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Effect of Vasectomy on Sperm Nuclear Chromatin Condensation in the Rabbit

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ABSTRACT: Histone-to-protamine exchange in haploid spermatids is known to play a central role for male fertility. The present study investigates, for the first time, the effects of vasectomy on the expression of protamines in the rabbit. During normal spermatogenesis, protamine-1 and protamine-2 mRNA were expressed from step 5 round spermatids to step 11 elongated spermatids. In unilaterally vasectomized animals, control testes revealed normal spermatogenesis with normal protamine expression, while vasectomized testes exhibited both normal spermatogenesis and spermatogenic arrest. Some testes with normal spermatogenesis revealed delayed expression of both protamine-1 and protamine-2. Furthermore, multinucleated round spermatids were a regular finding in these testes. In both treated and untreated animals, a higher percentage of spermatozoa from the cauda epididymis had highly condensed chromatin when compared with those from the testis. The percentage of spermatozoa with highly condensed chromatin from testes and epididymides from the vasectomized side of treated animals remained unchanged from controls. As the integrity of nuclear chromatin is important for oocyte fertilization, especially in intracytoplasmic sperm injection (ICSI), where most of the natural selection mechanisms are bypassed, our data add valuable information for the treatment of infertility by ICSI, showing that vasectomy may affect nuclear chromatin integrity of testicular spermatids but not epididymal spermatozoa. Microsurgical epididymal sperm aspiration (MESA), therefore, may be superior to testicular sperm extraction (TESE) in vasectomized patients.

Key words: Protamine, spermatogenesis, vasectomy.
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Due to an increasing demand for reversal of vasectomy, there is a growing interest in research on the effects of vasectomy on the histology of the testis/epididymis. To date, there is no unanimity in opinion among authors. While some did not find alterations in rat (Flickinger, 1972; McDonald and Scothorne, 1988), rabbit (Paufler and Foote, 1969; Flickinger, 1975a,b), and monkey (Chapman et al, 1978; Hadley and Dym, 1983), others reported marked changes in mouse (Singh and Chakravarty, 2000), rat (Flickinger et al, 1986, 1987, 1990; McDonald et al, 1996; Sarrat et al, 1996; Aydos et al, 1998; Whyte et al, 2000), hamster (Hamasaki et al, 1991; Sun et al, 1992), guinea pig (Hutson et al, 1976; Tung and Alexander, 1977), rabbit (Swanson and Hafs, 1969; Hooker, 1980), dog (Antypas et al, 1994), and monkey (Lohiya et al, 1987).

In men, several studies have suggested that testicular/epididymal changes may follow vasectomy; however, mechanisms contributing to testicular/epididymal damage are far from clear. While Abdelmassih et al (2002) reported that pregnancy and implantation rates after intracytoplasmic sperm injection (ICSI) with sperm from vasectomized men are negatively correlated with the time interval from vasectomy, Borges-Junior et al (2003) demonstrated that the interval between the vasectomy and the sperm-retrieval procedure has no effect on the outcome of ICSI until the interval of 14 years.

In infertile men, an abnormal persistence of histones in spermatozoa resulting in sperm nuclear instability has been reported. Aniline-blue staining for the assessment of excessive histones has been suggested as a marker to improve the assessment of fertility (Auger et al, 1990; Foresta et al, 1992; Hammadeh et al, 2001). Altered chromatin condensation is a ubiquitous defect in spermatids of nonobstructed azoospermic men submitted to testicular sperm extraction (TESE) followed by ICSI (Francavilla et al, 2001). Furthermore, poor chromatin packaging may contribute to failure of sperm decondensation after ICSI and result in failure of fertilization (Sakkas et al, 1996). Therefore, changes in the structure of sperm chromatin might be particularly worth considering in the context of in vitro fertilization (IVF) and ICSI.

The protamine-1 to protamine-2 ratio in spermatozoa has been reported to play an essential role for male fertility and, in addition, for the fertilizing capacity of sper-

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matozoa in TESE-ICSI treatments (Balhorn et al, 1988; Steger et al, 2001, 2003). During spermiogenesis, DNA-binding histones are removed from the chromatin and are replaced by protamines (reviewed in Steger, 1999, 2001). Protamine-DNA interactions result in chromatin condensation representing a prerequisite for the production of fertile spermatozoa (reviewed in Steger, 2003).

