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Two functional assays of sperm responsiveness to progesterone and their predictive values in in-vitro fertilization

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We have recently reported, in a small cohort of subjects, that acrosome reaction (AR) and intracellular free calcium ($[Ca^{2+}]_i$) increase in response to progesterone were significantly correlated with in-vitro fertilization (IVF) rate. In the present study we extended these results to 90 subjects undergoing IVF. We confirm that both parameters were highly significantly correlated with the fertilization rate ($P < 0.001$). In particular, significantly lower responses to progesterone were detected in subjects with a fertilization rate <50%, further enlightening the functional significance of sperm responsiveness to progesterone with respect to the process of fertilization. Moreover, we report here that both tests are highly discriminant of fertilization success, with positive predictive values >90% for $[Ca^{2+}]_i$ values which increase by >1.2-fold and AR inducibility >7% (cut-off values). Conversely, AR following challenge with the calcium ionophore A23187 was less significantly correlated with the percentage fertilization rate ($P < 0.05$), and showed lower predictive values than response to progesterone. All these tests ($[Ca^{2+}]_i$ increase in response to progesterone, AR in response to progesterone and to A23187) appear highly sensitive and moderately specific. The positive predictive value may rise to >95% when the combination of two tests ($[Ca^{2+}]_i$ and inducibility of AR in response to progesterone) is considered. No correlation with fertilization rate has been found for spontaneous AR or basal $[Ca^{2+}]_i$. In conclusion, we propose that assessment of human sperm responsiveness to progesterone may be clinically useful in predicting fertilizing ability *in vitro*.

Key words. A23187/acrosome reaction/ intracellular free calcium/IVF/progesterone

Introduction

Although in-vitro fertilization (IVF) is an important therapeutic option for many infertile couples, the percentage of fertilization failure is rather high in cases where the male partner is subfertile. In view of the fact that other assisted reproduction techniques, more specific for the treatment of male factor, are now available [such as intracytoplasmatic sperm injection (ICSI)], the possibility of predicting sperm fertilizing ability would be of great help in choosing the most appropriate technique. It is well established that routine semen analysis provides very little information on sperm fertilizing ability (Avery, 1992), and thus more specific and sensitive tests are necessary. In the past few years several tests have been proposed (for review, see Liu and Baker, 1992); however, since the success of fertilization at IVF is dependent on several variables, the predictive value of a single test is quite limited, with the exception of hemizona assay (Franken *et al.*, 1993; Gamzu *et al.*, 1994), which requires human oocytes and thus cannot be used as a routine pre-IVF assay. Limitation of single sperm functional tests is mainly related to the fact that sperm dysfunctions can be multiple and/or present at different steps of the fertilizing process [expression of hyperactivated motility pattern; sperm–zona binding, acrosome reaction (AR), zona penetration and fusion with oolemma] and thus the simultaneous or 'step-by-step' evaluation of different functional parameters can give a much more accurate prediction. Since AR is an essential event for the process of fertilization, the detection of the ability of spermatozoa to undergo AR has been proposed as a potential test of sperm function (for review, see De Jonge, 1994). In addition, since AR is dependent on an increase of intracellular calcium ($[Ca^{2+}]_i$), the simultaneous measurement of $[Ca^{2+}]_i$ increase and AR may give additional information on the integrity of signal transduction mechanisms leading to AR (Shimizu *et al.*, 1993).

