

## Decreased Responsiveness to Progesterone of Spermatozoa in Oligozoospermic Patients

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**ABSTRACT:** Spermatozoa from oligozoospermic subjects are characterized by a reduced *in vitro* ability to penetrate hamster oocytes and by a decreased responsiveness to physiological stimuli that trigger the acrosome reaction. One of the first steps in the induction of the acrosome reaction is an increase of intracellular free calcium concentrations ( $[Ca^{2+}]_i$ ). It has been recently shown that progesterone (P) is able to increase  $[Ca^{2+}]_i$  in capacitated human sperm at concentrations similar to those found in follicular fluid. We evaluated sperm  $[Ca^{2+}]_i$  increase in response to P (0.1  $\mu\text{g/ml}$ ) in 19 normo- and 17 oligozoospermic subjects. The average percentage of  $[Ca^{2+}]_i$  increase over the basal level was significantly lower in spermatozoa from oligozoospermic subjects when compared to normozoospermic subjects ( $136.7 \pm 8.22\%$  increase in oligo- versus  $263.3 \pm 39.7\%$  increase in normozoospermic subjects;  $P < 0.001$ ). Progesterone-

stimulated  $[Ca^{2+}]_i$  increase was significantly correlated with sperm motility ( $r = 0.54$ ), sperm concentration ( $r = 0.96$ ), and sperm morphology (% of normal forms) ( $r = 0.49$ ). In addition, P induced a significant increase of acrosome-reacted spermatozoa in normospermic patients ( $n = 10$ ), whereas no significant effect was observed in spermatozoa from oligozoospermic men ( $n = 7$ ). Taken together, these results indicate that spermatozoa from oligozoospermic men have a reduced ability to initiate the cascade of events that lead to the acrosome reaction in response to a physiological stimulus, such as P, and might contribute to explaining the reduced fertilizing capacity of these patients.

Key words: Oligozoospermia, progesterone, sperm intracellular calcium concentration, acrosome reaction, capacitation.

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Infertility associated with oligozoospermia is believed to be due to defective sperm function rather than defective sperm number. The defective sperm function is related to multiple, often concomitant, alterations. Past studies on this disease indicated several enzymatic defects, including an increased production of reactive oxygen species (Aitken et al, 1989), increased creatine-*N*-phosphotransferase (CPK) activity (Huszar et al, 1988), and increased LDH and LDH-X activities (Casano et al, 1991). However, it has been recently suggested that the increase of these enzymatic activities is due to the larger cytoplasmic volume, and thus enzyme contents, characteristic of a great percentage of spermatozoa from oligozoospermic men (Huszar et al, 1991). From a functional point of view, spermatozoa from oligozoospermic patients are much less responsive to stimuli that trigger the acrosome reaction *in vitro* (Aitken et al, 1984; Calvo et al, 1989). Moreover, in cases of severe oligozoospermia,

no acrosome-reacted spermatozoa were detected in response to follicular fluid (Calvo et al, 1989). These alterations may account for the reduced *in vitro* penetration ability of hamster oocytes (Aitken et al, 1984) characteristic of spermatozoa of oligozoospermic patients.

Recently, progesterone (P), present in high levels in follicular fluid, has been indicated as a physiological stimulus for initiation of the acrosome reaction in human spermatozoa (Osman et al, 1989). Progesterone induces an increase of intracellular free calcium concentration ( $[Ca^{2+}]_i$ ) in capacitated and capacitating human sperm (Thomas and Meizel, 1989; Blackmore et al, 1990; Baldi et al, 1991), which is the first event of the acrosome reaction (Thomas and Meizel, 1988). We hypothesize that a decreased responsiveness to P might be a biochemical feature of spermatozoa from oligozoospermic patients. To verify this hypothesis we evaluated sperm  $[Ca^{2+}]_i$  increase and induction of acrosome reaction in response to P in normo- and oligozoospermic subjects.

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### Materials and Methods

#### Chemicals

Fura 2/AM and A23187 were obtained from Calbiochem (San Diego, California). Progesterone and all other reagents were from

Table 1. Standard parameters of semen analysis in the two groups of patients (mean  $\pm$  SEM)

	Normozoospermic (n = 29)	Oligozoospermic (n = 24)
Volume (ml)	3.92 $\pm$ 0.3	3.9 $\pm$ 0.3
Sperm concentration (spermatozoa $\times$ 10 <sup>6</sup> /ml)	105.4 $\pm$ 14.4	11.3 $\pm$ 1.1*
Total sperm count (spermatozoa $\times$ 10 <sup>6</sup> /ejaculate)	389.8 $\pm$ 66.03	46.7 $\pm$ 5.7*
Motility at 60 minutes (% of progressive and non- progressive motile sperm)	49.1 $\pm$ 2.7	30.9 $\pm$ 2.7*
Morphology (% of normal forms)	62.2 $\pm$ 2.2	51.8 $\pm$ 2.1**

\*  $P < 0.001$ .\*\*  $P < 0.005$ .

