Quantitation of sperm disposal and phagocytic cells in the tract of short- and long-term vasectomized mice

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Summary. By calculating the number of spermatozoa produced by the mouse testis after vasectomy, and actually counting the number of spermatozoa present in the epididymides and vasa deferentia, the number of spermatozoa resorbed at different times was quantified. The contributions of sperm phagocytosis and intraluminal dissolution of spermatozoa (separate sperm heads and tails) in sperm disposal were examined. Sperm resorption was clearly demonstrated, with about 100×10^6 spermatozoa and 426×10^6 spermatozoa having been resorbed by 6 weeks and 6 months after vasectomy, respectively. A characteristic of the vasectomized tract was the high proportion of degenerating spermatozoa, and small lymphocytes, but very few intraluminal phagocytes were observed. The results suggest that spermatozoa are resorbed after vasectomy and that intraluminal sperm dissolution, rather than phagocytosis, is a prominent mechanism of sperm disposal in the tract of the vasectomized mouse.

Introduction

The majority of evidence, from primate and non-primate species, suggests that spermatogenesis continues after vasectomy (brief review in Barratt & Cohen, 1986). These authors showed that mouse sperm transport to the caput, corpus and cauda epididymidis proceeds at the same rate in the normal and vasectomized tract, at least for 3 months after vasectomy. As spermatozoa are still produced and transported along the tract after vasectomy, the crucial question concerns the fate of these spermatozoa. The orthodox reply is that spermatozoa are resorbed in the reproductive tract (see Flickinger, 1982); however, there are surprisingly few quantitative studies on the actual number of spermatozoa resorbed, or the relative importance of the different sperm disposal mechanisms involved in this resorption. Kwart & Coffey (1973) attempted to quantify sperm resorption in the rat: they estimated that granuloma formation may account for up to 6 months of sperm production. However, they did not estimate daily sperm production, but quoted values for daily sperm output, which is likely to have introduced a high degree of error into the results because losses in the tract were not considered (see Barratt & Cohen, 1986). Moore & Bedford (1978) calculated the daily sperm production, from numbers of testicular spermatozoa and spermatid reserves, and counted the actual number of spermatozoa in the tract of vasectomized rabbits. They showed only a very small degree of sperm resorption until granulomata appeared at about 6 months. The expansion of the tract, which is a common post-vasectomy response in many animals, served to store the spermatozoa produced.

There are probably 4 major mechanisms of sperm resorption after vasectomy: (1) phagocytosis, by intraluminal phagocytes (Phadke, 1964; Alexander, 1972; Galle & Friend, 1977; Flickinger,

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1982) or by a special population of cells in the epididymal epithelium (Tingari & Lake, 1972; Hoffer *et al.*, 1975); (2) intraluminal dissolution of spermatozoa (Kuwahara & Frick, 1975; Jean *et al.*, 1979; Sharlip *et al.*, 1984); (3) granuloma formation (Kwart & Coffey, 1973); and (4) the passage of spermatozoa into the lymphatic system—spermatolymphia (Ball & Setchell, 1983; Barratt & Cohen, 1986). Despite many studies providing evidence of such mechanisms little quantitative data are available on the number or proportion of spermatozoa resorbed by each mechanism. Evidence for sperm phagocytosis usually consists of micrographs of spermatozoa or sperm fragments in phagocytes (see Alexander, 1972). Intraluminal dissolution of spermatozoa, the first obvious stage of which is the separation of sperm heads from sperm tails (Kuwahara & Frick, 1975; Jean *et al.*, 1979), is reported by some authors but few data are available on the proportions of whole spermatozoa: heads and tails. Granuloma formation, which is a common response in many vasectomized animals (Ball, 1984), is often cited in the literature, but again only the presence and size of these structures is reported; there are few quantitative data on the number of spermatozoa that are resorbed by them. In contrast, semi-quantitative studies on sperm passage to the lymph system have been published (Ball & Setchell, 1983; Barratt & Cohen, 1986).

