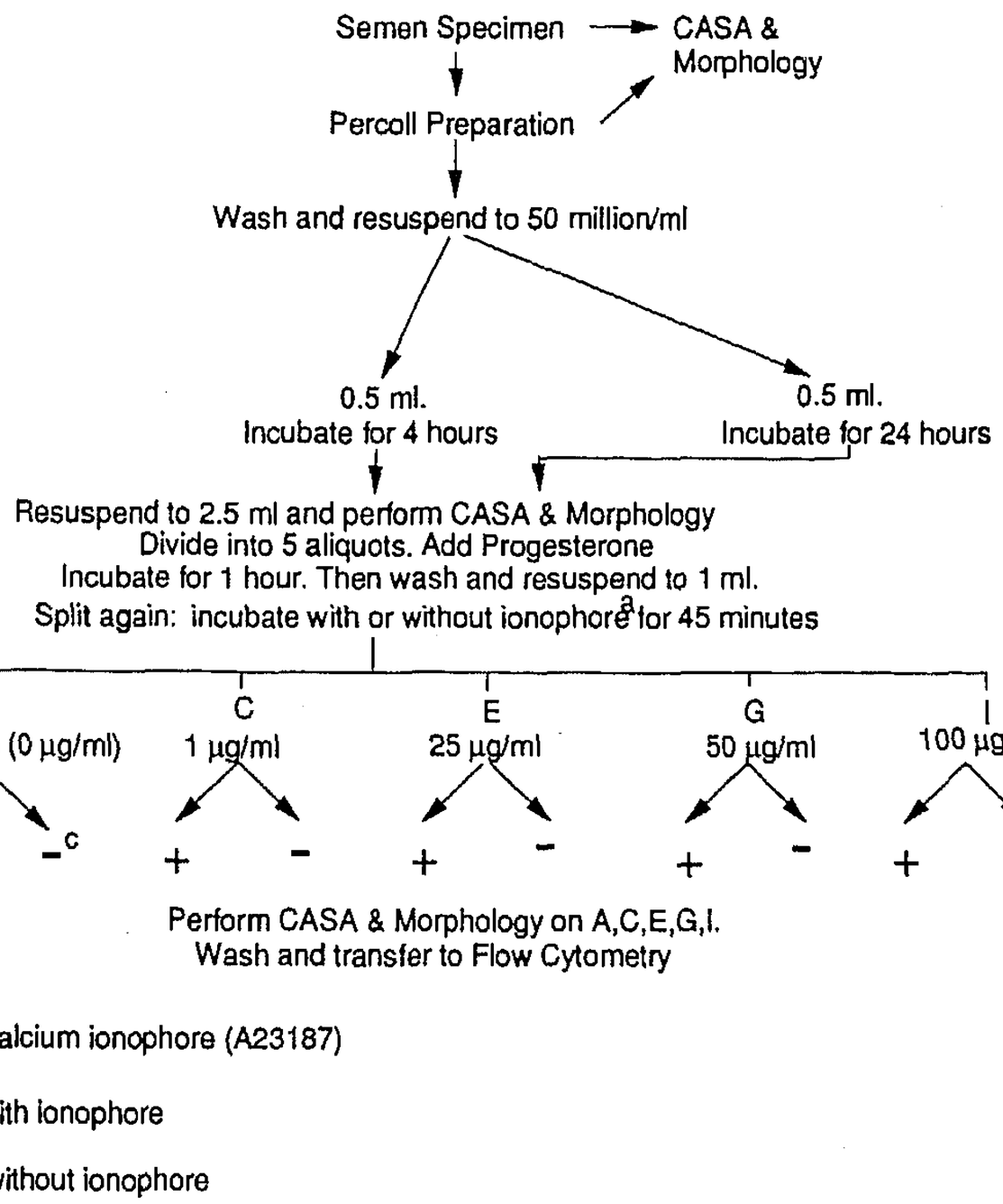

Figure 1. Flow chart to show the allocation of specimen treatments.

and divided again into two 0.5 ml aliquots. The second of each pair of aliquots received 10 µl A23187 (final concentration 10 µM); the first of each treatment pair received HTF-BSA alone. All tubes were again incubated, as above, for 45 min. After termination of incubation, volumes were made up to 5 ml with HTF-BSA and centrifuged at 300 *g* for 10 min to remove the A23187. Supernatants were discarded and the pellets resuspended to 0.5 ml with HTF-BSA and transported to the flow cytometry laboratory for further processing.

