

## Fertility of Rat Epididymal Sperm after Chemically and Surgically Induced Sympathectomy<sup>1</sup>

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### ABSTRACT

Guanethidine, a chemical that selectively blocks sympathetic noradrenergic neurons, was used to investigate the role of sympathetic innervation in the fertility of rat epididymal sperm, using both natural mating and in utero insemination protocols. This animal model correlates, at least in part, with spinal cord injury (SCI) in men. Adult male rats were treated daily by i.p. injections, for 21 or 42 days, with 0 or 6.25 mg/kg guanethidine. To compare the effects of guanethidine-induced sympathectomy with those following surgically induced sympathectomy, the inferior mesenteric ganglion and the proximal hypogastric nerves were removed in another group of rats. Both chemically and surgically induced sympathectomy increased the weight of the epididymis and seminal vesicles/coagulating glands as well as the number and the transit time of cauda epididymal sperm. Neither serum testosterone levels nor LH was affected by treatment with guanethidine. Using natural mating, no litters were produced by guanethidine-treated rats. Chemically denervated rats failed to produce copulatory plugs or ejaculate into the uterus. However, distal cauda epididymal sperm from chemically or surgically denervated rats displayed normal fertilization ability (80%) using in utero inseminations. In addition, the sperm of denervated rats did not show abnormal sperm chromatin structure using an assay that detects DNA damage. We conclude that sympathectomy delays the transit of sperm through the cauda epididymidis and produces ejaculatory dysfunction but does not compromise sperm quality in the distal cauda epididymidis. Moreover, these data provide compelling evidence that there is no association between the prolonged transit time of sperm within the epididymis, i.e., pre-ejaculatory sperm aging, and the fertility of those sperm, which has important implications for artificial insemination using sperm from men with SCI.

### INTRODUCTION

While the importance of autonomic innervation in neurovascular and neuromuscular events within male reproductive tissues is well established, the specific roles of autonomic innervation on the fertility of epididymal sperm are poorly understood. The innervation of the epididymis

includes adrenergic, cholinergic, and nonadrenergic noncholinergic systems and has been shown to be important for the regulation of sperm transport through the excurrent duct system [1], as well as muscle contraction and secretion within the sex accessory glands [2–4]. The sympathetic fibers that reach the pelvic organs via the hypogastric nerves are mainly preganglionic, ending on ganglion cells in close vicinity to the target organs [2].

Selective destruction of adrenergic nerves has been used as a tool to study the relative importance of sympathetic innervation in male reproductive function. Chemical denervation can be accomplished by treatment of rats with guanethidine. For example, chemical sympathectomy produced by daily administration of guanethidine in low doses (5 or 10 mg/kg) results in severe, long-lasting depletion of the neuronal catecholamine stores in the male sex accessory organs of rats and in accumulation in the vas deferens of a viscous material consisting of masses of sperm fragments. The same treatment produces only minor effects on adrenergic neurons in other parts of the body, including those innervating the vasculature of the genital organs [5]. We have found that selective denervation of the rat epididymis by short-term (1–3 wk) treatment with low levels of guanethidine (6.25 mg/kg per day) results in increased numbers of sperm in the cauda epididymidis and increased epididymal transit time, without apparent effect on sperm production as measured by testicular histology, daily sperm production, and hormone measures or on sperm viability or motion (see the companion paper [6]).

Surgical denervation has also been used to produce selective sympathectomy of the epididymis, and this procedure resulted in enlarged seminal vesicles and also abolished normal peristalsis of the epididymis and vas deferens, as well as the contraction and secretion of the prostate [7, 8]. Surgical denervation is technically difficult, especially with organs where intramural adrenergic ganglion cells are present; it can be disadvantageous for experimental purposes in that the period of denervation is brief, and reinnervation by sympathetic nerves from neighboring organs or vessels occurs soon after denervation is complete [9]. Billups et al. [10] have shown that surgical removal of the rat inferior mesenteric ganglion (IMG) results in a 50% loss of sympathetic innervation in the epididymis, as indicated by levels of tissue norepinephrine, and that after the loss of epididymal innervation, sperm transport through the epididymis was altered such that spermatozoa accumulated in the cauda epididymidis. These retained spermatozoa exhibited significantly reduced straight-line and curvilinear swimming velocities subsequent to IMG removal, although

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the percentage of motile sperm was unchanged [11]. In addition, after IMG denervation, the protein composition of cauda epididymal luminal fluid [12] and epithelial cell histology [13] were altered. Ricker et al. [14] found that the fertility of surgically denervated rats, investigated after natural mating, was severely compromised, due, at least in part, to inhibited ejaculatory competence. To investigate the fertilizing ability of those sperm independent of ejaculation, sperm were also inseminated directly into the uterus. Although the sperm derived from surgically denervated rats and inseminated in utero could fertilize eggs at a level equal to that observed for control sperm, fewer gestation Day 9 implants were reported compared to the number found after insemination of control sperm. Recent experiments suggested that the altered fertility may be associated with abnormal sperm chromatin structure (possibly secondary to stasis of the sperm in the cauda epididymidis) as evidenced by their failure to decondense fully in an in vitro sperm decondensation assay [15].

