Reduced sperm yield from testicular biopsies of vasectomized men is due to increased apoptosis

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Objective: To compare sperm yields, apoptotic indices, and sperm DNA fragmentation from vasectomized men and fertile men undergoing vasectomy.
Design: Testicular biopsies from vasectomized (n = 26) and fertile men (n = 46), were milked to calculate sperm/gram and also formalin-fixed to determine the numbers of developing sperm and incidence and intensities of testicular FasL, Fas, Bax, and Bcl-2. Testicular sperm DNA fragmentation was assessed using the alkaline Comet assay.
Setting: An ART unit.
Patient(s): Twenty-six men attending for intracytoplasmic sperm injection (ICSI) and 46 men attending for vasectomies.
Main Outcome Measure(s): Spermatocyte, spermatid and sperm yields, Fas, FasL, and Bax staining.
Result(s): Sperm yields from men vasectomized >5 years previously were markedly reduced compared to fertile men. Increased intensities of FasL and Bax staining were observed in the seminiferous tubules of vasectomy men. FasL positivity (percentage) also increased in Sertoli cells, and both FasL and Fas positivity (percentage) increased in primary spermatocytes and round spermatids of vasectomized men. Sperm DNA fragmentation, an end point marker of apoptosis, increased significantly in vasectomized men compared to fertile men.
Conclusion(s): Reduced sperm yields after vasectomy are associated with increased apoptosis through the Fas–FasL and Bax pathways. Sperm after vasectomy displayed increased DNA fragmentation. (Fertil Steril® 2007;87:834–41. ©2007 by American Society for Reproductive Medicine.)

Key Words: Apoptosis, ICSI, sperm yield, spermatids, vasectomy, DNA fragmentation

During the past 30 years there has been a marked increase in vasectomy procedures, particularly in more affluent parts of the world. Currently, more than 40,000 men have a vasectomy in the U.K. every year, accounting for more than 50% of sterilizations. Traditionally, vasectomy has been considered an irreversible form of contraception. However, with advances in assisted reproduction (1), men now have the choice of restoring their fertility by surgery or using intracytoplasmic sperm injection (ICSI) with testicular or epididymal sperm. As society changes and marital breakdown increases, more men are requesting reversal with a view to starting a second family. Recent studies report 2%–9% of men worldwide request a reversal each year (2–5).

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In our experience, about 10% of the men attending for ICSI with testicular sperm have had vasectomies with a previous partner. This may also be a more cost effective method (6). Further vasovasostomies have been reported to be only ~50% successful (7). It has also been reported that as the interval between vasectomy and reversal increases, the chances of pregnancy occurring decrease (8). This group showed that in men where vasectomy to reversal time is less than 2 years, there was a 50% chance of spontaneous pregnancy, whereas in partners of those where vasectomy to reversal time was more than 15 years, there was only a 30% chance of spontaneous pregnancy.

Apoptosis plays an important role in the testis by controlling germ cell number (9, 10) with 50%–70% of germ cells undergoing apoptosis at different stages of spermatogenesis (11–13). Testicular apoptosis can be initiated through the Fas/FasL pathway (9) or p53 up-regulation (14). The type 1 transmembrane receptor protein, Fas, is believed to be a key initiator of apoptosis in the testis (9). FasL has been located in the Sertoli cells and Fas in the germ cells of the testis (15). The binding of Fas to its ligand FasL triggers the caspase cascade that results in the programmed death of the Fas-positive cell.

In somatic cells apoptosis is also regulated by many other protein interactions, including those of the Bcl-2 family,

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before the cells commit to the process by the caspases and endonucleases. Bax can homodimerize and heterodimerize with Bcl-2 (16). The ratio of proapoptotic to antiapoptotic Bcl-2 family proteins is critical in the cell fate, with a predominance of Bcl-2 resulting in cell survival and Bax resulting in cell death. These molecules compete through dimerization, but it is not known whether antiapoptotic or proapoptotic members are dominant in determining the key survival-promoting decision (17).

To our knowledge no comparisons of testicular sperm yields from vasectomized men relative to fertile men have been reported, nor have there been any studies to compare the incidence of apoptosis in fertile with vasectomized men. The aim of this study was to determine the effects of vasectomy on sperm yield, the incidence and intensity of the apoptotic markers FasL, Fas, Bax, and Bcl-2 in the testis using immunohistochemistry, and optical density imaging and of testicular sperm DNA fragmentation using the alkaline Comet assay.