Flow cytometry

This was performed as previously described (Carver-Ward *et al.*, 1994). Briefly, all suspensions were made up to 2 ml with PBS and centrifuged at 500 *g* for 5 min. Pellets were resuspended in 20 µl anti-human CD46 monoclonal antibody (final concentration 10 µg/ml) and mixed by light vortexing. Tubes were incubated at room temperature for 30 min followed by centrifugation with 2 ml PBS. Supernatants were discarded and 4 µl FITC-conjugated goat anti-mouse Ig added to each pellet and mixed by light vortexing. Tubes were incubated for 30 min at room temperature in the dark, followed by two washes in PBS (as above). Final pellets were resuspended in 1 ml PBS and then analysed on the FACScan flow cytometer (Becton Dickinson). Immediately prior to analysis, 20 µl ethidium bromide (50 µg/ml) was added to give a final concentration of 1 µg/ml.

Data were collected in duplicate in list mode on a minimum of 5000 cells, using the FACScan Research Software, and stored on disk for later analysis. The parameters for data collection were: forward scatter, side scatter, green fluorescence, and red fluorescence. A gate was set to exclude cells that exhibited red fluorescence (ethidium bromide positive) – these cells had permitted influx of the label and were concluded to be dead. After setting the gate, the number of cells, above background, that fluoresced green (CD46 positive) were counted and expressed as a percentage of the live population. It was further noted that the majority (>90%) of the dead cells were CD46 positive, thus confirming that dead and dying spermatozoa exhibit a false acrosome reaction.

Data analysis

Data regarding sperm motility parameters, morphology, and acrosome reaction were analysed using the JMP-SAS statistical package (SAS Institute, NC, USA). The method of choice was two-way analysis of variance where the factors for analysis were progesterone dose (0, 1, 25, 50, 100 µg/ml) and capacitation time (4 and 24 h). Thus, dose and time were regarded as fixed effects and not random effects. Further, an interaction between dose and time was tested for.

The interaction model employed was: $y_{ijk} = \mu + \delta_i + \tau_j + (\delta\tau)_{ij} + \varepsilon_{ijk}$, based on examining variability in subgroups and between subgroups, where μ is the overall mean, δ_i is the dose deviation, τ_j is the time deviation, $(\delta\tau)_{ij}$ is the interaction between dose and time, ε_{ijk} is the error term; the dependent variable, y_{ijk} is a function of the effects; i refers to doses 1 to 5; j refers to times 1 and 2; k refers to replicates 1–24.

Results

Sperm parameters analysed were: percent motility (%mot), velocity (vcl: µm/s), linearity (lin,%), maximum amplitude of lateral head displacement (ALHMx,µm), mean ALH (ALHMn, µm), beat cross frequency (BCF, Hz), percent normal morphology (morph), acrosome reaction [%AR and the derived acrosome response to ionophore challenge (ARIC) score], and percent live spermatozoa.

Results from the 24 specimens are shown in Tables Ia and b. Each specimen was treated according to the schedule in Figure 1.

