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ORIGINAL ARTICLE

Male Reproductive System

Morphometric study of the testis and reproductive tract (including sperm granuloma) after vasectomy in mature rats

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By utilizing the rabbit model, previous studies have found good evidence indicating that vasectomy-induced spermatogenic damage is pressure-mediated: the damage occurs when the occluded reproductive tract is unable to accommodate additional spermatozoa produced by the testis. More studies with the more commonly used rat model have shown, however, controversial results on whether and why the damage occurs. In this study, 12 mature male Sprague-Dawley rats were subjected to unilateral vasectomy: double ligation (without severing) of the vas deferens exposed via a small inguinal incision; 37 days after the operation, the testes, epididymides, vasa deferentia (juxta-epididymal segments), and sperm granulomas (at the vasectomy site) were removed to obtain methacrylate resin-embedded sections and morphometric studies carried out with light microscopy. Marked spermatogenic damage with spermatids and spermatocytes depleted in the seminiferous epithelium in 43% of the seminiferous tubule profiles was demonstrated in 5 of the 12 testes on the vasectomized side, and the damage was associated with smaller or absent sperm granulomas; in the other 7 testes with essentially normal spermatogenesis, there was an increase (by 111% on average) in the volume of the tubule lumen, associated with larger granulomas or granulomas containing more spermatozoa. There was an overall increase (by 66%) in the thickness of the rete testis in the 12 testes; the epididymis or vas deferens showed no distension. It seems therefore that the spermatogenic damage induced by vasectomy in rats is pressure-mediated as well, and that variation in the damage depends mainly on the postoperative development of the sperm granuloma.

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INTRODUCTION

In the field of male reproduction, perhaps no studies are as controversial or results so variable as those on the spermatogenic damage of vasectomy, with many studies showing the postvasectomy damage while others not.^{1,2} In the rat – the most commonly used experimental animal, for example, vasectomy may be associated with normal spermatogenesis^{3–7} or testicular size^{8–14} 1–6 months after the operation, or with impaired spermatogenesis^{13,15–24} or reduced testicular size^{25–28} 1–18 months postoperation.

Our recent study on the rabbit demonstrated that damage to spermatogenesis did not occur when the reproductive tract (epididymis and vas deferens) was substantially distended and filled with spermatozoa continually produced by the testis 6 months after distal vasectomy, but when the juxta-epididymal segment of the occluded vas deferens was too short or small for storage of spermatozoa with proximal vasectomy, damage ensued.^{29,30} This is good evidence that spermatogenic damage is pressure-mediated, occurring when the reproductive tract is unable to accommodate additional spermatozoa and testicular fluid produced by the testis,³⁰ that is, when the intra-testicular pressure has probably increased owing to failure of sperm transport out of testis.

Unlike the rabbit, the rat often develops sperm granuloma at the vasectomy site owing to escape of spermatozoa, and this will relieve the intra-tract pressure.^{13,31} Many researchers have ignored these “appendages” or their likely effect on the other reproductive organs. Specifically, researchers have rarely performed quantitative studies of the testis together with that of the reproductive tract including sperm granuloma. To clarify whether the pressure-mediated hypothesis could also explain what happens in the rat after vasectomy, this study was undertaken to obtain and analyze morphometric changes (histological changes in quantitative terms) of the testis as well as the reproductive tract (including sperm granuloma) after vasectomy, which was designed to be ligation rather than severing of the vas deferens to minimize granuloma formation and thus increase any pressure-mediated changes.

MATERIALS AND METHODS

Rats and vasectomy

Rats were obtained from the Animal Center of North Sichuan Medical College. Experiment protocols were approved by the research section of the college, and ethical guidelines constituted by the college were followed during experiment.

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Twelve normal mature male Sprague-Dawley rats (aged 10–11 weeks) were subjected to unilateral vasectomy; 37 days after the operation, bilateral reproductive organs (including sperm granulomas around the vas deferens at the vasectomy site) were removed for preparation of tissue blocks and sections. The body weights of the animals were $209 \text{ g} \pm 12 \text{ g}$ and $302 \text{ g} \pm 11 \text{ g}$ (mean \pm standard error of the mean) at the beginning and end of the experiment, respectively.

After anesthesia by intraperitoneal injection of pentobarbital sodium (50 mg kg^{-1} bodyweight), as previously described,³² the vas deferens on one side (randomly chosen) was exposed via a longitudinal incision (approximately 8 mm in length) in the inguinal region (disinfected with povidone-iodine), and then doubly ligated, not severed, with the ligatures being approximately 2 mm apart. Care was taken to ensure that only a short segment of the vas was exposed, and the exposure was via a small incision away from the scrotal sac or epididymis. The operative procedure was thus designed to (i) prevent the formation of the sperm granuloma due to vascular transection (severing) and (ii) minimize postoperative adhesion and inflammation around the testis that might affect spermatogenesis.^{29,30}

Blocks and sections

Upon removal, organs were immersion fixed in Bouin's fluid for 48 h and then stored in 70% ethanol at 4°C. Approximately 1 month (for testis and epididymis) or 6 months (for other organs) later, the weight and density of each organ were measured to estimate its volume.^{2,33} (The densities of sperm granulomas, not measured in this study, were assumed to be 0.93 g cm^{-3}).³³ Tissue blocks were then obtained and embedded in glycol methacrylate (i.e., 2-hydroxyethyl methacrylate, Histo-resin by Leica Microsystems Nussloch GmbH, Germany). One section (for study of the testis, epididymis, vas deferens or sperm granuloma) was or two sections (for study of the rete testis) were cut from each block at $20 \mu\text{m}$ with a microtome (RM2235, Leica Biosystems) and then stained with periodic acid-Schiff's reagent and hematoxylin (for sections of the testis and rete testis) or hematoxylin alone (for other sections).