Ejaculation can be divided into three distinctive phases: emission, closure of the bladder neck, and antegrade ejaculation. The first two phases are controlled by the sympathetic nervous system originating from spinal segments T10–12. The third phase involves the contraction of the muscles surrounding the urethra to forcefully expel the semen in a forward (antegrade) direction out through the end of the penis. The nerves connecting these muscles exit through the sacral segments (S2–4) of the spinal cord. There are also sensory nerves that travel from the penis back to the sacral spinal cord. These nerves then go up the spinal cord to an ejaculation reflex coordination center that is located somewhere around T12, responsible for proper neurological coordination of the ejaculation [16]. In men, when a spinal cord injury (SCI) occurs at the level of T10–12, then either the ejaculatory center or the sympathetic nervous system in this region will be damaged. If the injury occurs below T12, then the ejaculation center and the sympathetics may be intact but the pathways back from the penis and out to the muscles around the urethra may not be “connected” to the ejaculatory center. After SCI, most men experience impairments in erectile and ejaculatory function. Semen can be obtained from almost all men with SCI through the use of vibratory or electrical stimulation. However, reduced fertility typically is associated with these sperm [17]. We sought to determine whether compromised sperm quality in such instances reflects perturbation at the level of the epididymis.

If autonomic denervation does indeed perturb the structure and function of the epididymis, specifically the transit of cauda epididymal sperm, we hypothesized that the fertility of those sperm, assessed with in utero insemination, might be affected by the “aging” induced by guanethidine-induced denervation. For this study, 6.25 mg/kg guanethidine was chosen for dosing, since our other work (presented in the companion paper [6]) indicated that in a 21-day exposure paradigm, it was the selectively efficacious dose to promote epididymal sympathectomy. In addition, we compared fertility following chemical denervation with fertility following surgical denervation and examined sperm from denervated rats for nuclear integrity using the sperm chromatin structure assay. This flow cytometric assay can detect altered chromatin structure in sperm from numerous species, including rats, after exposures to heat (fever), chemotherapeutic agents, mutagens, and testicular toxicants [18, 19].

## MATERIALS AND METHODS

### Animals

Adult male (380–430 g, 90–120 days old) and female (220–300 g, 60–90 days old) Sprague-Dawley rats were purchased from Charles River, Raleigh, NC, and housed in same-sex pairs in clear plastic cages (20 × 25 × 47 cm) with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperature (22°C), humidity (40–50%), and lighting conditions (14L:10D photoperiod, lights-out 0900 h EST). Purina (Ralston-Purina, St. Louis, MO) laboratory rat chow and tap water were provided ad libitum. Females were acclimated to lighting on the top of the cage racks for at least 2 wk before the experiment. Males were allowed to adapt for a similar period before treatment began. At the end of all experiments, rats were killed by decapitation (when blood was needed for hormone measures), CO<sub>2</sub> asphyxiation, or halothane anesthesia followed by cervical dislocation. Animals were maintained and handled according to IACUC/LAPR protocols approved by the Institute Animal Care and Use Committee.

### Experimental Protocols (Fig. 1)

*Experiment 1: Fertility after natural breeding.* Male rats were randomly assigned to two groups of 8 animals each: 0 (saline vehicle) or 6.25 mg guanethidine sulfate (G-8395; Sigma Chemical Co., St. Louis, MO)/kg BW. Animals were weighed daily, and dosing solutions, which were made fresh every week (for all experiments), were administered daily via i.p. injection for 21 days (0.1 ml/100 g BW).

*Natural mating procedure.* At the end of the treatment period, each male was housed with a single virgin female for 4 h/day (0900 h to 1300 h) for a maximum of 1 wk. After mating, males were separated from the females, and vaginal smears of each female were examined for the presence of sperm. Each sperm-positive female was returned to the males' cage until the next morning. Male rats were dosed with guanethidine until at least one sperm head was found in the vaginal smear of the cohabiting female. The day when sperm were found in the vaginal smear was considered Day 0 of gestation. Females were killed on gestation Day 20 to evaluate fertility (see below).

*Experiment 2: Ejaculated sperm counts after natural breeding.* Male rats were randomly assigned to two groups of 8 animals each: 0 (saline vehicle) or 6.25 mg guanethidine/kg BW; treatment occurred for 21 days according to the same protocol described for experiment 1.

*Number of copulatory plugs and ejaculated sperm.* At the end of the treatment, shortly after the room lights were turned off (0900 h EST), each male cohabited for 4 h in a wire-bottom cage with a proestrous virgin female. The number of copulatory plugs was recorded. The females were killed and uterine sperm were enumerated (see below). A fine curved forceps was used to elevate the cervix, and the cervix was ligated. The uterine horns were excised, trimmed, washed in Dulbecco's PBS, transferred to a 35-mm Petri dish containing 2 ml of warm Medium 199 (M 3769; Sigma), and opened using small scissors. The dish was shaken gently for 5–10 min of dispersion and placed in the incubator at 35°C, thereby allowing the uterine sperm to disperse. The sperm suspension was then transferred to a 15-ml conical tube, and the total volume was recorded. A sample of 100 µl was diluted 1:10 with fixative (10% formalin in PBS with 10% sucrose, pH 7.4), and spermatozoa were counted using a hemacytometer.

