CASE REPORT

Single and twin ongoing pregnancies in two cases of previous ART failure after ICSI performed with sperm sorted using annexin V microbeads

Ester Polak de Fried, M.D., and Flavia Denaday, M.D.

Department of Reproductive Medicine, CER Medical Institute, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina

Objective: To treat couples with intracytoplasmic sperm injection (ICSI) after annexin V sperm sorting.
Design: Two case reports.
Setting: Department of Reproductive Medicine at a private medical institute.
Patient(s): Couples on infertility treatment, donor oocytes.
Intervention(s): Sperm sorted with annexin V magnetic microbeads before ICSI, day 3 embryo transfer; case 1: ovum donation; case 2: patient oocytes.
Main Outcome Measure(s): 1) Sperm DNA fragmentation (terminal deoxynucleotide transferase–mediated dUTP nick-end labeling [TUNEL]) and active caspase-3 (immunocytochemistry); 2) fertilization rate, embryonic quality, blastocyst development of nontransferred embryos, and pregnancy outcome after ICSI of sorted sperm.
Result(s): Case 1: Premature ovarian failure patient with previous fertilization failures: asthenoteratozoospermia, abnormal DNA fragmentation (TUNEL 30%; normal <20%). ICSI with annexin V–treated sperm done on six donated metaphase II (MII) oocytes; four fertilized, and a 5-cell/grade-2 and a 6-cell/grade-2–3 embryo were transfered. A day 5 blastocyst was cryopreserved. The patient was in the last trimester of gestation. Case 2: Couple with >4 years of primary infertility and recent ICSI failure. Semen with teratozoospermia (5% normal forms [Kruger]) and abnormal active caspase-3 (16%; normal <11%). ICSI with annexin V–treated sperm done on 9 MII oocytes. All fertilized; a 7-cell/grade-1 and an 8-cell/grade-1–2 embryo were transferred. A day 5 expanded blastocyst was cryopreserved. The patient was in the second trimester of a twin normal pregnancy.
Conclusion(s): Sperm sorting with annexin V columns was effective in the treatment of two cases of ICSI failure, resulting in a single and a twin pregnancy after transfer of two embryos in each case. (Fertil Steril® 2010;94:351.e15–e18. ©2010 by American Society for Reproductive Medicine.)

Key Words: Fertilization failure, ICSI, human spermatozoa, apoptosis, DNA fragmentation, externalization of phosphatidylserine, TUNEL, caspases, caspase-3, immunomagnetic cell depletion, annexin V microbeads, MACS

Of all available ART procedures, intracytoplasmic sperm injection (ICSI) has become the most powerful alternative in the treatment of male factor infertility (1). Sperm selection for ICSI is based on subjective selection of a motile cell with the best possible morphologic appearance (2); however, spermatozoa may have genetic abnormalities (3, 4) that are not identified by that selection criterion (5). In addition, spermatozoa have been reported to display features of programmed cell death (apoptosis) (6, 7), which may affect their performance. Among them, DNA damage, evident by the presence of abnormally high levels of DNA fragmentation, has been related to abnormal semen parameters in spermatozoa from male-factor infertility patients (8–11); moreover, its presence has been associated with low fertilization rates despite the use of ICSI (8, 12), with alterations in early embryonic development (12), and with pregnancy abnormalities and loss (8, 9, 13).

One of the early features of cell apoptosis is the externalization of the phospholipid phosphatidylserine (PS) that is normally present on the inner leaflet of the plasma membrane (14). Moreover, PS externalization is associated with activation of caspases, the cytosolic cysteine-containing aspartate-specific proteases, which are main effectors in the apoptotic process (15). Phosphatidylserine has been detected on the surface of spermatozoa with altered membranes (16); in addition, among members of the caspase family, caspase-3 has been detected in the midpiece of human spermatozoa (17) and has been associated with alterations in sperm parameters and decreased sperm performance (18). Because PS has high and selective affinity for the phospholipid-binding protein annexin V (19), spermatozoa with externalized PS will bind to microbeads coupled with annexin V; recent studies have demonstrated the effectiveness of performing cell sorting with colloidal supramagnetic microbeads conjugated with annexin V (magnetic activated cell sorting [MACS]) in the elimination of dead and apoptotic-like spermatozoa (20–22).