Barratt & Cohen (1986) emphasized that the mechanisms of sperm disposal in operation after vasectomy are not unique, but are an extension of the normal mechanisms of sperm disposal. All four sperm disposal mechanisms occur in the normal animal, i.e. intraluminal dissolution (Flickinger *et al.*, 1978), intraluminal phagocytosis (Holstein, 1978), granuloma formation (Ball & Mitchinson, 1984) and especially sperm disposal to the lymph nodes (Barratt & Cohen, 1986).

To quantify sperm resorption after vasectomy, we estimated the number of spermatozoa produced by the testes and counted the number of spermatozoa still present in the tract after vasectomy, as outlined by Moore & Bedford (1978). We also counted the number of non-spermatogenic cells and the number of separated sperm heads and sperm tails from the tracts of normal and vasectomized mice so that the roles of sperm phagocytosis and sperm dissolution in sperm disposal could be assessed.

Materials and Methods

All mice (Strong A, Birmingham Strain YP) were maintained in an Animal House (temperature controlled 19–22°C; humidity $\approx$ 50%) under a lighting regimen of 12 h dark (20:00–08:00 h) and 12 h light. They were kept in boxes (37 cm $\times$ 20 cm; WKP Cages Ltd, Dartford, U.K.) with no more than 5 to a box. Sawdust (Pilsbury's Dietary Animal Foods, Birmingham) with shredded paper on a Kapok bedding was placed in the boxes. The animals were fed (mouse diet 41B pellets, Pilsbury's) *ad libitum* and watered on alternate days.

To obtain a baseline for studying the resorption of spermatozoa in the tract, the extra-gonadal reserves of spermatozoa needed to be stabilized and estimated (Amann, 1970; Berndston, 1977; Moore & Bedford, 1978) with the hope of achieving 'standard' reserves in every mouse. The extra-gonadal reserves of 22 mice were therefore exhausted by frequent ejaculation, i.e. placing the males (aged 3 months) into cages with females (also aged 3 months) so that regular mating could take place. Checks were made for vaginal plugs, and females were replaced when such were found.

After 2 weeks of this 'mating regimen' the males were taken out of the cages and treated in the following manner: 4 males were killed immediately; 10 mice were bilaterally vasectomized using the method of Lipshultz & Benson (1980); and the rest were used as age-matched controls. After 6 weeks, 4 bilaterally vasectomized mice were killed ('short-term vasectomized') along with 4 age-matched controls, and after 6 months, the remaining 6 bilaterally vasectomized mice ('long-term vasectomized') and 4 age-matched control mice were killed. The reproductive tracts and the testes were removed and cleaned of fat. The testes were placed in Bouin's fluid, then dehydrated in graded alcohols, cleared in xylene and embedded in paraffin wax. Sections were cut at 7 μ m and stained with PAS, counterstained with Ehrlich's haematoxylin (Bradbury, 1973). The daily sperm production rates (DSPR) of the testes were estimated histologically using the method of Amann (1970) as expanded by Sharma & Gupta (1979): i.e.

$$\text{DSPR} = (\text{corrected testis volume}) \times (\% \text{ seminiferous tubules in the testis}) \times (\text{corrected number of spermatids/stage 7 tubule cross-section})$$

$$(\text{duration of one cycle of the seminiferous epithelium}) \times (\text{area of stage 7 tubule cross-section}) \times (\text{thickness of histological section})$$

This is one of the most reliable techniques to estimate DSPR (Berndston, 1977; Russell, 1983).