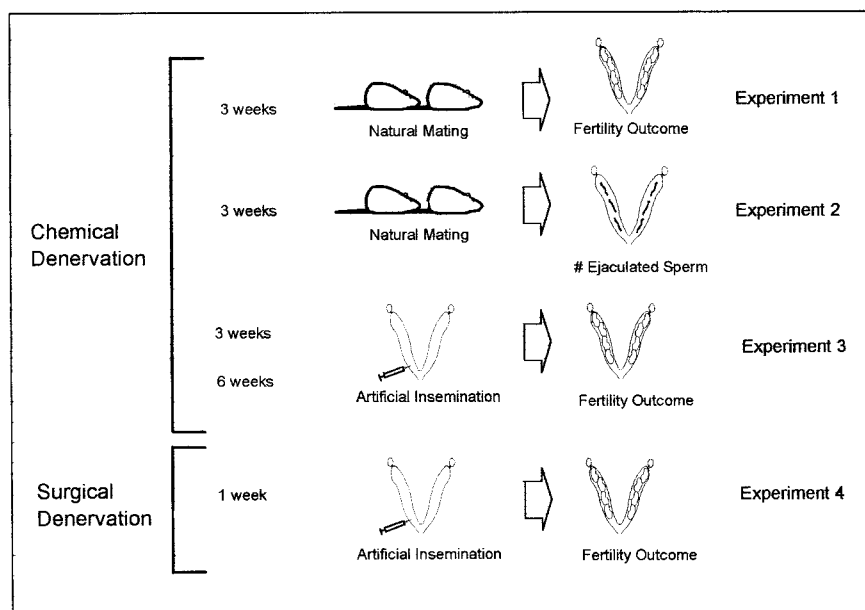


FIG. 1. Outline of experiments. Chemically denervated rats received 6.25 mg/kg guanethidine i.p. for 3 or 6 wk. Surgically denervated rats had the IMG ablated.

*Experiment 3: Fertility outcome after in utero insemination.* Male rats were randomly assigned to four groups of 8 animals each: 2 groups of 0 (saline vehicle) and 2 groups of 6.25 mg guanethidine/kg BW, treated according to the same protocol as described for experiment 1, for 21 or 42 days, in an attempt to further age sperm accumulated in the cauda epididymidis of the denervated rats.

After 1 wk of guanethidine treatment, animals in the 42-day treatment groups were placed with proestrous females for 4 h, and the numbers of copulatory plugs produced were counted. Then females were killed to evaluate uterine sperm numbers as described above.

*In utero insemination.* The procedure for in utero insemination was similar to that described previously [20, 21]. Briefly, a cohort of females were synchronized with 80  $\mu$ g s.c. of LHRH agonist (L-4513; Sigma) 115 h prior to insemination. Shortly after the room lights were turned off on the day of proestrus, the synchronized females were paired with sexually experienced, vasectomized males of proven sterility for 1 h. Typically, a copulatory plug could be found at the bottom of the wire-bottom cage if repeated intromissions had occurred. Receptive females (50–70% of those injected with LHRH) were selected for insemination.

The isolation and preparation of distal cauda sperm for insemination was the same as described previously [20, 21], with the exception that only 10 nM testosterone and dihydrotestosterone were added. Just prior to the inseminations, 0.25 mg/ml bovine lipoprotein (L 3626; Sigma) was added to the medium. Sperm were allowed to diffuse after the epididymal tubule was pierced with a no. 11 scalpel blade. The dish was allowed to shake gently and, after 5 min of dispersion, an aliquot of sperm was diluted 1:10 with fixative (10% formalin in PBS with 10% sucrose, pH 7.4) and counted using a hemacytometer; sperm concentration ranged from 100 to 280  $\times 10^6$ /ml. Within 15 min, each uterine horn was injected with a volume containing 5  $\times 10^6$  sperm, a value that results in 75% fertility using control males [20].

One female was inseminated per male. All inseminations were performed while the recipient female was in a surgical plane of halothane anesthesia. The bifurcation of the uterine horns was exposed through a low, midventral incision. Fine

curved forceps were used to elevate each horn while the insemination volume was injected through the wall of each horn via an 18-gauge i.v. catheter attached to a 0.5-ml syringe. Each injection site was cauterized immediately upon withdrawal of the needle. When insemination was complete, the abdominal musculature was sutured with 3–0 chromic gut, and the skin layer was drawn in close apposition and closed with Nexaband S/C adhesive (Tri-Point Medical, Raleigh, NC). Females were killed 9 or 20 days later, and the uteri and ovaries were examined to evaluate fertility as described below.

*Experiment 4: Fertility of surgically denervated rats after in utero insemination.* Surgical removal of the IMG (IMG denervation). Surgical IMG denervation was performed according to the method of Billups et al. [10, 11]. Briefly, male rats ( $n = 10$ ) were anesthetized with halothane. The abdominal area was shaved, and, through a midline laparotomy, the IMG and bilateral hypogastric nerves were identified on the anterior surface of the aorta and dissected away from surrounding vasculature. After resection of the IMG and the proximal 1.5–2 cm of both hypogastric nerves, the abdominal muscle layer was closed with absorbable suture (Ethicon Coated Vicryl, 4.0; Johnson and Johnson Inc., Somerville, NJ), and the skin was closed using stainless steel wound clips (9 mm, Clay Adams Brand; Becton Dickinson and Co., Sparks, MD). Sham-operated control rats ( $n = 10$ ) underwent similar surgical procedures except that the IMG and hypogastric nerves were left intact. One week after the surgery, the animals were killed, and the cauda epididymidis sperm were collected for artificial insemination as described above. A 1-wk postoperative assessment interval was chosen to allow for significant sperm accumulation in the cauda [10] without significant reinnervation [9]. Fertility was assessed by examination of the females on gestation Day 9, as described below.