The present study has a direct bearing on this issue, investigating, for the first time, the effect of vasectomy on the integrity of nuclear chromatin, namely the expression of protamines, in testicular spermatids and epididymal spermatozoa applying an animal model.

Materials and Methods

Animal Experiment

Animal experimentation was approved by the institutional animal use and care committee, Giessen, Germany. Eighteen New Zealand white male rabbits, 6 months old, were divided into 2 groups comprising 9 animals each. Group 1 served as the control. Animals of group 2 were unilaterally (right side) vasectomized and sacrificed 6 months after vasectomy. In the course of the experiment, 2 animals of group 2 died because of an otitis.

Tissue Samples

From each rabbit, testes and epididymides were divided into 2 parts. While 1 part was homogenized, the other part was fixed by perfusion in Bouin fixative and embedded in paraffin using standard techniques. For histological evaluation, 5-μm paraffin sections were stained with hematoxylin-eosin. The stages of the seminiferous epithelial cycle were evaluated on at least 100 cross-sectioned seminiferous tubules per animal.

In Situ Hybridization

In situ hybridization with digoxigenin-labelled cRNA-probes for protamine-1 and protamine-2 was performed as already reported (Steger et al, 1998, 2000). Briefly, 5-μm paraffin sections were partially digested with proteinase K. After prehybridization in 20% glycerol, sections were covered with the DIG-labeled sense or antisense cRNA-probes. Both cRNAs were used at a dilution of 1:100 in hybridization buffer containing 50% deionized formamide, 10% dextran sulfate, 2× SSC, 1× Denhardt solution, 10 μg/mL salmon sperm DNA, and 10 μg/mL yeast t-RNA. Hybridization was performed overnight at 37°C in a humidified chamber containing 50% formamide in 2× SSC. Following posthybridization washes, sections were blocked with 3% bovine serum albumin and then incubated with an anti-DIG Fab-antibody conjugated to alkaline phosphatase overnight at 4°C. Staining was visualized by developing sections with nitroblue-tetrazolium/5-bromo-4-chloro-3-indolylphosphate in a humidified chamber protected from light. For each test, negative controls were performed using DIG-labeled cRNA sense-probes.

Aniline-Blue Staining and Classification of Spermatozoa

Chromatin condensation was examined on smears fixed in 3% glutaraldehyde in 0.2 M phosphate buffer for 25 minutes and stained with 5% aniline blue at pH 3.5 for 8 minutes (Terquem and Dadoune, 1983).

Spermatozoa were classified, according to the intensity of the aniline-blue staining of their heads: unstained (class A), weakly stained (class B), and strongly stained (class C). For class A spermatozoa, the histone-to-protamine exchange was considered complete. The exchange was considered incomplete in class B spermatozoa and not yet begun in class C spermatozoa (modified from Henkel et al, 1994). Data are given as fractions in percent.

Statistical Analysis

Statistical analysis was carried out using the statistical program package BMDP (Dixon, 1993). As data were not normally distributed, an arcsine transformation was performed prior to the analysis to get stabilized variances and nearly normal distributions. According to the design of the experiment, for each class of spermatozoa, a 3-way analysis of variance (ANOVA) with repeated measures in the factors localization (testis; caput, corpus, and cauda epididymis) and side (right and left) was performed. Because there were some statistically significant interactions between the tested effects, groups 1 and 2 were compared separately for the right and left side by two-way ANOVA with repeated measures in the factor localization for further separation of the effects. In a further step, only for group 2, the 2 sides (control vs vasectomized) were compared by a 2-way ANOVA with repeated measures with respect to side and localization. All ANOVAs were done with the program BMDP2V.