MATERIALS AND METHODS

Subjects

Testicular biopsies were obtained from two groups of subjects. The first group consisted of men who had a vasectomy between 4 and 22 years previously (median 13 years) and attended the Northern Ireland Regional Fertility Centre for treatment (n = 26). The second group were men of proven fertility, biopsied at the time of vasectomy (n = 46). The mean age of the men was 46.5 ± 0.8 years in the vasectomized group and 39.0 ± 1.1 years in the fertile group. Informed written consent was obtained from patients taking part in the study. The project was approved by the Queen's University Belfast Research and Ethics Committee and was in accordance with The Declaration of Helsinki as revised in 1983.

Anesthesia

The left spermatic cord was located, and 10 mL of 0.5% bupivacaine (without adrenaline) was injected on either side of it with use of a 21-guage needle. After 10 minutes anesthesia was confirmed by firm testicular palpation. If the patient was aware of pain or pressure, a further bolus was injected. The scrotal skin over the lower pole of the testicle was anesthetized with 0.5 mL of 1% linocaine.

Testicular Biopsy

The testicular biopsy was performed as previously described (18).

Effects of Vasectomy on Sperm Yield

The first biopsy was weighed. The seminiferous tubules were teased apart and milked by stabilizing the left end of each seminiferous tubule with forceps and drawing the tubule through a second pair of jeweler's forceps in a left to right direction to force the contents from the free end into the culture medium (Biggers, Whitten, and Whittingham [BWW]). The tubule contents were centrifuged at 1000 × *g* for 10 minutes. Subsequently, the pellet was resuspended in 200 μL of BWW, and 10 μL was placed on a hemocytometer to calculate the sperm/gram of biopsy using the following formula: 10,000 × Dilution factor × Hemocytometer count/Biopsy weight.

Immunocytochemistry: Effects of Vasectomy on FasL, Fas, Bax, and Bcl-2

A second biopsy was not milked but was immersion-fixed, intact, without milking, in 10% formalin fixative and embedded in paraffin wax. Sections, 3-μm thick, were cut, air dried, dewaxed, and rehydrated. Those to be immunostained for Bcl-2 were immersed in 0.01 M citric acid buffer, pH 6.0, and microwaved for 25 minutes (5, 10, and 10 minutes), whereas the remaining slides stained for FasL, Fas, and Bax were placed in 0.1 M phosphate-buffered saline (PBS) solution at pH 7.2.

The LSAB+ kit (Dako, Glostrup, Denmark) was used and modified as follows. All sections were blocked in 3% hydrogen peroxidase for 5 minutes and rinsed in PBS. Avidin and biotin block were added to sections for 15 minutes to block the natural biotin in testicular tissue. Polyclonal antibodies to FasL (N-20, Santa Cruz Biotechnologies, Inc., Santa Cruz, CA), Fas (CD95 clone UB2, Beckman Coulter, Marseille, France), Bax (Epitope specific polyclonal, Biogenex, San Ramon, CA), and a monoclonal antibody to Bcl-2 (Clone 124, monoclonal, Dakopratts, Glostrup, Denmark) were diluted in 1% bovine serum albumin (BSA) (Sigma, Dorset, England) and applied as primary antibodies for 1 hour for FasL, Fas, and Bcl-2, and for 30 minutes for Bax, at room temperature. Antibody dilutions were 1:50, 1:8, 1:20, and 1:100, respectively. These methods were optimized for this study based on immunohistochemical techniques discussed by both Fukuzawa et al. (19) (FasL, Fas) and Strik et al. (20) (Bax, Bcl-2).

A secondary biotinylated antigoat (for the polyclonal antibodies) or antimouse (for the monoclonal antibody) was added for 30 minutes, rinsed, and streptavidin (conjugated to horseradish peroxide) was applied for another 30 minutes. A substrate–chromagen solution, made up of hydrogen peroxide and 3,3′-diaminobenzidine (Dakopatts) was then added for 3 minutes (for FasL and Bcl-2), 2 minutes (Bax), or 1 minute (Fas) to visualize each of the labeled antigens. All sections were counterstained with hematoxylin (Sigma-Aldrich) for 15 minutes.

Negative controls were carried out for each probe by eliminating the antibody and by substituting the antibody with nonimmune serum. Positive tissue controls: liver (catalogue no. 70321-3; Novagen, Madison, WI) (21), lung adenocarcinoma (22, 23) (catalogue no. 70365-3; Novagen),