#### Analysis of Fertility Outcome

The day when spermatozoa were found in the vaginal smear, or were inseminated in utero, was considered Day 0 of gestation. Females were subjected to cesarian section on gestation Day 9 (experiment 3, 6-wk treatment; experiment

4) or gestation Day 20 (experiments 1 and 3, 3-wk treatment). The ovaries were removed, and the corpora lutea were counted. The uteri were opened and the numbers of implantation sites were determined. Preimplantation loss, calculated for each female, was determined by calculating the difference between the number of corpora lutea and the number of implantation sites; the results were expressed per number of corpora lutea. For dams killed on gestation Day 20, the numbers of resorptions and live fetuses were also determined. Postimplantation loss was determined by calculating the difference between the number of implantation sites and the number of live fetuses; the results were expressed per number of implantation sites. Fetuses were blotted dry, weighed, sexed, and examined for external alterations. Fertility was calculated as the ratio between the number of gestation Day 9 implantation sites, or gestation day 20 fetuses, and the number of corpora lutea.

#### *Male Reproductive Organ Weights*

Soon after male rats were killed (except in experiment 2), the left testis, epididymis, and seminal vesicles (including coagulating glands) of all animals were trimmed of fat and fascia and weighed.

#### *Hormone Assays*

**Testosterone levels.** Blood collected in serum separator tubes at necropsy from the decapitated trunk of the animals in experiments 1 and 2 was held 45 min at room temperature, and serum was obtained after centrifugation ( $3000 \times g$ , 15 min,  $4^{\circ}\text{C}$ ). Serum was stored at  $-70^{\circ}\text{C}$  until testosterone was assayed using a Coat-A-Count kit (Diagnostic Products Corp., Los Angeles, CA). The minimum expected detectable limit was 0.20 ng/ml, and inter- and intraassay coefficients of variation were 10.8% and 5% [22].

**LH levels.** LH levels were analyzed by RIA. Materials were kindly provided by the National Hormone and Pituitary Program (Rockville, MD): iodination preparation, I-9; reference preparation, RP-3; and antiserum, S-11. Iodination materials were radiolabeled with  $^{125}\text{I}$  (New England Nuclear, Boston, MA) as described previously [23]. The assay was performed according to the recommendations provided, with the sensitivity for LH optimized by a 24-h incubation of sample and first antibody prior to the addition of  $^{125}\text{I}$ -labeled hormone. The sensitivity and inter- and intraassay coefficients of variation were 12 pg/tube, 10.3%, and 9.6%, respectively.

#### *Catecholamine Assays*

Soon after the decapitation of each rat in experiment 2, the brain was removed and quickly frozen at  $-80^{\circ}\text{C}$ . After thawing, the posterior hypothalamus was removed and weighed. Norepinephrine and dopamine levels were determined by HPLC according to the procedure described in the companion paper [6].

#### *Enumeration of Sperm Numbers, Daily Sperm Production, and Epididymal Transit*

Homogenization-resistant testicular spermatids and sperm in the caput/corpus epididymidis and cauda epididymidis from animals from experiments 1, 3, and 4 were enumerated as described previously [24]. The caput/corpus and cauda tissue were frozen at  $-70^{\circ}\text{C}$  until homogenization in saline-triton-merthiolate and subsequent counting. Before thawing was complete, the tissues were cut into small pieces

with scissors. Sperm numbers determined from the suspension used for artificial insemination in experiment 2 (see above), and from a sample taken to count sperm, were combined with the number of sperm in the remaining frozen tissue. Daily sperm production and epididymal sperm transit time were also calculated as described previously ([25] and the companion paper [6]).

#### *Sperm Chromatin Structure Assay (SCSA)*

The sperm remaining after in utero inseminations were pelleted by centrifugation, resuspended in 400  $\mu\text{l}$  of Tris buffer (50 mM, pH 8.0) containing 10 mM iodoacetamide, frozen on dry ice, and stored frozen until assayed using the SCSA [19] at South Dakota State University. Upon thawing, sperm were treated with a buffer, pH 1.2, for 30 sec to potentially denature nuclear DNA in situ and then stained with the metachromatic dye, acridine orange (AO). This dye fluoresces green when intercalated into native, undenatured DNA and red when associated with denatured, single-stranded DNA. Using flow cytometry, intensities of green (Green X) and red fluorescence (Red X) were measured for each of 5000 sperm per sample. The level of denaturation of DNA was determined for each sperm by calculating "alpha-t" ( $\alpha_t$ ), i.e., the intensity of red fluorescence divided by the sum of the intensities of red plus green fluorescence (Total X). In addition, the percentage of sperm outside the main population for  $\alpha_t$  (COMP) and the percentage of sperm with abnormally high green (% High Green) are derived. Practically, COMP indicates the percentage of abnormal cells, and  $\text{SD}\alpha_t$  indicates the extent of the abnormalities. An increase in red fluorescence has been correlated with increased DNA strand breaks, while changes in green fluorescence may reflect altered accessibility of DNA to AO due to altered chromatin packaging.