Sigma (St. Louis, Missouri). Progesterone was dissolved in DMSO at an initial concentration of 2 mg/ml and further diluted in a medium (see later) containing 7 mg/ml bovine serum albumin (BSA) according to Thomas and Meizel (1989). As variability in the capacitating ability between different albumin preparations has been reported (Aitken et al, 1984), BSA from the same lot was always used (Sigma #A-7888).

### Subjects

Intracellular calcium was investigated in 19 normo- and 17 oligozoospermic men. Due to the low amount of semen available for research purposes, the acrosome reaction was studied in a different group of 10 normo- and 7 oligozoospermic subjects. All the subjects performed semen analysis in our laboratory for couple infertility. Subjects with leucocyte and/or immature germ cell concentrations  $> 10^6$ /ml were not included in the study.

### Preparation and Incubation of Spermatozoa

Human semen was collected by masturbation after 3–4 days of sexual abstinence. According to WHO criteria (1987), sperm motility was assessed at 60 minutes by determining the percentages of progressive, nonprogressive, and static spermatozoa by optical microscope, and morphology was assessed by determining the percentage of normal forms by optical microscope. Standard parameters of semen analysis (measured in the original semen samples) in the two groups of patients are reported in Table 1.

After complete liquefaction, semen samples were layered on top of a discontinuous Percoll gradient (40 and 80%) and centrifuged for 30 minutes at 1,600  $\times$  g. Cells separated on 40 and 80% Percoll were collected, mixed together, washed once in an equal volume of BWW medium (Biggers et al, 1971) containing 3 mg/ml BSA, and resuspended in the same medium at a concentration of 5  $\times$  10<sup>6</sup>/ml. Spermatozoa were capacitated by incubation for 2 hours at 37°C in BWW medium before adding 2  $\mu$ M Fura 2/AM. Previous studies from our laboratory indicated that 2 hours capacitation were sufficient to determine maximal response to P (Baldi et al, 1991). Incubation proceeded for a further 45 minutes at 37°C, according to the protocol previously described (Baldi et al, 1991). Briefly, following centrifugation,

spermatozoa were resuspended in a medium (Thomas and Meizel, 1988) containing 125 mM NaCl, 10 mM KCl, 2.5 mM CaCl<sub>2</sub>, 0.25 mM MgCl<sub>2</sub>, 19 mM Na lactate, 2.5 mM Na pyruvate, 2 mM Hepes, and 3 mg/ml BSA and were further incubated in this medium for 15 minutes at 37°C. Finally, spermatozoa were centrifuged and resuspended in the same medium at a concentration of 5  $\times$  10<sup>6</sup>/ml.

For fluorescence measurements, 2.5  $\times$  10<sup>6</sup> spermatozoa were transferred to a quartz cuvette in a total volume of 2 ml. Fluorescence was measured using a spectrofluorometer (University of Pennsylvania, Biomedical Group) set at 340 nm excitation with emission at 510 nm. In preliminary experiments, we verified that Fura 2/AM was completely hydrolyzed in loaded spermatozoa of both normo- and oligozoospermic subjects, by evaluating the excitation spectra (emission = 510 nm) of Fura 2 loaded and unloaded cells using a Shimadzu RF5000 (Kyoto, Japan) fluorimeter. In spermatozoa from both normo- ( $n = 4$ ) and oligozoospermic ( $n = 4$ ) men, two peaks were present in Fura 2 loaded cells, at 291 and at 350 nm excitation, whereas a single peak at 291 nm was present in unloaded cells. Fura 2 spectra were also evaluated after calibration of the cells with digitonin and EGTA (see later). In these calcium-free conditions, the Fura 2 peak shifted to 375 nm, whereas the peak at 291 nm was still present (data not shown). No other peaks were detected, suggesting that no partially hydrolyzed Fura 2 was present in the cells and that cell autofluorescence was not interfering with Fura 2 fluorescence at 340 nm.

