Analysis of early meiotic events and aneuploidy in nonobstructive azoospermic men: a preliminary report

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Objective: To study the events of spermatogenesis in azoospermic men by examining meiosis I spermatocytes and postmeiotic spermatozoa.
Design: A preliminary analysis of synaptonemal complex (SC), recombination, and chromosomal constitution (meiotic and postmeiotic) in testicular tissue.
Setting: Academic research environment.
Patient(s): Three men with nonobstructive azoospermia and one fertile man (vasectomy reversal: control) who underwent testicular sperm extraction in preparation for intracytoplasmic sperm injection (ICSI).
Intervention(s): Testicular tissue specimens were processed by immunofluorescence analysis with antibodies against proteins associated with SC and recombination events. Fluorescence in situ hybridization (FISH) for chromosomes X, Y, and 18 was done on spermatocytes in prophase I and on postmeiotic spermatozoa.
Main Outcome Measure(s): SC formation and recombination in meiosis I, aneuploidy.
Result(s): The number of autosomal recombination foci in each patient was not statistically significantly different from control. The frequencies of XY bivalents with at least one recombination focus were statistically similar in the patients and control (74.2% vs. 82.6%, respectively). All observed cells in pachytene had normal XY constitutions. In spite of this, the rate of sex-chromosome aneuploidy in spermatozoa was statistically significantly higher in the patients compared with the control (1.89% vs. 0.83%).
Conclusion(s): The combination of immunocytologic technology with FISH can add a level of precision in etiologic investigations of severe male factor infertility: men can have normal pairing and recombination but still yield aneuploid spermatozoa. (Fertil Steril® 2006;85:646–52. ©2006 by American Society for Reproductive Medicine.)

Key Words: Synaptonemal complex, meiosis, recombination, aneuploidy, fluorescence in situ hybridization (FISH), SCP3, nonobstructive azoospermia, intracytoplasmic sperm injection (ICSI)

Intracytoplasmic sperm injection (ICSI) has become the treatment of choice for severe male factor infertility, allowing even men with virtually no mature sperm in the ejaculate to conceive. With the use of a micropipette, a single sperm is directly injected into the oocyte; because of this, ICSI can use sperm retrieved from the epididymis or testis (1–4). As a result, ICSI can potentially use genetically abnormal sperm that would not have had the capacity to fertilize an oocyte under natural processes. Thus, ICSI has been found to increase the risk of introducing aneuploidy in conceptions (5, 6). Researchers have suggested that these increases originate from insufficiencies in the meiotic processes, and that these insufficiencies are also related to male infertility (7, 8), but the effect of abnormal meiosis on fertility has yet to be fully appreciated.

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Different approaches that have attempted to examine meiotic events in humans include genetic linkage analysis of human pedigrees and cytologic analysis of chiasmata in diakinesis preparations (9). Although genetic linkage analysis has been valuable in identifying the frequency recombination events in the normal population, it is an indirect method and is not applicable to the infertile population. Diakinesis preparations are limited to only cells in that stage of division and are difficult to analyze because chromosomes are highly condensed, making localization of chiasmata to specific chromosomal regions difficult. As a result, the technique is limited in the amount of information that it can provide (10, 11).

To more accurately examine the events of pairing and recombination in meiosis, techniques that use fluorescently labeled antibodies to specific meiotic structures in situ have been developed (12). Antibodies to synaptonemal complex proteins such as SCP2 and SCP3 (lateral/axial elements tethering sister chromatids), SCP1 (transverse elements linking lateral elements), MLH1 protein (a mismatch repair protein localized to events of recombination), and CREST (localized to the centromeric regions on all chromosomes), when applied to spreads of testicular cells, can be used to

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identify the events of pairing and recombination in cells at different stages of meiosis. When used in conjunction with fluorescence in situ hybridization (FISH), the synaptonemal complexes and number of recombination foci can be examined for specific chromosomes of interest. In addition, FISH analysis of the population of mature sperm in the testicular biopsied samples would also allow for an investigation of whether there is any change in chromosomal constitution between cells in early meiosis to those at completion of meiosis.

MATERIALS AND METHODS

Clinical Information

We report on three patients who were diagnosed with nonobstructive azoospermia. All patients had normal somatic karyotypes and an absence of sperm in their semen. Biopsy results revealed spermatogenic hypoplasia in patient 1 (43 years old) who also had elevated serum follicle-stimulating hormone (FSH) levels (17.5 IU/L). Patient 2 (34 years old) had incomplete spermatogenic maturation arrest and a normal hormonal profile. Testicular biopsy or hormonal measurement was not done for patient 3 (30 years old), who had fathered three children and subsequently had a vasectomy. He had a history of anabolic steroid use and had had two vasectomy reversal attempts that were deemed to be failures after the subsequent absence of spermatozoa in the epididymis. Our control individual was 52 years of age, fertile (with two children from previous marriage), and had undergone testicular epithelial sperm extraction (TESE) for failure of vasectomy reversal. University of British Columbia ethics committee approval was obtained before initiating the study.