#### *Statistics*

Organ weight, hormone, sperm number, and fertility data from control and experimental treatment groups were compared using the nonparametric Mann-Whitney test. SCSA data were analyzed by the Statistical Analysis System (SAS Institute, Cary, NC). Results were considered statistically different at  $p < 5\%$ .

## RESULTS

As expected, guanethidine (6.25 mg/kg) did not alter catecholamine levels within the central nervous system. For norepinephrine, the respective hypothalamic concentrations for control and guanethidine-treated animals were  $12.00 \pm 1.07$  and  $12.37 \pm 0.71$  ng/mg protein  $\pm$  SEM, while for dopamine the concentrations were  $2.41 \pm 0.20$  and  $2.39 \pm 0.19$  ng/mg protein.

#### *Experiment 1*

The animals appeared healthy and active throughout the experiment. During treatment, body weight gains were similar between control and treated groups (data not shown). Final body weights and organ weights are shown in Table 1. Testis weight of males receiving guanethidine was comparable to that of control animals, while there was a significant increase in the weight of the epididymis and seminal vesicles/coagulating glands in the treated group.

Table 2 shows that the number of homogenization-resis-

TABLE 1. Body and organ weights after sympathectomy of the rat male reproductive system (mean  $\pm$  SEM).

Experimental group	n	Final body weight (g)	Testis (g)	Epididymis (g)	Seminal vesicles/coagulating glands (g)
Experiment 1	8				
Vehicle		506 $\pm$ 13.1	1.70 $\pm$ 0.03	0.54 $\pm$ 0.01	1.32 $\pm$ 0.11
6.25 mg/kg		488.5 $\pm$ 12.0	1.67 $\pm$ 0.03	0.62 $\pm$ 0.02**	3.46 $\pm$ 0.13***
Experiment 3: 21 days	8				
Vehicle		494.2 $\pm$ 12.5	1.68 $\pm$ 0.04	0.60 $\pm$ 0.03	2.19 $\pm$ 0.14
6.25 mg/kg		484.4 $\pm$ 7.3	1.70 $\pm$ 0.05	0.71 $\pm$ 0.02**	3.62 $\pm$ 0.21***
Experiment 3: 42 days	8				
Vehicle		502.8 $\pm$ 12.87	1.62 $\pm$ 0.03	0.60 $\pm$ 0.01	1.95 $\pm$ 0.08
6.25 mg/kg		511.8 $\pm$ 12.5	1.75 $\pm$ 0.03*	0.68 $\pm$ 0.01**	3.79 $\pm$ 0.19***
Experiment 4	10				
Sham		509.3 $\pm$ 8.8	1.63 $\pm$ 0.04	0.63 $\pm$ 0.02	1.92 $\pm$ 0.09
IMG-ablated		469.8 $\pm$ 9.2**	1.63 $\pm$ 0.04	0.71 $\pm$ 0.02**	2.81 $\pm$ 0.09***

\* Significantly different from the control group: \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001.

tant spermatids and the daily sperm production per testis were unchanged after 21 days of guanethidine treatment. While the number of sperm in the caput/corpus epididymidis was similar to that for controls, the number of sperm in the cauda epididymidis of the guanethidine-treated rats was significantly increased. Moreover, cauda transit time was increased significantly in the treated group compared to that in the control cauda. On the other hand, sperm caput epididymidis transit was unchanged (Fig. 2). Testosterone and LH levels were not affected by treatment with guanethidine (data not shown).

The effects of guanethidine on fertility and progeny outcome are shown in Table 3. Only control rats reproduced successfully; no litters were sired by treated rats.

#### Experiment 2

As in experiment 1, there was no effect on the body weight gain due to guanethidine treatment. The final body weights, expressed as mean  $\pm$  SEM, were 491.2  $\pm$  11.9 and 468.5  $\pm$  8 for the control and treated groups, respectively.

After 4 h of cohabitation with a female in proestrus, control rats produced 5.6  $\pm$  0.51 copulatory plugs. In contrast, only a single small plug was seen under the cage of 1 of 8 guanethidine-treated rats, resulting in a mean  $\pm$  SEM for this group of 0.2  $\pm$  0.2 copulatory plugs. The number of sperm in the uterine horns of females mated with control males was 157.11  $\pm$  10.85  $\times$  10<sup>6</sup>/ml. In the horns of all females mated with guanethidine-treated rats, occasionally one or two spermatozoa could be seen in the hemacytometer, although they were out of the counting field, resulting in an ejaculated sperm count of zero.

#### Experiment 3

No differences in body weight were found between control and treated animals over the course of treatment (data not shown); final body and organ weights are shown in Table 1. Testis weight of guanethidine-treated males was not significantly different from that of control animals after 21 days of treatment but was significantly increased after 42 days. There were also significant increases in the weights of the epididymis and seminal vesicles/coagulating glands compared to those of controls at both time points.

Table 2 shows that the number of homogenization-resistant spermatids, the daily sperm production, and number of sperm in the caput/corpus epididymidis were unchanged following 21 days of treatment with guanethidine. On the other hand, there was a significant increase in the number of sperm in the cauda epididymidis of treated animals. As in experiment 1, guanethidine treatment promoted a delay in sperm transit in the cauda epididymidis with no corresponding difference in the caput/corpus (Fig. 2).

As in experiment 1, neither testosterone nor LH levels were affected by 21 days of treatment (data not shown).