Results

The percentage of class A spermatozoa representing high nuclear chromatin condensation due to complete histone-to-protamine exchange was estimated in testes, as well as in the caput, corpus, and cauda of epididymides and exhibited a statistically significant global increase (testis vs cauda epididymis, $P < .0001$), shown by 3-way ANOVA. No significant differences could be found by interindividual comparison (right testes from control group and right testes from vasectomized group) and intraindividual comparison (left and right testes from control group). For untreated testes/epididymides from control group and vasectomized group (interindividual comparison), a statistically significant interaction ($P = .003$) between localization and group could be observed. For left/right testes/epididymes from the vasectomized group (intra-individual comparison), no significant interaction ($P = .06$) between localization and side could be demonstrated. Both effects were caused by the low chromatin condensation in the untreated testis of the vasectomized group and the high chromatin condensation in the caput epididymis of the same origin. Results are summarized in Figure 1.

In the control group, all testes (n = 18) revealed normal spermatogenesis and exhibited a stage-dependent expression of protamine-1 and protamine-2 mRNA from step 5 round spermatids (stage I) to step 11 elongated spermatids

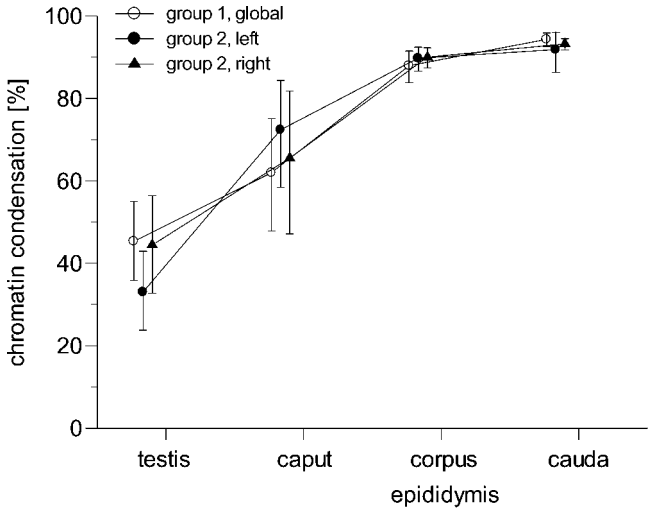

Figure 1. The percentage of class A spermatozoa representing high nuclear chromatin condensation due to complete histone-to-protamine exchange during their passage from testis to cauda epididymis, with global average over left and right side for the control group. Retransformed mean and standard deviation of arcsine-transformed data are given.

(stage VII). In all cases, signals were stronger for protamine-1 than for protamine-2. No in situ hybridization signals could be observed in stage VIII (Figures 2A and B and 3).

In the vasectomized group, untreated testes (n = 7) revealed normal spermatogenesis and exhibited a stage-dependent expression of protamine-1 and protamine-2 mRNA from step 5 round spermatids (stage I) to step 11 elongated spermatids (stage VII). This was identical to our findings in the control group. In contrast, treated testes of the vasectomized group displayed normal spermatogenesis (n = 3), round spermatid maturation arrest (n = 1), and spermatogenic arrest at the level of spermatocytes (n = 2) and spermatogonia (n = 1). In the 3 testes with normal spermatogenesis, 1 showed normal expression of protamines, while 2 revealed a delayed expression of both protamine-1 and protamine-2. Here, 83.7% (protamine-1 in animal 4), 30.1% (protamine-1 in animal 5), 33.3% (protamine-2 in animal 4), and 12.5% (protamine-2 in animal 5) of the seminiferous tubules showing stage I of the seminiferous epithelial cycle contained negative step 5 spermatids. Protamine expression started, with temporal delay, in step 6 spermatids of stage II (Figures 2C and D) and remained present to step 11 elongated spermatids (stage VII). Furthermore, multinucleated round spermatids represented a general phenomenon in testes with both normal spermatogenesis and round spermatid maturation arrest. These cells exhibited weak in situ hybridization signals for protamine-1 and protamine-2 (Figure 2E). Weak signs of inflammation could be observed in the intertubular tissue of 1 testis (not shown).

