Pronuclear Abnormalities and Cytoskeletal Organization During Assisted Fertilization in a Patient with Multifollicular Ovarian Response

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***Purpose*:** To analyze the distribution of α tubulins and acetylated α tubulins and the chromatin configuration in abnormally fertilized zygotes from a patient with a multifollicular ovarian response after in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI).

***Methods*:** Immunofluorescence and phase contrast microscopy was performed in abnormally fertilized zygotes.

***Results*:** After phase contrast microscopy analysis, immunofluorescence staining was performed in 20 oocytes that developed ≥ 3 pronuclei (PN) and karyomeres after IVF–ICSI. Around 80% of the abnormal zygotes from IVF were the consequence of monospermic fertilizations. Retention of the second polar body (PB) and the presumptive split of ≥ 1 PN within the cytoplasm were the main events present in most oocytes after IVF–ICSI.

***Conclusions*:** Fluorescence labeling of selected sperm and oocyte components affords a unique view of abnormal fertilized zygotes. Surprisingly, anomalies detected after IVF–ICSI showed similar etiologies in this special group of zygotes.

KEY WORDS: Abnormal fertilization; immunofluorescence; multifollicular response; poor quality oocytes.

INTRODUCTION

Multipronuclear zygotes resulting from IVF may arise from dispermic fertilization and represent around 4% of the inseminated oocytes. Multipronuclear zygotes after ICSI, on the other hand, are thought to be digynic in origin (1). In general, the pronuclear number as well as its size depends on the number of chromosomes involved and whether they remain in groups or not. When oocytes are postmature or are exposed to heat, some of the "pronuclei" observed under light microscopy after IVF can be due to the development of small karyomeres instead of one normal female pronucleus.

In this report we describe the etiology of severe abnormal fertilization in a patient with multifollicular ovarian response. This patient presented several characteristic features similar to those observed in Polycystic Ovarian Syndrome (PCO). PCO is a very heterogeneous disease and may present a wide range of symptoms. IVF results in PCO patients have been questioned during the last few years, but most authors (2,3) have found a higher number of oocytes retrieved per cycle and a lower fertilization rate compared with 'normovulatory patients.' Hypersecretion of luteinizing hormone (LH) is present in about 40% of PCO patients and has been associated with an increased risk of infertility and miscarriage. A deleterious effect of high tonic LH secretion has been suggested in this group of patients

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affecting the endometrium and/or the oocytes. High LH levels may affect oocytes directly (4), promoting early resumption of meiosis and abnormal oocyte maturation leading to fertilization failure or miscarriage if fertilization and implantation occurs. There is a consensus that a common feature in PCO patients is arrested follicular development at the stage when selection of the dominant follicle should normally occur (5).

In this report 20 abnormally fertilized oocytes after IVF–ICSI were studied using immunofluorescence to detect the distribution of α tubulins and acetylated α tubulins and the chromatin configuration. Zygotes with ≥ 3 pronuclei (PN) and/or karyomere development, with or without extrusion of a second polar body (PB), were included in this investigation.

MATERIAL AND METHODS

Patient

A 29 years old woman complaining of 4 years of primary sterility and a history of an unicornuate uterus. Partner's semen analysis was normal. Previous attempts in another center consisted of three intrauterine inseminations, one IVF cycle, and the combination of IVF–ICSI in a second attempt in which low fertilization rates and high rates of abnormal fertilization were observed, both after IVF and ICSI. Because the patient had moderate ovarian hyperstimulation in both cycles, embryos and normally fertilized zygotes were cryopreserved, thawed, and transferred in artificial cycles with no pregnancy achieved.

Assisted Reproduction

A Day 3 hormone profile revealed the following: TSH: 1.24 μIU/mL; fasting blood Insulin: 10 μU/mL; blood glucose: 79 mg%; estradiol (E_2): <20 pg/mL; FSH: 6.6 mIU/mL; LH: 10.9 mIU/mL; PRL: 22 ng/mL; androstenedione: 0.80 mg/mL; SHBG: 73 nmol/L. Pelvic ultrasound showed several follicles <8 mm in diameter around a central echo-dense stroma compatible with polycystic ovaries and a left unicornuate uterus. Physical examination showed body mass index to be within the normal range and the waist to hip ratio as 0.81. Metformin therapy (800 mg/day) was begun before ovarian suppression. GnRH analogue (Leuprolide Acetate, Reliser™, Serono Laboratories) was administered from Day 21 of the cycle