Among the different in-vivo inducers of the AR, zona glycoprotein ZP3 (which mediates sperm–oocyte interaction) (Cross *et al.*, 1988; Chamberlin and Dean, 1990), follicular fluid (Thomas and Meizel, 1988), cumulus–oophorus complex (Tesarik, 1985) and progesterone (Baldi *et al.*, 1991; Parinaud *et al.*, 1992; Uhler *et al.*, 1992) are the most specific, since they are present in high concentrations in the proximity of the oocyte. Although ZP3 is potentially the more appropriate agent to induce AR with the aim of studying sperm fertilizing ability, the lack of commercially available recombinant ZP3 limits the possibility of developing a routine test. Progesterone, present in high concentrations in the follicular fluid and in the cumulus matrix, has been shown to be able to enhance several sperm functions including the AR, hyperactivated motility, sperm–zona pellucida penetration, sperm–oocyte penetration (for review, see Baldi *et al.*, 1995). The clinical significance of these observations has recently been confirmed by studies on oligoasthenoteratozoospermic/subfertile versus normozoospermic men (Falsetti *et al*, 1993; Oehninger *et al.*, 1994) Moreover, in some cases of unexplained male infertility, Tesarik and Mendoza (1992) found, as a sole sperm anomaly,

a defective function of the putative sperm surface progesterone receptor. Recently, in a small cohort of patients, a highly significant correlation between sperm responsiveness to progesterone (in terms of $[Ca^{2+}]_i$ increase and AR) and fertilization rate *in vitro* has been reported by our group (Krausz *et al.*, 1995). In the present paper we extended this study to 90 infertile couples. We used progesterone to stimulate $[Ca^{2+}]_i$ while AR was induced by two distinct stimuli: a physiological one (progesterone), and a pharmacological one (the ionophore A23187). We studied the relationship between progesterone responsiveness assays, A23187-induced AR, parameters of routine semen analysis and fertilization rate. In order to establish the clinical significance of these assays we calculated their sensitivity, specificity, positive and negative predictive values.

Materials and methods

Chemicals

Fura 2/AM, A23187, ionomycin and fluorescein isothiocyanate (FITC)-labelled *Arachis hypogea* (peanut) lectin were obtained from Calbiochem (San Diego, CA, USA). Progesterone and FITC-labelled lectin were obtained from Sigma (St Louis, MO, USA). IVF medium was obtained from Medi Cult (Denmark). Progesterone and A23187 were dissolved in dimethyl sulphoxide (DMSO) at an initial concentration of 10 mg/ml and 10 mM respectively and further dilutions were made in IVF medium.

Subjects

The study population consisted of 90 unselected couples undergoing IVF therapy. The simultaneous measurement of all four parameters was not always possible, therefore the measurement of $[Ca^{2+}]_i$ was performed in 69 subjects, AR following progesterone stimulation in 60 subjects and AR following A23187 stimulation in 53 subjects

Preparation of spermatozoa

Spermatozoa were washed from seminal plasma; motile spermatozoa were separated using a 1 h on pellet swim-up procedure in IVF medium, washed, counted in a Makler chamber three times, and divided into two aliquots, one for subsequent oocyte insemination (100 000 spermatozoa/oocyte) and the other for calcium and AR studies. Oocytes were collected under transvaginal ultrasound guidance from women previously treated according to the following protocol (Krausz *et al.*, 1995): s.c buserelin acetate (Suprefact, Hoechst, 600 μg/day) starting from day 21 of the last menstrual cycle to desensitize the pituitary gland. When pituitary desensitization had been achieved as shown by a concentration of serum oestradiol <50 pg/ml, buserelin acetate treatment was continued together with purified follicle-stimulating hormone (FSH, 225 IU/day, Metrodin, Serono) Ovarian stimulation was monitored by daily ultrasound assessment of follicular growth and estimation of serum oestradiol concentration. When serum oestradiol was >600 pg/ml, women were treated with 10 000 IU human chorionic gonadotrophin (HCG, Profasi HP, Serono) and oocyte retrieval performed 34 h later. An average of 7.6 ± 0.94 ova per woman was recovered during this procedure Oocytes were graded (from 1 to 6) based on morphological criteria of cumulus and corona radiata according to Veeck (1986). All the oocytes ranged from grade 3 to 6. All the available oocytes were used for insemination.