For the study of testicular structures, each testis was first sliced into 6 pieces, perpendicular to the long axis and with approximately equal thickness. Two pieces were then sampled in a systematic manner, that is, the 1st and 4th, 2nd and 5th, or the 3rd and 6th pieces were chosen in turn; a half (block) of each piece was further cut for embedding. For study of the rete testis, all the other testicular pieces and blocks left were embedded as well, with care taken to ensure that complete section around the testicular capsule, perpendicular to the long axis, would be obtained from each of the six pieces.

For the study of the epididymis, two, one and two blocks (transsectional surfaces perpendicular to the long axis) were obtained in a systematic manner from the epididymal head, body, and tail, respectively.

For study of the vas deferens, the length of the juxta-epididymal occluded segment of the vas deferens on the vasectomized side was first measured between the epididymal end and the point approximately 2 mm away from the juxta-epididymal ligature along the vas deferens. Then two vasal blocks (transsectional surfaces perpendicular to the long axis) were obtained in a systematic manner from this segment. The same length of the juxta-epididymal vas deferens was obtained, and two blocks were sampled from the vas on the contralateral (nonvasectomized) side.

Similarly, for study of the sperm granuloma, two blocks (transsectional surfaces perpendicular to the long axis) were obtained and embedded from each newly formed tissue lump or mass around

the vas deferens. For the two animals in which sperm granulomas were not seen or obtained around the vas deferens (see "Sperm granuloma" in the Results), the segment (block with ligatures) of the vas deferens at the vasectomy site was additionally obtained and embedded, and 60–70 serial sections were then cut and stained to see if smaller sperm granulomas had developed.

Histology and morphometry

Sections were observed in a light microscope for qualitative changes of histology and measured by morphometric (stereological) methods for quantitative changes of key histological structures.

For morphometry of testicular structures, sections were observed on a computer screen (final magnification $\times 224$) attached to a stereology image system (Visiopharm, Hørsholm, Denmark) including an Olympus BX51 microscope (Tokyo, Japan). Fields of view on each section were sampled in a systematic manner, with distances between fields being set at 2.0 and 1.6 mm along the X and Y axes on the screen, respectively. Nine (or 20 for separate measurement of the smaller structure – tubule lumen) test points (uniformly distributed) and a rectangular frame ($500 \mu\text{m} \times 379 \mu\text{m}$) generated by a software were superimposed on the screen. Points hitting different structures were counted on each field to estimate the volume fractions (proportions) of the structures in the testis, and their total volumes were further estimated in combination with the volume of the testis.^{2,33} The frame was used, according to the forbidden line rule, to sample round or elliptical tubule profiles with a clear lumen, and then the short axis (i.e., the Feret diameter along the short axis) of the tubule profile was measured as a diameter of the seminiferous tubule.^{2,33} Assuming the seminiferous tubule and the tubule lumen to be two concentric cylinders, the total length of the tubule per testis and the mean thickness of the seminiferous epithelium were calculated according to the total volumes of the relevant structures and the mean diameter of the tubule.^{2,33}

Morphometry of epididymal structures was performed in the same way as for the testicular structures, except that 16 test points without a frame were superimposed on each field.

For morphometry of the vas deferens, the sperm mass (the whole profile) in the vas deferens was first observed in one field (magnification on screen $\times 224$) to estimate its area by counting test points (distances between points along the X and Y axes being 112 and $84 \mu\text{m}$, respectively). Then the vas deferens (the whole profile) was observed (in one field) at a lower magnification ($\times 89.6$) to estimate its area with another point counting (distances between points along the X and Y axes being 274 and $203 \mu\text{m}$, respectively). The ratio of the two areas was taken as an estimate of the volume fraction of the sperm mass in the vas deferens, which was used in combination with the volume of vas deferens to estimate the total volume of sperm mass in the vasal segment.^{2,33}

For morphometry of the rete testis, all sections from all testicular pieces and blocks were carefully observed beneath the capsule to find the rete, which was seen in 1–3 (2.2 ± 0.1) blocks (pieces) per testis. One of the two sections with the rete from each block, either the one clearer in microscopic quality or one randomly chosen, was used for further analysis. A complete micrograph (magnification on screen $\times 448$) of the rete was first taken using the image system and then printed in color (final magnifications of the rete $\times 59$ –123). Test points (distance between points along the X or Y axis 2.5 mm) and lines (distance between lines 5 mm) printed on a transparent binding film were superimposed onto the rete to estimate (i) the area of the rete lumen with point counting³³ and (ii) the thickness of

the rete lumen by measuring the widths of the lumen starting from the intersections between the test lines and the luminal boundary, assuming the mean width obtained on the sections approximately equaled the mean thickness.³⁴ The total volume of the rete lumen was estimated by multiplying the mean area (per testicular piece) of the rete by the length (along the long axis) of the testis according to the Zu's (i.e., Cavalieri's) principle.³³

For morphometry of the sperm granuloma, its complete picture (magnification on screen $\times 89.6$) was taken and printed (final magnifications $\times 19-48$). Point counting (distance between points along the X or Y axis 10 mm) was then performed on the picture, as described for morphometry of testicular structures, to estimate the area of the granulomatous epithelioid layer on section and the volumes of the granulomatous cavity and the epithelioid layer. And the section was observed through an oil lens ($\times 100$, UPlanSApo, NA 1.40) and the number of all profiles of capillary blood vessels in the epithelioid layer was directly counted on all fields (whole profile) of the layer. The ratio of the number of capillary profiles to the area of epithelioid layer was then calculated to obtain an objective impression of the capillary length density or vascular supply in the epithelioid layer.³³

Statistical analyses

To detect the overall effect of vasectomy, the vasectomized side was compared with the contralateral side in all of the 12 unilaterally vasectomized rats with the paired *t*-test (Table 1). To determine whether the effect occurred in some animals with a certain spermatogenic status, the paired *t*-test was separately performed in either of the two subgroups of animals: one ($n = 7$) with apparently

normal spermatogenesis and one ($n = 5$) with marked spermatogenic damage on the vasectomized side (Table 1). Statistical significance was set at $P \leq 0.05$.