The corrected testes volume was determined, taking into consideration (a) testis weight, (b) testis density, (c) correction for the shrinkage of the tissue and (d) correction for the non-productive area of the testis (see Amann, 1970). The percentage of seminiferous tubules in the testis was determined by Chalkley's technique (Chalkley, 1943). A correction factor for the number of spermatids was calculated using the method devised by Davies (1973). The duration of one cycle of the seminiferous epithelium of short- and long-term vasectomized mice was determined in a separate experiment by using [^3H]thymidine and autoradiography, as described by Clermont & Trott (1969). The area of stage 7 tubule cross-sections was calculated using the formulae πr^2 . The thickness of the histological section was verified to the nearest μm , using the calibrated fine focus on a Leitz SMLUX microscope with Nomarski interference contrast to give depth of field.

The reproductive tracts were separated into paired capita, corpora, caudae and vasa, and each pair was then placed into Petri dishes ($50 \times 20 \text{ mm}$) which contained 2 ml warm PBS (37°C , Dulbecco A, Oxoid, Basingstoke, U.K.). The pairs were finely minced, agitated and left for 20 min. The sperm suspension was then gently taken up several times into a 5-ml syringe; a second wash of the debris yielded relatively few spermatozoa, thus validating this extraction procedure. Five drops (usually 25 μl) taken separately from the suspension were each diluted and counted in a Neubauer counting chamber. The s.d. between these 5 dilutions was always $< 10\%$ mean; we assumed, therefore, that the sperm suspension was fairly uniform. The numbers of whole spermatozoa, separate sperm heads and sperm tails, and the numbers of 'non-spermatogenic cells' (see below) were counted using phase-contrast microscopy at a magnification of $\times 500$. 'Non-spermatogenic cells' excluded erythrocytes, epithelial cells and immature germ cells, i.e. included only white blood cells. To aid identification of these 'non-spermatogenic cells' before experimental samples were scored, smears were made of the reproductive tract, mouse blood and fluid from the mouse peritoneal cavity. These were stained with Wright's blood stain (to identify erythrocytes and white blood cells, including lymphocytes and macrophages) or Papanicolaou stain (to identify immature testicular germ cells). Scarlet red (Phadke, 1963) was also used in an attempt to identify macrophages specifically. When examining experimental samples frequent comparisons of wet films (by phase contrast or Nomarski interference contrast) to stained preparations were made.

It was unclear from previous studies whether degenerate spermatozoa were counted as 'having been resorbed' or not. In our study, as separate sperm heads/tails are thought to be in the process of resorption, but not yet actually having been resorbed, whole but damaged spermatozoa or separate sperm heads/tails in the tract are not counted as being resorbed. The number of spermatozoa resorbed is therefore taken as the difference between the number of spermatozoa expected and the number of spermatozoa (whole and degenerate) counted in the tract (see Table 1).

Statistical comparison, using the Student's t test, of the number of spermatozoa produced, the number of whole and degenerate spermatozoa, and the number of non-spermatogenic cells was made between vasectomized and control mice using log transforms of the arithmetical data (Parker, 1983).

Results

Conspicuous changes were observed in the reproductive tract after vasectomy: the whole system, especially the cauda and the vas deferens, was distended even after 6 weeks; this distension was more obvious after 6 months. No mouse which had not been vasectomized showed any similar macroscopic abnormality. Very small granulomata, $\approx 2 \text{ mm}$, were present in the epididymides of short- and long-term vasectomized mice, usually one on each side. With reference to the calculation of the DSPR of one testis, a worked example of a control mouse (Table 1) is:

corrected testis volume = 0.31 cm^3 ;

% seminiferous tubules in the testis = 74% ;

corrected number of spermatids/stage 7 tubule cross-section = 65 ;

duration of one seminiferous epithelial cycle = 8.3 days ;

area of stage 7 tubule cross-section = $17\,436 \mu\text{m}^2$;

thickness of histological section = $7 \mu\text{m}$;

final DSPR of that testis = 1.4×10^6 .

Table 1 shows that, although there was no change in the DSPR of short-term vasectomized mice, the DSPR of long-term vasectomized mice was significantly lower than in controls ($P < 0.01$). Table 1 also shows that the mean total extra-gonadal reserves of the normal male mice that had been frequently mated was about 19×10^6 whole spermatozoa. Only slightly more whole spermatozoa were counted in the tract of short-term vasectomized mice, and this was less than 50% of the number in the age-matched controls ($P < 0.01$). In long-term vasectomized mice, significantly fewer whole spermatozoa were counted in the tract than were present in the age-matched controls ($P < 0.01$).