Tables IIa and b demonstrate that treatment with various doses of progesterone over two capacitation times (4 h = t1, 24 h = t2) has no effect on any of the measured sperm motility parameters, morphology or ARIC score. Furthermore, neither treatment with progesterone alone (Figure 2), nor prior to

Table 1a. Semen parameters versus progesterone (P) dose after 4 h capacitation time

P dose	0 µg/ml	1 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
Motility (%)	74.2 ± 3.1	70.7 ± 2.8	66.6 ± 2.9	71.1 ± 2.7	73.2 ± 3.2
Velocity (µm/s)	45.5 ± 2.1	50.1 ± 1.4	45.9 ± 1.4	45.8 ± 1.7	45.4 ± 2.0
Linearity (%)	45 ± 1.2	49 ± 1.6	44 ± 1.8	44 ± 2.2	45 ± 1.4
ALHMx (µm)	3.1 ± 0.2	3.6 ± 0.1	3.4 ± 0.1	3.2 ± 0.2	3.2 ± 0.2
ALHMn (µm)	2.6 ± 0.2	3.0 ± 0.1	2.9 ± 0.1	2.8 ± 0.2	2.8 ± 0.2
BCF (Hz)	16.5 ± 0.5	17.1 ± 0.3	16.5 ± 0.4	17.5 ± 0.3	16.9 ± 0.5
%Normal (%)	69 ± 2.4	67 ± 1.5	67 ± 1.6	66 2.0	68 ± 2.0
ARIC score %	27.6 ± 1.9	28.1 ± 2.0	27.7 ± 1.9	28.3 ± 1.9	27.9 ± 1.9
%Live (%)	88.8 ± 1.4	87.6 ± 1.2	86.5 ± 1.4	87.0 ± 1.2	87.5 ± 1.3

ALMHX = maximum amplitude of lateral head displacement.
ALHMN = mean amplitude of lateral head displacement.
BCF = beat cross frequency.
ARIC = acrosome response to ionophore challenge.

Table 1b. Semen parameters versus progesterone dose after 24 h capacitation time

P dose	0 µg/ml	1 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
Motility (%)	46.9 ± 4.1	47.6 ± 3.3	49.0 ± 3.6	48.8 ± 4.2	47.6 ± 4.0
Velocity (µm/s)	46.9 ± 1.9	50.3 ± 1.1	48.4 ± 1.5	43.2 ± 5.2	47.8 ± 2.0
Linearity (%)	46 ± 2.4	50 ± 1.6	50 ± 1.9	45 ± 1.7	47 ± 1.7
ALHMx (µm)	3.4 ± 0.1	3,7 ± 0.1	3.9 ± 0.1	3.6 ± 0.2	3.6 ± 0.1
ALHMn (µm)	2.9 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	3.0 ± 0.2	3.0 ± 0.1
BCF (Hz)	17.1 ± 0.4	17.0 ± 0.3	17.3 ± 0.4	16.3 ± 0.4	17.6 ± 0.3
%Normal (%)	59 ± 2.0	58 ± 2.1	55 ± 2.6	58 ± 1.9	57 ± 2.3
ARIC score %	31.5 ± 2.9	31.0 ± 2.8	31.0 ± 1.6	31.0 ± 1.8	31.4 ± 1.9
%Live (%)	69.5 ± 1.9	70.3 ± 2.3	68.5 ± 2.6	68.7 ± 2.8	69.2 ± 3.1

NB: All results are statistically not significant, i.e. none of the treatment groups differs from the control, at either 4 h or 24 h. Results are expressed as values ±SEM.
ALHMx = maximum amplitude of lateral head displacement.
ALHMn = mean amplitude of lateral head displacement.
BCF = beat cross frequency.
ARIC = acrosome response to ionophore challenge.

calcium ionophore caused any significant alterations in the ARIC score. These data may be compared with our previous findings with pentoxifylline (Carver-Ward *et al.*, 1994), where pentoxifylline significantly potentiated the acrosomal response to calcium ionophore. Further observations of the raw acrosome reaction scores demonstrate (Table IIb) a significant increase in the spontaneous acrosome reaction after 24 h of incubation ($P < 0.0002$), which is already a well established fact (Brucker *et al.*, 1994). Notwithstanding, there was no observable effect caused by progesterone.

