BIOLOGY OF REPRODUCTION **59,** 890–896 (1998)

Rat Epididymal Sperm Quantity, Quality, and Transit Time after Guanethidine-Induced Sympathectomy[1]

Wilma De G. Kempinas,[2,3] Juan D. Suarez,[4] Naomi L. Roberts,[4] Lillian Strader,[4] Janet Ferrell,[4] Jerome M. Goldman,[4] and Gary R. Klinefelter[4]

Departamento de Morfologia,[3] Instituto de Biociências - UNESP, Botucatu, SP, Brazil
United States Environmental Protection Agency,[4] Reproductive Toxicology Division, National Health and Environmental Effects Research Laboratory, Research Triangle Park, North Carolina 27711

ABSTRACT

Guanethidine, a chemical that selectively abolishes peripheral noradrenergic nerves, was used to investigate the role of sympathetic innervation in the maintenance of epididymal sperm quantity and quality. Four groups of 10 adult male rats each were treated daily for 21 days, by i.p. injections, with either 0 (saline vehicle), 6.25, 12.5, or 25 mg/kg guanethidine. Norepinephrine content was reduced to undetectable levels in the cauda epididymidis in all guanethidine groups after 3 wk of treatment and was reduced to 7.4% of the control values after 1 wk of 6.25 mg/kg treatment. While body weight gain was significantly decreased at 12.5 and 25 mg/kg compared to that in controls, there was a significant increase in the weights of the seminal vesicles/coagulating glands in all treated groups. The number of homogenization-resistant spermatids per testis and the daily sperm production per testis remained unchanged. The weight of the epididymis was significantly increased at 6.25 and 12.5 mg/kg. Moreover, the number of cauda epididymal sperm and the transit time were increased significantly at 6.25 mg/kg (10.2 days) compared to values in the control cauda (6.3 days). Neither serum testosterone levels nor LH was affected in a dosage-related manner. There were no effects of guanethidine treatment on cauda epididymal sperm motility or morphology. A quantitative analysis of detergent-extracted cauda epididymal sperm proteins by SDS-PAGE revealed no differences, but there were diminutions in seven proteins in homogenates of caput/corpus tissue. Histologic analysis of testis and epididymis sections revealed no differences between control and denervated animals. In a subsequent experiment the lowest effective dosage (6.25 mg/kg) was given to rats for 1 wk, and an increased number of cauda epididymal sperm and a delay in sperm transit were observed. Our results indicate that low-dosage guanethidine exposure denervates the epididymis within 1 wk, thereby delaying epididymal transit; however, neither 1- nor 3-wk exposure produces qualitative changes in the sperm.

INTRODUCTION

The male reproductive tract receives abundant innervation from the autonomic nervous system. This innervation, which encompasses adrenergic, cholinergic, and nonadrenergic noncholinergic (NANC) systems, has been shown to influence vasoactivity within the testis [1], sperm transport through the excurrent duct system, [2] and muscle contraction and secretion within the sex accessory glands [3–5]. While the effects of autonomic innervation on neurovascular and neuromuscular events within male reproductive tissues are well established, the effects of autonomic innervation on reproductive capacity and fertility are poorly understood.

Both surgical and chemical approaches have been used to study in vivo the role of sympathetic innervation. One advantage of chemical sympathectomy, when mechanistic specificity can be maintained, is that it is relatively noninvasive. For denervation of noradrenergic sympathetic targets, guanethidine has been frequently employed, since it does not cross the blood-brain barrier and has not been found to affect cholinergic or sensory neurons or adrenomedullary cells [6]. Guanethidine is able to block norepinephrine uptake into adrenergic neurons while being itself actively and selectively taken up by the catecholamine uptake pump. This results in a reduction in tissue norepinephrine concentrations [7] and leads ultimately to a cytotoxic immune-mediated destruction of the neuron [8].

Morphological studies have shown that the testicular autonomic innervation of most mammals seems to be restricted to the capsule and most superficial blood vessels, thus strongly suggesting its role in the control of blood flow and temperature (for references, see [9]). Unlike the classical concept of sympathetic innervation, the sympathetic supply to the vas deferens and the accessory glands has been shown to be innervated chiefly by ''short'' postganglionic adrenergic neurons, located close to the target organs [3]. Collectively these nerves have been termed the ''short adrenergic neuron system'' or the ''urogenital short neuron system'' [10].

Chronic treatment of adult rats with low dosages (5–10 mg/kg) of guanethidine causes long-lasting damage to the short noradrenergic innervation of the male internal genitalia but only minor effects on adrenergic neurons in other parts of the body, including those innervating the vasculature of the genital organs and those that innervate the testis [11, 12]. Widespread sympathectomy, involving the degeneration of over 95% of peripheral noradrenergic neurons (''short'' and ''long''), occurs after chronic treatment with high dosages (20–25 mg/kg, 2–6 wk) [13, 14]. Consequently, it has appeared that administration of low concentrations of guanethidine to rats would effectively target only a single cell type: the postganglionic sympathetic neuron.

The epididymis is an organ in which sperm undergo final maturation and storage prior to ejaculation. It receives autonomic innervation (adrenergic, cholinergic, and NANC nerves) from the inferior mesenteric ganglion (IMG), major pelvic ganglion, and pelvic accessory ganglion [15]. Within the epididymis, nerve fibers are immunolocalized to peritubular and subepithelial regions [16, 17]. Studies using

Accepted June 1, 1998.
Received February 4, 1998.

[1]W.D.G.K. is an EPA Visiting Scientist supported by Foundation for the Promotion of Science of State of São Paulo (FAPESP) - Brazil. Although the research described herein has been funded in part by the U.S. Environmental Protection Agency, it does not necessarily reflect the views of the agency and no official endorsement should be inferred.

