Use of *Xenopus laevis* Frog Egg Extract in Diagnosing Human Male Unexplained Infertility

DAVID B. BROWN, Ph.D.,[a] AND MANUBAI NAGAMANI, M.D.[b]

[a]*Department of Human Biological Chemistry and Genetics,* [b]*Department of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, Texas*

Approximately one in six married couples find themselves involuntarily infertile. This ratio translates to between two and four million U.S. couples. Although numerous tests are available for diagnosing infertility problems, 5–10 percent of all couples who seek medical treatment are diagnosed with unexplained infertility. Several tests are presently available for diagnosing male infertility; however, none of the present procedures test for activation of the sperm nucleus following entry into the fertilized egg, a series of events critical for the entry of the zygote into the developmental program. We have developed an *in vitro* human sperm activation assay, using *Xenopus laevis* frog egg extract. When normal human sperm is permeabilized and then mixed with frog egg extract, the sperm nuclei decondense, synthesize DNA, and recondense during a three-hour time course. We have tested this assay's utility in diagnosing previously unexplained infertility. We found that 20 percent of the male infertility patients produced sperm that responded abnormally in the assay (95 percent confidence interval, 4–48 percent; $n = 15$), while sperm samples from 15 fertile males showed no abnormal responses ($p = 0.112$). These preliminary results indicate that the human sperm activation assay may be a useful tool for diagnosing some cases of human infertility.

INTRODUCTION

During fertilization, the sperm nucleus is activated by the egg cytoplasm. The activated sperm undergoes nuclear envelope breakdown and chromatin dispersion; the male pronucleus is formed and surrounded by a new nuclear envelope; DNA is synthesized in both the male and female pronuclei; and mitosis ensues, with nuclear envelope breakdown and chromosome formation [1,2]. In order to identify the factor(s) required for sperm activation, Lohka and Masui developed an *in vitro* frog sperm activation assay [3]. They demonstrated that incubating demembranated *Xenopus laevis* frog sperm in *Rana pipiens* frog egg extract induced sperm activation, including chromatin decondensation, pronuclei formation, DNA synthesis, and chromatin recondensation. These results were extended to the human system when Gorden et al. [4] and Brown et al. [5] demonstrated that mixing permeabilized human sperm with *Xenopus laevis* frog egg extract promoted the early events of human sperm activation, including chromatin decondensation, pronuclei formation, DNA synthesis, and chromatin recondensation. We now present results from the first clinical application of the human sperm activation assay (HSAA), analyzing the

29

sperm from 15 fertile males and 15 male unexplained infertility patients. An abstract of this study has appeared elsewhere [6].

MATERIALS AND METHODS

Unexplained Infertility Patients

At the University of Texas Medical Branch Infertility Clinic, a couple is diagnosed as having unexplained infertility when the following conventional tests have been completed without finding a cause for the couple's infertility: the female partner will have undergone complete infertility evaluations, including post-coital tests (to rule out sperm motility loss in cervical mucus), a timed endometrial biopsy (to exclude luteal phase defect), a hysterosalpingogram (to establish tubal patency), and laparoscopy (to rule out pelvic pathology); in the male partner, semen analysis should have been normal on two occasions with sperm concentrations greater than 20 million/ml, total sperm numbers of 40 million or more, sperm motility greater than 60 percent, and normal morphology in more than 60 percent of the sperm [7]. The inexplicably infertile couples must also have been involuntarily infertile for over two years. In this study, sperm was obtained from 15 unexplained infertility patients who have been involuntarily infertile for more than five years (average of 7.3 years, range of five to ten years). All of the semen samples from the unexplained infertility patients that were analyzed in this study had normal sperm concentrations, total sperm counts, motility, and morphology.

Human Sperm Preparation

All semen donors abstained from ejaculation for at least two days prior to the collection of the semen samples that were obtained by masturbation.