at a daily dose of 1 mg. When ovarian suppression was confirmed the dose was decreased to 0.5 mg/day and ovarian stimulation was begun with 200 IU/day of recombinant FSH (Puregon™, Organon Laboratories) in a step-down protocol. After 9 days of ovarian stimulation, with an E_2 serum level of 4000 pg/mL and pelvic ultrasound showing at least two follicles ≥ 18 mm, 10000 IU of hCG (Profasi HP 10.000™, Serono Laboratories) was indicated. Follicular aspiration was performed 34 h post–hCG-administration. The patient received daily vaginal progesterone gel (Crinone gel 8%™, Serono Laboratories) from the day following follicular retrieval. The patient didn't have signs or symptoms of ovarian hyperstimulation syndrome. A total of 40 oocytes were obtained. In several oocytes, the presence of cytoplasmic granules and vacuoles was evident. IVF was performed in 24 Metaphase II (MII) oocytes and ICSI in nine MII oocytes as previously described (6). The remaining oocytes (Metaphase I and Prophase I) were not inseminated. A total of 23 inseminated or injected oocytes (19 from IVF and 4 from ICSI), discarded as "abnormally fertilized" 16–18 h postinsemination, were the studied material obtained from the couple who have had previously signed an informed consent. Abnormally fertilized zygotes were defined as those that developed ≥ 3 PN and/or karyomeres. Of 23 zygotes, 20 (86.9%) were informative after fluorescence staining.

Cytoskeletal, Sperm Tail, and Chromatin Labeling

An initial assessment of pronuclear development and PB extrusion was done under phase contrast microscopy, in 20 abnormal zygotes. After that, zona pellucida was removed and the denuded zygotes were fixed and permeabilized in a microtubule stabilizing buffer (6). Zygotes were incubated overnight with anti–acetylated α tubulin and at room temperature with anti–α tubulin monoclonal antibodies to detect the sperm tail and cytoskeleton respectively. After that, the material was further incubated in fluorescein-conjugated goat antimouse immunoglobulin G (IgG), counterstained with Hoescht 33258 (1 μg/mL) to detect DNA, and then examined using an Olympus epi-fluorescent microscope. Images were photographed (Ektachrome film, 1600ASA) and processed using Adobe Photoshop 5.0 software (Adobe System). For control staining, PBS + BSA replaced the specific antibody solution. Monoclonal antibodies and reagents used were purchased from Sigma (St. Louis, MO, U.S.A.).

RESULTS

At the time of pronuclear visualization, only five oocytes from IVF and three from ICSI showed two pronuclei. Nineteen oocytes from IVF developed multiple pronuclei and/or karyomeres (abnormal fertilization rate: 79.2%). After ICSI, four oocytes showed signs of multipronuclear and/or karyomere development (44% of the injected oocytes) and the remaining two oocytes degenerated after injection. It is worth noting that the rate of karyomere formation in monospermic fertilization after IVF was 75% (12/16), results that are not comparable to that found after ICSI (see Discussion). Forty-eight hours after ovum pick up, three good-quality embryos were generated by IVF and ICSI, without any sign of multinucleation, and less than 50% of the fragmentation was transferred to the patient. Out of the five remaining normally fertilized embryos one arrested its development at the two-pronuclei stage and four were cryopreserved. After 12 days of embryo transfer, a positive β hCG level was detected and 38 weeks later a healthy child was born after cesarian section.

Microtubules, Chromatin, and Sperm Tail Characterization

A total of 20 abnormal zygotes (16 from IVF and 4 from ICSI) were classified under phase contrast microscopy as multipronucleated and showing karyomere development (Fig. 1), and then further processed for immunofluorescence staining.

Microtubules were analyzed to know if their cytoskeletons were severely affected in these particular poor-quality oocytes. The number of spermatozoa within the oocytes, as well as the chromatin structure of each pronucleus or subnucleus, allowed us to understand the origin of each abnormal development. Independently of the number of pronuclei found, α tubulins were uniformly distributed all around the cytoplasm of the abnormal zygotes. Multipronuclear development ($\geq$3 PN) occurred in six zygotes after IVF and none after ICSI. Four of these six zygotes had extruded the second PB; two of the six had only one spermatozoon within the cytoplasm, one had three, and three zygotes had two decondensed spermatozoa in the cytoplasm. On the other hand, multipronucleation and karyomere development ($\geq$3 PN + karyomeres) was observed in 10 and 4 zygotes, after IVF and ICSI respectively. All of these abnormal zygotes showed a presumable retention of the second PB (since only one PB was

observed by phase contrast microscopy before insemination until zygotes were classified as "abnormal") and had only one spermatozoon within the oocytes (Fig. 1).

