

Discriminant Analysis Indicates a Single Sperm Protein (SP22) Is Predictive of Fertility Following Exposure to Epididymal Toxicants

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ABSTRACT: In a previous study, we found that ethane dimethanesulphonate (EDS) compromised the fertilizing ability of proximal cauda epididymal sperm from the rat within 4 days of exposure, an effect that persisted in castrated, testosterone (T)-implanted animals, establishing direct action on the epididymis. This EDS-induced reduction in fertilizing ability was highly correlated with a quantitative decrease in specific sperm protein. Here we sought to determine whether the fertility of proximal cauda epididymal sperm recovered from animals exposed to a variety of male reproductive toxicants could be predicted by assessing quantitative changes in specific sperm protein(s), or whether more common endpoints (e.g., sperm motility, sperm morphology, serum and epididymal tissue T, cauda epididymal sperm reserves) also are required to predict fertility. Intact adult male rats were dosed with EDS (25 or 50 mg/kg), chloroethylmethanesulphonate (CEMS; 12.5 or 18.75 mg/kg), or epichlorohydrin (EPI; 3 or 6 mg/kg) daily for 4 days. Castrated, T-implanted rats were dosed with hydroxyflutamide (HFLUT; 12.5 or 25 mg/kg) daily for 5 days. On day 5, proximal cauda epididymal sperm

were inseminated *in utero* into receptive, cervically stimulated adult females, and on day 9, fertility (implants/corpora lutea) was assessed. Fertility was decreased by the higher dose of each toxicant ($P < 0.05$) and also by the lower dose of EPI and HFLUT. Likewise, an acidic 22 kDa sperm protein (SP22) was decreased quantitatively ($P < 0.05$) in silver-stained two-dimensional gels by the higher dose of each toxicant as well as by the lower dose of EPI and HFLUT. Although sperm motility and serum T were altered by specific exposures, these endpoints were not useful in predicting fertility. In contrast, SP22 was highly correlated ($P < 0.0001$; $r^2 = 0.83$) with fertility. Indeed, the amount of SP22 correctly predicted 90% and 94% of the fertile (>50% fertility) and subfertile (<50% fertility) animals, respectively, when discriminant analysis was performed. Thus, the amount of SP22 in a cauda epididymal sperm sample may be a useful predictor of fertility in toxicant-treated animals.

Key words: Epididymis, biomarker.

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We recently demonstrated, using *in utero* insemination, that a single intraperitoneal (i.p.) injection of 50 mg/kg ethane dimethanesulphonate (EDS) decreased the fertilizing ability of sperm from the proximal cauda epididymidis of adult male rats within 4 days (Klinefelter et al, 1994b). When animals were castrated and implanted with testosterone (T)-filled Silastic capsules, which maintained serum T at control levels, fertilizing ability remained normal. In contrast, when castrated, T-implanted rats received 50 mg/kg EDS, fertilizing ability was reduced to a level that was similar to that observed in EDS-treated, intact animals. These data established that EDS acts directly on the epididymis to compromise sperm mat-

uration. Moreover, we demonstrated that both the concentration of T in the caput/corpus epididymidis and the amount of an acidic 18-kDa sperm protein were significantly correlated with fertilizing ability.

The objectives of the present study were twofold. First, we wished to determine if the known male reproductive toxicants, chloroethylmethanesulphonate (CEMS), epichlorohydrin (EPI), and hydroxyflutamide (HFLUT) also would render proximal cauda epididymal sperm infertile within 4 days. Second, we hoped to determine which, if any, of the measured endpoints were correlated with, and predictive of, fertility. These chemicals were selected based on their ability to alter epididymal structure and/or function. That is, exposure to CEMS produces lesions in the epididymis that are similar to those produced by EDS (Klinefelter et al, 1994), e.g., the disappearance of clear cells in the proximal cauda epididymidis, a decrease in specific sperm motion parameters, and a diminution in specific proximal cauda sperm proteins. Exposure to EPI has been shown to compromise both fertility (Jones et al, 1969; Cooper et al, 1974; Toth et al, 1989) and sperm motion (Slott et al, 1990). Finally, HFLUT is a known

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antiandrogen capable of perturbing androgen-dependent tissues such as the epididymis (Dhar et al, 1982; de las Heras et al, 1988). Of these three chemicals, only EPI had previously been shown to alter fertility within a few days of exposure (Cooper et al, 1974).