Measurement of $[Ca^{2+}]_i$

After swim-up procedure, spermatozoa were resuspended at a concentration of 5×10^6/ml in IVF medium, incubated at 37°C with fura 2/AM for 45 min, washed, resuspended in FM buffer (125 mM NaCl, 10 mM KCl, 2.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, 19 mM sodium lactate, 2.5 mM sodium pyruvate, 20 mM HEPES/NaOH; Thomas and Meizel, 1988) and further incubated for 15 min. $[Ca^{2+}]_i$ was measured as previously described using a spectrofluorimetric method (Baldi *et al.*, 1991; Falsetti *et al*, 1993). Spermatozoa were transferred to a quartz cuvette in a total volume of 2 ml containing a total of 5×10^6 spermatozoa Fluorescence was measured using a spectrofluorometer (University of Pennsylvania Biomedical Group, Philadelphia, PA, USA) set at 340 nm excitation with emission at 510 nm. Spermatozoa were stimulated with 0 1 and 1 μg/ml progesterone which was added directly in the cuvette. Fluorescence measurements were converted to $[Ca^{2+}]_i$ by determining maximal fluorescence (F_{max}) with ionomycin (8 mM final concentration) followed by minimal fluorescence (F_{min}) with 10 mM EGTA, pH 10. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz *et al.* (1985) assuming a dissociation constant of fura 2 for calcium of 224 nM. Addition of ionomycin yielded similar increases in fluorescence in all the samples examined, indicating a similar fura 2/AM incorporation. Autofluorescence of the cells was assessed by measuring fluorescence of unloaded cells, autofluorescence subtraction did not modify basal or stimulated $[Ca^{2+}]_i$ as previously reported (Falsetti *et al.*, 1993).

Determination of AR

Acrosome-reacted spermatozoa were evaluated using the fluorescent probe FITC-labelled *A.hypogea* lectin, as described previously (Aitken *et al.*, 1993, Krausz *et al.*, 1994) Briefly, aliquots (125 μl) of spermatozoa (10×10^6/ml) recovered after swim-up procedure were incubated with an equal volume of progesterone (4 μg/ml), A23187 (10 μM) or DMSO control solvent for 1 h at 37°C, centrifuged and further incubated in 0.5 ml hypo-osmotic swelling medium for 1 h. After centrifugation, spermatozoa were finally suspended in 50 μl ice-cold methanol, layered on a slide, stained with fluorescent lectin and scored using a fluorescent microscope (Leitz Wetzlar, Germany). The AR was evaluated on a total of 100 spermatozoa/slide. Only curly tailed spermatozoa were scored. Control solvent gave identical results to IVF medium alone. The difference between percentage progesterone- or ionophore-induced and percentage spontaneous acrosome reacted spermatozoa was considered to be the percentage of spermatozoa in the population capable of responding to progesterone or ionophore [and thus called, respectively, AR following progesterone challenge (ARPC) and AR following ionophore challenge (ARIC)].

Statistical analysis

Results are expressed as mean ± SEM The distribution of the raw data was examined by scattergram analysis as well as calculations of kurtosis and frequency distribution plots Where appropriate, the data were normalized by $\log([Ca^{2+}]_i)$, or square root (AR) transformation to reduce skews. The *t*-test was used to assess the statistical significance of differences between groups after normalization of the data distribution. The data were back-transformed for presentation.

Results

Reduced responsiveness to progesterone and A23187 in samples exhibiting <50% fertilization rate (FR)

Of the 90 couples admitted to the study, 54 (59.3%) had female factor, eight (8.7%) male factor, 21 (23%) mixed and eight (8.7%) unexplained infertility.

The mean percentage oocytes fertilized (average FR) for the overall population was 46 ± 3.6% (n = 90). This value is slightly lower than that reported by other authors in different

study populations but in the present study no oocyte selection was performed before IVF; furthermore, a relatively high number of subjects whose spermatozoa did not fertilize any egg were included in the study population (see below)

Subjects were arbitrarily divided into two groups on the basis of average FR: group 1 with FR ≥50% (average FR, 75.3 ± 2.5, n = 47), and group 2 with a FR <50% (average FR, 13.6 ± 2.3, n = 43). In group 2, 20 subjects out of 43 did not fertilize any egg. No significant differences were observed concerning oocyte number and grade of maturation between the two groups of subjects (data not shown).