Spermatozoa were stimulated with 0.1  $\mu$ g/ml P; this P concentration has been previously shown to elicit maximal stimulation of [Ca<sup>2+</sup>]<sub>i</sub> in capacitated human spermatozoa (Baldi et al, 1991). Fluorescence measurements were converted to [Ca<sup>2+</sup>]<sub>i</sub> by determining maximal fluorescence ( $F_{max}$ ) after lysing the cells with 0.01% digitonin followed by minimal fluorescence ( $F_{min}$ ) with 10 mM EGTA, pH 10. [Ca<sup>2+</sup>]<sub>i</sub> was then calculated according to Grynkiewicz et al (1985), assuming a dissociation constant of Fura 2 for calcium of 224 nM. Addition of digitonin yielded similar increases in fluorescence in all the samples examined, indicating a similar Fura 2/AM incorporation. In preliminary experiments, autofluorescence of the cells was assessed by two different methods: (1) measuring fluorescence of unloaded cells and (2) quenching total Fura 2 fluorescence with Mn<sup>2+</sup> after the

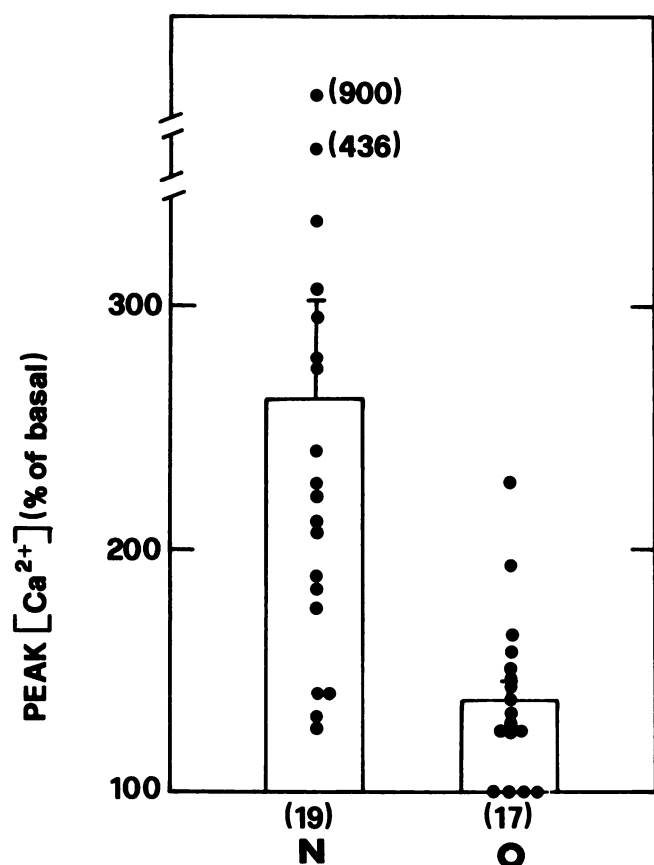


FIG. 1. Average and single values of P-stimulated  $[Ca^{2+}]_i$  (% increase over basal) in spermatozoa from normozoospermic (N) and oligozoospermic (O) subjects. The mean values of the two groups are significantly different ( $P < 0.001$ ). In parentheses is the number of subjects.

addition of ionomycin. Autofluorescence levels were almost identical with both methods, and thus, in the following experiments, autofluorescence was measured with the  $Mn^{2+}$  method. Autofluorescence subtraction did not modify basal or stimulated  $[Ca^{2+}]_i$ , as assessed in eight normo- and in six oligozoospermic men (not shown). Response to P was evaluated at least twice in each sample (same ejaculate) and the mean of the two values was used for the statistical analysis.

#### Determination of Acrosome Reaction

Acrosome-reacted spermatozoa were evaluated using the fluorescent probe fluorescein isothiocyanate (FITC)-labeled *Arachis hypogea* (peanut) lectin. Spermatozoa, prepared as described above with exclusion of Fura 2/AM loading, were stimulated with progesterone ( $4 \mu\text{g/ml}$ ) or A23187 ( $10 \mu\text{M}$ ) for 1 hour at  $37^\circ\text{C}$ . After incubation, spermatozoa were centrifuged at  $1,000 \times g$  for 5 minutes and further incubated in  $0.5 \text{ ml}$  hyposmotic swelling medium for 1 hour at  $37^\circ\text{C}$ . After centrifugation at  $400 \times g$  for 5 minutes, the pellet was resuspended in  $50 \mu\text{l}$  of ice-cold methanol, layered on an immunofluorescence slide, dried at  $37^\circ\text{C}$ , and stored at  $-20^\circ\text{C}$ . The slides were stained with fluorescent lectin at  $4^\circ\text{C}$  for 15 minutes, washed with phosphate-buffered saline, and stored at  $4^\circ\text{C}$  in the dark for up to 1 week

before scoring. Fluorescence was observed under a fluorescence microscope, and the acrosome reaction was evaluated on a total of 100 spermatozoa/slide.