[2]Correspondence: Wilma De Grava Kempinas, Departamento de Morfologia, Instituto de Biociências - UNESP, 18618 000 Botucatu, SP, Brazil. FAX: 55 14 821 3744; e-mail: kempinas@surfnet.com.br

both chemical and surgical denervation have confirmed that autonomic innervation plays a significant role in epididymal functions [12, 18–21]. Billups et al. [22] have shown that surgical removal of the rat IMG caused a significant increase in the number of spermatozoa present in the cauda epididymidis; alterations in epididymal histology, luminal fluid protein, and sperm motion parameters were also reported.

If autonomic denervation does indeed perturb the structure and function of the epididymis, we hypothesized that sperm reproductive capacity would be affected by the loss of the short, postganglionic sympathetic innervation induced by low-dosage guanethidine exposures. The present study investigated the effects of guanethidine treatment on sperm number, sperm quality (sperm motility and protein composition), and epididymal morphology.

MATERIALS AND METHODS

Animals

Adult (380–430 g, 90–120 days old) male Sprague-Dawley rats (Charles River, Raleigh, NC) were housed one per cage (clear plastic, 20 × 25 × 47 cm) with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperature (22°C), humidity (40–50%), and light (14L:10D, lights-out 0900 h EST) conditions and were provided with Purina (Ralston-Purina Co., St. Louis, MO) laboratory chow and tap water ad libitum. Animals were allowed to adapt for at least 2 wk before beginning treatment. Animals were maintained and handled according to IACUC/LAPR protocols approved by the Institute Animal Care and Use Committee.

Experimental Protocols

Experiment 1. Rats were randomly assigned to four different groups of 10 animals each: 0 (saline vehicle), 6.25, 12.5, or 25 mg guanethidine sulfate (G-8395; Sigma Chemical Co., St. Louis, MO)/kg body weight. Animals were weighed daily, and dosing solutions, prepared weekly, were administered daily for 21 days via i.p. injection.

Histology

After 21 days of treatment, 4 of 10 rats from each group were anesthetized with halothane, and the left testis and epididymis were isolated and collected following hemicastration prior to whole-body perfusion. A 20-gauge needle was inserted into the right atrium of the heart, and blood (3–5 cc was aspirated into a 5-cc syringe. A 16-gauge blunted needle was then inserted through the left ventricle, into the aorta, and the right atrium was incised. Once vasculature was flushed with Dulbecco's PBS, tissues were perfused-fixed with 5% glutaraldehyde in 0.05 M collidine buffer containing 0.1 M sucrose, pH 7.4. When fixation was complete, the testis and epididymis were removed, and small cubes of testis, mid-caput, mid-corpus, proximal, and distal cauda epididymidis [23] were immersed in fixative overnight at 4°C. Tissues were subsequently washed with buffer, postfixed in 1% aqueous osmium tetroxide, and processed for light microscopy [24]. Animals not used for histology were killed by CO_2 asphyxiation and cervical dislocation. Blood (5–8 cc) was removed by cardiac puncture, and organs and fluids were collected and analyzed as described below.

Organ Weights

The left testis and epididymis of all animals, and seminal vesicles (including coagulating glands, with fluid) of nonperfused rats, were trimmed of fat and fascia and weighed.

Hormone Assays

Testosterone levels. Blood collected at necropsy was held 45 min at room temperature, and serum was obtained after centrifugation (3000 × *g*, 15 min, 4°C) in serum separator tubes (Vacutainer; Becton Dickinson, Rutherford, NJ). Serum was stored at −70°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%, respectively [25].

In vitro testosterone production. The left testis of each animal was removed and decapsulated, and the parenchyma was sliced into ~50-mg pieces. Each piece was weighed and placed into a 1.5-ml microfuge tube containing 1.0 ml Medium 199 (M199). The M199 was buffered with 0.71 g/L sodium bicarbonate ($NaHCO_3$) and 2.1 g/L Hepes, and contained 0.1% BSA (Schwartz-Mann, Orangeburg, NY) and 25 mg/L soybean trypsin inhibitor, pH 7.4. Testosterone production was assessed by incubating parenchyma in duplicate, either with or without maximal hCG stimulation (100 mIU/ml), for 2 h at 34°C [26]. After centrifugation (5 min, 10 000 × *g*), medium was frozen at −70°C until testosterone assay.

LH levels. LH levels were analyzed by RIA. Materials kindly provided by the National Hormone and Pituitary Program (Rockville, MD) were as follows: iodination preparation, I-9; reference preparation, RP-3; and antiserum, S-11. Iodination materials were radiolabeled with ^{125}I (New England Nuclear, Boston, MA) as described previously [27]. The assay was performed according to provided recommendations, with the sensitivity for LH optimized by a 24-h coincubation of sample and first antibody prior to the addition of ^{125}I-labeled hormone. The sensitivity and inter- and intraassay coefficients of variation were 12 pg/tube, 10.3%, and 9.6%, respectively.

Norepinephrine Determination

Cauda epididymidis norepinephrine levels were determined by HPLC. Weighed left caudae from all rats were first minced and then sonicated (over ice) in a 1:11 dilution of cold HPLC mobile phase (pH 3.1), consisting of 58 mM dibasic sodium phosphate (Fisher Chemical, Fairlawn, NJ), 0.09 mM EDTA (Mallinckrodt, Paris, KY), 1.5 mM 1-heptanesulfonic acid sodium salt (Eastman Kodak, Rochester, NY), and 8% acetonitrile (Burdick & Jackson, Muskegon, MI) in HPLC-grade water previously filtered and deionized through a Milli-Q system (Millipore, Marlborough, MA). The samples were centrifuged (20 min, 13 000 rpm, 4°C), and each supernatant was drawn off and filtered (Gelman Acrodisc LC13, 0.2; Ann Arbor, MI), prior to HPLC injection. The HPLC system consisted of a Waters model 510 isocratic pump, Waters model 717 plus refrigerated autosampler, Waters Bondapak C_{18} (3.9 × 300 mm) reversed-phase column (Marlborough, MA), and an ESA Coulochem II electrochemical detector (Bedford, MA) with ESA model 5014 high-performance analytical and model 5021 conditioning cells. A flow rate of 1.5 ml/min was employed for all separations. Norepinephrine standard was obtained from Sigma. The norepinephrine recovery was 95%.