Table 1. Daily sperm production rates (DSPR), actual number and expected number of whole spermatozoa in the tract, the number of sperm heads and sperm resorption in vasectomized and respective control mice

Interval after vasectomy	No. of mice	Mean DSPR (both testes) $\times 10^{-6}$	No. of whole spermatozoa in tract $\times 10^{-6}$		Actual no. of sperm heads in tract $\times 10^{-6}$ (C)	Approximate no. of spermatozoa resorbed $\parallel$ ($\times 10^{-6}$)
			Expected $\dagger$ (A)	Actual (B)		
0 (frequently mated mice)	4	2.98 ± 0.21	— $\ddagger$	19 ± 4.1	0.05 ± 0.002	—
6 weeks						
Control	4	2.86 ± 0.20	139	45.2 ± 13.1	0.2 ± 0.05	— $\P$
Vasectomized	4	2.90 ± 0.20	140	19.7** ± 5.7	20.5*** ± 7.7	100
6 months						
Control	4	3.00 ± 0.12	566	58.0 ± 21.9	0.51 ± 0.1	— $\P$
Vasectomized	6	2.25** ± 0.27	461** $\S$	11.9** ± 12.6	23.2*** ± 9.7	426

Values are mean $\pm$ s.d.

$\dagger$ DSPR over time period plus extra gonadal reserves (taken as 19×10^6 whole spermatozoa).

$\ddagger$ No data available.

$\S$ DSPR taken as 3.0×10^6 up to 6 weeks after vasectomy and 2.62×10^6 (mean of 3.0×10^6 and 2.25×10^6) from 6 weeks to 6 months after vasectomy.

$\P$ Number could not be estimated as spermatozoa may have been lost in urine or by masturbation (see text).

$\parallel$ Approximate no. of spermatozoa resorbed = column A - (B + C) columns.

Values significantly different from respective controls: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 2 shows the high proportion of degenerating spermatozoa (sperm heads separated from sperm tails) in the vasectomized tracts. To aid clarification, as the numbers of separate sperm heads and sperm tails were very similar, only the numbers of sperm heads are reported. The ratio of degenerating:whole spermatozoa was very different in vasectomized and normal tracts: in the cauda epididymidis of 6-month controls, the ratio was 1:114, compared to 1:0.2 in long-term vasectomized mice. There were significantly more separate sperm heads in the vasectomized tracts compared with the respective short- and long-term controls ($P < 0.001$). Surprisingly, very few 'classical' phagocytes (macrophages or polymorphonuclear leucocytes) were observed in the tracts of short- or long-term vasectomized mice; on rare occasions spermatozoa were seen to be phagocytosed in the normal, as well as in the vasectomized, tract. Most non-spermatogenic cells were classified as small lymphocytes. There were significantly more non-spermatogenic cells in short-term vasectomized mice than in the normal control mice ($P < 0.001$), with even higher numbers of these cells in long-term vasectomized mice ($P < 0.001$; Table 3). At 6 months after vasectomy patchy degeneration of the seminiferous tubules was apparent, but no specific testicular abnormality was regularly found.