RESULTS

Animals did not become ill during experiment and inflammation at the incision site was not seen after the operation. Adhesion around the testis or epididymis was not seen at removal of the organs; there was some adhesion around the vas deferens at the vasectomy site but the juxta-epididymal vas deferens or the sperm granuloma was removed without difficulty. The length of the whole vas deferens on the vasectomy side was $3.5 \text{ cm} \pm 0.1 \text{ cm}$ and that of the juxta-epididymal vas deferens $1.9 \text{ cm} \pm 0.1 \text{ cm}$.

Testis

Thirty-seven days after the unilateral vasectomy, spermatogenesis on the nonvasectomized side in all 12 animals was normal (Figure 1a and Table 1). Spermatogenesis on the vasectomized side appeared normal as well, like that on the nonvasectomized side, in seven (designated as subgroup-normal in this paper) of the 12 animals. In contrast, marked spermatogenic damage on the vasectomized side occurred in the other five animals (designated as subgroup-damaged): (i) spermatids and spermatocytes were depleted in the seminiferous epithelium (i.e., with almost no spermatids or spermatocytes seen in the epithelium) and a few or many immature spermatogenic cells sloughed into the tubule lumen in 43% and 32% (average) of the seminiferous tubule profiles, respectively, and (ii) the total volume of seminiferous tubules per testis decreased

Table 1: Morphometric results (mean \pm s.e.m. obtained from the testis, rete testis, epididymis, and vas deferens)

	Rats without spermatogenic damage ($n=7$) ^a		Rats with spermatogenic damage ($n=5$) ^a	
	Nonvasectomized side	Vasectomized side	Nonvasectomized side	Vasectomized side
The testis				
Volume of testis (cm ³) [#]	1.52 \pm 0.06	1.51 \pm 0.06	1.71 \pm 0.09	0.81 \pm 0.12*
Total volume of the seminiferous tubules in the testis (cm ³) [#]	1.13 \pm 0.05	1.11 \pm 0.05	1.26 \pm 0.08	0.46 \pm 0.10*
Total volume of the interstitial tissue in the testis (cm ³)	0.39 \pm 0.03	0.39 \pm 0.02	0.45 \pm 0.02	0.35 \pm 0.02*
Total volume of the tubule lumen in the testis (mm ³)	22.1 \pm 3.3	40.7 \pm 5.4*	46.2 \pm 9.5	17.6 \pm 1.6*
Total length of the seminiferous tubules in the testis (m)	18.2 \pm 0.8	18.5 \pm 1.0	18.3 \pm 1.5	13.2 \pm 1.3*
Mean diameter of the seminiferous tubules (μm) [#]	279 \pm 5	276 \pm 5	295 \pm 7	203 \pm 10*
Mean thickness of the seminiferous epithelium (μm) [#]	120 \pm 2	112 \pm 3*	120 \pm 4	80 \pm 7*
Tubule profiles ^b without spermatids and spermatocytes (%)	0	0	0	43.3 \pm 10.0
Tubule profiles ^b with immature germ cells sloughed (%)	0	0	0	32.1 \pm 5.3
The rete testis				
Total volume of the lumen of the rete testis (mm ³)	0.590 \pm 0.230	0.719 \pm 0.156	0.881 \pm 0.347	0.837 \pm 0.089
Mean width of the rete lumen (μm) [#]	59.8 \pm 13.0	78.8 \pm 12.9	77.3 \pm 14.0	109.4 \pm 9.4
The epididymis				
Volume of epididymis (mm ³)	442 \pm 26	438 \pm 40	461 \pm 30	429 \pm 67
Volume of the epididymal duct ^c in the epididymis (mm ³) [#]	296 \pm 19	273 \pm 31	304 \pm 22	212 \pm 34*
Volume of the interstitial tissue in the epididymis (mm ³)	146 \pm 11	165 \pm 14	157 \pm 12	217 \pm 35
Volume of sperm mass ^d in the epididymal duct (mm ³) [#]	147 \pm 15	134 \pm 18	172 \pm 15	16 \pm 9*
Volume of cellular mass ^e in the epididymal duct (mm ³)	0	0	0	36 \pm 12
The vas deferens				
Length (mm) of the juxta-epididymal vasal segment ^f	19.3 \pm 1.4	19.3 \pm 1.4	19.2 \pm 1.0	19.2 \pm 1.0
Volume (mm ³) of the vas deferens	29.7 \pm 2.6	31.7 \pm 2.6	32.2 \pm 1.7	27.2 \pm 0.8*
Total volume (mm ³) of sperm mass in the vasal cavity	2.46 \pm 0.63	2.54 \pm 0.82	1.61 \pm 0.51	2.07 \pm 1.10

* $P \leq 0.05$ compared with the nonvasectomized sides of the same rats without or with spermatogenic damage (paired *t*-test); [#] $P \leq 0.05$ for comparison between the nonvasectomized and vasectomized sides of all (12) rats (paired *t*-test). ^aMarked damage to spermatogenesis on the vasectomized side occurred in 5 of the 12 adult Sprague-Dawley rats at 37 days after unilateral vasectomy; ^bPercentage of seminiferous tubule profiles with spermatids and spermatocytes depleted in the seminiferous epithelium or with immature spermatogenic cells sloughed into the tubule lumen; ^cThe duct included efferent ductules in this study; ^dSperm agglomerate mainly composed of densely packed spermatozoa; ^eCellular agglomerate (seen and measured in one sub-group only) mainly composed of immature round spermatids (among some spermatozoa); ^fThe segment of the vas deferens between the epididymis and the vasal ligature on the vasectomized side and the same length of the juxta-epididymal vasal segment on the nonvasectomized side were obtained from each rat. s.e.m.: standard error of the mean

to 24%–59% ($36\% \pm 6\%$ as calculated from individual differences between the vasectomized and contralateral sides) of the control (the contralateral testis) (Table 1 and Figure 1).