Among the different routine semen analysis parameters, the highest degree of statistical significance between the two groups of patients was found for basal (not shown) and post-swim-up total motility (74 ± 3 and 85 ± 2% in groups 1 and 2 respectively, $P < 0.005$). Although a statistically significant correlation between percentage motility after swim-up and FR was found (r = 0.413; $P < 0.001$, n = 90), 50% of patients whose spermatozoa failed to fertilize had sperm motility >80%, indicating a poor predictive value of this parameter with respect to IVF outcome, as already reported (for review, see Liu and Baker, 1992). Similar results were obtained when basal and post-swim-up sperm concentrations were considered (results not shown). Moreover, although the percentage of cells with normal morphology, assessed according to WHO (1992) procedure, was slightly lower in group 2 (49 ±3%) with respect to group 1 (54 ± 2%), the difference was not significant and no correlation was found between FR and percentage normal sperm forms. These results confirm that assessing sperm characteristics with routine semen analysis is poorly indicative of fertilizing ability.

Basal $[Ca^{2+}]_i$ was similar in the two groups of patients (results not shown). Average percentage sperm $[Ca^{2+}]_i$ increase in response to both 0.1 and 1.0 μg/ml progesterone was significantly lower in subjects with a FR < 50% compared to subjects with a FR ≥ 50% (Figure 1), confirming previous results obtained by our group (Krausz *et al.*, 1995). In six out of the 15 patients (40%) that did not fertilize any egg where $[Ca^{2+}]_i$ increase in response to progesterone was tested, no response was observed. In the remaining patients, mean $[Ca^{2+}]_i$ increased in response to progesterone by a factor of 1.6 ±0.12 (n = 9). It is worth noting that three of the latter patients were included in another IVF cycle, and in two of them fertilization was achieved on the second attempt.

Basal percentage AR was similar in the two groups of patients (Figure 2). After stimulation with 4 μg/ml progesterone and 10 μmol/l A23187 a significantly lower response was detected in subjects with FR < 50% (Figure 2). In particular, the significance between the two groups was more pronounced when progesterone was used to stimulate spermatozoa ($P < 0.001$ compared with $P < 0.05$ for A23187).

Responsiveness (measured as $[Ca^{2+}]_i$ and AR) to A23187 and relationship to percentage fertilization rate

The $\log[Ca^{2+}]_i$ increases in response to both 0.1 and 1 0 μg/ml progesterone, √ARPC and √ARIC were all significantly correlated to FR (Table I). Among these parameters, the most significant relationship was found between √ARPC and FR

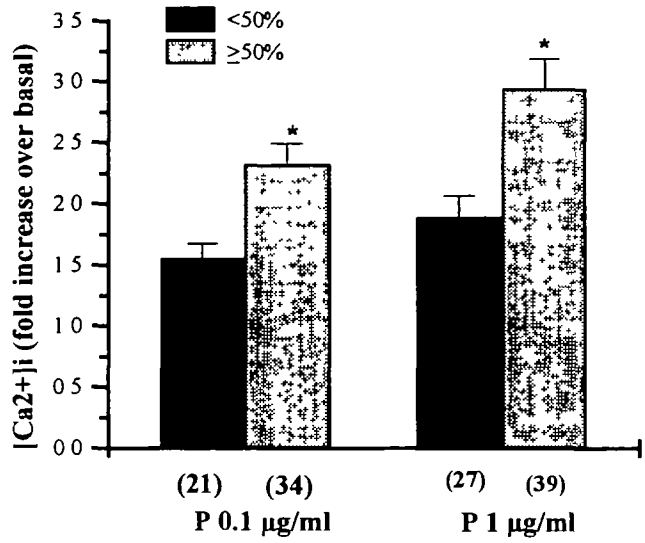

Figure 1. Intracellular calcium $[Ca^{2+}]_i$ increase in response to two different concentrations (0.1 and 1 μg/ml) of progesterone (P) in fura 2-loaded spermatozoa of patients with a fertilization rate <50% or ≥50% Number of patients shown in parentheses *Significantly different from corresponding group 2 measurement, $P < 0.005$