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Reprint requests to: Ester Polak de Fried, M.D., Department of Reproductive Medicine, CER Medical Institute, Humboldt 2263, Buenos Aires, ZIP CODE: 1425, Argentina (FAX: 54-11-4778-0011; E-mail: ester_polak@cermed.com).

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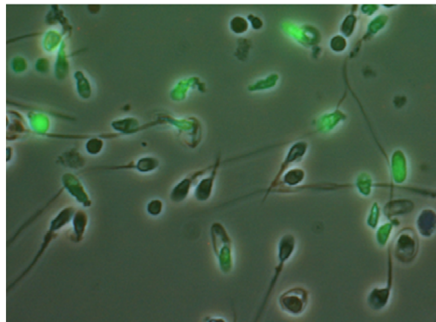
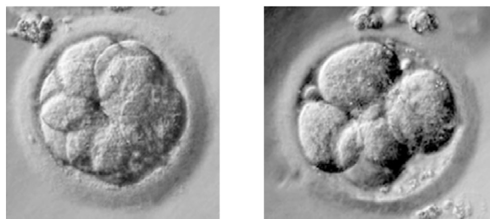
FIGURE 1

Case 1: (**A**) DNA fragmentation analysis done using terminal deoxynucleotide transferase–mediated dUTP nick-end labeling (8); a typical image of fluorescent staining in the sperm head is shown. (**B**) Micrographs of the day 3 embryos selected for transfer: 5 cell/grade 2 and 6 cell/grade 2–3.

A



B

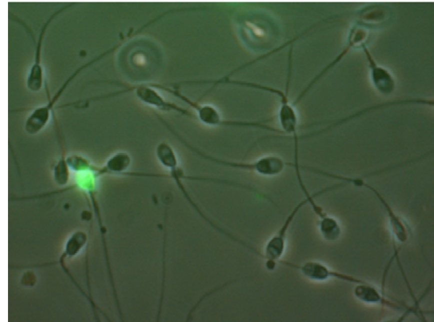


Polak de Fried. Pregnancies with annexin V–sorted sperm. Fertil Steril 2010.

FIGURE 2

Case 2: (**A**) Immunodetection of active caspase-3 (17), showing a typical protein staining in the sperm midpiece. (**B**) Micrographs of day 3 embryos selected for transfer: 8 cell/grade 1–2 and 7 cell/grade 1. In both cases, embryo classification was done following Lucinda Veek's grading system (26).

A



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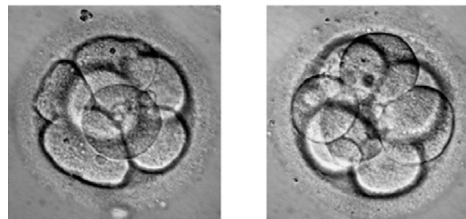

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The present report describes the use of the annexin V MACS technology for sperm selection before the ICSI procedure and its effect on sperm fertility potential in two cases of previous conception failure. Case 1 describes an ongoing single pregnancy achieved with a couple using oocyte donation and ICSI of annexin V–sorted sspermatozoa from a semen sample with mild asthenoteratozoospermia but abnormally high percentage of DNA fragmentation as determined by the terminal deoxynucleotide transferase–mediated dUTP nick-end labeling (TUNEL) technique. Case 2 reports an ongoing twin pregnancy achieved in a couple after transfer of two embryos obtained after ICSI of patients' oocytes with annexin V–sorted sspermatozoa from a semen sample with low percentage of normal sperm forms and high levels of active caspase-3. Informed consent forms approved by the review board of our institutional Human Subjects and Ethics Committee were signed by oocyte donors and members of couples from both cases.