After 1 wk of experiment and 4 h of cohabitation with a proestrous female, control rats produced 4.62  $\pm$  0.60 copulatory plugs. No plugs were found under cages of guanethidine-treated rats. The number of sperm in the uterine horns of females mated with control males was 165.31  $\pm$  40.53  $\times$  10<sup>6</sup>/ml. In the uterine horns of females mated with guanethidine-treated rats, no spermatozoa were found.

The effects of treatment with guanethidine on fertility and pregnancy outcome are shown in Table 3. Control and treated groups displayed the same implantation efficiency. In addition, no differences were seen in litter size, fetal

TABLE 2. Sperm parameters after sympathectomy.

Experimental groups	n	Spermatid number ( $\times$ 10 <sup>6</sup> /testis)	Daily sperm production ( $\times$ 10 <sup>6</sup> /testis/day)	Caput/corpus epididymal sperm number ( $\times$ 10 <sup>6</sup> /organ)	Cauda epididymal sperm number ( $\times$ 10 <sup>6</sup> /organ)
Experiment 1	8				
Vehicle		208.06 $\pm$ 8.45	34.11 $\pm$ 1.39	105.05 $\pm$ 4.59	75.13 $\pm$ 7.96
6.25 mg/kg		184.74 $\pm$ 8.99	30.28 $\pm$ 1.47	94.69 $\pm$ 7.36	279.67 $\pm$ 3.79***
Experiment 3: 21 days	8				
Vehicle		197.35 $\pm$ 11.41	32.35 $\pm$ 1.87	174.02 $\pm$ 5.55	247.42 $\pm$ 12.70
6.25 mg/kg		196.19 $\pm$ 11.59	32.16 $\pm$ 1.90	160.34 $\pm$ 12.32	314.98 $\pm$ 16.85**
Experiment 4	10				
Sham		190.22 $\pm$ 10.45	31.18 $\pm$ 1.71	123.75 $\pm$ 11.34	199.86 $\pm$ 14.53
IMG-ablated		183.02 $\pm$ 15.03	30.00 $\pm$ 2.46	109.00 $\pm$ 5.50	265.88 $\pm$ 15.13**

\*Significantly different from the control group: \*\* $p$  < 0.01; \*\*\* $p$  < 0.001.

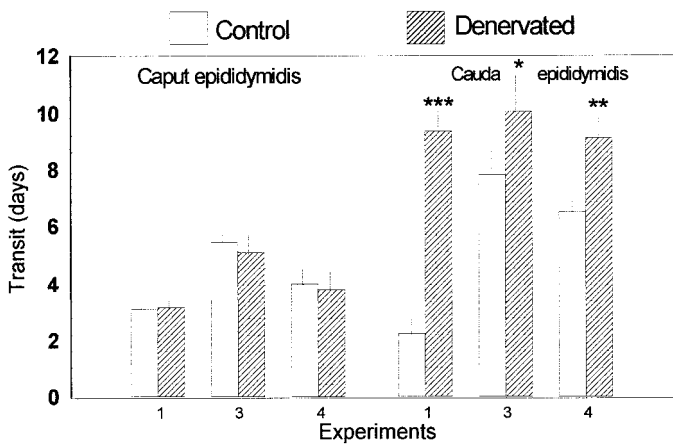


FIG. 2. Caput/corpus and cauda epididymidis sperm transit time in the control and 21-day guanethidine-treated groups (experiments 1 and 3) and in the sham and IMG-ablated groups. Values are expressed as mean  $\pm$  SEM ( $n = 8$ , experiments 1 and 3;  $n = 10$ , experiment 4). Significantly different from the control group: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

weight, external aspect of the gestation Day 20 fetuses, or pre- and postimplantation loss.

#### Experiment 4

The final body weight of the IMG-ablated rats was significantly lower ( $p < 0.01$ ) than that of the sham-operated rats:  $469.8 \pm 9.2$ ,  $509.3 \pm 8.8$ , respectively. Testis weight was no different from that of sham-operated rats; however, there was a significant increase in the weight of epididymis and seminal vesicles/coagulating glands in the denervated group (Table 1).

Table 2 shows that the number of homogenization-resistant spermatids, the daily sperm production, and the number of sperm in the caput/corpus epididymidis were unchanged after surgical denervation. On the other hand, there was a significant increase in the number of sperm in the cauda epididymidis of denervated animals. As in the previous experiments, surgical denervation promoted a delay in sperm transit in the cauda epididymidis with no corresponding difference in transit time through the caput/corpus (Fig. 2).

The effects of IMG ablation on fertility rate and progeny outcome are shown in Table 3. Control and denervated groups displayed the same fertility rate and implantation efficiency. Moreover, no differences were seen in the number of implantation sites and preimplantation loss.

#### SCSA—Experiments 3 and 4

Sperm nuclei from denervated rats did not show any evidence of DNA damage (i.e., increases in red fluorescence,  $\alpha_t$ , or COMP) when compared with those from control rats (Table 4). A slight but statistically significant increase in total green fluorescence was found in sperm from both groups of sympathectomized rats, and this was sufficient to lower  $\alpha_t$  in the guanethidine group (without an increase in red fluorescence) and increase total fluorescence in the surgically denervated rats.