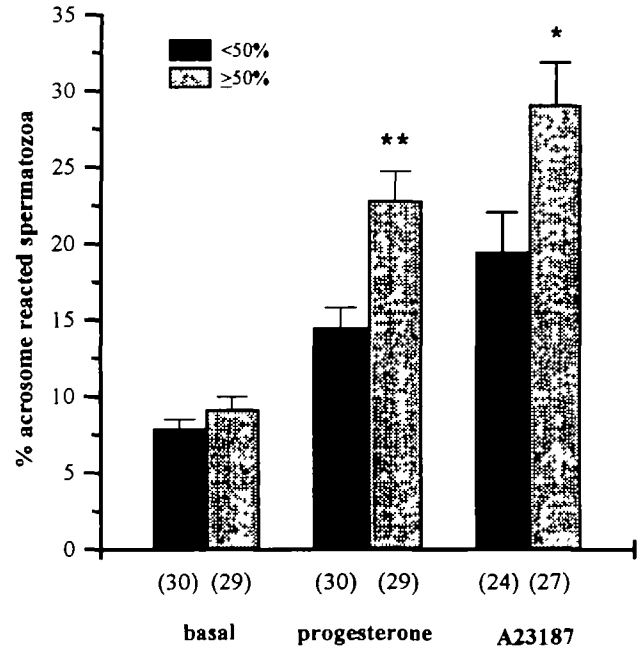

Figure 2. Basal, progesterone- and A23187-stimulated acrosome reaction in spermatozoa of patients with fertilization rate <50% or ≥50% Number of patients shown in parentheses. Significantly different from corresponding group 2 measurements at *$P < 0.05$ and **$P < 0.001$

(Table I). When the subjects are divided into three groups on the basis of ARPC, the relationship between ARPC and FR is evident (Figure 3) Indeed, the mean percentage of eggs fertilized at IVF increased as the percentage of spermatozoa capable of acrosome discharge became greater (Figure 3). √ARPC was significantly correlated to $\log[Ca^{2+}]_i$ increase in response to progesterone (r = 0.45; $P < 0.007$), indicating a close relationship between the two parameters.

Table I. Correlation coefficients by simple regression analysis of the different parameters evaluated and the fertilization rate obtained

Parameter	r^a	P^b	n^c
Log[Ca^{2+}]$_i$ – 1 0 µg/ml progesterone	0 456	0.0005	70
Log[Ca^{2+}]$_i$ – 0 1 µg/ml progesterone	0.445	0.0005	56
√AR (basal)	0.146	NS	60
√ARIC	0.308	0 005	53
√ARPC	0 493	0 0001	59

NS, not significant, [Ca^{2+}]$_i$, intracellular calcium; AR, acrosome reaction; ARIC, AR following ionophore challenge; ARPC, AR following progesterone challenge.
[a]Regression coefficient.
[b]Significance of probability
[c]Number of couples.