CASE REPORT 1

A 35-year-old woman and her partner (41 years old) came to the CER Institute to receive infertility treatment. The patient had been diagnosed with premature ovarian failure at the age of 25 years, and was previously treated with two cycles of oocyte donation, although with a very low fertilization rate and failure to achieve pregnancy. Evaluation of the partner's semen sample (23) within a month of treatment revealed a moderate asthenoteratozoospermia (31 million/mL sperm concentration, 45% progressively motile spermatozoa, and 9% normal sperm forms by the Kruger criteria; normal values $\geq$20 million/mL, $\geq$50% motile spermatozoa, >14% normal forms), and presence of 3 million round cells/mL (normal values <1 million/mL). After evaluation, the couple was offered ovum donation with ICSI of the partner spermatozoa. During the first treatment at our center, the couple received five oocytes from a 25-year-old fertile donor, three of which were classified as metaphase II (MII)–stage oocytes. The ICSI procedure resulted in total fertilization failure; major abnormalities in the oocytes were ruled out, because 100% fertilization rate was achieved in another recipient of the same oocyte cohort, and transferred embryos implanted and resulted in an ongoing pregnancy.

Based on the results of the ICSI procedure, further evaluation of the semen sample was performed, which involved assessment of DNA fragmentation by the TUNEL technique (8) and immunocytochemical detection of active caspase-3 (17) in selected motile spermatozoa; a total of 400 sperm cells were scored. The studies revealed abnormal DNA fragmentation (30%; normal value <20%) (Fig. 1A). Abnormal DNA damage was also observed in a spermatozoa recovered from a cryopreserved sample of the patient obtained 7 days earlier (DNA fragmentation 58%), and the thawed sample also showed abnormal high levels of active caspase-3 (25%; normal value $\leq$11%). The increased levels of caspase-3 detected in frozen-thawed spermatozoa are in agreement with earlier studies (24); these findings led us to propose that sperm cryopreservation before ART, a procedure that is extensively performed, particularly in oocyte donation programs, should be avoided.

A second procedure of ovum donation and ICSI with the partner spermatozoa was carried out starting February 2009. Based on the results of the semen studies, motile spermatozoa selected by density-gradient centrifugation were subjected to a procedure of depletion of apoptotic sperm cells using MACS with annexin V microbeads (Miltenyi Biotec, Auburn, CA) before oocyte microinjection. Oocytes were obtained from a 23-year-old donor that received controlled ovarian stimulation consisting of a long protocol of GnRH agonist (0.2 mL daily of subcutaneous leuprolide acetate [Lupron; Abbott Laboratories, Buenos Aires, Argentina]) starting in the midluteal phase of the previous cycle in association with gonadotropin therapy (Puregon; Organon Laboratories, and Menopur; Ferring Laboratories, Buenos Aires, Argentina) up to the day before the hCG administration (Gonacor; Ferring Laboratories, Buenos Aires, Argentina). Monitoring and ICSI procedures were performed as previously reported (25). Six MII oocytes were microinjected, four of which were fertilized and developed. During donor ovarian stimulation, the patient was taking increasing doses of micronized oral E_2 according to the standard protocol (25). At the time the endometrium reached an ultrasound trilaminar pattern of 13.6 mm thickness, two embryos classified as 5 cell/grade 2 and 6 cell/grade 2–3 (26) (Fig. 1B) were transferred using a soft catheter (Wallace Embryo Replacement Catheter; Smiths Medical International, Kent, U.K.). The other embryos were placed in culture, and one blastocyst on day 5 was cryopreserved. Fourteen days later, β-hCG serum levels of 635.6 mIU/mL were obtained, and 10 days later, an ultrasound showed a single gestation with positive cardiac activity. The patient's pregnancy evolved free of complications, and at the time of writing, the patient was in the 37th week of gestation of a normal baby boy. In November 2009 a healthy male baby was born and weighed 3,100g.