TABLE 1. Body and organ weights after 21-day (experiment 1) or 7-day (experiment 2) guanethidine treatment (mean ± SEM).

	Dosages (mg/kg)					
	Experiment 1[a]				Experiment 2[b]	
Body/organ	0	6.25	12.5	25	0	6.25
Final body weight (g)	493.5 ± 7.1	484.6 ± 10.6	438.8 ± 5.5*	398.2 ± 5.76*	483.6 ± 9.4	480 ± 7.3
Testis (g)	1.69 ± 0.10	1.78 ± 0.05	1.82 ± 0.05	1.77 ± 0.11	1.38 ± 0.16	1.55 ± 0.06
Epididymis (g)	0.60 ± 0.03	0.69 ± 0.02*	0.67 ± 0.02*	0.58 ± 0.01	0.59 ± 0.01	0.67 ± 0.02*
Caput/corpus (g)	0.34 ± 0.02	0.36 ± 0.01	0.35 ± 0.01	0.32 ± 0.01	0.34 ± 0.01	0.35 ± 0.01
Cauda (g)	0.25 ± 0.01	0.33 ± 0.01*	0.31 ± 0.01*	0.25 ± 0.01	0.25 ± 0.00	0.32 ± 0.01*
Seminal vesicles/coagulating glands (g)	1.78 ± 0.09	2.88 ± 0.12*	2.89 ± 0.15*	2.25 ± 0.12*	1.78 ± 0.08	2.86 ± 0.09*

[a] Experiment 1, n = 10 for all measures, except seminal vesicles/coagulating glands weight (n = 6).
[b] Experiment 2, n = 8 for all measures.
* Significantly different from the control group ($p < 0.05$).

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 nonperfused rats (right side) were enumerated as described previously [28]. Cauda epididymal sperm numbers determined from the suspension used for motility (see below) were combined with the number of sperm in the remaining cauda tissue. Daily sperm production (i.e., DSP) was derived by dividing the total number of homogenization-resistant spermatids per testis by 6.1 days, the number of days of the seminiferous cycle in which these spermatids are present. Transit time through the caput/corpus epididymidis or cauda epididymidis was calculated by dividing the number of sperm within each of these regions by the DSP.

Sperm Motility

Sperm were recovered from the epididymis for sperm motion analysis as previously described [29]. Briefly, the right cauda epididymidis of all nonperfused rats was nicked with a no. 11 scalpel blade in order for sperm to disperse in 2 ml of Hanks' Balanced Salt Solution (HBSS) buffered with 4.2 g/L Hepes and 0.35 g/L $NaHCO_3$ and containing 2.0 g/L BSA, 0.9 g/L D-glucose, 0.1 g/L sodium pyruvate, and 0.025 g/L soybean trypsin inhibitor, pH 7.4, at 34°C. Sperm were diluted with HBSS just prior to analysis on a Hamilton-Thorne (Beverly, MA) IVOS using the Toxicology software version 10.6. A minimum of 200 motile sperm were analyzed per sample. For each field, 60 frames were analyzed at a rate of 60 frames/sec. Sperm were considered motile when average path velocity (VAP) exceeded 20 μm/sec and considered progressively motile if straightness (VSL [straight line velocity]/VAP) was greater than 50.

Sperm Morphology

The cauda sperm suspensions used for the motility assays were diluted 1:10 with 10% neutral buffered formalin in Dulbecco's PBS with 5% sucrose, and the spermatozoa were evaluated for individual sperm morphology. Two hundred spermatozoa (heads only or intact sperm) per animal were evaluated for head and/or flagellar defects by phase-contrast microscopy (×200, total magnification) in wet preparations. Classifications of individual spermatozoa were: a) normal, b) normally shaped head separated from flagellum, c) misshapen head separated from flagellum, d) misshapen head with normal flagellum, e) misshapen head with abnormal flagellum, f) degenerative flagellar defect(s) with a normal head, and g) other flagellar defects(s) with a normal head. Spermatozoa obtained through a tiny incision in the right caput epididymidis of nonperfused rats were similarly processed and classified.

Sperm and Caput/Corpus Protein Analysis

Quantitative analysis of epididymal sperm and tissue proteins has been recently described elsewhere [30, 31]. Briefly, sperm remaining in the HBSS after videotaping of motility were washed twice in buffer without BSA and centrifuged (1000 × *g*, 15 min, 4°C). After the final wash, sperm were extracted for 1 h at room temperature with 1 ml of 80 mM *n*-octyl-B-glucopyranoside (OBG) in 10 mM Tris, pH 7.2, containing freshly added PMSF. After a final centrifugation (10 000 × *g*, 5 min), the supernatant was removed and frozen (−70°C) until concentration and dialysis. Left caput/corpus epididymal tissues (~100 mg) from all rats were minced with scissors and detergent extracted in 1 ml of 10 mM Tris buffer containing 1 mM EDTA, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 0.25% OBG, pH 7.2, to which 0.2 mM PMSF was freshly added. The extract was homogenized on ice in a ground-glass homogenizer and allowed to sit for 1 h, and after centrifugation (10 000 × *g*, 5 min), the supernatant was removed and frozen (−70°C).