#### Statistical Analysis

Results are expressed as mean  $\pm$  SEM. Statistical comparisons were made using Student's *t*-test after log transformation of the data. For correlation analyses, log-transformed data were analyzed using Pearson's correlation coefficient.

## Results

### Basal and Progesterone-Stimulated $[Ca^{2+}]_i$ in Spermatozoa from Oligozoospermic and Normozoospermic Patients

There was no significant difference in average basal  $[Ca^{2+}]_i$  between spermatozoa from normozoospermic ( $414.6 \pm 40.3 \text{ nM}$ ,  $n = 19$ ) and oligozoospermic ( $404.5 \pm 55.8 \text{ nM}$ ,  $n = 17$ ) patients. Such values were higher than the basal  $[Ca^{2+}]_i$  we reported in a previous paper in capacitated spermatozoa after 2 hours incubation ( $250 \pm 41 \text{ nM}$ ; Baldi et al, 1991). However, in the present study spermatozoa were capacitated under different conditions. In particular, capacitation was obtained in BWW medium and before incubation with Fura 2/AM. These conditions might be more effective in obtaining sperm capacitation, thus explaining the higher  $[Ca^{2+}]_i$  found.

Progesterone-stimulated  $[Ca^{2+}]_i$  increase (expressed as percentage  $[Ca^{2+}]_i$  increase over basal) was significantly higher in normozoospermic than oligozoospermic patients ( $263.3 \pm 39.7\%$  versus  $138.7 \pm 8.22\%$ ,  $P < 0.001$ ,  $n = 19$  and 17). Average and single values of  $[Ca^{2+}]_i$  percent increases in response to P in the two groups of patients are shown in Figure 1. Interestingly, in four cases of severe oligozoospermia, we could not detect any response to P (Fig. 1).

Progesterone-stimulated  $[Ca^{2+}]_i$  increase was significantly correlated with sperm motility at 60 minutes ( $r = 0.54$ ,  $P < 0.001$ ; Fig. 2), with sperm concentration ( $r = 0.96$ ,  $P < 0.001$ ; data not shown), and with sperm morphology ( $r = 0.49$ ,  $P < 0.001$ ; data not shown).

### Progesterone-Stimulated Acrosome Reaction in Spermatozoa from Normozoospermic and Oligozoospermic Men

As shown in Figure 3, spermatozoa from normospermic men respond to P ( $4 \mu\text{g/ml}$ ) and A23187 ( $10 \mu\text{M}$ ) with a significant increase of acrosome-reacted forms. In contrast, spermatozoa from oligozoospermic men did not show any significant increase of acrosome-reacted forms in response to P, whereas a significant increase of acrosome reaction, of a similar magnitude to that obtained in normospermic subjects, was present following exposure to A23187 (Fig. 3).