CASE REPORT 2

A 34-year-old patient and her 38-year-old partner arrived at our clinic with >4 years of primary infertility. The woman's work-up included a normal hysterosalpingogram in 2006. Failed intrauterine inseminations were performed in other institutions after ovulation induction using clomiphene citrate or recombinant FSH (rFSH), despite the results on multiple semen analysis revealing a male factor, in addition to a varicocele surgically treated in 2007, which anticipated the need of an ART procedure with ICSI. In February 2009 an ICSI procedure involved patient stimulation with rFSH in association with GnRH analogue from the previous luteal phase; 250 IU rFSH was provided during 8 days. Eight follicles were retrieved, and of them, six MII oocytes were injected and showed normal fertilization; 72 hours later, two embryos were transferred. The remaining four embryos were placed in culture, but none of them reached blastocyst stage. The patient did not achieve pregnancy.

In April 2009 the couple consulted our institution. A cycle day 3 hormonal profile showed normal values, and 16 follicles were identified on the count of antral follicles by ultrasound. A semen analysis showed 5% normal sperm forms by Kruger strict morphology. Because of the earlier failed fertility treatments, a diagnosed varicocele and the presence of abnormal sperm morphology, the assessment of DNA fragmentation and active caspase-3 levels were carried out using the same procedure as described in case report 1. Ten million motile sperm/mL were recovered from a fresh ejaculate (40 million sperm cells/mL, 51% progressively motile spermatozoa) by the swim-up procedure; scores for DNA fragmentation of 2% and active caspase-3 of 16% were obtained (Fig. 2A). Even though DNA fragmentation was found to be within accepted values, the abnormal results for caspase-3 prompted us to indicate motile sperm filtration using the MACS annexin V microbeads before the ICSI procedure. The patient received a luteal-phase GnRH analogue protocol in association with a step-down gonadotropin therapy protocol. The starting doses were 225 IU rFSH (Puregon) in association with 150 IU purified urinary gonadotropins (Menopur) for 5 days, followed by 150 IU rFSH and 75 IU purified urinary hMG for 5 days. On day 11, E_2 level was 2,032 pg/mL and the endometrial lining formed a trilaminar pattern of 10 mm diameter. The size of the follicles ranged from 10 mm to 22 mm (six follicles >17 mm). Ovulation was triggered with 10,000 IU hCG (Gonacor), and 16 oocytes were retrieved 37 hours later by ultrasound-guided transvaginal aspiration. Nine MII oocytes were microinjected after sperm sorting. All oocytes showed normal fertilization (presence of two pronuclei), and nine embryos developed 72 hours later. On day 3, two embryos classified as 8 cell/grade 1–2 and 7 cell/grade 1 (26) (Fig. 2B) were transferred using the same procedure described for case 1. One of the remnant embryos reached the expanded blastocyst stage on day 5 and was cryopreserved by vitrification (27). Fourteen days after embryo transfer, serum β-hCG testing was performed, and a serum concentration of 447.6 mIU/mL was determined. The first transvaginal ultrasound performed 15 days after β-hCG assessment revealed two normal inserted gestational sacs with presence of embryos and normal cardiac activity in both of them. At the time of writing, the patient's pregnancy was evolving normally in the second trimester of pregnancy. The estimated date of delivery is March 2010.

CONCLUSION

To the best of our knowledge, this is the first study describing one single and one twin ongoing pregnancy after applying annexin V MACS procedure before ICSI in couples with several previous conception failures. In these cases, donor and patient oocytes were used, and semen from both partners had mild abnormalities in sperm parameters and abnormal levels of apoptotic markers (i.e., DNA fragmentation and active caspase-3). In case 1, a single ongoing pregnancy was achieved after a two-embryo transfer obtained from the institutional oocyte donor program; in case 2, a twin ongoing pregnancy from a two-embryo transfer was obtained with the patient's oocytes. Effectiveness of the selective filtration procedure of sperm suspensions with signals of apoptosis (DNA fragmentation by TUNEL and active caspase-3 by immunocytochemistry) was observed in both cases.

The results from this study suggest that annexin V columns improve pregnancy rates in couples with repeated IVF failure by selecting sperm with lower levels of DNA fragmentation; implementation of this procedure in a large group of patients is currently underway. These preliminary results also suggest that the use of the annexin V columns appears to be a safe procedure.

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