Upon thawing, extracts (sperm, tissue) were desalted and concentrated with 1 mM Tris buffer by two centrifugations (3000 × *g*, 45 min, 4°C) in Centricon-10 units (Amicon, Danvers, MA). Protein concentration was determined using a Pierce protein assay kit (Rockford, IL), and a volume equivalent to 30 μg was solubilized for 30 min at room temperature in 20 μl of sample solubilization buffer consisting of 5.7 g urea, 4 ml 10% Nonidet P-40, 0.5 ml ampholytes (Serva, Heidelberg, Germany; 3–10), and 0.1 g dithiothreitol per 10 ml. A mini 2-dimensional electrophoresis system (Bio-Rad, Richmond, CA) was used. Isoelectric focusing (750 V, 3.5 h) was carried out in gels consisting of 6.24 g urea, 1.5 g acrylamide, 2.25 ml 10% Nonidet P-40, and 0.65 ml ampholytes (Serva; 3–10) per 10 ml. Molecular weight separation was carried out in 14% acrylamide gels (200 V, 45 min). Gels were silver stained using a Daiichi silver-staining kit (Integrated Separation Systems; Daiichi Pure Chemical Co. Ltd., Highpark, MA). A Kepler 2-dimensional gel analysis system (Large Scale Biology Corp., Rockville, NM) was used for background correction, spot matching, and spot area quantitation. Images were acquired by transmittance at 80-μm spatial resolution and 4096 gray levels on an Ektron 1412 scanner (Kepler 2D gel system) and converted to 256 gray levels. Quantitation was done by fitting 2-dimensional gaussian

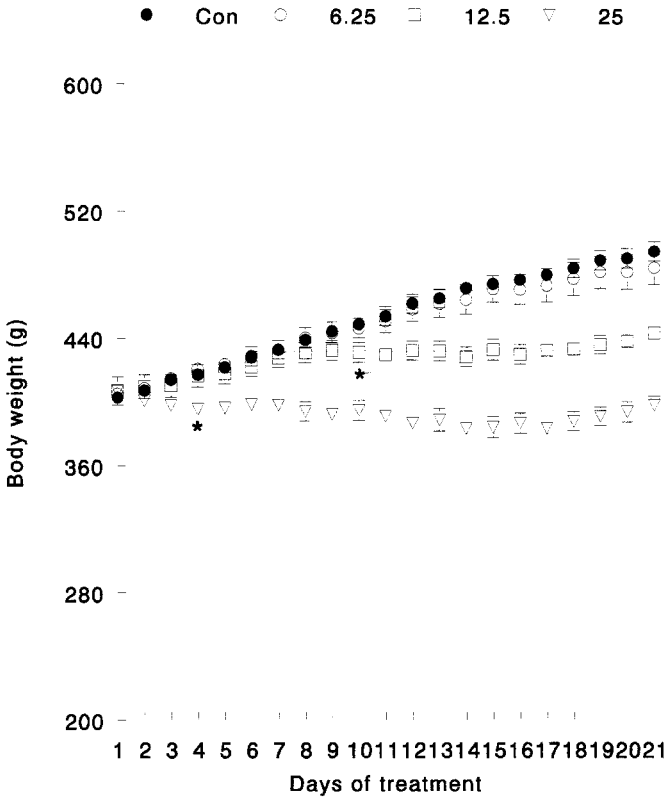

FIG. 1. Effect of sympathectomy on the body weight gain of control rats and rats treated with guanethidine for 21 days. Means ± SEM (n = 10 per group). The asterisks represent the day of treatment on which when the results started to be different from those for the control group ($p < 0.05$).

distributions to the density distribution of the spot area after background subtraction.

Experiment 2

In an attempt to correlate changes in cauda epididymidis norepinephrine levels and sperm transit time in a shorter period of treatment, rats were randomly assigned to two groups of 8 animals each: 0 (saline, vehicle) or 6.25 mg guanethidine/kg BW, administered daily for 7 days, via i.p. injection. At the end of the treatment, the animals were killed by CO_2 inhalation, and the testis, epididymis, and seminal vesicles/coagulating glands were weighed. The right testis and epididymis were used for spermatid and sperm counts, and the left cauda epididymidis was used for norepinephrine determination, as described above.

Statistics

Organ weight, hormone, and sperm number and morphology data from control and experimental treatment groups were compared using the nonparametric Kruskal-Wallis and Mann-Whitney tests. The differences were considered statistically significant when the probability was less than 5%. Sperm protein and motility results were analyzed using the General Linear Models procedure (one-way ANOVA) of the Statistical Analysis System [32]; and if significant differences were found ($p < 5\%$) in the overall ANOVA, the least-square means were compared to determine differences between the vehicle- and guanethidine-treated groups.

RESULTS

Experiment 1

While the animals appeared healthy and active throughout the experiment, body weight gain was significantly decreased at the two higher dosages as compared to that in controls. No difference was observed at 6.25 mg/kg (Table 1). For the highest dosage the decrease was detectable as early as the 4th day of treatment, and in the animals treated with 12.5 mg/kg it was detectable by the 10th day (Fig. 1).

There was a lack of dose responsiveness in organ weight increases (Table 1). Testis weight was unaffected by guanethidine treatment, but there was a significant increase in the weight of epididymis at 6.25 and 12.5 mg/kg. This increase in epididymis weight was attributed to the increase in the weight of the cauda region, as caput/corpus weight was unaffected. The seminal vesicles/coagulating glands were significantly heavier in all treated groups.

Norepinephrine levels were reduced to undetectable levels in the cauda epididymidis at all dosage levels (Table 2). Neither serum testosterone nor LH levels were affected in a treatment-related manner, but serum LH levels were significantly decreased in the animals treated with 12.5 mg/kg guanethidine (Table 2).