Although the selection of effective chemicals was critical to successfully addressing the two objectives of this study, the post-exposure sampling time, the sampling site, and the manner in which fertility was assessed were equally important. Sperm require 4 days to move through the caput/corpus epididymidis (Robb et al, 1978). Thus, sperm recovered from the proximal cauda epididymidis 4 days after toxicant exposure resided in the epididymis during exposure. Moreover, sperm in the proximal cauda epididymidis are more homogeneous with respect to age than those in the distal cauda epididymidis, thereby providing greater sensitivity for detection of a toxicant-induced alteration (Klinefelter et al, 1990). Finally, the likelihood of detecting a toxicant-induced alteration in the fertility of proximal cauda sperm was maximized by inseminating *in utero* a threshold number of sperm, i.e., a number that falls on the linear portion of the dose-response curve, beneath maximum fertility (Amann, 1986; Klinefelter et al, 1994b). To ensure that equal numbers of progressively motile sperm were inseminated regardless of treatment and to increase the likelihood of detecting other endpoints that might be correlated with the fertility of epididymal sperm, we selected doses of each test chemical that would exert negligible, if any, effect on sperm motion.

In addition to the above experimental considerations, a castrated, T-implanted animal model was selected to evaluate the impact of the antiandrogen HFLUT. The high endogenous androgen concentrations (T and its 5 α -reduced metabolite, dihydrotestosterone [DHT]) in the epididymis of an intact animal, together with the fact that DHT has a tenfold greater affinity for the androgen receptor than T, prompted the use of castration and T replacement to increase the sensitivity of the epididymis to antiandrogen exposure. Indeed, the relative insensitivity of the epididymis in the intact animals to antiandrogen has been described previously (Dhar et al, 1982; de las Heras et al, 1988). It is now well documented that when sperm are isolated within 4 days from the proximal cauda epididymidis of castrated, T-implanted animals and inseminated *in utero*, fertility is completely normal (Dyson and Orgebin-Crist, 1973; Klinefelter et al, 1994b). Thus, castrated, T-implanted controls would be comparable to intact controls with respect to their fertility and sensitivity to toxicant exposure.

Herein we report that each of the test chemicals (EDS, CEMS, EPI, and HFLUT) significantly decreases fertility of sperm from the proximal cauda epididymidis within 4 days of exposure. The only other endpoint that was equally affected by treatment was the quantifiable area of an acidic 22-kDa protein (SP22) resolved on silver-stained, two-di-

mensional (2-D) gels of detergent extracts of these sperm. Moreover, the amount of SP22 was used in discriminant analysis to correctly predict the fertility of 46 of 50 animals when the cutoff for fertility/subfertility was set at 50%.

Methods

Animals

Both adult (90–120-day-old) male and (60–90-day-old) female Sprague-Dawley rats were purchased from Harlan-Sprague-Dawley, Inc. (Indianapolis, Indiana) and housed two per cage (clear plastic, 20 × 25 × 47 cm) with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperature (22°C) and humidity (40–50%), and were given Purina laboratory rat chow and tap water *ad libitum*. Males receiving toxicants were maintained in a 14-hour-light:10-hour-dark (14L:10D) schedule, with lights out at 7 PM eastern standard time (EST). The females and vasectomized males were housed in a 14L:10D reversed light schedule with lights out at 9 AM EST.

Intact males were numbered and randomly assigned to one of the following treatment groups: VEH-I (intact vehicle controls 30% dimethylsulfoxide [DMSO] in water), 25 mg/kg EDS, 50 mg/kg EDS, 12.5 mg/kg CEMS, 18.75 mg/kg CEMS, 3 mg/kg EPI, and 6 mg/kg EPI. Castrated males were numbered and randomly assigned either to the VEH-C (castrated vehicle controls; 15% ethanol in Dulbecco's phosphate buffered saline [DPBS]), 12.5 mg/kg HFLUT, or 25 mg/kg HFLUT treatment group. The higher doses for each of the toxicants were selected based on preliminary data indicating efficacy in reducing fertility. Each of the chemicals was administered via i.p. injection, with EDS given as a single dose on day 1, CEMS and EPI given as four daily doses, and HFLUT given as five daily doses. EDS was kindly provided by the Sterling Research Group and HFLUT was provided by Schering Corporation.