Overall, in all 12 animals, vasectomy induced a significant reduction (around 25% on average) in the (total) volume of the testis or seminiferous tubules, and a reduction (around 15% on average) in the mean diameter of the seminiferous tubule or the mean thickness of the seminiferous epithelium. The reductions were primarily contributed by the subgroup-damaged – with significant reductions in the subgroup-damaged not the subgroup-normal – in all four parameters except the mean thickness of the seminiferous epithelium (Table 1). The thickness decreased significantly in both subgroups, with decreases of $33\% \pm 6\%$ and $7\% \pm 2\%$ in the subgroup-damaged and the subgroup-normal, respectively (Table 1).

In the subgroup-damaged, the vasectomy-induced spermatogenic damage was associated with shortening of the seminiferous tubule and atrophy of the inter-tubular interstitial tissue (Table 1). Interestingly, the tubule lumen shrank (decreased by $53\% \pm 12\%$) with spermatogenic damage (in the subgroup-damaged) while it was enlarged (increased by $111\% \pm 41\%$) when damage to spermatogenesis did not occur (in the subgroup-normal).

Rete testis

In all 12 animals, the mean width of the rete lumen increased by $66.1\% \pm 29.4\%$ after vasectomy, although its total volume remained unchanged (Table 1 and Figure 2). In both the subgroup-normal and subgroup-damaged, the rete lumen appeared to be wider (by 64%–68%) on the vasectomized side than on the contralateral side, but statistical significance was not detected ($P > 0.16$) because of the large variation in the measurements.

Epididymis

After vasectomy, there was an overall reduction in the volumes of the epididymal duct and the sperm mass stored in the duct, and this reduction was primarily the result of the volume reduction of the sperm mass in the subgroup-damaged. In these five animals, the volume of sperm mass decreased by $90\% \pm 6\%$ (85%–99%) after vasectomy; more cellular mass, composed of mostly immature spermatogenic cells (especially round spermatids) with some scattered mature spermatozoa, was stored in the epididymal duct (Table 1 and Figure 3). Few neutrophils (leukocytes) were seen in the epididymal duct or interstitium in any epididymis.

Vas deferens

Overall, there was no postvasectomy change in the size of the juxta-epididymal vas deferens. In the subgroup-damaged, the basal volume decreased slightly by $15\% \pm 5\%$, but the volume of sperm mass stored in the vas remained unchanged (Table 1). The sperm mass observed in all vasa deferentia consisted of densely packed spermatozoa, with the exception of one vasectomized vas deferens (Rat 9 in the subgroup-damaged in Table 2): in the proximal (closer to the epididymis) section from the juxta-epididymal vas deferens, many (perhaps around 50%) round spermatids were seen among spermatozoa; in the other distal (closer to the basal ligature) section, some (perhaps around 5%) round spermatids were seen among spermatozoa.

Sperm granuloma

Newly formed tissue lumps or masses were not seen around the epididymis or testis. One (and only one) tissue lump was observed around the vas deferens at the vasectomy site (Figure 4), identified as a sperm granuloma from light microscopy in ten of

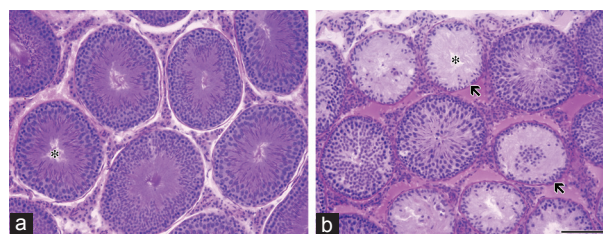


Figure 1: Typical testicular micrographs taken from a testis on the nonvasectomized side (a) and a testis on the vasectomized side (b, with marked spermatogenic damage). *, lumen of seminiferous tubule. ⚡, seminiferous tubules with seminiferous epithelium depleted of spermatids and spermatocytes; in one tubule, many immature spermatogenic cells sloughed into the tubule lumen. Scale bar, 150 μ m.

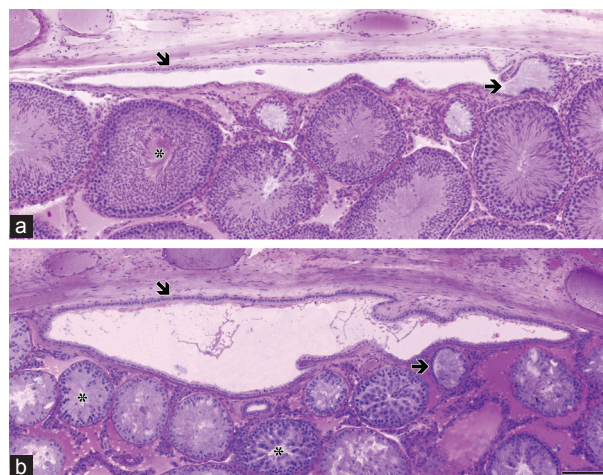


Figure 2: Typical micrographs of the rete testis taken from a testis on the non-vasectomized side (a) and a testis on the vasectomized side (b, with marked spermatogenic damage). ⚡, rete testis; →, straight tubule; *, lumen of seminiferous tubule. Scale bar, 150 μ m.