Table 3 shows that the number of homogenization-resistant spermatids per testis and the daily sperm production per testis were unchanged in all treatment groups. Neither the sperm number (Table 3) nor the sperm transit time (Fig. 2) in the caput/corpus regions showed significant differences when compared to the control values. However, at the level of the cauda epididymidis, both sperm number and sperm transit time were significantly increased at 6.25 mg/kg (Table 3 and Fig. 2). Neither sperm morphology nor any of the sperm motion parameters were significantly altered by guanethidine exposure (Table 4). However, there was a tendency in the treated groups toward an increased number of decapitate sperm. The histologic analysis of tes-

TABLE 2. Catecholamine, serum and parenchyma testosterone (without and with hCG stimulation), and serum LH levels after 21-day treatment with guanethidine (mean ± SEM; n = 10).

	Dosages (mg/kg)[a]			
Catecholamine/Hormone	0	6.25	12.5	25
Norepinephrine (ng/mg)	0.93 ± 0.10	ND	ND	ND
Serum testosterone (ng/ml)	2.16 ± 0.42	3.21 ± 0.74	1.36 ± 0.44	1.27 ± 0.24
Parenchyma testosterone (ng/g)	505.34 ± 82.63	609.73 ± 67.11	364.54 ± 43.57	297.75 ± 36.49*
Parenchyma testosterone (hCG stimulated) (ng/g)	1576.33 ± 285.24	2038.92 ± 230.15	1361.66 ± 195.62	994.55 ± 171.52
Serum LH (ng/ml)	0.23 ± 0.05	0.18 ± 0.03	0.10 ± 0.02*	0.23 ± 0.06

[a] ND, nondetectable.
* Significantly different from the control group ($p < 0.05$).

TABLE 3. Sperm numbers after 21-day (experiment 1) or 7-day (experiment 2) guanethidine treatment (mean ± SEM).

	Dosages (mg/kg)					
	Experiment 1[a]				Experiment 2[a]	
Sperm number	0	6.25	12.5	25	0	6.25
Spermatid number (× 10^6/testis)	174.04 ± 11.74	174.47 ± 14.04	200.72 ± 10.35	153.65 ± 14.67	208.25 ± 9.02	196.43 ± 12.97
Daily sperm production (× 10^6/testis/day)	28.53 ± 1.93	28.60 ± 2.30	32.91 ± 1.70	25.19 ± 2.40	34.14 ± 1.48	32.20 ± 2.13
Caput/corpus epididymal sperm number (× 10^6/organ)	95.21 ± 11.18	84.24 ± 7.72	66.90 ± 12.58	71.28 ± 14.62	121.03 ± 7.49	108.94 ± 10.41
Cauda epididymal sperm number (× 10^6/organ)	176.21 ± 11.78	283.09 ± 9.90*	235.41 ± 23.06	178.78 ± 19.98	218.24 ± 8.47	293.82 ± 24.47*

[a] n = 6 of 10 (experiment 1) or 8 (experiment 2).
* Significantly different from the control group ($p < 0.05$).

tis and epididymis sections revealed no obvious differences between control and guanethidine-treated groups.

A quantitative analysis of detergent-extracted cauda epididymal proteins by SDS-PAGE revealed no differences for cauda sperm (not shown) but showed treatment-related diminutions in seven proteins in homogenates of caput/corpus (Fig. 3). Proteins CC3 (45 kDa, pI 4.4), CC8 (40 kDa, pI 4.6), and CC20 (44 kDa, pI 5.4) were diminished significantly ($p < 0.05$) in the caput/corpus homogenate in all guanethidine-exposed animals. Proteins CC21 (16 kDa, pI 4.5), CC25 (15 kDa, pI 4.7), CC27 (26 kDa, pI 5.1), and CC29 (65 kDa, pI 4.2) were diminished significantly only in the 12.5 and 25 mg/kg groups.

Experiment 2

Norepinephrine levels in the cauda epididymidis of the 7-day guanethidine-treated animals were 7.4% of the control values (data not shown). There was a significant increase in the weights of the seminal vesicles/coagulating glands and epididymis (Table 1) and a significant increase in the number of sperm in the cauda epididymidis (Table 3) of the denervated rats. Sperm transit time also was significantly increased in the cauda epididymidis as compared with that in the control group (Fig. 2).

DISCUSSION

In the present study the levels of norepinephrine in the cauda epididymidis of guanethidine-treated animals were either undetectable (after 3 wk of treatment) or markedly lower than control values (1 wk treatment), confirming that denervation was effective.

There was a significant increase in the number of sperm and in the sperm transit time in the cauda epididymidis of rats treated with 6.25 mg/kg. This is consistent with the loss of innervation in this region, accounting for decreased contractility of the duct, which delayed sperm transit, even after 1 wk of treatment. It has been suggested that in addition to regulating seminal emission, adrenergic neurotransmitters may be involved in the control of epididymal sperm transit [2, 5, 21, 33], thus explaining the increased concentration of spermatozoa observed in denervated animals.

Among the multiple epididymal functions there is a resorption of fluids, resulting in a progressive concentration of sperm within the lumen [34]. The process of fluid resorption in the epididymis has also been shown to be under the control of testicular androgens as well as of adrenergic neurotransmitters (for references, see [20]). In the present study, an increased concentration of sperm (i.e., sperm/g tissue, not shown) at 6.25 mg/kg in the cauda epididymidis was observed in rats presenting normal testosterone levels, reinforcing the notion that sympathetic nerves may be involved in the control of epididymal mechanisms of fluid resorption. The lack of dose responsiveness in the weight of the epididymis, and the failure to observe increases in cauda sperm number and transit time at 12.5 and 25 mg/kg, are most likely related to systemic alterations and a possible toxicity associated with these higher exposures, as evidenced by the decreases in body weight gain.

TABLE 4. Effect of 21-day guanethidine administration on sperm motion parameters (mean ± SEM; n = 6).