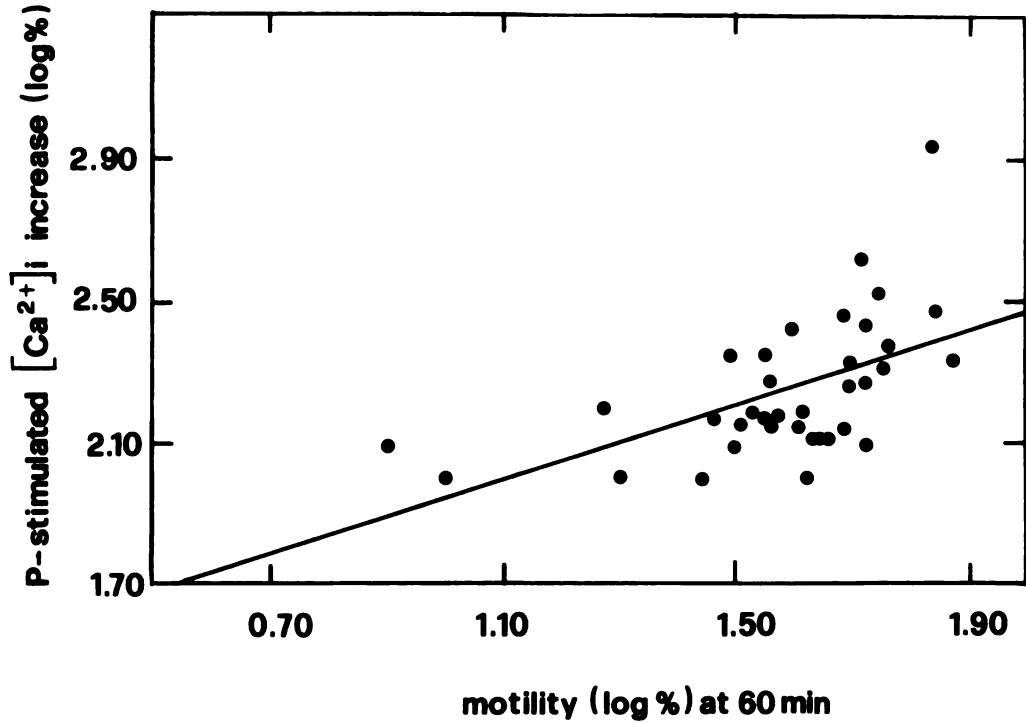


FIG. 2. Correlation between sperm motility at 60 minutes and P-stimulated  $[Ca^{2+}]_i$  increase in spermatozoa from 19 normo- and 17 oligozoospermic men. Regression analysis indicates a statistically significant relationship, with a correlation coefficient of 0.54 ( $P < 0.001$ ).

**Discussion**

Spermatozoa from oligozoospermic patients are characterized by a reduced *in vitro* fertilization rate of human

oocytes (Matson et al, 1989) and penetration of hamster oocytes (Aitken et al, 1984). Furthermore, they are less responsive to nonphysiological (A23187 and ionomycin) and physiological (follicular fluid) stimuli of acrosome

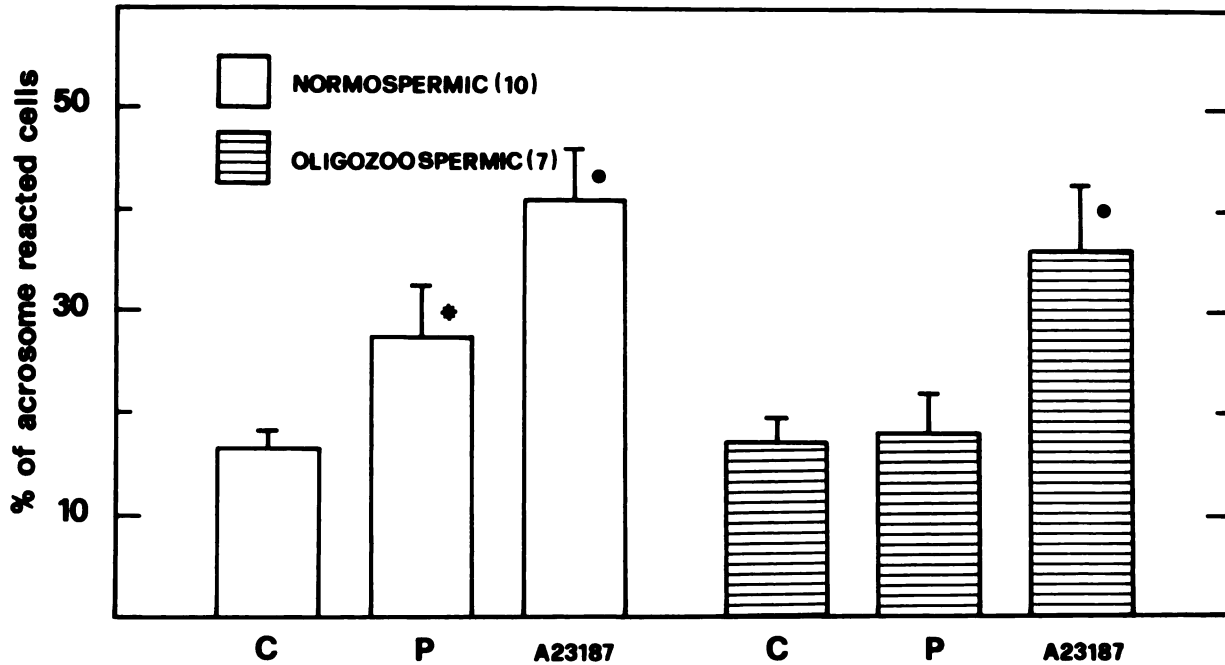


FIG. 3. Average percentage acrosome-reacted forms in basal (C) and P- ( $4 \mu\text{g/ml}$ ) and A23187- ( $10 \mu\text{M}$ ) stimulated spermatozoa from 10 normo- and 7 oligozoospermic subjects. ●  $P < 0.01$ ; \*  $P < 0.05$  versus C.