Discussion

Unilateral vasectomy has been demonstrated to have no effect on the contralateral testis/epididymis even 6 months after vasectomy. Histological evaluation of the treated testes revealed 3 testes with normal spermatogenesis, while 4 testes displayed spermatogenic impairment, including appearance of intraepithelial vacuoles and multinuclear spermatids. Spermatogenic impairment is not caused by increased intratubular pressure, as direct measurements by micropuncture of epididymal and seminiferous tubule hydrostatic pressure demonstrated that increased pressure in the distal epididymis after vasectomy is not generally transmitted to the seminiferous tubules (Flickinger et al, 1985, 1995). Although infiltration by lymphocytes was a regular finding in vasectomized rabbits (Bigazzi et al, 1976; Alexander and Tung, 1977; Tumboh-Oeri and Roberts, 1979; Hooker 1980), rats (Herr et al, 1987; Flickinger et al, 1988; Handley et al, 1991), mice (Nashan et al, 1990), and men (Jarow et al, 1994), in the present study, it was observed solely in 1 out of 7 testes, suggesting also no immunological reason for spermatogenic impairment.

Interestingly, the time period after vasectomy seems to play a critical role for testicular histology. Human and canine spermatogenesis has been reported to remain unchanged 2 to 3 weeks after vasectomy, while 3 to 6 weeks after vasectomy, progressive spermatogenic arrest at the level of spermatocytes were observed (Derrick et al, 1974; Urry et al, 1976). In rabbit, testicular alterations were found 6 months after vasectomy (Flickinger, 1975b). In mice, vasectomy caused a statistically significant decrease in the number of preleptotene spermatocytes, pachytene spermatocytes, and step 7 spermatids 5 weeks after surgery (Croft and Bartke, 1976).

While vasectomy has been reported to have no significant effects on quantitative data obtained from the testis/epididymis of rabbits (Lohiya et al, 1983) and monkeys (*Macaca fascicularis* and *Macaca mulatta*) (Hadley and Dym, 1983; Peng et al, 2002), morphometric analyses on testicular biopsy specimens from men undergoing vasectomy reversal revealed a 100% increase in the thickness of the seminiferous tubular walls, a 50% increase in the mean cross-sectional tubular area, and a significant reduction in the mean number of Sertoli cells and spermatids per tubular cross-section.

Summarized, alterations in testicular/epididymal histology due to vasectomy seem to depend on the animal species, the peculiarities of techniques, and the time passed after surgery. In general, both number and extent of vasectomy-induced alterations within the testis/epididymis are greater in long-term vasectomized than in short-term vasectomized individuals (Sarrat et al, 1996).