Animals that were castrated and given T implants were brought to a surgical plane of anesthesia with halothane, the testes were removed, and a 2.5-cm T-filled silastic capsule was placed in the subscapular space (Ewing et al, 1977) just prior to the injection of toxicant. During castration, each testis was exposed individually via an abdominal incision, taking care to avoid trauma and bleeding. A ligature of 4-0 silk was tightly drawn around the testicular artery and efferent ducts, fascia between the ligature and testis was cut, the testis was removed, and the epididymis was returned to the scrotum. 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, North Carolina).

On day 5, individual males representing the various treatment groups within a particular experimental block were killed approximately every 20 minutes. Each animal was anesthetized with halothane and killed by cervical dislocation. The *in utero* insemination of epididymal sperm, collection of serum and tissue, and sperm motion and morphology analyses were performed as described below.

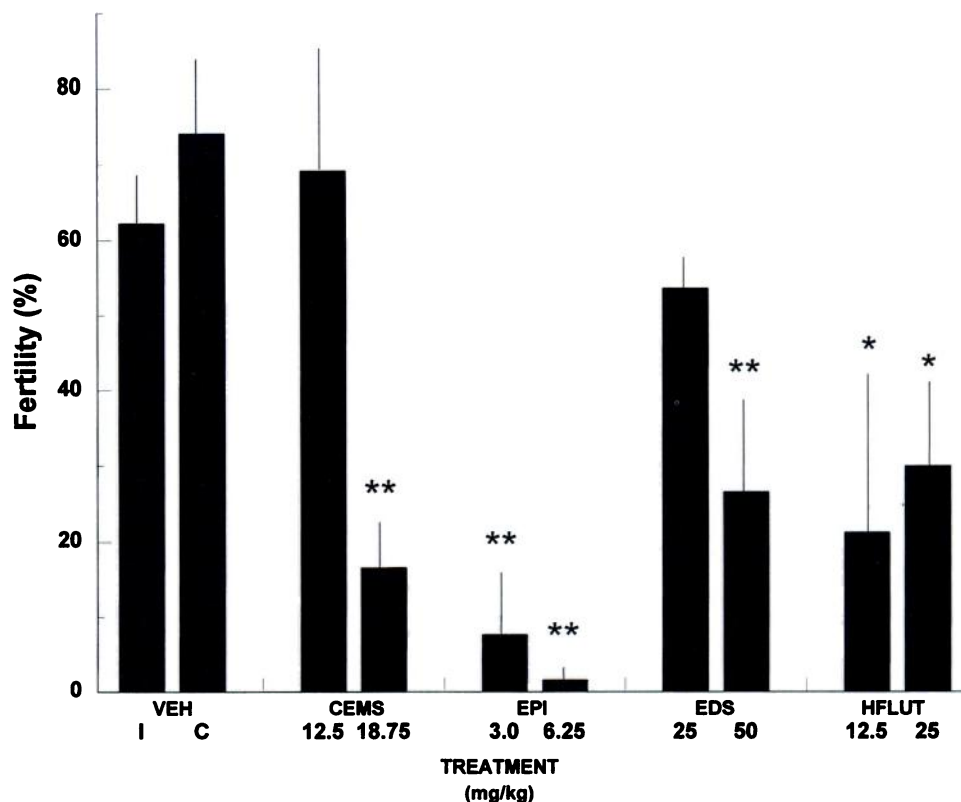


FIG. 1. Graph showing the treatment-related changes in fertility of proximal cauda epididymal sperm expressed as a percentage equivalent to the total number of fetal implants on day 9 of gestation divided by the total number of corpora lutea of pregnancy. Vehicle controls (intact [I] [$N = 6$] and castrated with T implant [C] [$N = 5$]), chloroethylmethanesulphonate (CEMS; 12.5 mg/kg [$N = 4$] and 18.75 [$N = 7$]), epichlorohydrin (EPI; 3 mg/kg [$N = 3$] and 6 mg/kg [$N = 7$]), ethane dimethanesulphonate (EDS; 25 mg/kg [$N = 3$] and 50 mg/kg [$N = 5$]), hydroxyflutamide (HFLUT; 12.5 mg/kg [$N = 3$] and 25 mg/kg [$N = 7$]). EPI, CEMS, and EDS were compared with intact vehicles (VEH-I) and HFLUT was compared with castrated vehicles (VEH-C). The number of corpora lutea per pregnant female ranged from 6 to 14. Significant reduction in fertility compared to appropriate controls is indicated by ** ($P < 0.01$) and * ($P < 0.05$).