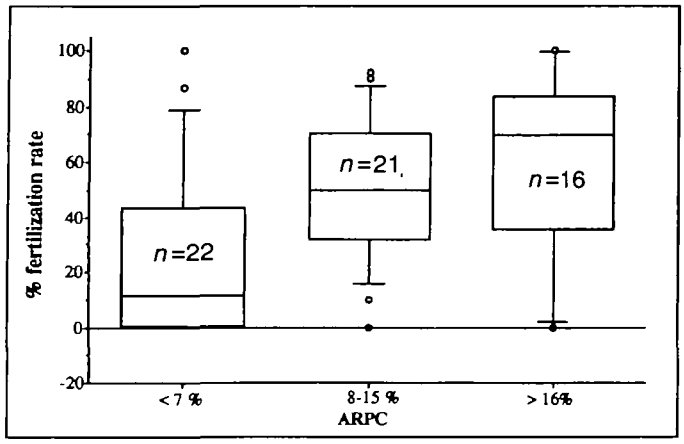

Figure 3. Average response curve for the percentage fertilization rate at IVF by patient population grouped according to acrosome reaction following progesterone challenge (ARPC) values. Boxes indicate 25th and 75th percentiles while the horizontal line within the box indicates the 50th percentile value (median). Vertical lines give 10th and 90th percentile limits of the data, while single points indicate extreme values outside this range.

Predictive values of the single tests

In order to calculate specificity, sensitivity, and positive and negative predictive values, two different limiting (cut-off) values were chosen for each parameter (Table II): the lower one because of the higher sensitivity associated with the highest negative predictive value, and the higher one because of the highest positive predictive value. The percentage of patients with values over the cut-off whose spermatozoa fertilized at IVF (positive predictive value) was >80% for all parameters for both limiting values (Table II). The best positive predictive values were found for 0.1 µg/ml [Ca^{2+}]$_i$ and ARPC (Table II). Conversely, the percentage of patients with values under the cut-off that did not fertilize (negative predictive value) was <80% for all parameters, the most sensitive and specific being [Ca^{2+}]$_i$ increase in response to 0.1 µg/ml progesterone and ARPC (Table II).

In order to establish if the combination of the two tests (ARPC and [Ca^{2+}]$_i$ increase in response to progesterone) which showed the higher correlation with respect to FR could give a better predictive model, we assigned 1 or 0 points for each test respectively, when the value was above or below the higher cut-off value. We found that when both tests gave

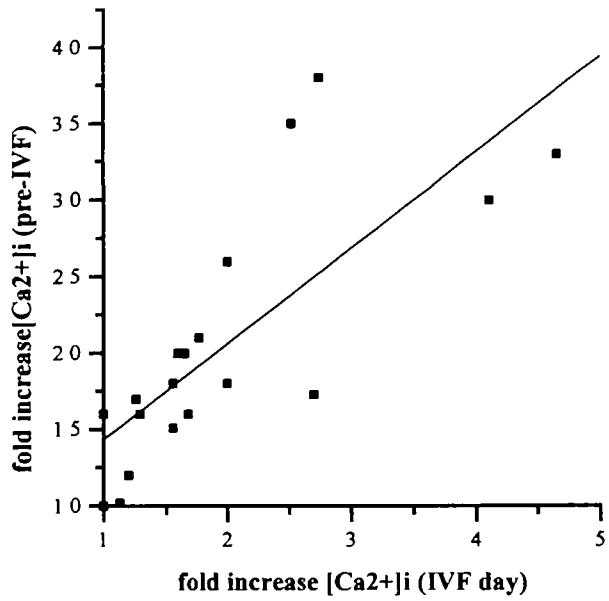

Figure 4. Correlation curve (r = 0 77, $P < 0.001$, n = 19) between factor of increase over basal value of intracellular calcium ([Ca^{2+}]$_i$) in spermatozoa in response to 1.0 µg/ml progesterone in the same patients on semen analysis 1 month prior to in-vitro fertilization and on the day of IVF.

positive results (2 points, n = 23), 96% of patients achieved fertilization, whereas when only one test was positive (1 point, n = 13), the percentage of patients achieving fertilization was reduced (63.6%). Although only two subjects showed negative results for both tests (0 points), it is worth noting that in these two subjects no fertilization was achieved.