Table 2a. ARIC data versus progesterone concentration after 4 h capacitation time

P dose	0 µg/ml	1 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
− ION*	2.82 ± 0.46	2.93 ± 0.42	3.23 ± 0.48	3.00 ± 0.38	3.06 ± 0.50
+ ION**	30.41 ± 1.99	31.03 ± 2.09	30.94 ± 2.06	31.30 ± 2.03	30.94 ± 1.99
ARIC score	27.59 ± 1.94	28.10 ± 2.03	27.71 ± 1.94	28.3 ± 1.95	27.87 ± 1.87

ARIC = acrosome response to ionophore challenge.

Table 2b. ARIC data versus progesterone concentration after 24 h capacitation time

P dose	0 µg/ml	1 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml
− ION*	21.87 ± 2.43	22.30 ± 2.40	21.83 ± 2.35	22.55 ± 2.42	22.87 ± 2.44
+ ION**	53.34 ± 2.49	53.27 ± 2.33	53.05 ± 2.47	53.48 ± 11.49	54.18 ± 2.46
ARIC score	31.47 ± 1.94	30.97 ± 1.79	30.99 ± 1.59	30.97 ± 1.78	31.35 ± 1.86

* = without calcium ionophore (ION).
** = with calcium ionophore.
Values are expressed as means ± SEM.
ARIC = acrosome response to ionophore challenge.

