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# Determination of human sperm calcium uptake mediated by progesterone may be useful for evaluating unexplained sterility

Sperm samples from couples who underwent assisted reproduction were classified according to the World Health Organization (WHO) criteria of concentration, motility, and morphology, in normal and subnormal cases (oligozoospermic, asthenozoospermic, and teratozoospermic). The percentage of spermatozoa that increased $[Ca^{2+}]_i$ in response to progesterone (P) was determined by means of flow cytometry. The evaluation of the P-mediated intracellular calcium increase by flow cytometry may be a fast and objective tool for the diagnosis of human sperm samples, especially in cases of unexplained sterility. (Fertil Steril® 2004;82: 738–40. ©2004 by American Society for Reproductive Medicine.)

No unique, reliable method allows the prediction of the fertilizing potential of a human semen sample. However, sperm response to progesterone (P) has been considered a good marker of sperm physiology (1) because semen samples from infertile patients show a scarce or null P response (2–8). Moreover, Krausz et al. (4, 5) suggested the measurement of the level of intracellular calcium and the acrosome reaction in response to P as predictive value for semen quality. Unfortunately, the fluorescence microscopy evaluation of the acrosome reaction is subjective and cumbersome. In addition, as the sperm sample is heterogeneous, the measurement of calcium concentration in the whole sperm sample is not representative because only a subpopulation of spermatozoa is able to increase the intracellular calcium (9).

By using a fast and objective flow cytometry technique, we measured the proportion of spermatozoa that increases the intracellular calcium in response to P in normospermic samples (9). The aim of the present work was to determine whether the P calcium uptake by means of flow cytometry allows us to evaluate the functional state of human sperm samples showing different sperm quality according to the World Health Organization criteria, with emphasis on cases of unexplained sterility.

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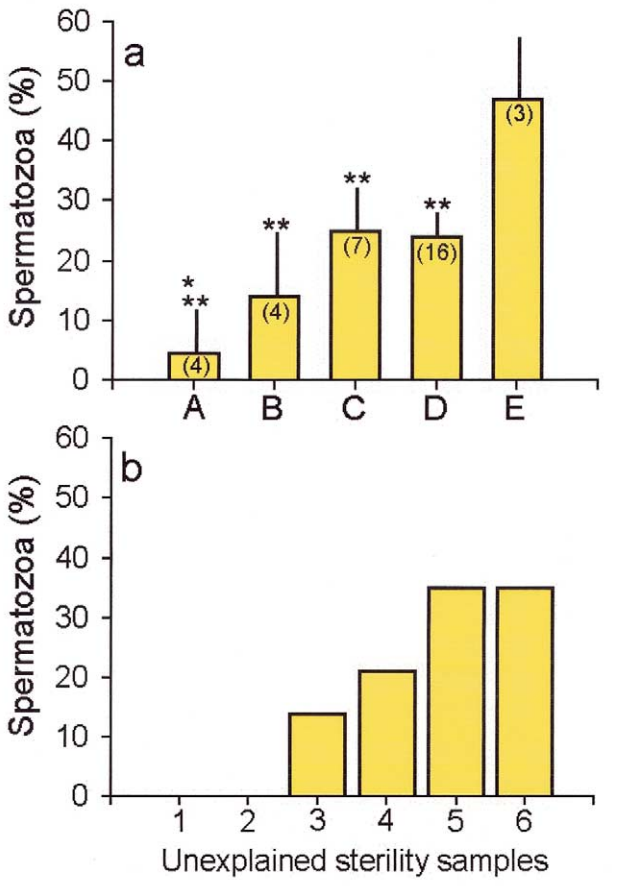
The study was carried out in accordance to the Helsinki Declaration of 1975 on human experimentation, in a blinded assay with samples from 35 couples who underwent in vitro fertilization (IVF) performed according to routine procedures. In addition, samples undergoing intrauterine insemination (IUI) protocols were also included. Semen samples were collected on the day of oocyte retrieval after a recommended 48 to 96 hours of sexual abstinence, and were included in this study if there was a sufficient quantity of sperm left over after insemination. Sperm quality was evaluated according to the WHO criteria of concentration, motility, and morphology, classifying the samples as normal and subnormal (oligozoospermic, asthenozoospermic, or teratozoospermic). For data analysis, the samples were divided into the following groups: A, showing all three pathologies; B, two pathologies; C, only one pathology; D, normal for IVF (female factor infertility); E, normal for IUI; F, normal but with unexplained sterility.