	Dosages (mg/kg)			
Parameter	0	6.25	12.5	25
Motile sperm (%)	65.87 ± 3.19	63.67 ± 5.77	67.75 ± 1.14	60.77 ± 4.88
Progressive motile sperm (%)	50.67 ± 2.14	49.87 ± 7.18	38.67 ± 0.87	33.48 ± 8.60
Average cell path velocity (mm/s)	123.98 ± 2.50	131.32 ± 4.69	120.86 ± 3.27	112.45 ± 6.71
Straight line velocity (μm/s)	79.37 ± 3.54	86.62 ± 7.54	78.80 ± 2.91	64.50 ± 8.32
Point-to-point track velocity (mm/s)	304.77 ± 9.37	303.82 ± 9.91	284.75 ± 11.39	258.37 ± 24.33
Amplitude of lateral head displacement	20.33 ± 0.40	21.78 ± 0.26	19.86 ± 0.78	18.35 ± 1.25
Beat/cross frequency (Hz)	24.35 ± 0.90	22.02 ± 0.76	21.72 ± 1.08	26.10 ± 1.98
Straightness (%)	64.23 ± 1.95	65.27 ± 3.64	62.49 ± 1.53	56.53 ± 4.12
Linearity (%)	25.77 ± 0.76	27.97 ± 1.62	26.29 ± 0.72	24.20 ± 1.09
Normal shaped sperm (%)	91.84 ± 2.59	82.83 ± 9.16	84.08 ± 7.93	79.08 ± 9.29
Sperm with head defects (%)	2.33 ± 0.59	1.50 ± 0.62	2.92 ± 0.85	0.75 ± 0.66
Sperm with tail defects (%)	5.83 ± 3.03	15.67 ± 8.82	13.00 ± 7.96	20.17 ± 8.98

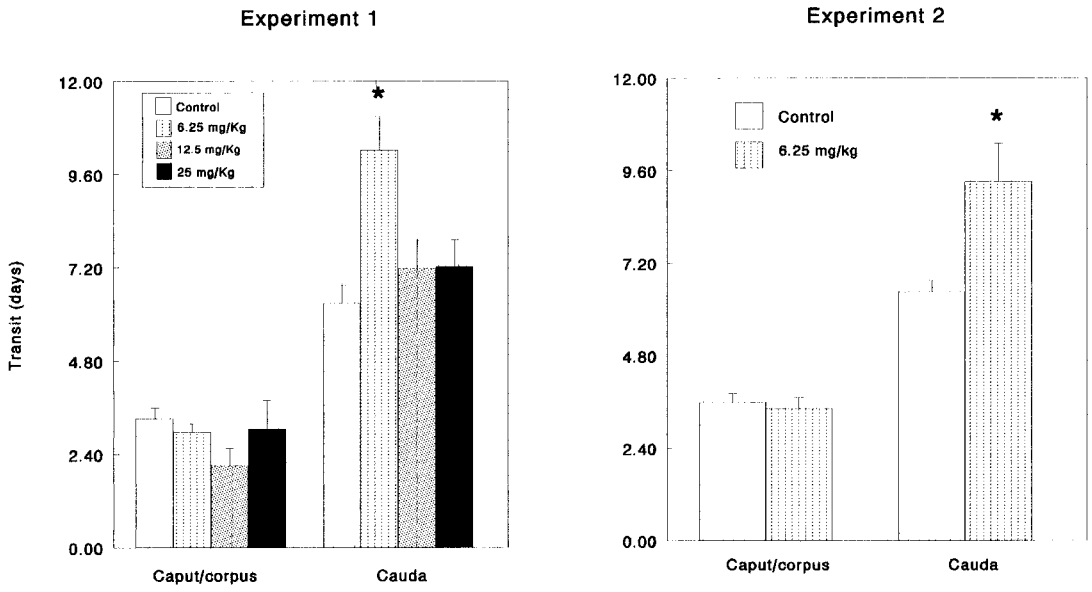

FIG. 2. Caput/corpus and cauda epididymidis sperm transit time in control and 21-day (experiment 1, n = 6 of 10) or 7-day (experiment 2, n = 8) guanethidine-treated groups. Values are expressed as mean ± SEM. *Significantly different from the control group ($p < 0.05$).

Although epididymal function was altered, sperm proteins, motility, and morphology of the sperm were not significantly affected by guanethidine-induced denervation. These results are similar to those of another study [22] in which no differences were found between control and IMG-ablated rats in the percentage of motile cauda epididymal spermatozoa. Similarly, Ricker et al. [35] failed to produce any significant change in cauda epididymal sperm-associated proteins after sympathetic denervation by surgical removal of the IMG. These results are in agreement with the lack of an effect on fertility after in utero insemination with sperm retrieved from the cauda epididymidis of guanethidine-treated or surgically denervated rats (presented in the companion paper [36]).

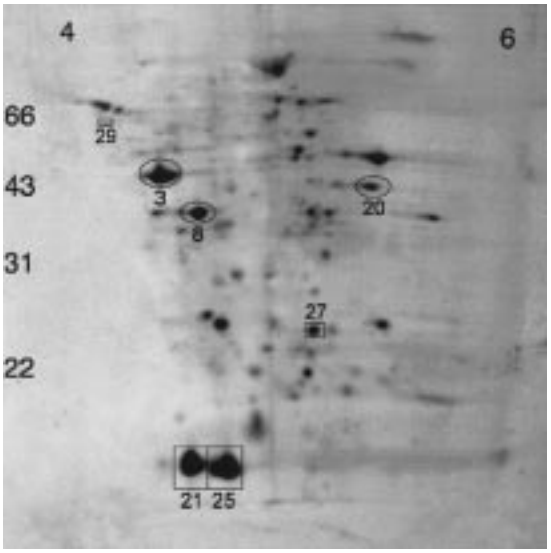

FIG. 3. Silver-stained 2-dimensional profiles of proteins from the caput/corpus epididymidis; y-axis: molecular weights ($\times 10^{-3}$), x-axis: pI range. Proteins numbered 3, 8, 20, 21, 25, 27, and 29 refer to as CC3, CC8, CC20, CC21, CC25, CC27, and CC29 in the text. Note that proteins surrounded by circles are diminished at all guanethidine exposures. Proteins surrounded by squares are diminished with the two highest dosages only (12.5 and 25 mg/kg).