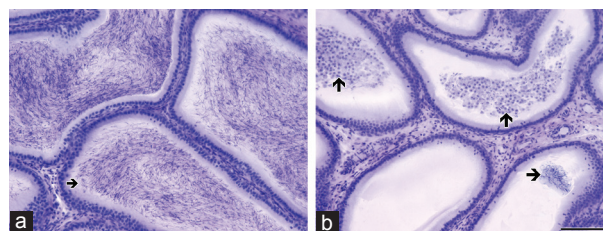


Figure 3: Typical epididymal micrographs taken from a nonvasectomized side (a) and a vasectomized side (b, with marked damage to spermatogenesis in the ipsilateral testis). →, sperm mass composed of densely packed spermatozoa; ↑, cellular mass composed of densely packed round spermatids with some scattered spermatozoa. Scale bar, 150 μ m.

the 12 unilaterally vasectomized rats (Table 2 and Figure 5). In the other two rats, (i) a small tissue lump (volume 31 mm³), probably a sperm granuloma, was seen around the vas deferens at the vasectomy site in one rat (Rat 11 in Table 2), but was lost during tissue processing, before embedding; (ii) microscopic observation of serial sections of the basal blocks with ligatures demonstrated: in one rat (Rat 11 in Table 2), five small sperm granulomas (diameters 130 μ m–400 μ m) were observed in the block, with no spermatozoa in the granulomatous cavity and no capillary blood vessel profiles in the epithelioid layer; in the other rat (Rat 12 in Table 2), one sperm granuloma (diameter 450 μ m) was seen in the block, with

Table 2: Morphological and morphometric characteristics of the sperm granuloma formed around the vas deferens at the vasectomy site

	Without spermatogenic damage ^a							With spermatogenic damage ^a				
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Rat 8	Rat 9	Rat 10	Rat 11 ^b	Rat 12 ^b
Volume of the sperm granuloma (mm ³)	327	237	209	151	175	124	113	130	47	23	0	0
Volume of the granulomatous cavity (mm ³)	226	122	137	91	119	69	63	51	24	5	-	-
Volume of the epithelioid layer (mm ³)	46	38	26	28	24	27	16	18	10	9	-	-
With numerous spermatozoa in the cavity	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No*	Yes	Yes	-	-
Number of nonsperm cells in the cavity ^c	Numerous	Many	Many	Numerous	Few	Few	Few	Numerous	Few	Few	-	-
Lightly stained zone in inner part of the epithelioid layer	Disjunctive	Disjunctive	Disjunctive	Disjunctive	Uniform	Uniform	Uniform	- [#]	Uniform	Uniform	-	-
Density of capillary vessels in the epithelioid layer ^d	6.9	19.9	8.0	17.3	1.1	0.0	1.8	3.1	4.8	6.6	-	-

^aFilled with nonsperm nuclei, with sparse spermatozoa; [#]The lightly stained zone, neither disjunctive nor uniform, was hardly formed in the inner part of the epithelioid layer. ^{*}Marked damage to spermatogenesis on the vasectomized side occurred in 5 (Rats 8-12) of the 12 adult Sprague-Dawley rats at 37 days after unilateral vasectomy; ^bIn Rats 11 and 12, microscopic granulomas were observed in the segment of vas deferens with ligatures; ^cNumerous (in Rats 1, 4 and 8) or some (in Rats 2-3) typical neutrophils were seen among the nonsperm cells; ^dNumber of capillary blood vessels (profiles on section) per area of the granulomatous epithelioid layer (mm⁻²)

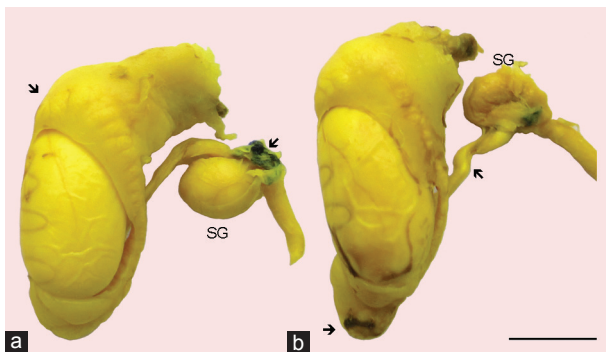


Figure 4: Typical appearance (after immersion fixation with Bouin's solution) of the sperm granuloma (SG) formed around the vas deferens at the vasectomy site (↘). (a) from Rat 3; (b) from Rat 7 (Table 2). ↘, epididymal head; →, epididymal tail; ↖, occluded juxta-epididymal vas deferens. Scale bar, 10 mm.

spermatozoa filling the granulomatous cavity and a few capillary blood vessel profiles seen in the epithelioid layer.

The size of the sperm granulomas was variable, but granulomas in seven animals with normal spermatogenesis (in the ipsilateral testis) were generally larger than those in the other five animals (including two animals in which only very small microscopic granulomas were observed in the segment of ligated vas deferens) with spermatogenic damage (Table 2). (χ^2 test [with Yates correction for continuity] or Fisher exact test for the Contingency Table data [7, 0, 0, 5] representing the numbers of animals with larger or smaller granulomas that were associated with normal or damaged spermatogenesis: $P < 0.005$). In other words, larger granulomas were more likely to be associated with normal spermatogenesis while smaller or no granulomas were more likely associated with spermatogenic damage. In addition, the larger they were, the more sperm (mass) they contained in the granulomatous cavity (Table 2).