Semen samples from 15 unexplained infertility patients were obtained at the time of the regularly scheduled procedures at the infertility clinic (e.g., sperm penetration assay, SPA; *in vitro* fertilization-embryo transfer, IVF-ET; and gamete intrafallopian transfer, GIFT). The portion of the sample not used for these procedures (usually discarded) was used for the HSAA. These samples were kept in a 4°C refrigerator for up to seven days for parallel weekly assays on samples collected throughout the week. Semen samples from 15 fertile males were stored from one to seven days, mimicking the storage conditions of the semen samples from the unexplained infertility patients. Fresh samples are not needed for the HSAA, as stored semen samples from a fertile male were found to respond the same in the human sperm activation assays as did sperm collected from the same fertile male on the day the assay was performed, even after 4°C storage of up to a month (data not shown).

On the day of the experiment, semen samples were incubated for 30 minutes at 37°C; each sample was then suspended in 10 ml of nuclear isolation medium (NIM, 200 mM sucrose, 2.4 mM $MgCl_2$, 10 mM Tris-HCl, and 5 mM maleic acid, pH 7.4). A sperm count was taken and an aliquot (containing 20 million sperm) was pelleted in a centrifuge for ten minutes at 400 g, then resuspended in 10 ml of NIM containing 0.05 percent lysolecithin and 1 μg/ml soybean trypsin inhibitor. The mixture was kept at room temperature for five minutes and the sperm repelleted as before, then washed, first in NIM with 3 percent BSA and then with NIM containing 0.4 percent BSA, as described by Lohka and Masui [3]. Sperm were finally suspended in *Xenopus* extract isolation medium (XEIM, 10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 100 mM KCl, and 50 mM DTT) at a concentration of 25,000 sperm/μl and kept on ice for 45

minutes before mixing with the frog egg extract. This XEIM (DTT) pre-treatment was found to enhance the decondensation and DNA synthesis events (unpublished observations), presumably by reducing the protamines that in mammalian sperm are cross-linked by intermolecular disulfide bonds [8]. *Xenopus laevis* sperm contain "intermediate" protamines [9] that are not cysteine-rich [10], so the frog egg extract lacks the reducing factor(s) found in mammalian eggs that are required for reducing the disulfide bridges of the human sperm; we must thus reduce the disulfide bonds experimentally.

Xenopus laevis *Egg Extract Isolation*

To promote oocyte maturation, ovulation, and egg laying, adult female *Xenopus laevis* frogs were injected with 500 units of human chorionic gonadotropin in the morning and evening of the day preceding the experiment. Mature eggs were collected and dejellied with 2 percent cysteine-HCl, pH 7.6. The eggs were then rinsed and incubated for one hour at room temperature in Barth's medium composed of 88 mM NaCl, 1.0 mM KCl, 0.83 mM $MgSO_4$, 0.34 mM Ca $(NO_3)_2$, 0.41 mM $CaCl_2$, 7.5 mM Tris-HCl, pH 7.6, 10 μg/ml penicillin, 10 Mg/ml streptomycin, and 2.4 mM $Na(CO_3)_2$. The eggs were washed three times in XEIM. Excess buffer was removed and the eggs lysed by centrifugation at 10,000 g, 4°C, for 15 minutes in a swinging bucket rotor. The resulting middle layer between the lipids and the egg pellet was removed from the centrifuge tube, re-centrifuged, and the resulting middle layer used as the stock extract solution. The extract was stored on ice until used for the HSAA.

Human Sperm Activation Assay (HSAA)