#### DISCUSSION

The epididymis is an organ in which sperm undergo maturation and storage prior to ejaculation. In both rats and men, sperm are fully capable of successfully fertilizing an egg by the time they reach the proximal cauda epididymidis. Collectively, the biochemical changes (i.e., protein acquisition and modification, lipid turnover, thiol oxidation) that sperm undergo during their transit to the proximal cauda epididymidis increase the velocities of progressively motile sperm and confer upon them the ability to fertilize a mature oocyte. However, the mechanisms underlying these changes are not yet understood. Studies using both chemical and surgical denervation have confirmed that autonomic innervation plays a significant role in epididymal functions [7, 8, 14, 25–27]. However, a clear, mechanistic linkage between autonomic innervation of the epididymis and the fertility of epididymal or ejaculated sperm remains unclear.

In the present study, the lack of effect of systemically administered guanethidine at this dosage on hypothalamic norepinephrine is consistent with previous findings [28]. Compared to the marked depletions seen in the cauda epididymidis (reported in our companion paper [6]), the data indicate that the gonads and accessory sex organs are the principal sites of action of guanethidine within the hypothalamic-pituitary-gonadal axis. The weights of both the seminal vesicles/coagulating glands and epididymis were markedly increased in the guanethidine-treated and IMG-ablated groups, presumably due to excretory failure and retention of sperm, respectively [1, 4, 25, 27, 29]. Indeed, there were significant increases in the number of sperm and the transit time in the cauda epididymidis of all denervated rats. This is consistent with the loss of innervation in this region, which would decrease contractility of the duct, thereby delaying sperm transit.

TABLE 3. Fertility in male rats after sympathectomy (mean  $\pm$  SEM).<sup>a</sup>

Parameters	Experiment 1 (natural insemination) 21-day treatment		Experiment 3 (in utero insemination)				Experiment 4 (in utero insemination) 7-day postsurgery	
	0	6.25	0	6.25	0	6.25	Sham	IMG ablated
Sperm positive females	8	8	N/A	N/A	N/A	N/A	N/A	N/A
Fertility (%) <sup>b</sup>	86.81 $\pm$ 2.60	0	70.77 $\pm$ 11.52	64.76 $\pm$ 9.41	94.42 $\pm$ 2.84	91.71 $\pm$ 2.48	82.60 $\pm$ 5.38	87.67 $\pm$ 3.91
Fetuses per litter								
Total	13.29 $\pm$ 0.64	—	10.14 $\pm$ 1.65	9.43 $\pm$ 1.76				
Males	6.29 $\pm$ 0.47	—	5.00 $\pm$ 1.00	4.29 $\pm$ 0.71	N/A	N/A	N/A	N/A
Females	7.00 $\pm$ 0.72	—	5.14 $\pm$ 0.88	5.14 $\pm$ 1.34				
Fetal weight (g)	2.58 $\pm$ 0.03	—	2.45 $\pm$ 0.10	2.48 $\pm$ 0.10	N/A	N/A	N/A	N/A
Implantation sites	14.86 $\pm$ 0.70	—	11.71 $\pm$ 2.26	10.71 $\pm$ 1.82	15.50 $\pm$ 1.18	13.67 $\pm$ 1.20	13.67 $\pm$ 1.20	13.67 $\pm$ 1.20
Preimplantation loss (%)	2.99 $\pm$ 2.10	—	29.23 $\pm$ 11.52	35.24 $\pm$ 9.41	5.58 $\pm$ 2.84	8.29 $\pm$ 2.48	17.40 $\pm$ 5.38	12.24 $\pm$ 3.91
Postimplantation loss (%)	10.37 $\pm$ 2.74	—	9.27 $\pm$ 4.73	11.07 $\pm$ 5.95	N/A	N/A	N/A	N/A

<sup>a</sup> N/A, not applicable.

<sup>b</sup> Implantation sites or fetuses/corpora lutea.

TABLE 4. SCSA results after chemically and surgically induced sympathectomy of rats (mean  $\pm$  SEM).

SCSA endpoints	Experiment 3 <sup>a</sup>		Experiment 4 <sup>a</sup>	
	0	6.25	Sham	IMG-Ablated
$\alpha_r$	200.82 $\pm$ 3.26	189.85 $\pm$ 3.26*	169.23 $\pm$ 1.67	167.45 $\pm$ 1.67
SD $\alpha_r$	40.98 $\pm$ 2.71	37.70 $\pm$ 2.71	43.98 $\pm$ 3.30	43.85 $\pm$ 3.30
COMP	1.01 $\pm$ 0.14	0.80 $\pm$ 0.14	1.83 $\pm$ 0.33	1.78 $\pm$ 0.33
Red X	117.35 $\pm$ 1.71	112.25 $\pm$ 1.71	95.28 $\pm$ 1.17	95.92 $\pm$ 1.17
Green X	464.12 $\pm$ 3.59	476.11 $\pm$ 3.59*	459.31 $\pm$ 2.34	467.76 $\pm$ 2.34*
Total X	293.65 $\pm$ 1.65	297.75 $\pm$ 1.65	278.21 $\pm$ 1.34	282.73 $\pm$ 1.34*
% High Green	7.81 $\pm$ 0.47	8.61 $\pm$ 0.47	6.45 $\pm$ 0.36	7.43 $\pm$ 0.36

<sup>a</sup> n = 8 (experiment 3); n = 10 (experiment 4).

\* Significantly different from the control group: \**p* < 0.05.