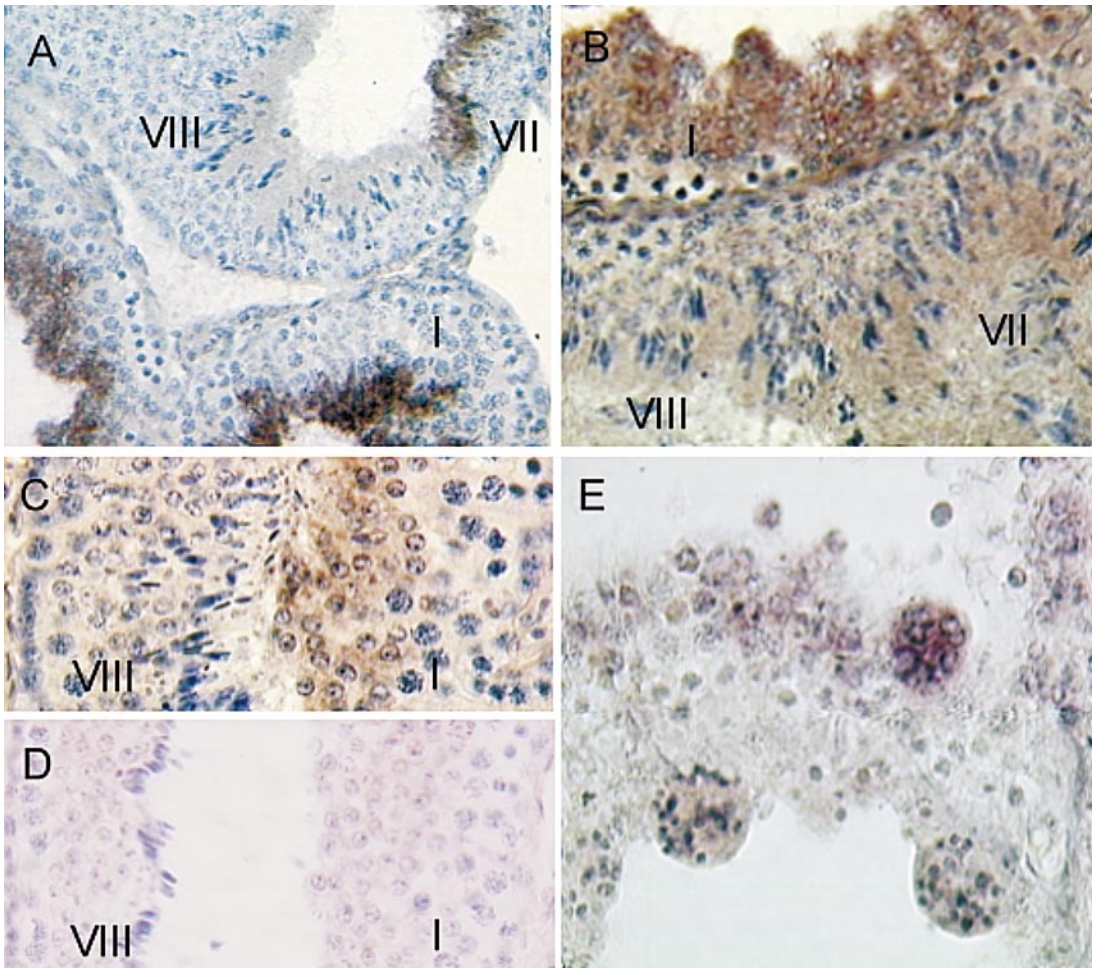

Figure 2. In situ hybridization for protamine-1 **(A)** and protamine-2 **(B)** during normal spermatogenesis of the rabbit (control group), showing a positive signal from step 5 round spermatids (stage I) to step 11 elongated spermatids (stage VII), while spermatids from stage VIII are completely negative. **C** and **D** show seminiferous tubules of stages VIII and I. Step 5 round spermatids of stage I exhibit positive signals for protamine-1 in animals of the control group **(C)**, but are completely negative in animals of the vasectomized group **(D)**. Two in 3 rabbits exhibits delayed expression of protamine-mRNA. **E** shows multinucleated round spermatids, a common finding in the treated testis of the vasectomized group. Primary magnification, 40×.

Stage-specific histone-to-protamine exchange is known to play a vital role for the differentiation of spermatids and the production of fertile spermatozoa. In the present study, we investigated whether vasectomy affects, in addition, protamine gene expression causing a spermatogenic arrest at the level of round spermatids. During normal spermatogenesis, transcripts of protamines occurred in step 5 to step 11 spermatids. In the vasectomized animals, 2 in 3 testes with normal spermatogenesis revealed delayed expression of protamine transcripts. A similar observation was reported in cryptorchid testes of infertile stallions (Steger et al, 2000; Bergmann et al, 2001). Delayed protamine gene expression may be due to decreased testicular cAMP concentrations (Tsang et al, 1979; Wang et al, 1994), as an aberrant cyclic adenosine monophosphate (cAMP) signal transduction pathway may result in delayed binding of cAMP-responsive element modulator (CREM) to cAMP-responsive element (CRE) within the promoter of the protamine genes. Round spermatid maturation arrest in 1 animal may be due to a complete lack of protamine transcription.