Permeabilized sperm from the normal controls and the infertility patients were mixed separately with frog egg extract at a concentration of 150,000 sperm/150 μl of extract. The permeabilized sperm were incubated at 19°C in extracts containing ^{3}H-TTP at a concentration of 80 μCi/ml. Chromatin decondensation, DNA synthesis, and chromatin recondensation were evaluated. Phase-contrast microscopy was used to assess chromatin decondensation and recondensation. Aliquots (5 μl) were taken, placed on glass slides, and covered with glass cover slips at five and ten minutes and at three hours following the addition of the sperm to the frog egg extract. At the five- and ten-minute time points, the percentage of sperm that had decondensed (Fig. 1B) was determined. Between 95–100 percent of the control sperm were normally decondensed within five minutes. At the ten-minute time point, between 95–100 percent normally have the appearance of the nucleus shown in Fig. 1B. At the three-hour time point, it was determined if the sperm nuclei had recondensed their chromatin as may be seen by the smaller nucleus shown in Fig. 1D when compared to the nucleus shown in Fig. 1B. Recondensation was typically observed in 95–100 percent of the control sperm. DNA synthesis in the sperm nuclei was assessed, using a 50 μl aliquot taken at the two-hour time point and diluted with 50 μl of phosphate-buffered saline. The sperm were then affixed to glass slides by centrifugation, dried, and fixed for five minutes in Carnoy's fixative (three parts ice-cold methanol to one part glacial acetic acid). These fixed cytopreps were then dipped in 42°C Kodak nuclear track emulsion (NTB-2), stored at 4°C for two weeks, and then, following development, were Giemsa-stained through the emulsion. We also made cytopreps at the 15-minute time point to observe chromatin smearing and at the three-hour time point to see if the chromatin had recondensed to the level
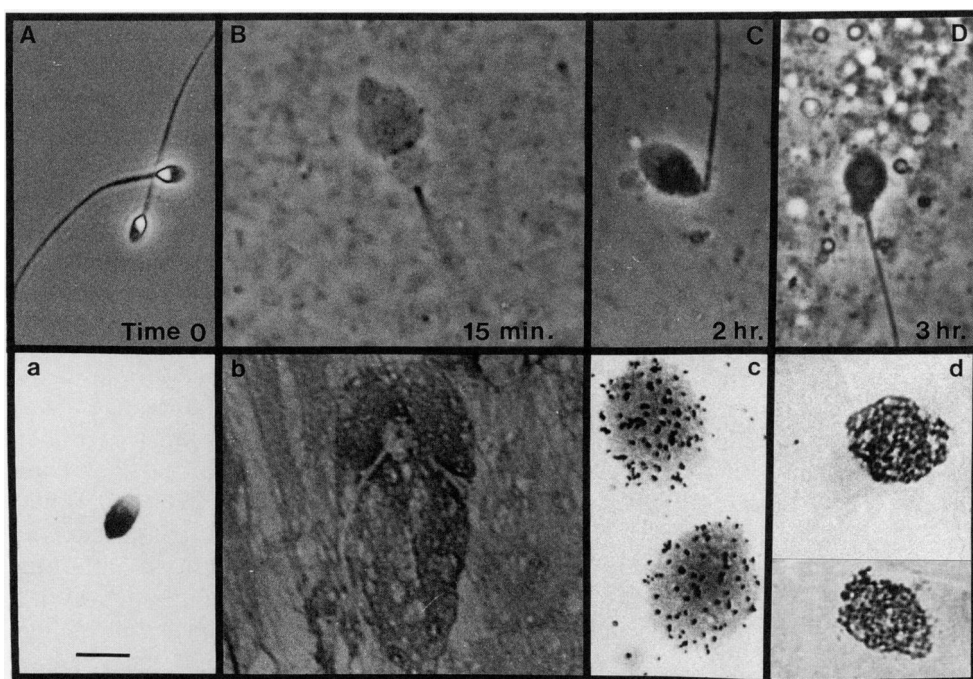
FIG. 1. Pictorial representation of permeabilized human sperm incubated in frog egg extract.   Figures 1A–1D are phase-contrast micrographs.   Figures 1a–1d are bright-field micrographs of Giemsa-stained nuclei.   Figure 1c is a Giemsa-stained autoradiograph.   A,a: Time zero.   B,b: 15-minute incubation.   C,c: Two-hour incubation.   D,d: Three-hour incubation.   Bar, 10 μm.

shown in Fig. 1d. The cytopreps were prepared and fixed as previously described, followed by Giemsa staining without autoradiography. All microscopy was done using a Leitz Orthoplan microscope. The micrographs in Figs. 1–4 were photographed at 800 X magnification, using a Zeiss Photomicroscope III. Two hundred nuclei were scored for labeling and the results with control sperm (percentage of the nuclei showing label above background) compared to those with the patients' sperm.

