ORIGNAL ARTICLE: ANDROLOGY

Proteomic identification of target proteins in normal but nonfertilizing sperm

Cynthia Frapsauce, M.D.,[a] Cedric Pionneau, M.D.,[b] Julien Bouley, Ph.D.,[c] Vanina Delarouziere, M.D.,[a] Isabelle Berthaut, Ph.D.,[a] Celia Ravel, Ph.D.,[a] Jean-Marie Antoine, M.D.,[d] Florent Soubrier, Ph.D.,[c] and Jacqueline Mandelbaum, M.D.[a]

[a] Department of Histology and Reproductive Biology, UMPC (Paris VI), Tenon Hospital, Assistance Publique des Hôpitaux de Paris; [b] Plateforme post-génomique de la Pitié-Salpêtrière, UPMC; [c] Inserm; and [d] Gynaecology, Obstetrics and Reproductive Medecine, Tenon Hospital, Assistance Publique des Hôpitaux de Paris, Paris, France

Objective: To identify the male molecular causes of failures of IVF (with a deficient binding of spermatozoa to the zona pellucida, without any obvious oocyte anomaly), which are undetected by classical sperm analysis.
Design: Case-control prospective study.
Setting: University hospital.
Patient(s): Proteomic profiles of spermatozoa in patients with a complete failure of fertilization and no spermatozoa bound to the zona pellucida were compared with those of controls (men with normal fertilization and cleavage rates after classical IVF for tubal indication).
Intervention(s): All samples were analyzed by two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) after being divided into three fractions according to their isoelectric point.
Main Outcome Measure(s): Differentially expressed proteins between infertile men and controls were identified by mass spectrometry.
Result(s): Seventeen proteins differentially expressed between cases and controls were found. Twelve of these proteins were identified by mass spectrometry, and two may influence gametes interaction: laminin receptor LR67 and L-xylulose reductase (P34H).
Conclusion(s): This study shows that 2D-DIGE might be useful in finding potential targets for diagnosis and prognosis of idiopathic infertility in IVF. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: Spermatozoa, proteome, fertilization, zona pellucida, two-dimensional electrophoresis

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/frapsaucec-proteomic-identification-target-proteins-sperm/

Use your smartphone to scan this QR code and connect to the discussion forum for this article now.*

* Download a free QR code scanner by searching for "QR scanner" in your smartphone's app store or app marketplace.

In conventional IVF, 5% of the attempts lead to complete and unexpected failure of fertilization, despite normal sperm parameters. In 56% of the cases, a severe decrease of sperm fixation to the zona pellucida is noticed without any impairment in the number, maturity, or quality of retrieved oocytes (1). These biological observations suggest an infertility of male origin and defective gametic interaction due to spermatozoa, as already described in the literature (2, 3).

Animal experiments with transgenic mice have improved the basic knowledge of the fertilization process. Mice deficient in germinal angiotensin converting enzyme have a similar phenotype: male factor infertility characterized by a failure of fertilization and a reduced number of spermatozoa binding to the zona pellucida, despite apparently normal sperm (4, 5). Others gene invalidations concerning the A-disintegrin and metalloprotease group of proteins (ADAM proteins) lead to the same phenotype. Male mice lacking fertilin β (ADAM 2) are infertile. Spermatozoa have a reduced capacity to bind to the zona pellucida and fuse with the oocyte cytoplasmic membrane (6). When cyritestin (ADAM 3) is disrupted, male mice are also infertile, with a poor fixation of spermatozoa to the zona pellucida but a preserved ability to fuse with zona-free oocytes (7).

Received August 31, 2013; revised April 23, 2014; accepted April 24, 2014.
C.F. has nothing to disclose. C.P. has nothing to disclose. J.B. has nothing to disclose. V.D. has nothing to disclose. I.B. has nothing to disclose. C.R. has nothing to disclose. J.-M.A. has nothing to disclose. F.S. has nothing to disclose. J.M. has nothing to disclose.
This study was supported by a grant from Assistance Publique des Hôpitaux de Paris (APHP-CRC 01020, P010605, FESFM).
Reprint requests: Cynthia Frapsauce, M.D., Reproductive Medecine and Biology Department, CHRU Bretonneau, 2 boulevard tonnellé, 37044, Tours cedex 9, France (E-mail: c.frapsauce@chu-tours.fr).

Fertility and Sterility® Vol. ■, No. ■, ■ 2014 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2014.04.039

New experimental technologies like proteomics allow significant progress in discovering target proteins involved in human fertilization and novel diagnostic biomarkers of human male factor infertility (8–10). Numerous high-resolution proteomic profiles of normal spermatozoa have been performed with two-dimensional electrophoresis (2D-E) or liquid chromatography associated with mass spectrometry (11–14). Only a few authors have focused on infertile patients and have analyzed the proteomic profiles of asthenozoospermic or oligospermic men (15–18). Others investigated the difference in protein expression between human round-headed and normal spermatozoa (19). In 2012, Xu et al., studied the proteomic characteristics of spermatozoa in normospermic patients with infertility. With 2D-E gels and mass spectrometry, they identified 24 proteins differentially expressed between fertile and infertile men, which concerned sperm motility, capacitation, acrosomal reaction, and sperm-oocyte proliferation (20). With regard to failure of fertilization in conventional IVF, Pixton et al. reported a case of complete failure of fertilization in a man with normal sperm parameters (21). After comparison by 2D-E of the sperm proteome profile of this patient with those of several semen donors, 20 proteins were found to be differentially expressed between the patients and the semen donors.