Granulomas (around the vas deferens) were characterized by formation of the epithelioid layer (surrounding the spermatozoa), consisting of large activated macrophages with abundant cytoplasm.^{22,35,36} The inner cytoplasmic region appeared to be a lightly stained zone, which was either a uniform layer or folded into disjunctive areas (Figure 5 and Table 2). Specifically, we observed capillary blood vessels in the epithelioid layer, with 6–240 capillary profiles being

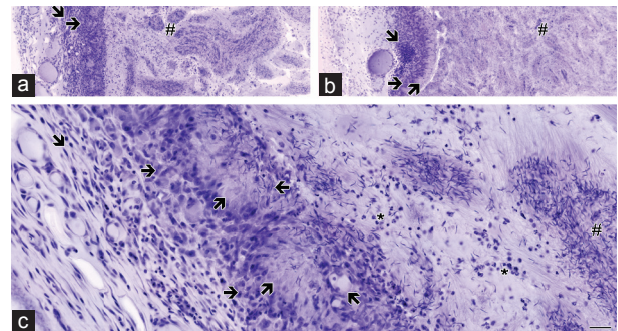


Figure 5: Micrographs of sperm granulomas from Rat 1 (a and c) and Rat 5 (b) as shown in Table 2. The granulomatous cavity is filled with sperm mass (#), densely packed spermatozoa; the granulomatous wall is composed of (i) the epithelioid layer (→), which has a lightly stained zone (↗, either disjunctive in a/c or uniform in b) on the luminal side interlaced with spermatozoa (↔), and (ii) the adventitia, the inner layer (↘) of which is rich in neutrophils and lymphocytes. *, numerous neutrophils in the cavity; ↖, capillary blood vessel in the epithelioid layer. Scale bars: 150 μ m (a and b) and 30 μ m (c).

counted per granuloma except the one from Rat 6 (Table 2). In addition, larger granulomas appeared more likely to have more inflammatory cells (typically neutrophils) in the cavity, a disjunctive lightly stained zone of the epithelioid layer, and more capillary blood vessels in the epithelioid layer (Figure 5 and Table 2).

DISCUSSION

Development of sperm granulomas after vasectomy is the result of sperm production (and leakage), since inhibition of sperm production before the operation, e.g. by testosterone treatment³⁷ or local heating,^{6,38} will suppress granulomatous development. In rats, sperm granulomas may be formed at the vasectomy site in some animals in 1 week after operation,^{3,11,16,35,39} and all animals by 1–3 months after operation.^{5,6,13,37,40} The granulomatous size may be (i) 2–3 cm in diameter by 12 months postoperation,^{26,41,42} (ii) close to the testicular size by 6 months postoperation⁴ or (iii) 2.0 g in weight by 18 months postoperation.²⁷ Moreover, in the longer term (e.g., 6 months and more after vasectomy), multiple granulomas along the vas deferens, and even in the epididymis, may be formed.^{4,13,27,43} It can therefore be assumed that the granulomas (weights 0.02–0.30 g) observed around the vas deferens in this rat study were at different developing stages, and the

larger they were, or the more spermatozoa they contained, the longer they had been formed. With larger development of the granulomas, as shown in this study, (i) they tended to have richer vascularity in the epithelioid layer and more leukocytes in the cavity, and (ii) the lightly stained area of the epithelioid layer tended to be more disjunctive or folded. The implication is that the larger they grow, the more active their immunity against spermatozoa becomes.

The current study demonstrated clearly that larger sperm granulomas, or granulomas containing more spermatozoa, were associated with essentially normal spermatogenesis while the absence of granulomas, or formation of smaller granulomas or granulomas containing fewer spermatozoa, was associated marked spermatogenic damage.

This result of association can be explained by a pressure-mediated mechanism:³⁰ with early formation of larger granulomas, the reproductive tract (including granulomas) is still spacious enough to accommodate the spermatozoa (and testicular fluid) continually produced by the testis, therefore, spermatogenesis remains normal. Smaller or no granulomas indicate that the reproductive tract is unable to accommodate the additional spermatozoa produced, which will result in higher intra-testicular pressure owing to fluid stasis or blockage of spermatozoa within the testis, bringing about spermatogenic damage (production of fewer spermatozoa) that relieves the pressure.

Such a correlation was previously noted by Kuwahara and Frick,¹⁶ who studied Wistar rats (unilaterally vasectomized with double ligation of the vas deferens) at 10 time-points and concluded: (i) spermatic cysts (sperm granulomas) were formed in most rats by the third week postvasectomy; (ii) “when the spermatic cyst was formed in the vas deferens, the size and the appearance of the testis on that side was almost normal on gross and histological examination,” and “when a spermatic cyst was not formed, degenerative changes took place promptly;” (iii) “these observations suggest that the seminiferous tubules may be very sensitive to the increase in intra-tubular pressure and in such instances the spermatic cyst acts as a “shock absorber” to prevent the abnormal increase of pressure within the ductal system, especially the seminiferous tubules.” (The authors did not say the exact number of vasa deferentia with or without granulomatous formation, nor did they describe the histological changes in the testis.) However, McDonald,¹ with much research on vasectomy, reviewed and concluded: “occasionally, workers have fallen into the trap of seeing degenerated testes and absent granulomas and falsely concluding that the testis had succumbed because intraluminal pressure was raised because of a failure of granuloma formation (Kuwahara and Frick, 1975; Sun *et al.* 1992). In these papers, it was much more probable that the granuloma had not formed because the testis had degenerated prior to a rise in intraluminal pressure.”

Why is this correlation so controversial? Because (i) few studies have measured the intra-tubular pressure; and the few which have, in golden hamsters and guinea pigs, did not suggest that there was direct transmission of increased pressure from the tract to the testis after vasectomy.³¹ (ii) Few studies have quantitatively studied the spermatogenesis, or the histological features of the epididymis, vas deferens and sperm granulomas. (iii) Sperm granulomas might have been ignored by many researchers. Some of those who did not overlook the granuloma might not have associated the granuloma with the testis, and others might only have correlated the presence or absence of granulomas with the testis, so the number of cases with, or more likely without, granulomas might be too small for efficient comparison.