RESULTS

Normal Sperm (Control) Response in the HSAA

A pictorial representation of a normal response of permeabilized human sperm to the frog egg extract is shown in Fig. 1. The sperm nuclei shown in Figs. 1A–D and 1a–d were photographed (800 × magnification) and printed at the same magnification, with the bar representing 10 μm. Phase-contrast microscopy was used when photographing the nuclei shown in Figs. 1A–D, while Figs. 1a–d are bright-field photographs of Giemsa-stained cytopreps, with the nuclei shown in Fig. 1c being Giemsa-stained autoradiographs. Permeabilized sperm without extract treatment are shown in Figs. 1A and 1a. Following a five-minute incubation in the frog egg extract, greater than 90 percent of the sperm are decondensing. After a 10- to 15-minute incubation in the extract, greater than 95 percent of the sperm have completely decondensed nuclei. A typical completely decondensed sperm nucleus is shown in Fig. 1B. We have observed that at the 15-minute time point, the sperm nuclei do not remain intact during the cytocentrifugation procedure used when

TABLE 1
Human Sperm Activation Assay Rating Scheme

	% of Control			
	<40	40–60	60–80	80–100
% Decondensing at five minutes	–	+	++	+++
% Decondensing at ten minutes	–	+	++	+++
% Synthesizing DNA at two hours	–	+	++	+++
% Recondensing at three hours	–	+	++	+++

making cytopreps; the nuclei become smeared on to the glass slide (Fig. 1b). Following a two-hour incubation in the frog egg extract, the sperm nuclei have begun to recondense their chromatin. Notice the decrease in size of the sperm nucleus shown in Fig. 1C as compared to the nucleus shown in Fig. 1B. The recondensed sperm nuclei also withstand the cytocentrifugation procedure (note intact nuclei in Fig. 1c). The labeling of these nuclei indicates that they have undergone DNA synthesis. Typically, >95 percent of the control nuclei are labeled following a two-hour incubation in frog egg extract containing tritiated thymidine, while >95 percent are fully recondensed following a three-hour incubation. Examples of recondensed nuclei are shown in Figs. 1D and 1d.

Use of the HSAA in the Analysis of the Sperm Obtained from Fertile Males and Unexplained Infertility Patients

In this study, sperm samples were obtained from 15 unexplained infertility patients and 15 fertile males and prepared for use in the HSAA as described in Methods. In each HSAA, a sperm sample from a fertile male (previously shown to produce sperm that have a normal response in the HSAA) was also assayed as a parallel control. We have developed a rating system (Table 1) comparing the HSAA responses of the control sperm sample to the sperm samples obtained from 15 unexplained infertility patients and 15 fertile males. The results of the pilot study are shown in Table 2. As can quickly be ascertained by looking at the data summarized in Table 2, three of the sperm samples from the infertility patients responded abnormally in the HSAA, whereas all sperm from fertile men responded normally in the assay.

Case Reports of Patients Producing Sperm That Responded Abnormally in the HSAA

Patient 4: Sperm from patient 4 sperm sample, although scored normal (60 percent) in the sperm penetration assay (SPA; [11–13]), had a diminished decondensation response (50 percent decondensed after five minutes of incubation in extract),

TABLE 2
Human Sperm Activation Assay Pilot Study Results

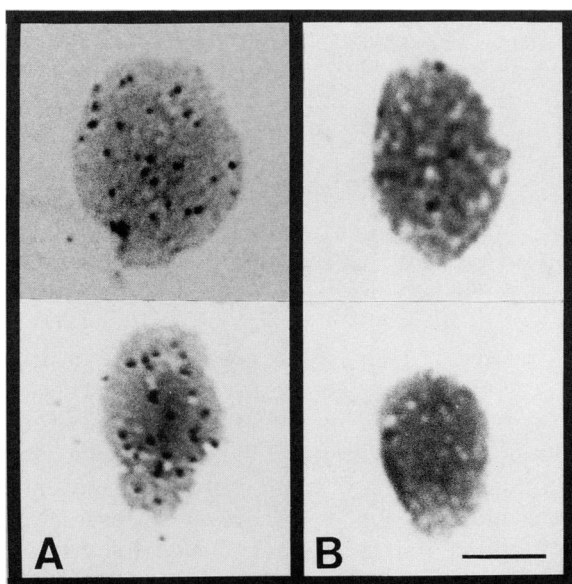|	Decondensation (5 minutes)	Decondensation (10 minutes)	DNA Synthesis (2 hours)	Recondensation (3 hours)
Fertile males 1–15	+++	+++	+++	+++
Infertility patients 1–3, 5, 7–14	+++	+++	+++	+++
Infertility patient 4	+	++	+++	+++
Infertility patient 6	+++	+++	–	+++
Infertility patient 15	+	+	+	?