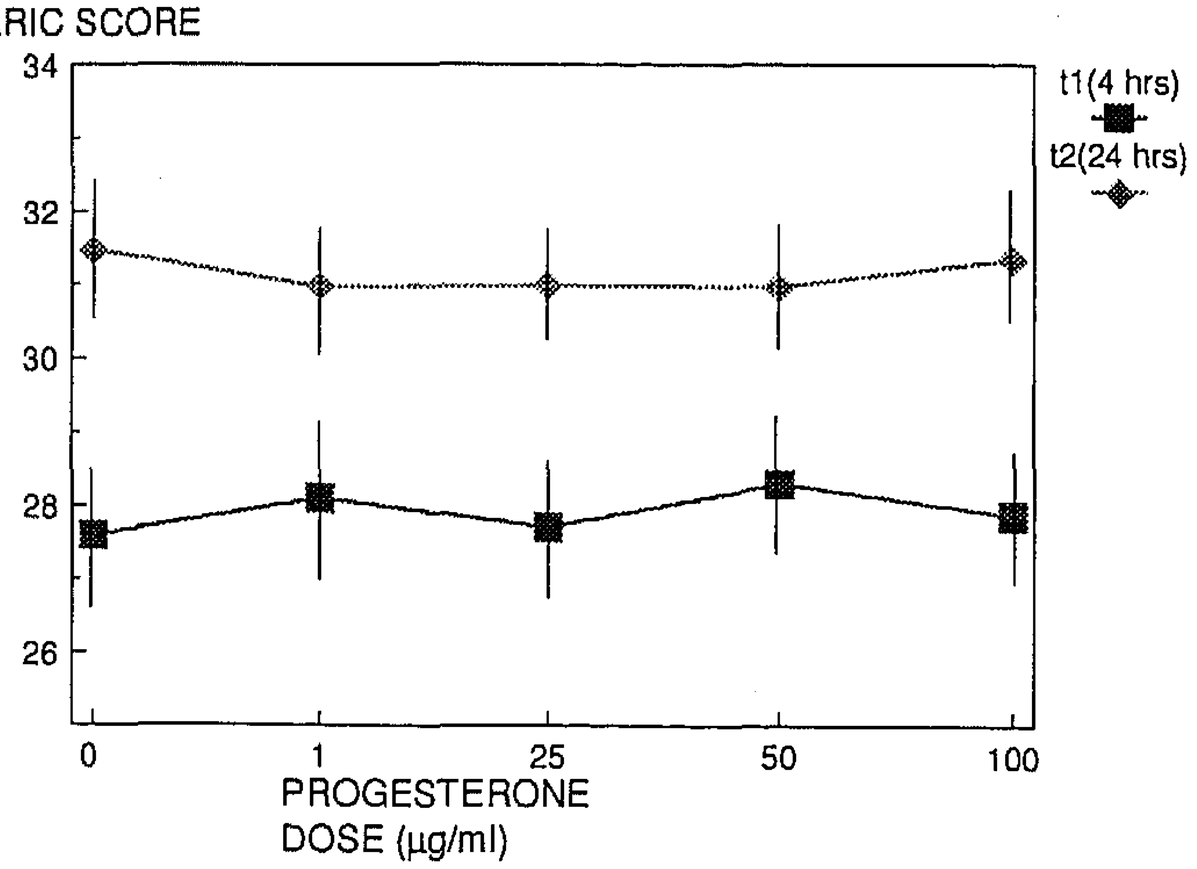

Figure 2. Graphical representation of acrosome response to ionophore challenge (ARIC) data. Effects of progesterone dose on ARIC scores after two different capacitation times. Values expressed are ± SEM. Values expressed are ±SEM.

Further analysis using multivariate analysis of variance (MANOVA) revealed no significant dose or time effects after examination of overall means for the sperm motility parameters, morphology, and the ARIC scores.

Discussion

We have previously reported a simple, routine, objective technique for the detection of the acrosome reaction in human spermatozoa, and utilized the assay to demonstrate the effects of pentoxifylline on the same (Carver-Ward *et al.*, 1994). Here we have described application of this technique to the

assessment of the actions of progesterone on the human sperm acrosome reaction. Previous studies have demonstrated conflicting data regarding the actions of progesterone. Brucker *et al.* (1994) demonstrate a stimulatory effect on the spontaneous acrosome reaction of spermatozoa subjected to 4 and 22 h of capacitation. Conversely, Uhler *et al.* (1992) found negative or minimal effects of progesterone on the spontaneous acrosome reaction. Several reports (Thomas and Meizel, 1989; Baldi *et al.*, 1991; Parinaud *et al.*, 1992; Mendoza and Tesarik, 1993; Shimizu *et al.*, 1993) have examined the effects of progesterone on calcium influx in human spermatozoa. However, evidence of an increased calcium influx cannot be taken to refer to concomitant shedding of the acrosomal matrix (Shimizu *et al.*, 1993); in fact, many events may induce reversible changes in sperm membrane permeability (thereby causing calcium channel fluctuations) without inducing actual loss of the organelle, i.e. the acrosomal membrane and matrix. Thus, we would suggest that the observations of rapid calcium influx be interpreted as a functional but reversible event related to sperm membrane permeability rather than an event terminating in the completion of the acrosome reaction. The initiation of an event should not be taken to imply the outcome unless it is combined with objective quantitation of the terminal event (Parinaud *et al.*, 1992). Similarly, observations of the sperm acrosome reaction are subject to individual variation and should be interpreted with care, for example, the concomitant use of a supravital stain is essential in all assays for sperm acrosome assessment (with the notable exception of transmission electron microscopy) due to the fact that dead and dying spermatozoa display a false positive acrosome reaction caused by breakdown of membrane integrity, thereby allowing influx of the dye labelling the acrosomal contents. Thus, assays not taking this effect into account (Gearon *et al.*, 1994) must be interpreted with caution, as any agent causing a loss of sperm viability (for example, the use of the ionophores, high centrifugation speeds, prolonged incubation times etc.) will demonstrate an artificially high 'acrosome reaction'. Notwithstanding, we observed no detrimental effects of progesterone with regard to sperm viability at all concentrations tested, whilst calcium ionophore treatment alone caused between 15 and 35% loss of viability (see Tables Ia and b), thereby agreeing with a previous report (Parinaud *et al.*, 1992) on the low toxicity of progesterone to spermatozoa.

In conclusion, we state that we observed no measurable effects of progesterone on the sperm acrosome reaction – the only observable difference (Figure 2) being a slightly increased acrosome reaction ascribable to 24 h of capacitation: however, this observation is not statistically significant. Further studies are in progress to examine the effects of other potential acrosome reaction inducers.

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