As shown by Voglmayr,⁶ all of the adult Sprague-Dawley rats developed sperm granulomas, mostly near the obstructed vas deferens, 30 days after bilateral vasectomy (with the proximal cut end of the vas deferens fulgurated and ligated), and the testis appeared

normal. Feller *et al.*⁴⁰ reported a 100% incidence of granulomatous development (at the proximal stump of the vas deferens) 2–8 weeks after unilateral double ligation of the rat vas, no less than that after unilateral open-ended vasectomy (with removal of a 5 mm segment of the rat vas and ligation of the distal vas only), without difference in the testicular or epididymal weights between the vasectomized and the contralateral sides in the groups. In our study, granulomas around the vas deferens were found in ten of the 12 unilaterally vasectomized rats; in the other two rats, we observed microscopic granulomas in the ligated vas deferens. Hence, all rats may have developed sperm granuloma soon after vasectomy at the operative site. With such a high incidence of granulomatous formation, simple correlation between the presence (or absence) of granulomas and status of spermatogenesis would not be possible or efficient.

This is the first rat study that has quantitatively studied the testis and the reproductive tract including sperm granulomas, and the first study that has correlated the size and histological characteristics of granulomas with the status of spermatogenesis.

The vasectomy performed was a double ligation of the vas deferens via a small inguinal incision without vasal transection, not the commonly used vasectomy with vasal transection (severing) that might induce larger vasal damage and likelier or quicker formation of sperm granulomas. Indeed this operation of vasal ligation might have prevented or slowed granulomatous formation, as granulomas at the vasectomy site were not observed or identified in two of the 12 vasectomized vasa deferentia postoperation and, in particular, the mean weight of the granulomas was low, only 54% of the mean weight of granulomas observed at 30 days after vasectomy in rats of similar ages.⁶ Hence, it may be that this slower or smaller granulomatous formation led to a higher intra-tract pressure, which prevented testicular spermatozoa from entering the tract, leading to higher intra-testicular pressure and then spermatogenic damage in five of the 12 cases. That is, the vasectomy-induced spermatogenic damage is pressure-mediated.

Unilateral vasectomy in this rat study and our previous rabbit studies^{30,44} induced spermatogenic damage on the vasectomized side, not the contralateral side. Therefore, the vasectomy-induced damage must be largely a local (rather than a systemic) effect of the operation.^{1,29,30} If not due to local postoperative complications (see below), the damage of vasectomy or vasal obstruction *per se* must result from sperm accumulation in the reproductive tract. Furthermore, the damage cannot be explained from the available evidence if it is not caused by intra-testicular sperm stasis and associated higher pressure from the intra-tract sperm accumulation.

A correlation between lack of granulomas and damage of spermatogenesis was also noted by Flickinger *et al.*¹⁸ 3 months after bilateral vasectomy in four adult Lewis rats: all the three testes that lacked ipsilateral sperm granulomas of the vas were small while all of the other five testes that possessed vasal granulomas were of normal weights, with the small testes showing extensive depletion of spermatogenic cells. The authors did not determine the cause of the relationship between the absence of granuloma and damage of spermatogenesis, probably because of (i) the small number of animals, (ii) the lack of morphometric analysis, and (iii) the disadvantage of the bilateral vasectomy that does not exclude the possibility of systemic effects.

Unlike the rabbit reproductive tract,³⁰ the rat epididymis and vas deferens, as shown in this morphometric study, do not distend after vasectomy. This is often attributed to the lack of distensibility of the rat excurrent ducts,^{1,31} but we speculate this could be because the tissue is prone to granulomatous development and because of the sensitivity of the testis to change in pressure. The raised intra-tract pressure may cause the valve-shaped rete testis at the testicular exit⁴⁵ to close and obstruct sperm

transport; or the rete may be sensitive to the increase in intra-seminiferous tubular pressure caused by obstructed sperm transport. As a result, spermatogenesis might have been damaged before the excurrent ducts were significantly distended. Although we did not measure the intra-tubular pressure, we did find evidences suggestive of increased pressure: the larger lumen of the seminiferous tubule and thinner seminiferous epithelium in the testes without spermatogenic damage, and the thicker rete testis in all the testes on the vasectomized side.

While vasectomy did not often result in changes in testicular histology, as reviewed by Flickinger,^{41,46} ligation of the efferent ductules or the epididymis did result in deleterious changes in testicular histology in several species. Later studies by Neaves,¹⁰ Kuwahara and Frick,¹⁶ and Kuwahara¹⁴ produced similar results, with marked testicular damage or atrophy after ligation of the corpus epididymidis or the ductuli efferentes, but not of the vas deferens. McDonald *et al.*²¹ reported enlargement and degeneration of testes 1–4 weeks after ligation of the epididymal head. These findings can be explained by a pressure-mediated mechanism: ligation of the excurrent duct closer to the testis induces a rapider and stronger transmission of intra-duct pressure to the testis, resulting in quicker and severer spermatogenic damage.

Using the rabbit model, we demonstrated that the vasectomy-induced spermatogenic damage was sloughing of immature (adluminal) spermatogenic cells in the seminiferous epithelium.^{30,44} The histological changes of the seminiferous epithelium on the vasectomized side with spermatogenic damage and of the structural components in the ipsilateral excurrent ducts, as observed and measured in this rat study, are consistent with the conclusion.