FIG. 2. Bright-field photographs of Giemsa-stained autoradiographs of sperm nuclei following two hours of incubation in frog egg extract.   A. Fertile control labeled nuclei.   B. Patient 6 unlabeled nuclei.   Bar, 10 μm.

by which time normal human sperm were 95 percent decondensed. Even after a ten-minute incubation, only 80 percent of the patient's sperm had decondensed. We have repeated the HSAA for patient 4 using the excess sperm from an unsuccessful GIFT attempt (four eggs transferred), finding 60 percent of the sperm decondensed after five minutes of incubation in the extract, with 98 percent of the normal sperm decondensing during the same incubation period. Following a ten-minute incubation in the egg extract, 90 percent of the patient's sperm had decondensed, with 98 percent of the control sperm being decondensed. When new samples of this patient's sperm were obtained for use in the HSAA at 4, 11, and 13 months following the initial analyses, this patient's sperm responded normally in the HSAA when compared to the control sperm. During the time period where the patient's sperm was responding normally in the HSAA, an IVF-ET attempt resulted in a successful pregnancy; however, the pregnancy ended with a spontaneous miscarriage at six weeks of pregnancy. The sperm is from the male member of a couple who have been trying to conceive for ten years and at present, with the exception of our assay, the couple has been found to be normal in every test performed.

  Patient 6:   Patient 6 has sperm that was seen to decondense and recondense in a normal fashion. Only 27 percent of the sperm nuclei were found to be labeled following autoradiography, however, while 100 percent of the control sperm nuclei were found to be labeled. Autoradiographs of typical labeled control nuclei and unlabeled patient nuclei are shown in Figs. 2A and 2B, respectively. This couple has been trying to conceive for eight years. The sperm penetration assay has not been performed for this patient.

  Patient 15:   Patient 15 has sperm that responded variably in the rate and extent of decondensation and DNA synthesis. Only 50 percent of the sperm had decondensed at both the five- and ten-minute time points with 45 percent showing partial decondensation and 5 percent showing full decondensation. The cytopreps from the 15-minute time point are shown in Fig. 3. In Fig. 3A the typical smearing of the
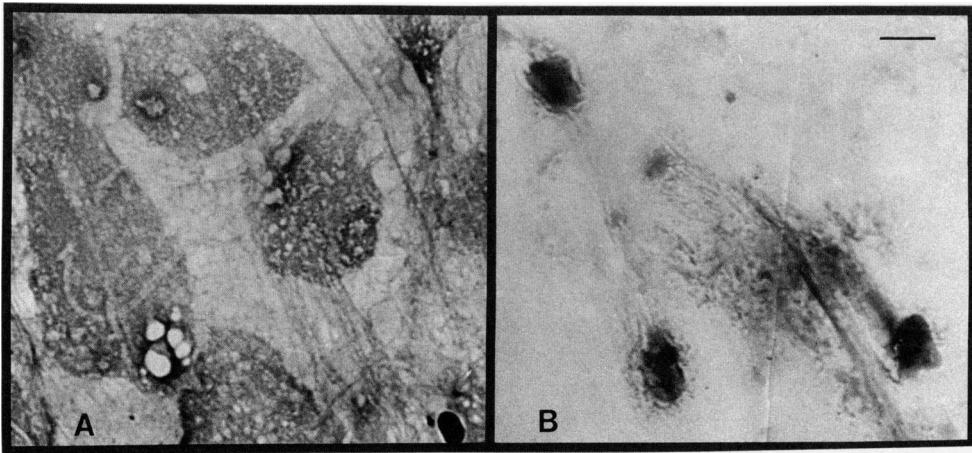
FIG. 3. Bright-field photographs of Giemsa-stained sperm nuclei following 15-minute incubations in frog egg extract.   A. Fertile control, decondensed and smeared nuclei.   B. Patient 15, three nuclei that are neither smeared nor decondensed by a decondensed smeared nucleus.   Bar, 10 μm.