Discussion

Sperm resorption after vasectomy was clearly demonstrated in this study, with approximately 33 days production (100×10^6 spermatozoa) and 140 days production (426×10^6 spermatozoa) being resorbed after 6 weeks and 6 months. The results also show intraluminal dissolution of

Table 2. Mean ($\pm$ s.d.) number of whole spermatozoa and sperm heads ($\times 10^{-6}$) in the reproductive tract of normal (0), short-term (6 weeks) and long-term (6 months) vasectomized mice with respective control mice

Interval after vasectomy	No. of mice	Caput			Corpus			Cauda			Vas			Total	
		Whole	Heads		Whole	Heads		Whole	Heads		Whole	Heads		Whole	Heads
0	4	1.7 ± 0.7	0.01 ± 0.008		2.1 ± 0.7	0.01 ± 0.005		11.7 ± 3.2	0.02 ± 0.01		3.4 ± 0.8	0.01 ± 0.006		19.0 ± 4.1	0.05 ± 0.002
6 weeks															
Control	4	3.6 ± 0.9	0.05 ± 0.02		4.2 ± 1.1	0.03 ± 0.01		29.9 ± 8.5	0.08 ± 0.01		7.3 ± 3.1	0.04 ± 0.02		45.2 ± 13.1	0.02 ± 0.05
Vasectomized	4	1.5* ± 0.9	0.07 ± 0.02		6.2 ± 1.7	0.5*** ± 0.21		9.6* ± 5.1	10.87*** ± 4.5		2.4* ± 1.4	9.1*** ± 3.2		19.7** ± 5.7	20.5*** ± 7.7
6 months															
Control	4	3.47 ± 2.0	0.045 ± 0.029		5.9 ± 1.8	0.065 ± 0.02		37.7 ± 1.5	0.33 ± 0.09		10.8 ± 3.7	0.07 ± 0.02		58.0 ± 21.9	0.51 ± 0.12
Vasectomized	6	2.5 ± 3.7	0.37 ± 0.43		3.5 ± 3.7	0.55** ± 0.3		4.0** ± 3.2	16.1*** ± 8.7		1.8** ± 3.3	6.8*** ± 3.9		11.9** ± 12.6	23.2*** ± 9.7

Values significantly different from respective controls: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 3. Mean ($\pm$ s.d.) number of non-spermatogenic cells ($\times 10^{-3}$) in the reproductive tract of normal, short-term (6 weeks) and long-term (6 months) vasectomized mice with respective controls

Time after vasectomy	No. of mice	Caput	Corpus	Cauda	Vas	Total
0	4	0.6 ± 0.01	0.3 ± 0.01	0.2 ± 0.01	0.3 ± 0.03	1.4 ± 0.14
6 weeks						
Control	4	0.8 ± 0.11	0.6 ± 0.07	0.4 ± 0.04	0.6 ± 0.07	2.4 ± 0.21
Vasectomized	4	7.9*** ± 1.2	9.0*** ± 0.98	80.1*** ± 15.1	70.7*** ± 13.1	168*** ± 26.3
6 months						
Control	4	0.8 ± 0.07	0.8 ± 0.13	9.0 ± 0.74	0.9 ± 0.07	11.6 ± 0.97
Vasectomized	6	10.8*** ± 3.6	30.5*** ± 9.7	200*** ± 60	79*** ± 31	325*** ± 74.9

Values significantly different from respective controls: *** $P < 0.001$.

spermatozoa as a predominant mechanism of sperm disposal in the vasectomized mouse. As few of the non-spermatogenic cells were obvious phagocytes, intraluminal phagocytosis is thought to play only a very minor role in sperm disposal.

It was possible to compare the results from the various groups of mice, as the results between mice were consistent, e.g. a standard error of $< 8\%$ between the numbers of whole spermatozoa in mice 6 weeks after vasectomy, although larger variations were observed between mice vasectomized for 6 months (Tables 2 & 3). The assay of spermatogenesis was apparently successful, on the criteria of Sharma & Gupta (1979) and Amann (1981), although we cannot say how the results relate to reality (see below). We could not estimate the number of spermatozoa resorbed in age-matched controls, as sperm loss may have occurred via the urine (Lino *et al.*, 1967) or by masturbation (Cohen, 1977). Consequently, these mice only serve as controls for DSPR estimates, the relative number of degenerating spermatozoa and the relative numbers of non-spermatogenic cells.