In the present study we have found differences between control and guanethidine-treated rats in some caput epididymal tissue proteins. Therefore, as sperm proteins in the cauda epididymidis are not altered, the changes in caput proteins must reflect proteins of epithelial or neuromuscular origin. At the lower dosage, the changes in proteins might be related to the sperm transit delay in the cauda epididymidis, particularly if the same proteins are altered in the cauda. On the other hand, with the higher dosages, the effect on the proteins might be related to more generalized guanethidine toxicity as discussed above. No significant changes in caput epididymal fluid proteins were detected 1–4 wk after surgical IMG removal [35].

Seminal vesicle/coagulating gland weights were markedly increased in all treated groups, and the epididymis weight was increased at 6.25 and 12.5 mg/kg, presumably due to persistent accumulation of unexpressed secretion and retention of sperm at the cauda epididymidis, respectively. Seminal emission is brought about by contraction of the smooth muscle of the epididymis and accessory sex organs, an autonomically regulated process. Of the genital organs, those having a thicker muscular coat (such the vas deferens, cauda epididymidis, and seminal vesicle) receive rather abundant amounts of noradrenergic terminals and are apparently designed for a rapid, forced ejection of their seminal contents [3, 37]. After sympathectomy, they are typically enlarged, showing signs of marked distention by retained sperm and secretory products [2, 12, 20, 33, 38].

Neither testis weight nor spermatid numbers and daily sperm production were changed by treatment with guanethidine. These results agree with those of other authors who failed to show any testicular effects in male rats after chemical (with guanethidine) [20] or surgical denervation [22].

While guanethidine has a proven selective effect on the peripheral postganglionic sympathetic neurons and there is evidence that it does not cross the blood-brain barrier [7], central neurotoxic effects may be induced by its action on central areas of the brain that lack this barrier [39]. However, no dosage-related effect on LH was observed; a significant decline was observed only at 12.5 mg/kg. Normal levels of plasma testosterone are a common finding after

guanethidine-induced sympathectomy [5, 38]. Indeed, testosterone was decreased significantly only when testicular parenchyma from the group treated with 25 mg/kg was incubated ex vivo without hCG. Thus, it does not appear as though guanethidine exerts effects in the hypothalamic-pituitary-gonadal axis.

We conclude that low-dosage guanethidine exposure denervates the epididymis within 1 wk, thereby delaying epididymal transit, but does not produce qualitative changes in the sperm, i.e., sperm motion, sperm morphology, or sperm protein. At higher dosages, guanethidine seems to produce systemic alterations, impeding the epididymal response to chemical sympathectomy. The relationship between delayed cauda epididymal transit and fertility is discussed in the companion paper [36].

ACKNOWLEDGMENTS

We express our sincere gratitude to W. Keith McElroy, Joan Metcalf Hedge, and Joy Hein for skilled technical assistance; to Dr. Gary Held for helping us in preparing the gel plate; and to Dr. Robert E. Chapin and Dr. L. Earl Gray Jr. for their comments on the manuscript.