Testicular Tissue Extraction

Spermatozoa were retrieved from the patients by TESE, and cryopreserved for future application in ICSI (3). A small amount of fresh testicular tissue was processed, with patient consent, for immunofluorescence analysis (12).

Testicular Tissue Preparation

We gently teased apart seminiferous tubules with fine scissors into 3- to 5-mm segments in PBS (pH 7.4) at room temperature. We incubated the tissue in freshly prepared hypo-extraction buffer (30 mM Tris, 50 mM sucrose, 17 mM citric acid, 5 mM EDTA, 0.5 mm of DTT, 0.1 mM PMSF) for 45 to 60 minutes; we then placed the tissue on a microscope slide containing 20 μL of 100 mM sucrose (pH 8.2) and squeezed out the contents of individual tubular segments with a pair of fine forceps. We transferred 10 μL of the germ cell/sucrose slurry to one side of a new slide overlain with 1%paraformaldehyde and rolled the slide to spread the germ cells evenly on the slide's surface. Afterward, we placed each slide in a humid chamber for 24 hours at room temperature and allowed them to air dry for 30 minutes.

Immunostaining

The dried slide was washed twice in 0.4% PhotoFlo (Kodak 200 solution) for 2 minutes, drained and air dried again. We soaked the slides in 1XADB (1% donkey serum, 0.3% BSA, 0.0005% Triton X, PBS; pH 7.2) at room temperature for 30 minutes, agitating every 5 minutes. We then drained the slide, applied primary antibody cocktail (rabbit anti-human MLH1, 1:37.5; SCP3 anti-mouse IgG1, 1:30; CREST antisera, 1:25; 1XADB), covered it with a plastic coverslip, and incubated it in a humid chamber at 37°C for 24 hours. The slide was washed in 1XADB once for 10 minutes at room temperature, and then in fresh 1XADB for 40 to 48 hours at 4°C. We applied the secondary antibody cocktail (fluorescein donkey anti-rabbit IgG [H+L], 1:50; rhodamine TRITC goat anti-mouse IgG (H+L), 1:50; Coumarin AMCA donkey anti-human IgG [H+L], 1:50) and incubated the slide in a humid chamber at 37°C for 90 minutes. Afterward we washed the slide three times in PBS at room temperature for 10, 20, and 30 minutes, agitating every 5 minutes. The slide was drained, and antifade was added with a glass coverslip.

FISH after Immunofluorescence Analysis

We removed the glass coverslip from each slide by soaking in 2XSSC, then fixed the slide in 4% formaldehyde at room temperature for 5 minutes. Each slide was washed in PBS for 5 minutes and then in 2XSSC for 5 minutes, both at room temperature. We air dried the slides in darkness. After the probe mixture was applied—CEPX (Spectrum Green), CEP Y (Spectrum Orange), and CEP 18 (Spectrum Aqua) (Vysis Inc., Downers Grove, IL)—the slide was covered with glass coverslip and sealed with rubber cement. The slides were codenatured and probed on a hot plate at 75°C for 5 minutes, then incubated in a humid chamber at 37°C overnight. After the coverslips were removed, the slides were washed in 4XSSC at room temperature for 10 minutes with constant agitation. We air dried the slides, then added DAPI II counterstain (Vysis Inc., Downers Grove, IL) and coverslips.

FISH Interpretation Criteria for Analysis of Spermatozoa

We scored the nuclei with the same criteria as we have used in the past (13). Scoring was only done in an area of the slide where consistent hybridization was evident on initial screening of the slide. Only nuclei with intact morphologic features and long sperm tails were scored to select for postmeiotic germ cells (spermatozoa) and to exclude any other cell type or artifact that was present on a slide. Any nuclei that had abnormal morphologic features would not be scored to avoid overlapping cells being scored as one. Two signals of the same color were scored as two copies of the corresponding chromosome when they were comparable in brightness and size and were separated from each other by a distance longer than the diameter of each signal. Nullisomy of any individual chromosome was considered when the sperm clearly contained at least one of the other chromosomal signals; complete nullisomic sperm, the absence of any signal, was scored as such.