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; Holdem 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).
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., 1985; Baldi et al., 1991). In DMSO was stored at −40°C in 0.25 ml aliquots. A fresh weekly working stock (2 mg/ml) solution was obtained by diluting 1:10 in unsupplemented human tubal fluid media (HTF-BSA and centrifuged at 3000 x g, 10 minutes to remove the progesterone. Pellets were resuspended up to 1 ml with HTF-BSA.
Effect of progesterone on spermatozoa

Semen Specimen → CASA & Morphology

Percoll Preparation

Wash and resuspend to 50 million/ml

0.5 ml. Incubate for 4 hours

0.5 ml. Incubate for 24 hours

Resuspend to 2.5 ml and perform CASA & Morphology

Divide into 5 aliquots. Add Progesterone

Incubate for 1 hour. Then wash and resuspend to 1 ml.

Split again: incubate with or without ionophor for 45 minutes

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: $y_{ijk} = \mu + \delta_i + \tau_j + (\delta \tau)_{ij} + \epsilon_{ijk}$, based on examining variability in subgroups and between subgroups, where $\mu$ is the overall mean, $\delta_i$ is the dose deviation, $\tau_j$ is the time deviation, $(\delta \tau)_{ij}$ is the interaction between dose and time, $\epsilon_{ijk}$ 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 (vel: 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 1a 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

<table>
<thead>
<tr>
<th>P dose (µg/ml)</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Motility (%)</strong></td>
<td>74.2</td>
<td>70.7</td>
<td>66.6</td>
<td>71.1</td>
<td>73.2</td>
<td></td>
</tr>
<tr>
<td><strong>Velocity (µm/s)</strong></td>
<td>± 3.1</td>
<td>± 2.8</td>
<td>± 2.9</td>
<td>± 2.7</td>
<td>± 3.2</td>
<td></td>
</tr>
<tr>
<td><strong>Linearity (%)</strong></td>
<td>± 2.1</td>
<td>± 1.4</td>
<td>± 1.4</td>
<td>± 1.7</td>
<td>± 2.0</td>
<td></td>
</tr>
<tr>
<td><strong>ALHMx (µm)</strong></td>
<td>45.5</td>
<td>50.1</td>
<td>49.9</td>
<td>45.8</td>
<td>45.4</td>
<td></td>
</tr>
<tr>
<td><strong>BCF (Hz)</strong></td>
<td>± 1.2</td>
<td>± 1.6</td>
<td>± 1.8</td>
<td>± 2.2</td>
<td>± 1.4</td>
<td></td>
</tr>
<tr>
<td><strong>ALHMn (µm)</strong></td>
<td>3.1</td>
<td>3.6</td>
<td>3.4</td>
<td>3.2</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td><strong>ALHMy (µm)</strong></td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td><strong>BCF (Hz)</strong></td>
<td>16.5</td>
<td>17.1</td>
<td>16.5</td>
<td>17.5</td>
<td>16.9</td>
<td></td>
</tr>
<tr>
<td><strong>%Normal</strong></td>
<td>69</td>
<td>67</td>
<td>67</td>
<td>66</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td><strong>ARIC</strong></td>
<td>± 2.4</td>
<td>± 1.5</td>
<td>± 1.6</td>
<td>± 2.0</td>
<td>± 2.0</td>
<td></td>
</tr>
<tr>
<td><strong>%Live</strong></td>
<td>27.6</td>
<td>28.1</td>
<td>27.7</td>
<td>28.3</td>
<td>27.9</td>
<td></td>
</tr>
<tr>
<td><strong>%Normal</strong></td>
<td>88.8</td>
<td>87.6</td>
<td>86.5</td>
<td>87.0</td>
<td>87.5</td>
<td></td>
</tr>
<tr>
<td><strong>%Live</strong></td>
<td>± 1.4</td>
<td>± 1.2</td>
<td>± 1.4</td>
<td>± 1.2</td>
<td>± 1.3</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>P dose (µg/ml)</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>25</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Motility (%)</strong></td>
<td>46.9</td>
<td>47.6</td>
<td>49.0</td>
<td>48.8</td>
<td>47.6</td>
<td></td>
</tr>
<tr>
<td><strong>Velocity (µm/s)</strong></td>
<td>± 4.1</td>
<td>± 3.3</td>
<td>± 3.6</td>
<td>± 4.2</td>
<td>± 4.0</td>
<td></td>
</tr>
<tr>
<td><strong>Linearity (%)</strong></td>
<td>± 1.9</td>
<td>± 1.1</td>
<td>± 1.5</td>
<td>± 5.2</td>
<td>± 2.0</td>
<td></td>
</tr>
<tr>
<td><strong>ALHMx (µm)</strong></td>
<td>3.4</td>
<td>3.7</td>
<td>3.9</td>
<td>3.6</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td><strong>ALHMn (µm)</strong></td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td><strong>ALHMy (µm)</strong></td>
<td>2.9</td>
<td>3.2</td>
<td>3.3</td>
<td>3.0</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td><strong>BCF (Hz)</strong></td>
<td>17.1</td>
<td>17.0</td>
<td>17.3</td>
<td>16.3</td>
<td>17.6</td>
<td></td>
</tr>
<tr>
<td><strong>%Normal</strong></td>
<td>59</td>
<td>58</td>
<td>55</td>
<td>58</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td><strong>ARIC</strong></td>
<td>± 2.0</td>
<td>± 2.1</td>
<td>± 2.6</td>
<td>± 1.9</td>
<td>± 2.3</td>
<td></td>
</tr>
<tr>
<td><strong>%Live</strong></td>
<td>69.5</td>
<td>70.3</td>
<td>68.5</td>
<td>68.7</td>
<td>69.2</td>
<td></td>
</tr>
<tr>
<td><strong>%Normal</strong></td>
<td>± 1.9</td>
<td>± 2.3</td>
<td>± 2.6</td>
<td>± 2.8</td>
<td>± 3.1</td>
<td></td>
</tr>
</tbody>
</table>

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.
ALMhx = 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 1b) 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.

Figure 2. Graphical representation of acrosome response to ionophore challenge (ARIC) data. Effects of progesterone dose on ARIC scores after two different incubation 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 in human spermatozoa may 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 1a 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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References