Reproducibility of [Ca^{2+}]$_i$ responsivity

In order to establish whether [Ca^{2+}]$_i$ increase in response to progesterone can be considered a stable parameter, we evaluated such response 1 month before and on the same day as IVF in 19 subjects. Figure 4 shows the correlation between responsiveness to progesterone in the two different assessments. The high correlation coefficient found between the two different measurements (r = 0.77, $P < 0.001$) suggests that the degree of responsiveness to progesterone is stable and can be considered a permanent feature of human spermatozoa from the same subject.

Discussion

Impaired responsiveness of human spermatozoa to progesterone has been recently reported in subjects with reduced fertilizing ability (Tesarik and Mendoza, 1992; Falsetti *et al.*, 1993; Oehninger *et al.*, 1994; Krausz *et al.*, 1995). In addition, we found, in a small cohort of patients, that response to progesterone of spermatozoa is highly correlated with fertilization rate (Krausz *et al.*, 1995), suggesting that measurement of sperm responsiveness to progesterone could be used as diagnostic tool for the evaluation of sperm fertilizing ability in patients undergoing assisted fertilization programmes. The present study provides additional evidence that sperm AR and increase of [Ca^{2+}]$_i$ in response to progesterone is useful for the assessment of in-vitro fertilizing ability. Indeed, this study,

Table II. Limiting values, sensitivity, specificity, positive (PPV) and negative (NPV) predicting values for the different parameters evaluated

Parameter	Limiting value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
$[Ca^{2+}]_i$ – progesterone 1 0 μg/ml	1 5[a]	81	69	91	47
	1 3[a]	96	53	89	77
$[Ca^{2+}]_i$ – progesterone 0 1 μg/ml	1 4[a]	83	78	95	41
	1 2[a]	93	55	91	62
ARPC	7[b]	85	77	93	59
	5[b]	89	69	91	64
ARIC	17[b]	57	75	88	35
	10[b]	85	58	87	53

For abbreviations, see Table I.
[a]Factor by which value is increased over basal $[Ca^{2+}]_i$
[b]Percentage value

conducted in a cohort of 90 patients, confirms the lower responsiveness to progesterone in subjects with reduced fertilizing ability as well as the presence of a highly significant correlation between responsiveness to progesterone and fertilization rate. More importantly, the discriminant analysis performed in our study indicates that an increase of $[Ca^{2+}]_i$ in response to 0.1 μg/ml progesterone of >1.4-fold and an ARPC >7% can predict fertilization success respectively in 95 and 93% of cases; when both parameters are over the threshold value, the positive predictive value rises to >95%. Conversely, we did not find a correlation between the degree of spontaneous AR and the fertilization rate, confirming that such a parameter is poorly predictive of IVF success (Henkel *et al.*, 1993). Moreover, AR induction by the calcium ionophore A23187 (ARIC), although showing a significant correlation with fertilization rate, was less predictive, sensitive and specific than ARPC and $[Ca^{2+}]_i$ increase in response to progesterone with respect to fertilization outcome. In agreement with our findings, Calvo *et al.* (1994a) recently reported, in a study population of 232 subjects, that AR in response to follicular fluid is highly predictive of IVF success. Moreover, this test showed similar specificity, sensitivity, negative and positive predictive values as those obtained in the present study (Calvo *et al.*, 1994a). Since progesterone is the main inducer of AR in the follicular fluid (Osman *et al.*, 1989; Morales *et al.*, 1992) it may be speculated that both tests, induction of AR by progesterone and by follicular fluid, basically evaluate a similar sperm characteristic. In addition, Shimizu *et al.* (1995) reported, in agreement with our data, preliminary results showing that progesterone-induced $[Ca^{2+}]$ increase is correlated with fertilization rate. It must be mentioned that in a study population of 117 subjects, Parinaud *et al.* (1995), among several AR inducers tested, found that only response to A23187 and to the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were discriminant of IVF success, whereas responsiveness to progesterone and follicular fluid were not correlated to fertilization rate. However, in their study progesterone and follicular fluid were demonstrated to be very weak stimulators of AR (Parinaud *et al.*, 1995), pointing out possible important methodological differences in determination of sperm AR or in sperm preparation between their and other studies (Calvo *et al.*, 1989, 1994a; Krausz *et al.*, 1995; present study)