sperm nuclei is shown, seen from control normal sperm in the HSAA. In Fig. 3B sperm nuclei from patient 15 are shown, with three intact sperm nuclei and one smeared nucleus. Sperm nuclei that are not decondensing maintain their nuclear structure during cytocentrifugation, while decondensing sperm smear on the glass slide. In Fig. 4 are shown the cytoprep-autoradiographs of sperm nuclei from a
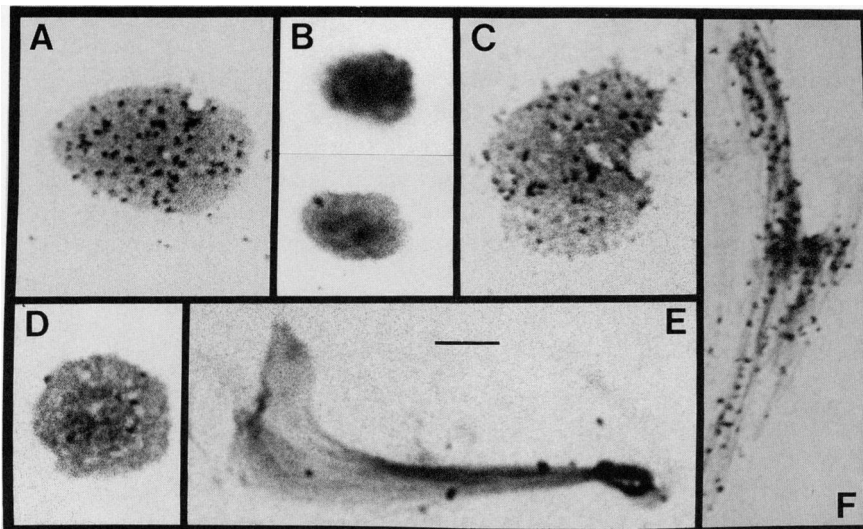

FIG. 4. Bright-field micrographs of Giemsa-stained autoradiographs of sperm nuclei following two hours of incubation in frog egg extract.   A. Fertile control, decondensed labeled sperm nucleus (96 percent).   Figures B–F show examples of the variable decondensation and labeling observed in patient 15 sperm nuclei.   B. Nuclei neither decondensed, nor labeled (41 percent).   C. Nucleus decondensed and labeled (14 percent).   D. Nucleus decondensed and unlabeled (8 percent).   E. Nucleus decondensed, smeared, and unlabeled (7 percent).   F. Nucleus decondensed, smeared, and labeled (30 percent).   Bar, 10 μm.

control fertile male (Fig. 4A) and from patient 15 (Fig. 4B–F) that had been incubated for two hours in egg extract containing tritiated thymidine. In the control labeled nuclei, 96 percent were found to be labeled. Nuclei from patient 15 had variable levels of decondensation, with 44 percent of the nuclei being labeled. Forty-one percent of the nuclei were neither decondensed nor labeled (Fig. 4B), 14 percent were decondensed and labeled (Fig. 4C), 8 percent were decondensed and unlabeled (Fig. 4D), 7 percent were smeared and unlabeled (Fig. 4E), and 30 percent were smeared and labeled (Fig. 4F). This couple has tried to conceive for seven years. Their sperm penetration assay score was zero. It is interesting to note that the couple decided to take part in the sperm donor program available at the UTMB clinic. When using donor sperm, the first attempt at artificial insemination resulted in a pregnancy.

DISCUSSION

The results from this study demonstrate that 20 percent of the males from couples having unexplained infertility produce sperm that respond abnormally in the HSAA (95 percent confidence interval of 4–48 percent; one-tailed Fisher exact test, $p = 0.112$). Abnormal responses that were expected included lack of sperm chromatin decondensation, diminished DNA replication, and/or abortive sperm chromatin recondensation. Abnormal responses that were detected included: *(1)* a slow rate of chromatin decondensation, *(2)* a low level of DNA synthesis, and *(3)* variability in the rate and extent of decondensation and DNA synthesis. We did not find any patients producing sperm that recondensed abnormally.