reaction (Aitken et al, 1984; Calvo et al, 1989). Recently, increases of creatine-phosphokinase (Huszar et al, 1988) and LDH and LDH-X (Casano et al, 1991) activities and increased production of reactive oxygen species (Aitken et al, 1989) have been reported in spermatozoa from oligozoospermic patients. However, these alterations are probably related to the relatively larger volume of cytoplasm present in spermatozoa from oligozoospermic patients, thus reflecting an increase of enzyme contents more than a true biochemical alteration of the cell (Huszar et al, 1991).

Recently, progesterone, present in high concentration in follicular fluid and in cumulus matrix, has been indicated as a possible physiological stimulus for initiation of the acrosome reaction in human capacitated spermatozoa (Osman et al, 1989). Indeed, P induces an increase of  $[Ca^{2+}]_i$  (Thomas and Meizel, 1989; Blackmore et al, 1990; Baldi et al, 1991) in human sperm at concentrations similar to those found in human follicular fluid (Thomas and Meizel, 1989). We now report a decreased responsiveness to P, measured as both an increase of  $[Ca^{2+}]_i$  and induction of acrosome reaction, in spermatozoa from oligozoospermic patients when compared to normozoospermic ones. Moreover, the increase of intracellular  $Ca^{2+}$  in response to P was significantly correlated with sperm concentration, sperm motility, and sperm morphology. These findings suggest a functional alteration in the ability of spermatozoa from oligozoospermic men to initiate the acrosome reaction in response to a physiological stimulus. Surprisingly, in our hands, spermatozoa from oligozoospermic men exhibited an increase of the acrosome reaction of a magnitude similar to that observed in normozoospermic subjects, when stimulated with  $10 \mu M$  A23187. Such a finding is in apparent contrast with the decreased responsiveness to A23187 observed in oligozoospermic men by Aitken et al (1984) and in a group of subfertile men by Cummins et al (1991). However, in the study by Aitken et al (1984), responsiveness to A23187 was tested as sperm penetration rate of hamster oocytes and increase of motility, but not acrosome reaction, whereas Cummins et al (1991) evaluated the acrosome reaction in response to A23187 with a different methodological approach (separation on Percoll gradient was performed after stimulation with A23178) and in a larger number of cases.

The decreased responsiveness to P can hardly be explained by the increased cytoplasmic volume characteristic of spermatozoa from oligozoospermic patients, as P action is due to an interaction with sperm surface, as discussed later, and does not involve the cytoplasm. Indeed, we believe that the decreased response to P is an effective biochemical alteration of the spermatozoon that might be related to the decreased fertilizing capacity, although the relationship between response to P and fertilizing capacity is yet to be defined.

The mechanism of P action on human sperm is still debated. Progesterone-binding sites are present on the sperm surface (Chang et al, 1981), although they appear to be different from classic nuclear P receptors (Baldi et al, 1991). On the other hand, P-stimulated  $[Ca^{2+}]_i$  increase in human sperm is mediated by an interaction with sperm surface, as recently demonstrated using BSA-conjugated P (Meizel and Turner, 1991), and is completely abolished in the presence of EGTA (Thomas and Meizel, 1989). Taken together, these data suggest the activation by P of a receptor-operated  $Ca^{2+}$  channel or, alternatively, a P-mediated increase of permeability to  $Ca^{2+}$  ions. The reduced responsiveness we found in sperm from oligozoospermic subjects might be due to a decrease of P-binding sites on the sperm surface and/or a reduced permeability to  $Ca^{2+}$  ions. However, basal  $[Ca^{2+}]_i$  were similar in the two groups of subjects, indicating similar permeability of sperm plasma membrane to  $Ca^{2+}$  ions.

In conclusion, our data demonstrated a decreased  $[Ca^{2+}]_i$  response to P of spermatozoa in oligozoospermic patients. As the increase of  $[Ca^{2+}]_i$  is considered the first event of acrosome reaction, our finding might contribute to explaining the reduced fertilizing capacity characteristic of these patients.

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