As mentioned above, the ideal inducer of AR is the zona glycoprotein ZP3, considered to be the physiological AR stimulus during the process of fertilization, while the physiological significance of sperm responsiveness to progesterone is questioned. However, the high concentration of progesterone in the cumulus matrix, which must be crossed by the spermatozoon before reaching the oocyte zona, and the fact that capacitated spermatozoa, i.e. those that possess the ability to fertilize the oocyte, are more responsive to progesterone (Baldi *et al.*, 1991, Mendoza and Tesarik, 1993; Meyers *et al.*, 1995; Shi and Roldan, 1995), suggest that the steroid may have a role in induction of AR during the process of fertilization. Roldan *et al.* (1994) recently demonstrated, in the mouse, that exposure to progesterone enhances sperm responsiveness to ZP3, providing evidence for a physiological role of progesterone in the fertilization process in mouse In addition, some of the pathways of signal transduction of progesterone are common to those of ZP3 (increase of $[Ca^{2+}]_i$, stimulation of tyrosine phosphorylation of proteins). In particular, both agonists increase tyrosine phosphorylation of a protein in the molecular weight range of 95–97 kDa (Leyton and Saling, 1989; Tesarik *et al*, 1993; Luconi *et al.*, 1995), which has been recently cloned and identified as sperm receptor for ZP3 (Burks *et al.*, 1995). The tight correlation between progesterone-induced $[Ca^{2+}]_i$ increase and AR and fertilization rate found in this study further elucidates the functional significance of sperm responsiveness to progesterone. Moreover, all this evidence suggests that assessment of sperm responsiveness to progesterone may be considered a good index of sperm function in the proximity of the oocyte, particularly when concerning the integrity of the mechanisms of signal transduction. However, it is possible that zona pellucida *in vivo* is more potent than progesterone or cumulus in inducing AR in human spermatozoa, thus justifying the presence of some false negative results and the relatively low negative predictive values. On the other hand, the presence of a few false positive results in our study may be due to some defective oocytes. Indeed, although oocyte maturity grade was similar in the two groups, other possible female gamete defects, such as genetic aberration and/or surface receptor deficiencies, which potentially alter the process of fertilization, cannot be excluded. Due to the low proportion of male factor patients according to the WHO (1992) criteria (<9%), it was not possible to distinguish the predictive value of these tests between normozoospermic and male factor patients It is noteworthy that, within the couples with pure female factor,

we were able to distinguish two subgroups of male patients on the basis of progesterone responsiveness: that with a positive response (over the cut-off value) had a significantly higher fertilization rate than the one with low response (data not shown), indicating that these tests may be predictive also for subjects with normal semen characteristics.

An important question concerns the variability of sperm responsiveness to progesterone from one ejaculate to another. We show here that $[Ca^{2+}]_i$ increase in response to progesterone is similar, in a small cohort of subjects, in two measurements performed within 1 month, suggesting a substantial reproducibility of the parameter. Intra-individual variability of progesterone-induced AR was not measured in our study; however, Calvo *et al.* (1994b) reported that intra-individual variability of follicular fluid-induced AR was low and response did not change with time, indicating also that AR inducibility can be considered a stable parameter.

In conclusion, our study indicates that assessment of sperm responsiveness to progesterone by measuring $[Ca^{2+}]_i$ increase and AR is highly predictive of fertilization success especially when the two parameters are measured simultaneously. Such assays are quite simple and highly specific, do not require expensive equipment and thus can be proposed as routine pre-IVF tests (subject to suitable cost-benefit analyses) in order to select patients for the most appropriate treatment.

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