It is difficult to compare the DSPR of the mouse, as calculated by our histological methods, to the results of other authors; there are no published data for the use of these methods with mice. In our mice the DSP/g is considerably less than in the rat and more similar to that in the human (see Amann, 1981). To confirm the low DSP/g in these mice it would be necessary to use other methods to estimate the DSPR, e.g. homogenization of spermatids (Johnson *et al.*, 1983). If our DSPR estimates are below the 'true' figures, then because our measured sperm number in the tracts is reliable, loss by resorption may well be much larger than we have supposed, but certainly is not less. The DSPR was not affected by short-term vasectomy, although a decrease in the DSPR was observed after 6 months (Table 1). This effect with time is a common observation in vasectomized animals (Flickinger, 1975; Alexander & Tung, 1977). In our animals granuloma formation and a distension of the tract did not prevent focal damage to the seminiferous epithelium, as has been suggested (Kuwahara & Frick, 1975; Bedford, 1976).

The mechanisms involved in sperm resorption in the mouse now need to be put into perspective. Phagocytes were found in the normal tract, but surprisingly there were not many more in short- or long-term vasectomized mice. Although these phagocytes may move in and out of the tract, it is hard to envisage how so few phagocytes can dispose of so many spermatozoa (epithelial phagocytosis, however, may play a part but is not reported in this study; further work is in progress). Although granulomata did form in short- and long-term vasectomized mice, they were very small

and rarely was there more than one per tract. To examine sperm resorption by granulomata, these structures need to be examined microscopically. Intraluminal phagocytosis or granuloma formation we think plays only a minor role in sperm disposal after vasectomy in the mouse. In contrast, because the high number of degenerating spermatozoa in the tract of short- and long-term vasectomized mice is an observation in keeping with humans (Sharlip *et al.*, 1984), rats (Mayorga & Bertini, 1981), guinea-pigs (Galle & Friend, 1977) and hamsters (Flickinger, 1982), we are confident that intraluminal dissolution is a major mechanism of sperm disposal after vasectomy.

The nature of the dissolution is uncertain. Sperm break-up may be due to the release of enzymes, acrosomal or otherwise (Galle & Friend, 1977; Mayorga & Bertini, 1981), from the spermatozoa themselves. Release of enzymes from phagocytes may also be important, perhaps leaving phagosomes, with their destructive enzymes, open to the luminal environment (Berger *et al.*, 1982). Moore & Bedford (1978), who showed storage and not resorption in the tracts of rabbits, found it hard to envisage sperm dissolution, emphasizing the resistant nature of the spermatozoa. However, a study by Jean *et al.* (1979) suggested that the crowding of spermatozoa after vasectomy creates a milieu that promotes dissolution, i.e. a co-operative effect. This 'crowding effect' warrants further investigation. The presence of small lymphocytes in the normal tract has been reported in other species, e.g. rats (Dym & Romrell, 1975) and humans (Ritchie *et al.*, 1984), although higher numbers in vasectomized tracts have not been reported before. Suggested functions for these lymphocytes can only be speculative, but it is suggested that in the male tract they may prevent an antisperm antibody response (Ritchie *et al.*, 1984). An increase in these lymphocytes after vasectomy may therefore seem paradoxical, because antisperm antibodies are then commonly produced (Isahakia & Alexander, 1984). It could be that there is a change in the ratio of the T cell subsets after vasectomy, leading to predominance of T helper/inducer subsets, which could aid the development of such antisperm antibody responses (see Barratt & Cohen, 1986). The mouse would appear to be a good experimental model for examining the effects of vasectomy on the human, as many of the responses of the mouse after vasectomy are similar to those in man, e.g. spermatogenesis (Jarow *et al.*, 1985), intraluminal dissolution of spermatozoa (Sharlip *et al.*, 1984), and granuloma formation (Ball & Mitchinson, 1984). However, mainly whole, not breaking up, spermatozoa have been observed in the human vas before vasovasostomy (C. L. R. Barratt, unpublished observations).

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