REFERENCES

1. Kumazawa T, Mizumura K, Sato J. Response properties of polymodal receptors studied using in vivo testis superior spermatic nerve preparations of dogs. J Neurophysial (Bethesda) 1987; 57:702–711.
2. Hodson N. Sympathetic nerves and reproductive organs in the male rabbit. J Reprod Fertil 1965; 10:209–220.
3. Sjöstrand NO. The adrenergic innervation of the vas deferens and the accessory male genital glands. Acta Physiol Scand 1965; 65(suppl 257):7–82.
4. Smith ER. The canine prostate and its secretion. In: Thomas JA, Singhal RL (eds.), Molecular Mechanisms of Gonadal Hormone Action. Baltimore: University Park Press; 1975: 167–204.
5. Lamano Carvalho TL, Petenusci SO, Favaretto ALV, Kempinas WG. Morphometric and biochemical evaluation of rat prostate and seminal vesicle following chemical sympathectomy with guanethidine. Arch Int Physiol Biochem 1990; 98:225–230.
6. Johnson EM Jr, O'Brien F, Werbitt R. Modification and characterization of the permanent sympathectomy produced by the administration of guanethidine to newborn rats. Eur J Pharmacol 1976; 37:45–54.
7. Johnson EM Jr, Manning PT. Guanethidine-induced destruction of sympathetic neurons. Int Ver Neurobiol 1984; 25:1–37.
8. Manning PT, Powers CW, Schmidt RE, Johnson EM Jr. Guanethidine-induced destruction of peripheral neurons occurs by an immune-mediated mechanism. J Neurosci 1983; 3:714–724.
9. Lamano Carvalho TL, Kempinas WG, Favaretto ALV. Morphometric evaluation of rat testis, epididymis and vas deferens following chemical sympathectomy with guanethidine. Ann Anat 1993; 175:453–457.
10. Setchell BP, Brooks DE. Anatomy, vasculature, innervation and fluids of the male reproductive tract. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction. New York: Raven Press; 1988: 753–836.
11. Gannon BJ, Iwayama T, Burnstock B, Gerkens J, Mashford ML. Prolonged effects of chronic guanethidine treatment on the sympathetic innervation of the genitalia of male rats. Med J Aust 1971; 2:207–208.
12. Evans B, Gannon BJ, Heath JW, Burnstock G. Long-lasting damage to the internal male genital organs and their adrenergic innervation in rats following chronic treatment with the antihypertensive drug guanethidine. Fertil Steril 1972; 23:657–667.
13. Burnstock G, Evans B, Gannon BJ, Heath JW, James V. A new method of destroying adrenergic nerves in adult animals using guanethidine. Br J Pharmacol 1971; 43:295–301.
14. Heath JW, Evans BK, Gannon BJ, Burnstock G, James VB. Degeneration of adrenergic neurons following guanethidine treatment: an ultrastructural study. Virch Arch (Cell Pathol) 1972; 11:182–197.
15. Ricker DD, Crone JK, Chamness SL, Klinefelter GR, Chang TSK. Partial sympathetic denervation of the rat epididymis permits fertilization but inhibits embryo development. J Androl 1997; 18:131–138.
16. El-Badawi A, Schenk EA. The distribution of cholinergic and adrenergic nerves in the mammalian epididymis. Am J Anat 1967; 121:1–14.
17. Kuntz A. The Autonomic Nervous System. Philadelphia: Lea and Febiger; 1953: 289–298.
18. Bacq ZM. Impotence of the male rodent after sympathetic denervation of the genital organs. Am J Physiol 1931; 96:321–330.
19. Simeone FA. A neuromuscular mechanism in the ductus epididymis and its impairment by sympathetic denervation. Am J Physiol 1933; 103:582–591.
20. Lamano Carvalho TL, Favaretto ALV, Petenusci SO, Kempinas WG. Prepubertal development of rat prostate and seminal vesicle following chemical sympathectomy with guanethidine. Braz J Med Biol Res 1993; 26:639–646.
21. Ricker DD, Chang TSK. Neuronal input from the inferior mesenteric ganglion (IMG) affects sperm transport within the rat cauda epididymis. Int J Androl 1996; 19:371–376.
22. Billups KL, Tillman SL, Chang TSK. Ablation of the inferior mesenteric plexus in the rat: alteration of sperm storage in the epididymis and vas deferens. J Urol 1990; 143:625–629.
23. Robaire B, Hermo L. Efferent ducts, epididymis, and vas deferens: structure, functions, and their regulation. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction. New York: Raven Press; 1988: 219–224.
24. Klinefelter GR, Laskey JW, Roberts NL, Slott V, Suarez JD. Multiple effects of ethane dimethanesulfonate on the epididymis of adult rats. Toxicol Appl Pharmacol 1990; 105:271–287.
25. Klinefelter GR, Laskey JW, Roberts NL. In vitro/in vivo effects of ethane dimethanesulfonate on Leydig cells of adult rats. Toxicol Appl Pharmacol 1991; 107:460–471.
26. Laskey JW, Klinefelter GR, Kelce WR, Ewing LL. Effect of ethane dimethane-sulphonate on adult and immature rabbit Leydig cells: comparison with EDS treated rat Leydig cells. Biol Reprod 1994; 50: 1151–1160.
27. Goldman JM, Cooper RL, Rehnberg GL, Hein JF, McElroy WK, Gray LE Jr. Effects of low, subchronic doses of methoxychlor on the rat hypothalamic-pituitary-reproductive axis. Toxicol Appl Pharmacol 1986; 86:474–483.
28. Robb GW, Amann RP, Killian GJ. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J Reprod Fertil 1978; 54:103–107.
29. Klinefelter GR, Gray LE Jr, Suarez JD. The method of sperm collection significantly influences sperm motion parameters following ethane dimethanesulphonate administration in the rat. Reprod Toxicol 1991; 5:39–44.
30. Klinefelter GR, Laskey JW, Ferrell J, Suarez JD, Roberts NL. Discriminant analysis indicates a single sperm protein (SP22) is predictive of fertility following exposure to epididymal toxicants. J Androl 1997; 18:139–150.
31. Klinefelter G, Suarez JD. Toxicant-induced acceleration of epididymal sperm transit: androgen-dependent proteins may be involved. Reprod Toxicol 1997; 11:511–519.
32. SAS. SAS User's Guide: Basics, Version 5. Cary, NC: Statistical Analysis System Institute Inc.; 1982.
33. Hodson N. Role of the hypogastric nerves in seminal emission in the rabbit. J Reprod Fertil 1964; 7:113–122.
34. Brooks DE. Epididymal functions and their hormonal regulation. Aust J Biol Sci 1983; 36:205–221.
35. Ricker DD, Chamness, SL, Hinton BT, Chang TSK. Changes in luminal fluid protein composition in the rat cauda epididymidis following partial sympathetic denervation. J Androl 1996, 17:117–126.
36. Kempinas WG, Suarez JD, Roberts NL, Strader LF, Ferrell J, Goldman JM, Narotsky MG, Perreault SD, Everson DP, Ricker DD, Klinefelter GR. Fertility of rat epididymal sperm after chemically and surgically induced sympathectomy. Biol Reprod 1998; 59:897–904.
37. Lamano Carvalho TL, Hodson N, Blank NLA, Watson PF, Mulderry PK, Bishop AE, Gu J, Bloom SR, Polak JM. Occurrence, distribution and origin of peptide containing nerves of guinea pig and rat male genitalia and the effects of denervation on sperm characteristics. J Anat 1986; 149:121–141.
38. Kempinas WG, Petenusci SO, Rosa e Silva AAM, Favaretto ALV, Lamano Carvalho TL. The hypophyseal-testicular axis and sex accessory glands following chemical sympathectomy with guanethidine of pre-pubertal to mature rats. Andrologia 1995; 27:121–125.
39. Newton BW, Melvin JE, Hamill RW. Central neurotoxic effects of guanethidine: altered serotonin and enkephalin neurons within the area postrema. Brain Res 1987; 404:151–161.