However, the DNA-packaging process in spermatozoa is known to be not completed during spermatid differentiation in the testis. Further chromatin condensation occurred during the passage of spermatozoa through the epididymal lumen (Evenson et al, 1989; Auger and Dadoune, 1993; Haidl et al, 1994; Yossefi et al, 1994; Hingst et al, 1995; Golan et al, 1996) and is due to the formation of disulphide bonds between protamines (Soawaros and Panyim, 1979; Yossefi et al, 1994). The global increase of chromatin condensation from testis to cauda epididymis has also been demonstrated in the present study applying aniline-blue staining. Interestingly, no significant differences in chromatin condensation could be observed in the cauda epididymis of animals exhibiting normal spermatogenesis. On condition that there is sufficient

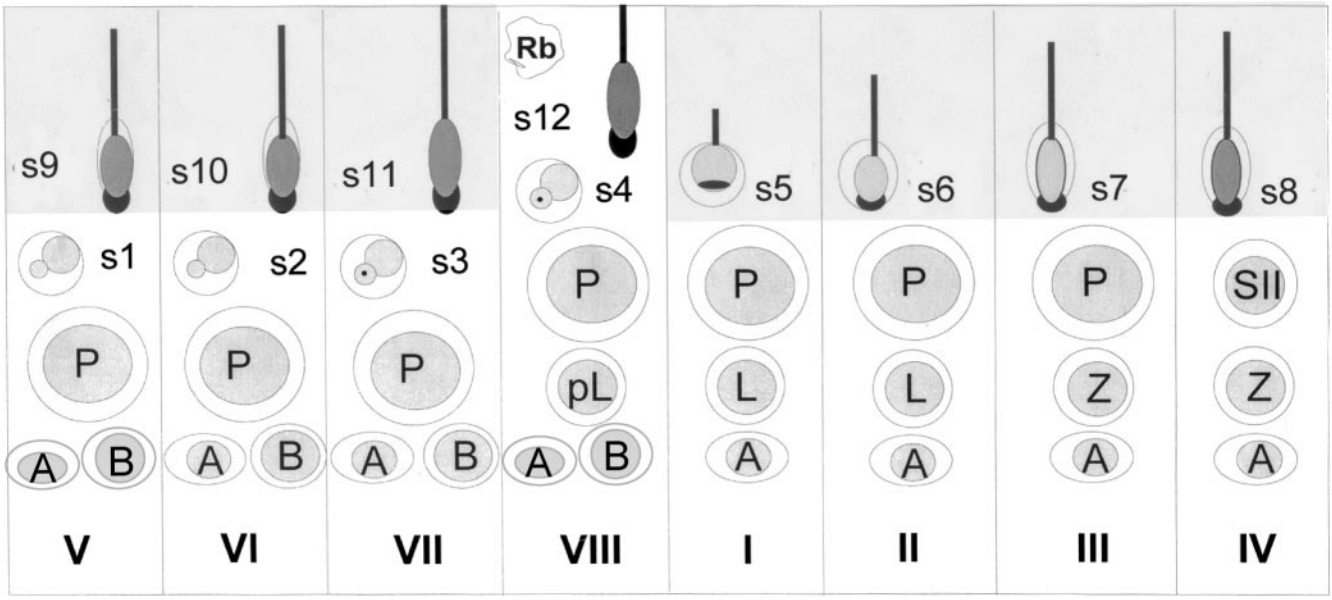

Figure 3. Stages of the seminiferous epithelial cycle of the rabbit, modified from Swierstra and Foote (1963). Transcripts for protamine-1 and protamine-2 are present in the cytoplasm from step 5 round spermatids (stage I) to step 11 elongated spermatids (stage VII). No in situ hybridization signals can be observed in stage VIII of the cycle.

protamine gene expression, delayed chromatin condensation due to delayed protamine gene expression seems to be compensated in the course of the passage through the epididymis.

In conclusion, our data demonstrate that vasectomy may be followed by spermatogenic impairment. However, unilateral vasectomy has been shown to reveal no negative effects on the contralateral testis. Although a delayed expression of protamines has been demonstrated in some testes 6 months after vasectomy, spermatozoa within the epididymides showed no significant differences in chromatin condensation. As the integrity of nuclear chromatin plays a vital role for oocyte fertilization, especially in ICSI, where most of the natural selection mechanisms are bypassed, our data add valuable information for the treatment of infertility by ICSI, showing that vasectomy may affect nuclear chromatin integrity of testicular spermatids but not epididymal spermatozoa. As a consequence, in vasectomized patients, microsurgical epididymal sperm aspiration (MESA) may be superior to TESE.

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