All these findings bring new understanding to the molecular process of fertilization in humans. With an innovative approach combining a protein prefractionation method based on the protein isoelectric point (pI) with two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), we have tried to analyze the spermatozoa proteome profile of patients with normal sperm parameters who failed to fertilize in conventional IVF. Their particular phenotype, with a lack of sperm binding to the zona pellucida, led us to hypothesize a molecular defect in the surface function of the gamete.

MATERIALS AND METHODS

Subjects and Sample Collection

This project was approved by the bioethics committee of Tenon Hospital (ARC APHP no. 01020) and runs between 2006 and 2008. During the study period, 2,022 IVF cycles were performed. Sixty percent of the cycles were intracytoplasmic sperm injection (ICSI). Informed consent was obtained from all the subjects. Included men had normal sperm parameters according to the 1999 World Health Organization's (WHO) criteria, which are more strict than new ones (22), and postcapacitation test on density gradient yielded at least 1 million motile spermatozoa. Complete failure of fertilization in conventional IVF was noticed with none or very few spermatozoa bound to the zona pellucida of mature oocytes (<10 spermatozoa). For IVF procedure, 80,000 spermatozoa with progressive mobility, per milliliter, were inseminated in each dish. Sperm motility was checked 18 hours postincubation and was normal in all cases and controls. Identifiable oocyte causes for failure of fertilization were excluded (less than four retrieved oocytes or immature, degenerative, or dysmorphic retrieved oocytes). Unexplained failures of fertilization with unexpected poor survival or normal binding to the zona pellucida were also excluded. In parallel, a control group was selected that was composed of men with normal and fertilizing sperm during a past conventional IVF.

Specific semen samples were collected for the proteomic study to eliminate albumin contained in IVF media. Semen samples were prepared in the laboratory, produced by masturbation into a sterile container, 2 or 3 days after the IVF attempt. After liquefaction, sperm parameters were evaluated (volume, sperm concentration, motility) according to the latest criteria (22). Spermatozoa were separated from seminal plasma by centrifugation (300 *g*, 20 minutes) through discontinuous density gradients (45%–90%, Puresperm, Nidacon) diluted in Ham's F12 medium (Gibco). The pellet was washed twice in phosphate-buffered saline (1×), by centrifugation (450 *g*, 10 minutes). The supernatant was totally removed. The dry pellet was frozen at −80°C. The proteins were extracted in lysis buffer (urea 8.3 M; thiourea 2 M; Chaps 40%; DTT 50 mM; spermin dihydrate base 24 mM). After new centrifugation (16,000 *g*, 30 minutes, 4°C), the supernatant was collected. All protein extracts were frozen at −80°C. Six samples (three cases and three controls) were analyzed in the study.

Prefractionation of Protein Extracts

Isoelectrofocusing (IEF) in liquid phase was used for the prefractionation of the samples. Proteins were separated according to their pI, with Zuo and Speicher's method in an IEF Zoom Fractionator (Invitrogen) (23). Protein extracts (patients and controls) were each separated into three fractions, corresponding to three reduced pH gradients: fraction 1 (3<pH<5.4), fraction 2 (5.4<pH<7), and fraction 3 (7<pH<10).

2D-DIGE (24)

Two-dimensional DIGE was performed as described elsewhere (25). All protein fractions were first precipitated with the Perfect Focus Kit (G-Biosciences) and resuspended in labeling buffer (urea 7 M; thiourea 2 M; Chaps 4%; Tris pH 8.8 30 mM). Protein fractions were then labeled using fluorescent cyanine dyes (Cy), according to the manufacturer's instructions for minimal labeling (GE Healthcare Life Sciences). The internal standard, a mixture of identical amounts of the six protein samples, was labeled with Cy2 and used for normalization and gel matching. Cy3 and Cy5 were alternatively used to label the cases and controls protein extracts according to the dye switch method. To compare more than two analytical samples, all normalized Cy3 and Cy5 images were matched across all gels using Cy2 images. Labeled proteins were separated on 24 cm Immobiline DryStrip gels (GE Healthcare Life Sciences) and rehydrated for at least 12 hours. Three linear pH gradients were used according to protein fractions (fraction 1, 4.5–5.5, fraction 2, 5.3–6.7, fraction 3, 6–9). Strips were focused at 20°C for a total of 95 kVh in fraction 1, 135 kVh in fraction 2, and 33 kVh in fraction 3 using the Ettan II IPGphor system (GE Healthcare Life Sciences). Once IEF was complete, the strips were equilibrated in SDS-containing buffer (reduction and alkylation) before being loaded onto SDS polyacrylamide gels for separation according to the