CONCLUSIONS

Results show that multipronuclear and karyomere development were the main abnormal fertilization event after IVF–ICSI in this patient. Because of the low normal fertilization rate and the high abnormal fertilization observed in previous cycles, we decided to make a combination IVF–ICSI cycle to see whether ICSI could bypass this problem. We observed a similar 2 PN development after both techniques and a very high abnormal fertilization rate after IVF and ICSI. These abnormal fertilization rates were higher after IVF than after ICSI, though some of it was to be attributed to polyspermia (see below); this finding, to our knowledge, has not been previously reported. It is clearly seen that the fertilization pattern observed differed highly from patients with multifollicular ovarian response, as in PCO patients, in whom low fertilization rate is the main feature. To elucidate the possible etiology of these abnormalities, we used immunofluorescence and we surprisingly found that monospermic fertilization was present in 12 of 16 abnormal zygotes (75%) after IVF. Usually, multipronuclear zygotes from IVF result from dispermic fertilization. The distribution of α tubulins in abnormal fertilized zygotes resembles the pattern observed in normal fertilized oocytes (V.Y. Rawe, "Cellular events during fertilization in humans," University of Buenos Aires, PhD Dissertation, 2000, 113 pp.), and it is different from that observed after fertilization failures. In the latter, for instance, α tubulins may be mostly associated with condensed sperm chromosomes (premature chromosome condensation) or found at the first embryonic metaphase plate (6). Immunofluorescence information indicated that the formation of abnormally fertilized zygotes after IVF was most frequently the product of dyginic triploidies (retention of the second PB) and the presumptive split of one or more pronuclei within the cytoplasm in this particular patient. Although abnormal fertilization rate after IVF was higher than after ICSI, the abnormal development observed after both assisted reproduction techniques was mostly associated with retention of second PB and karyomere formation. In the present case, independent of the fertilization technique used, the inability to lead a normal genome organization in
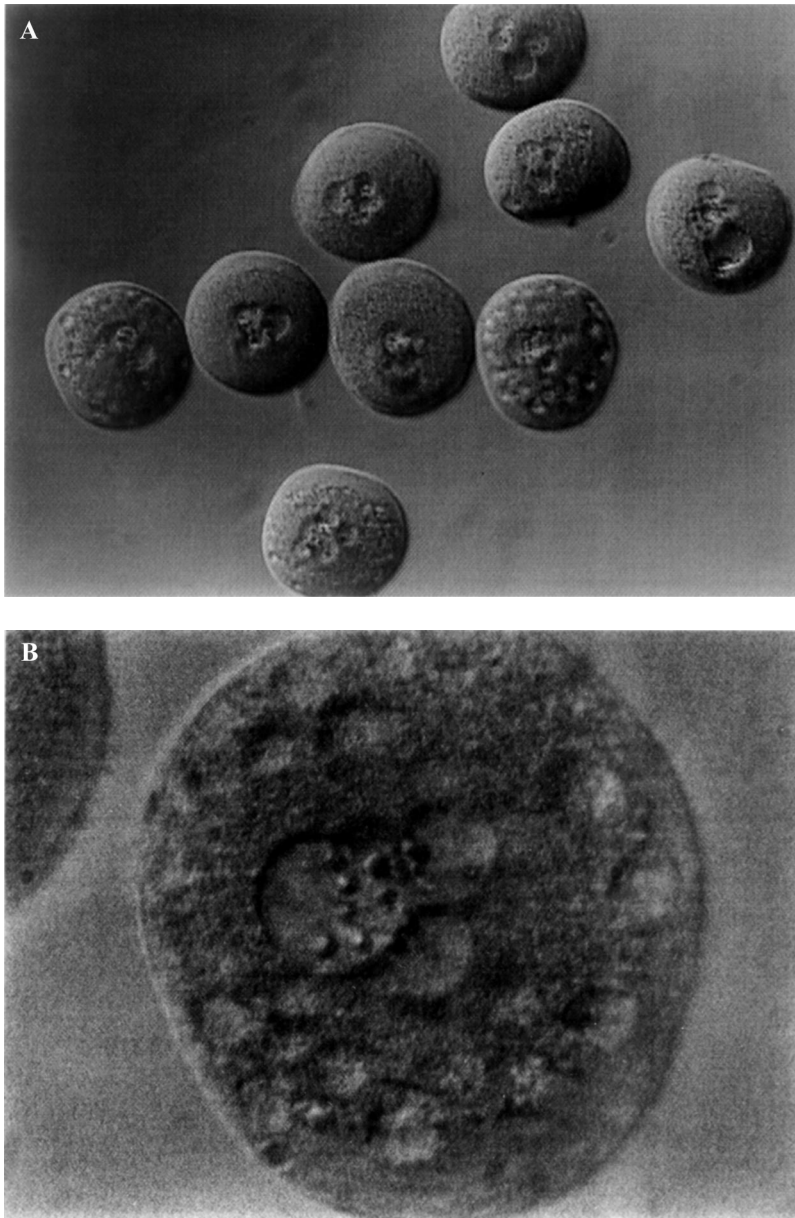
Fig. 1. Phase contrast and fluorescence microscopy of zygotes from a patient with multifollicular ovarian response after abnormal fertilization. (A) Three or more pronuclei and karyomere development after IVF. Mainly, retention of the second polar body and the presumptive split of one or more pronuclei within the cytoplasm were the causes of this pattern confirmed after immunofluorescence analysis. (B) Detail of a zygote with multipronuclear and karyomere development after IVF. Note also the presence of vacuoles in the cytoplasm of this particular zygote. Photographs have been taken after zona