Our results show that guanethidine-treated rats did not produce copulatory plugs and ejaculated only isolated sperm inside the female reproductive tract. Thus no litters were produced by denervated animals. The same kind of ejaculation problem, called “dry sex,” in which orgasms are experienced without emission of semen, has been reported in men taking guanethidine as an antihypertensive medicine [30] and in rats that have had the IMG surgically removed [14]. The effect of guanethidine on sexual behavior, penile reflexes, and spontaneous seminal emission in the rat was studied previously by means of acute (4 h prior to testing) and daily i.p. injections of a low (5 mg/kg) and moderately high (25 mg/kg) dose of the drug [5]. Acute low-dose treatment eliminated the expulsion of a seminal plug during ejaculation without affecting sex behavior, demonstrating a selective effect of the drug on the mechanism underlying plug production. Moreover, daily injection with low doses of guanethidine eliminated emission both in and ex copula for 4 wk, without altering sexual behavior or penile reflexes [5]. Since there was no evidence of retrograde ejaculation into the bladder, the seminal anorrhea associated with guanethidine seems to result from impairment of the normal mechanism underlying emission.

Billups et al. [10] previously showed that surgical removal of the rat IMG caused a significant increase in the number of spermatozoa present in the cauda epididymidis as early as 1 wk after surgery. These results were interpreted as evidence that sperm transit time through the cauda epididymidis was prolonged after IMG ablation and that spermatozoa in the IMG-ablated rats were thereby retained within the distal regions of the epididymis longer than those in sham-operated control rats. When cauda epididymal sperm accumulation similar to that observed after IMG denervation was induced without disruption of sympathetic innervation by partially constricting the vas deferens with a “cuff” of polyethylene tubing [14], and the cauda epididymal spermatozoa from cuff-constricted males were used for in utero insemination, no statistically differences in fertilization rates were found compared to control values. Low-level guanethidine exposure results in an accumulation of cauda sperm that is comparable to that observed following IMG removal, an effect that results from delayed cauda transit (see companion paper [6]).

As stated previously, sperm can be obtained from men after SCI by electroejaculation or vibratory ejaculation. These ejaculates often have normal sperm counts but more immotile sperm than found in men without SCI, and this condition does not seem to be caused by lifestyle factors but may be related to factors within the seminal plasma [31]. Therefore, in general, sperm production and quality are adequately maintained in the majority of men with SCI. What makes spontaneous fertility a problem, for the most

part, is ejaculatory dysfunction. However, the fertility of sperm recovered “artificially” from men with SCI is typically poor [17].

We have shown in previous work [4, 27] that guanethidine-induced sympathectomy prevents fructose secretion in the rat prostate and seminal vesicle, in addition to promoting reduced efficiency of delivery by the latter. It might be assumed that sympathetic innervation may be directly involved in the secretory activity of the male accessory sex glands, at least in rats.

By evaluating the fertility of distal cauda epididymal sperm after in utero insemination, the present study was designed to determine whether the delayed transit of cauda epididymal sperm resulting from guanethidine exposure exerts qualitative effects on sperm. The results, however, failed to reveal any fertility-related problem, even after 6 wk of drug exposure. Indeed, when cauda epididymal sperm were taken from either guanethidine-treated or surgically denervated rats for in utero insemination, no differences were found in fertility (i.e., the number of implants or fetuses on Day 9 or 20 of gestation relative to the number of corpora lutea compared to values for the control rats). While these results corroborate the absence of qualitative effects on sperm after guanethidine-induced sympathectomy as reported in the companion article [6] they are in contrast to findings from an earlier report [14] of significant decreases in the fertility of cauda epididymal sperm 1 and 4 wk after surgical denervation. At the present time we have no satisfactory explanation for this discrepancy.

It is noteworthy, however, that Ricker et al. [14] followed up on this observation by evaluating fertilization in these animals. Eggs recovered the day after breeding appeared to be fertilized normally; thus the observed effects on fertility were not apparently due to altered sperm fertilizing ability. On the basis of these results, they concluded that the lack of implantation after fertilization could be indicative of DNA damage in the sperm or of altered sperm chromatin. In our experiments, however, no evidence of DNA damage resulting from either guanethidine or surgical denervation was observed, at least as measured with the SCSA. There was no evidence of increased red fluorescence with AO staining, which is a biomarker of increased susceptibility to acid denaturation that can result from DNA damage or altered chromatin structure. Although sperm from both types of rats did show slightly elevated green staining, this increase is probably not biologically significant, since it was not accompanied by augmented red fluorescence. These SCSA results are in agreement with those of an earlier study in which rat sperm were detained in the epididymis by ligation for up to 7 days [32]. On the other hand, Cuasnicu and Bedford [33] observed altered fertilizing ability after efferent duct ligation, perhaps due to

compromised maturation of proximal epididymal sperm that were prevented from reaching the cauda in a timely fashion and with a normal milieu of luminal constituents.

The data we have presented herein provide compelling evidence that there is no association between the prolonged transit time (stasis) for sperm within the epididymis and the fertilizing ability of those sperm. Thus, the poor quality and low rate of fertilization of ejaculated sperm from men with SCI may not represent compromise at the level of the epididymis but may reflect effects that are secondary to lesions in the sex accessory glands. Low-level guanethidine or surgically induced sympathectomy delays the transit of sperm through the cauda and produces ejaculatory dysfunction but does not compromise sperm quality in the distal cauda epididymidis or impair the ability of those sperm to fertilize ova in vivo.

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