In the case of patient 4 (slow rate of chromatin decondensation), we determined that the HSAA could be used to follow the patient's sperm quality as regards fertilization potential when used in the IVF-ET procedure. While the sperm from patient 4 was responding normally in the HSAA, the sperm fertilized an egg *in vitro* and produced an embryo that was transferred into the patient's wife, resulting in a successful pregnancy. This correlation demonstrates that the HSAA may have potential for monitoring suitability of a particular semen sample for *in vitro* fertilization. As far as we know, the three different semen samples (two showing abnormal HSAA responses, one responding normally) were obtained from patient 4. It is not clear why the patient would begin to produce sperm that respond normally in the HSAA, following two abnormal responses. In the human male, spermatogenesis takes 74 days [14]. During these 74 days, various aspects of spermatogenesis might be sensitive to such environmental influences as temperature, tobacco and/or alcohol use, or changes in the diet. As the environmental influences change, patients whose spermatogenic environment changed might also undergo a change in semen quality. We have, however, no evidence that such changes occurred in patient 4's environment.

Patient 15 (variability in the rate and extent of decondensation and DNA synthesis) was producing abnormal sperm that did not respond properly in the HSAA, or in the SPA. This finding led us to conclude that the problem with this infertile couple was related to the male's sperm; the conclusion was substantiated by the successful pregnancy that resulted following the first attempt at artificial insemination using donor sperm.

We do not believe that the abnormal sperm activation responses observed in this study are a result of variability in the frog egg extracts used throughout the study. In

each experiment, the frog egg extract was tested for sperm activation activity using the sperm from a fertile male that had been previously shown to respond normally in the HSAA. No variability was seen in the sperm activation response of the control sperm when incubated in the different frog egg extracts.

We analyzed the sperm from 15 fertile males in order to determine what frequency of males in a fertile population produce sperm that responds abnormally in the sperm activation assay. All 15 samples from fertile males responded normally in the assay. We therefore believe that the abnormal responses observed in the infertile patients' samples suggest that the patients are infertile as a result of producing sperm deficient in fertilization capacity as regards the events that occur following the entry of the sperm nucleus into the egg cytoplasm.

Several tests are presently available for diagnosing male infertility; these assay sperm number, morphology, motility, and the ability to fuse with and then enter a zona-free hamster egg, as occurs during the SPA [11–13]. None of the standard procedures, with the exception of the SPA, test the activation of the sperm nucleus following entry into the fertilized egg. The SPA score is determined by the percentage of eggs that are penetrated by the sperm, as determined by counting the eggs that contain decondensed or activated sperm nuclei; however, one cannot use the SPA to study the efficiency of the decondensation process. During the SPA, the hamster egg routinely becomes bound with a large number of sperm that do not enter the egg and thus do not decondense. One cannot tell a non-decondensed sperm that is bound to the egg from a sperm that has entered the egg and not decondensed as a result of not responding to the egg activation signals. Interestingly, a zero SPA score may in some cases reflect the sperm's inability to decondense, instead of its penetration capabilities, as is presently assumed. As regards using the fertilized hamster eggs to follow DNA synthesis and recondensation, the labor and expense that would be associated with obtaining enough eggs to analyze one patient would prohibit the use of the SPA to study sperm activation as a routine clinical procedure. Conversely, the HSAA allows the investigator routine comparison of hundreds of a patient's activated sperm nuclei with those from a normal, fertile male and easily detects any abnormal response, thus making this the assay of choice for sperm activation.

Although the small sample size ($n = 15$) of this pilot study produced a wide 95 percent confidence interval, the sperm activation assay shows great promise in diagnosing a major class of currently unexplained infertility. Clearly, we must now use the HSAA to analyze a large number of sperm samples from unexplained infertility patients in order to decrease the 95 percent confidence interval and to determine more accurately the percentage of unexplained infertility patients who can be diagnosed by the assay results.