pellucida removal and most of the times PBs are lost. (C) Karyomere development after ICSI. Only one sperm tail can be seen associated with DNA. In this case three pronuclei, two karyomeres, and the retention of the second PB had been observed. (D) Development of multipronuclear zygotes as a consequence of dispermic fertilization after IVF. (E) Polyspermic fertilization after IVF. This zygote had developed 4 PN seen by phase contrast microscopy. Note three sperm tails in green in the middle of the microtubules. Cytoskeleton was identified by anti–α tubulin monoclonal antibodies; sperm tail was visualized using anti–acetylated α tubulin monoclonal antibodies. Both primary antibodies were recognized with a FITC conjugated anti-IgG secondary antibody. DNA was counterstained with Hoescht 33258.
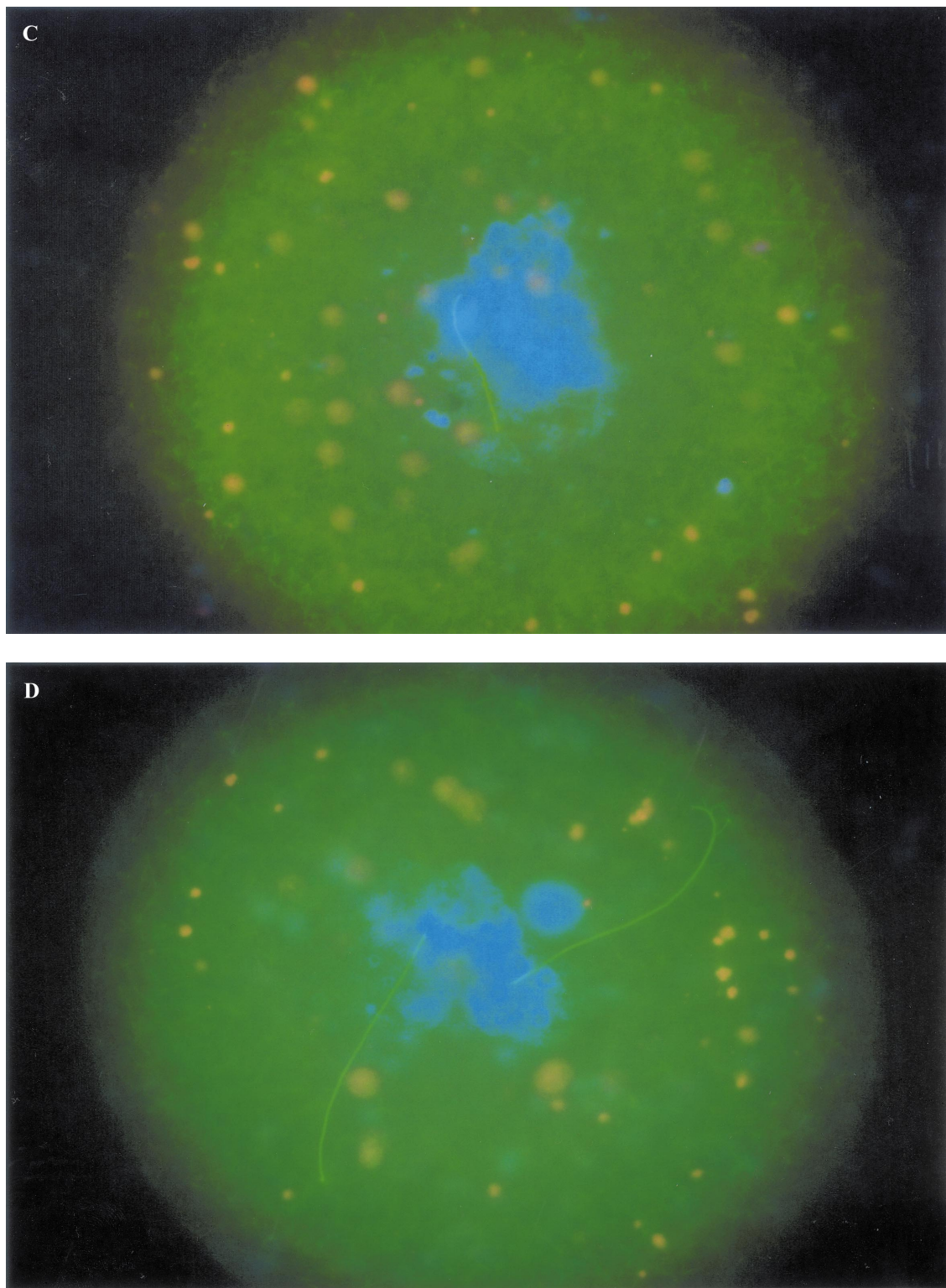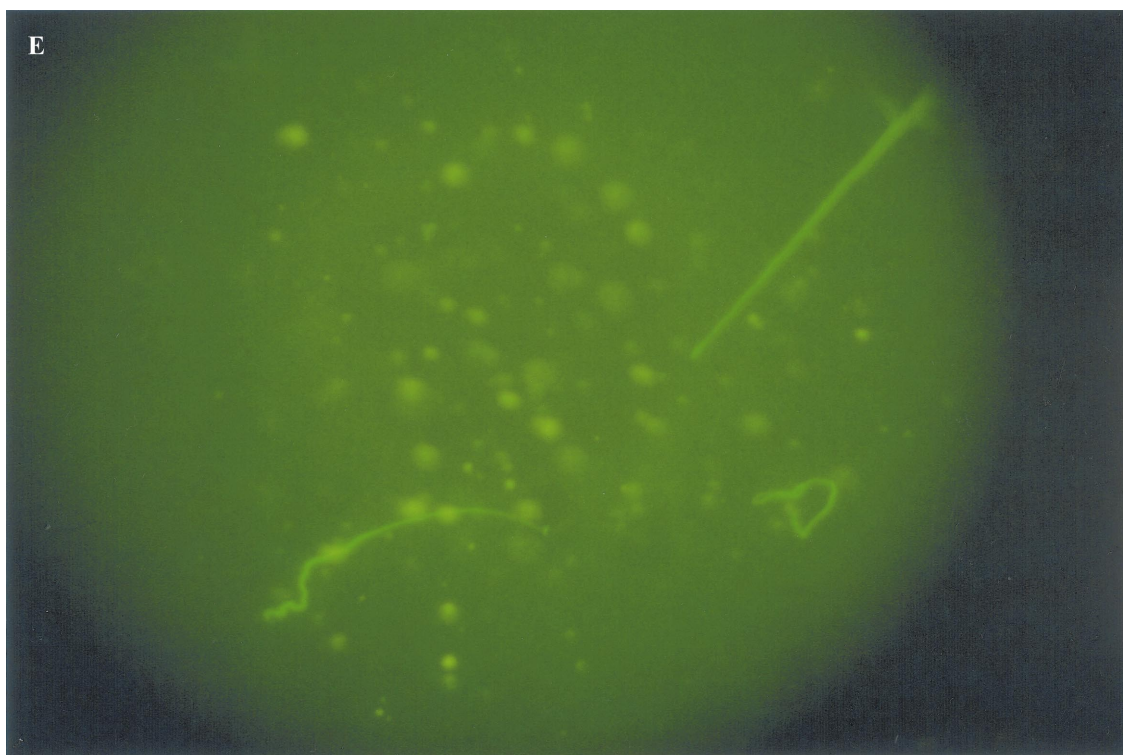
Fig. 1. (*Continued*)

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these poor-quality oocytes was the main factor responsible for abnormal fertilization and consequently the determinant of the low normal fertilization rate observed.

We found that the use of immunofluorescence was essential for a comprehensive understanding of the cellular events present in oocytes from this patient. It would be interesting to see if there is a relationship between the endocrine metabolic environment and the risk of abnormal fertilization.

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