The total number of spermatocytes and spermatids in the testis of normal mature Sprague-Dawley rats were estimated to be approximately 12–15 and 44–62 times more than the total number of spermatogonia, respectively.⁴⁷ Thus, sloughing of the immature germ cells would suggest a considerable reduction in sperm production by the testis with spermatogenic damage after vasectomy. With this reduction, together with possible elimination of spermatozoa by any granulomas, the intra-testicular and intra-tract pressure would be reduced or return to normal. As a result, the seminiferous epithelium left, with maintained spermatogonial numbers,⁴⁴ would reinitiate and restore the spermatogenesis. After this, pressure-related damage to the restored spermatogenesis might occur again, re-establishing the balance between sperm production and storage.²⁹

In the vasectomized rabbit, distension of the epididymis is attended by infiltration into the epididymal duct of leukocytes involved in sperm elimination.³⁰ In the current study of rats, however, appreciable epididymal distention or leukocyte infiltration was not observed after vasectomy. It is noteworthy that spermatogenesis was markedly damaged in all the testes in the subgroup-damaged on the vasectomized side, and in all the ipsilateral epididymides, the sperm mass also diminished markedly, with mostly sloughed round spermatids replacing the sperm mass in the epididymal duct. Taken together, these findings supported our hypothesis that some structure, most likely at the exit of the rete testis or the efferent ductules, was sensitive to the obstruction of sperm passage through the extra-testicular duct that it blocked sperm efflux from the testis soon after vasectomy, before the epididymis was appreciably distended. Therefore, (i) with immature spermatogenic cells sloughing off the seminiferous epithelium owing to pressure-mediated spermatogenic damage, detached immature spermatogenic cells would move into the posttesticular duct when the intra-tract pressure decreases following spermatogenic damage. The reduction in the volume of the occluded juxta-epididymal vas deferens is suggestive of a decrease in intra-tract pressure. (ii) With the

elimination of spermatozoa, through the reproductive tract and in the sperm granuloma, and the likely growth of the epididymis in parallel with the increase in body weight during the experiment, the number of epididymal spermatozoa would decrease at the end of the experiment.

Sperm granulomas are rarely formed, and the vas deferens is often distended after vasectomy in the rabbit,^{13,29,30} but granulomas formed in the epididymis have been reported more than half a year after vasectomy.¹³ Different scenarios seen in the rat reflects species differences in biology or histopathology. An important factor may be the size of the reproductive tract. The rat vas may be too small or thin, liable to injury or damage, leading to leakage of spermatozoa after vasal ligation or transection. Kwart and Coffey⁴ demonstrated that granulomas developed at more sites (e.g., the epididymal tail) other than the ligation site 6 months after vasectomy when rats were vasectomized at an immature age, that is, when there were few spermatozoa in the vas, sperm granulomas were unlikely to form and vasal injury would heal better soon after vasectomy. This suggests that granulomas would develop early at the vasectomy site in mature rats in which the vas deferens contains numerous spermatozoa that are liable to leak after the operation; and, consequently, more granulomas would subsequently develop at the same site. This might be why rat granulomas are mostly reported at the vasectomy site.

It should be mentioned that operative trauma or adhesion of the testis with the surrounding tissue might induce acute spermatogenic damage in rabbits.²⁹ Similarly, testicular damage or atrophy after vasectomy in rats is sometimes attributed to nonspecific local effects of the operation (rather than specific effects of the vasal obstruction *per se*) such as injury (especially damage to the vasculature and innervation), adhesion or infection around the testis,^{9,26,40,42,46,48} or to postoperative cryptorchidism.^{1,5,46} Thus, small atrophied testes or data from them might have been excluded in some studies.^{40,46,48,49} However, the vasectomy in this rat study, like the one in our previous rabbit studies,^{29,30} was via the inguinal canal, away from the scrotum, without adhesion around the testis, and even without much adhesion around the vas deferens, so it was unlikely that the spermatogenic damage observed was artificial or reflected iatrogenic effects of the surgery.

In summary, our previous study in rabbits demonstrated that the vasectomy-induced spermatogenic damage was pressure-mediated.³⁰ The results obtained in our recent study of older men long after vasectomy can be explained by the pressure-mediated mechanism.⁵⁰ In this study of rats, double ligation of the vas deferens away from the scrotum induced marked spermatogenic damage which was associated with smaller, later or no development of the sperm granuloma at the vasectomy site. This result, together with some other evidence such as larger tubule lumina and thicker rete testis, suggest that the damage was pressure-mediated as well, essentially consistent with the conclusion reached for the rabbit model.³⁰ Although we performed an extensive morphometric study of the reproductive organs (including sperm granuloma), we did not see morphological evidence of an increase in the intra-tract pressure: distension of the tract or sperm accumulation in the tract. This may be because the rat vas deferens is particularly liable to granulomatous formation after vasectomy, which will store and eliminate spermatozoa and thus relieve the intra-tract pressure. This will make it impossible to determine the number of spermatozoa produced by the testis from the number stored in the reproductive tract. In addition, the rat testis may be sensitive to an intra-tubule pressure increase: even a slight increase may result in spermatogenic damage. Unlike the rabbit model,³⁰ therefore, a short-term rat vasectomy may not be validly compared with a long-term one, or a proximal rat vasectomy with a distal one, because either would not necessarily increase sperm storage or hydrostatic pressure within the occluded reproductive tract because sperm granulomas would

drain away spermatozoa. Similarly, a rat vasectomy with a larger vascular injury (e.g., tightly ligated and then sectioned) may lead to earlier formation of granulomas associated with normal spermatogenesis, whereas the same vasectomy with a smaller vascular injury (e.g., lightly ligated without sectioning) may have later or smaller granuloma formation associated with spermatogenic damage. These features or limitations explain why such rat studies are so controversial. In conclusion, the rat vasectomy model is not ideal for study of pressure-mediated spermatogenic damage, as this will depend mainly on the development of sperm granuloma postvasectomy.

AUTHOR CONTRIBUTIONS

LM conducted the operations, and prepared, observed, and measured the sections for testicular and epididymal structures; YG prepared, observed, and measured sections of the vas deferens and sperm granulomas; YY prepared, observed, and measured sections of the rete testis; YGL and XZD assisted with the operations and sample processing; and ZWY designed and instructed the study, performed the statistics, and wrote the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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