In conclusion, the results from this pilot study indicate that the HSAA may be a useful tool for diagnosing some cases of male infertility. With the present procedures available for diagnosing infertility patients, it is estimated that 90 to 95 percent of the patients who seek treatment at an infertility clinic can be diagnosed [15]. We have developed, and now tested an HSAA in an attempt to diagnose some of the 5 to 10 percent of patients who are classified with unexplained infertility. Our assay allows analysis of sperm activation events, including chromatin decondensation, pronuclei formation, DNA synthesis, and chromatin recondensation. None of the other available infertility tests assesses activation of the sperm nucleus following entry into the fertilized egg, a series of events critical for the entry of the zygote into the

developmental program. The HSAA will allow diagnosis of otherwise unexplained infertility patients by determining that their infertility is a result of their sperm nuclei not responding properly to the egg cytoplasm following fertilization. Couples having unexplained infertility spend a large amount of money on current treatments. It is estimated that as much as a billion dollars a year is spent by from 300,000 to one million couples in pursuit of pregnancy [16]. As with patient 15, the HSAA can be used to identify patients who cannot benefit from the available treatments of infertility, thereby saving the associated expense and discomfort. Eventually, we hope to develop methods to identify and perhaps ultimately to replace the defective or missing components of the sperm that prevent normal sperm activation.

ACKNOWLEDGEMENTS

REFERENCES

1. Longo FJ: Fertilization: A comparative ultrastructural review. Biol Reprod 9:149–215, 1973
2. Longo FJ, Kunkle M: Transformation of sperm nuclei upon insemination. Curr Top Dev Biol 12:149–184, 1978
3. Lohka MJ, Masui Y: Formation in vitro of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. Science 220:719–721, 1983
4. Gordon K, Brown DB, Ruddle FH: In vitro activation of human sperm induced by amphibian egg extract. Exp Cell Res 157:409–418, 1985
5. Brown DB, Blake EJ, Wolgemuth DJ, Gordon K, Ruddle FH: Chromatin decondensation and DNA synthesis in human sperm activated in vitro by using Xenopus laevis egg extracts. J Exp Zool 242:215–231, 1987
6. Brown DB, Nagamani M: Use of frog egg extract in diagnosing human infertility. J Cell Biol 111:115a, 1990
7. World Health Organization: Appendix 1.A, Normal values of semen variables. In WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction. New York, Cambridge University Press, 1987, p 27
8. Perreault SD, Barbee RR, Slott VL: The role of disulfide bond reduction during mammalian sperm nuclear decondensation in vivo. Dev Biol 101:160–167, 1984
9. Kasinsky HE, Huang SY, Mann M, Roca J, Subirana JA: On the diversity of sperm histones in the vertebrates. IV. Cytochemical and amino acid analysis in Anura. J Exp Zool 234:33–45, 1985
10. Kasinsky HE: Specificity and distribution of sperm basic proteins. In Histones and Other Basic Nuclear Proteins. Edited by LS Hnilica, GS Stein, JL Stein. Boca Raton, FL, CRC Press Inc, 1989, pp 73–163
11. Yanagimachi R, Yanagimachi H, Rogers BJ: The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. Biol Reprod 15:471–476, 1976
12. Overstreet JW, Yanagimachi R, Katz DF, Hayashi K, Hanson FW: Penetration of human spermatozoa into the human zona pellucida and the zona-free hamster egg: A study of fertile donors and infertile patients. Fertil Steril 33:534–542, 1980
13. Wolf DP, Sokoloski JE: Characterization of the sperm penetration bioassay. J Androl 3:445–451, 1982
14. Johnson L: Spermatogenesis (Animal Species and Humans). In Gamete Physiology. Edited by RH Asch, JP Balmaceda, I Johnston. Norwell, MA, Serono Symposia, USA, 1990, pp 3–18
15. Webster BW, Cook AS, Garner CH: The infertility evaluation. In Handbook of the Laboratory Diagnosis and Treatment of Infertility. Edited by BA Keel, BW Webster. Boca Raton, FL, CRC Press Inc, 1990, pp 1–9
16. U.S. Congress, Office of Technology Assessment: Infertility: Medical and Social Choices. OTA-BA 358. Washington DC, U.S. Government Printing Office, 1988