Seminal plasma was removed by centrifugation and the final pellet was resuspended in BWW (10) at a concentration of $1 \times 10^6$ cells/mL, and incubated for 3 hours at 37°C under 5% $CO_2$ in air to induce sperm capacitation. The determination of the percentage of spermatozoa that increase the $[Ca^{2+}]_i$ in response to P was determined by means of flow cytometry according to Giojalas (9) with few modifications. The sperm population was loaded with Fluo-3 (which binds the calcium ions that enter the cell only in live spermatozoa [11]), at a final concentration of 5 $\mu$M. The Fluo-3 fluorescence of 3,000 cells was measured in an Ortho Cytoron Absolute flow cytometer (Ortho Diagnostic Systems, Raritan, NJ) with a 530-nm filter. Florescence emission of each sperm sample was analyzed in triplicate before and after a 30-second stimulation with P 3-(O-carboxy-methyl)oxime:BSA (P-BSA), at a final concentration of 0.5 $\mu$M.

The data analysis was done in the population of Fluo-3–labeled cells (the unlabeled cells were gated out) with the WinMDI 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA). The percentage of cells that increased $[Ca^{2+}]_i$ in each sperm population was calculated by the difference between unstimulated and P-stimulated; a mean of three repetitions was performed for each sperm sample. All reagents were obtained from Sigma-Aldrich (St. Louis, MO). Percentages were arcsin square root transformed before the analysis of variance (ANOVA); the statistically significant differences

**FIGURE 1**

Percentage of spermatozoa increasing intracellular calcium in response to P in samples with different sperm quality. (**a**) Sample types A–E (mean ± SE). The number of samples is represented between brackets on each bar. (**b**) Values of individual samples of unexplained sterility (sample F). *A:* Three pathologies. *B:* Two pathologies. *C:* One pathology. *D:* Normal samples for IVF. *E:* Normal samples for IUI. *F:* Unexplained sterility. *Statistically significant differences as compared with all the other groups ($P<.001$); **Statistically significant differences as compared with group E ($P<.001$).




*Giojalas. Progesterone response in unexplained sterility. Fertil Steril 2004.*

between treatments were determined with Student's *t*-test. The correlation analysis was performed with the Pearson test with a confidence level of 95%.

In this study we observed a statistically significant correlation between the proportion of spermatozoa that respond to P by increasing the intracellular calcium and the sperm quality of the sample ($r^2 = 0.63$; $P<.0001$; N = 35). The P response was significantly lower in the samples that showed the three pathologies (group A) compared with all the other groups ($P<.001$) (Fig. 1a), which was in agreement with previous reports (2–8). However, when the sample showed one or two pathologies (groups C and B), no difference was found compared with the normal group for IVF (group D). Samples from groups A through D showed a reduced response to P in relation to the normal sample for IUI (group E) ($P<.001$), indicating the lower performance of normal samples that follow an IVF procedure in comparison with samples destined for IUI.

The sperm response to P in samples of unexplained sterility (group F) was varied; samples 1 and 2 had a null response, 3 and 4 had values similar to normal samples for IVF, and samples 5 and 6 were similar to normal samples for IUI (see Fig. 1b). This result suggests that the evaluation of the P response may help to further investigate the physiological state of a sample from a donor with unexplained sterility. Notwithstanding, Tesarik and Mendoza (3) reported a defective P response in the five cases of unexplained sterility they analyzed.

Taking into account that samples from subfertile patients showed a low response to P, a possible correlation between the percentage of IVF and the percentage of spermatozoa that augmented the intracellular calcium in response to P was investigated. However, no correlation was observed between these parameters ($r^2 = 0.08$; $P>.24$; N = 35). With the exception of oligo-astheno-teratozoospermic samples, where the response to P was almost null and the percentage of IVF was zero in all the cases, many samples that showed a good P response (higher than 20%) failed to fertilize the egg in vitro. Moreover, in cases of unexplained sterility, two samples showed a null response to P and 0% of IVF; the two that had the highest proportion of P responding cells (35%), showed 0% and 50% of IVF. Because the success of an IVF procedure depends on both gametes, the correlation between any sperm parameters and the IVF outcome should be interpreted with caution, especially when the purpose is to recommend a semen parameter as an IVF predictor.

In summary, the use of flow cytometry to evaluate the ability of human sperm to respond to P by increasing the intracellular calcium may be a fast and objective tool for the diagnosis of the human sperm quality, especially in cases of unexplained sterility.

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