Artificial Insemination

The procedure for *in utero* insemination is similar to that described previously (Klinefelter et al, 1994b). Briefly, a cohort of females were synchronized with 80 μ g subcutaneous (s.c.) of luteinizing hormone releasing hormone (LHRH) agonist (Sigma Chemical Co., St. Louis, Missouri; L 4513) 115 hours 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 for 30 minutes. Typically a copulatory plug could be found at the bottom of a wire-bottom cage if repeated intromissions occurred. Receptive females (50–70% of those injected with LHRH) were moved, along with toxicant-treated males, to the surgical suite.

The isolation and preparation of proximal cauda sperm for insemination were the same as described previously (Klinefelter et al, 1994b). Briefly, the cauda epididymides of each treated male were placed in a 35-mm culture dish containing 2 ml of medium 199 (M199; Sigma, M 3769 with Earle's salts and phenol red-free), buffered and supplemented as described previously (Klinefelter et al, 1992) with the exception that only 10 nM T and DHT were added. Just prior to the inseminations, 0.25 mg/ml bovine lipoprotein (Sigma, L 3626) was added to the medium. Sperm were allowed to diffuse after piercing the epididymal tubule with

a #11 scalpel blade. The dish was allowed to shake gently and, after 5 minutes of dispersion, an aliquot of sperm was diluted with fixative (10% formalin in phosphate-buffered saline with 10% sucrose, pH 7.4) and counted using a hemacytometer; sperm concentrations ranged from 20–40 $\times 10^6$ /ml. Within 15 minutes, each uterine horn was injected with a volume containing 5×10^6 sperm, a value that results in 75% fertility in control males (Klinefelter et al, 1994b).

A single 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, mid-ventral incision. A fine, curved forceps was used to elevate each horn while the 0.1 to 0.2-ml 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.

Nine days later, each inseminated female was anesthetized again with halothane and killed via cervical dislocation. The uteri were exposed and implanted fetuses were enumerated. The ovaries were excised and the number of corpora lutea of pregnancy was count-

Table 1. The effect of various epididymal toxicants on serum testosterone (T), caput/corpus tissue T, and cauda sperm reserves

| | VEH-I | VEH-C | CEMS 12.5 mg/kg | CEMS 18.75 mg/kg |
|---------------------------------------|------------------|-----------------|--------------------|---------------------|
| Serum T (ng/ml) | 2.12 ± 0.49* (6) | 3.32 ± 0.55 (5) | 0.725 ± 0.028† (4) | 0.640 ± 0.133† (7) |
| Caput/corpus T (ng/g) | 8.50 ± 2.86 (6) | 7.62 ± 3.80 (5) | 6.10 ± 3.70 (4) | 2.86 ± 0.76 (7) |
| Cauda reserved (×10 ⁶) | 266 ± 40 (6) | 257 ± 41 (5) | 173 ± 30 (4) | 153 ± 21 (7) |

Chloroethylmethanesulphonate (CEMS), epichlorohydrin (EPI), and ethane dimethanesulphonate (EDS) were compared with intact vehicles (VEH-I) and hydroxyflutamide (HFLUT) was compared with castrated vehicles (VEH-C).

* Values represent the mean ± standard error of the mean (SEM) and in parentheses, *N*.

† *P* < 0.01.

ed. The fertility of each male was expressed as a percentage equivalent; number of implants/number of corpora lutea × 100.

Serum and Caput/Corpus Epididymidis Testosterone

Blood (2 ml) was collected from the left ventricle of each anesthetized animal just prior to cervical dislocation. Serum was obtained following centrifugation (3,000 × *g*, 15 minutes, 4°C) in serum separator tubes and stored at -70°C until the T assay was performed using a Coat-A-Count kit (Diagnostic Products Corp., Los Angeles, California) procedure.

The right caput/corpus epididymidis was removed, weighed, and frozen at -70°C until steroid extraction. Upon thawing, steroids were extracted as described previously (Klinefelter et al, 1994b). Briefly, the tissue was minced in 25% methanol (10 ml/g) and homogenized on ice with a tissumizer (Tekmar, Cincinnati, Ohio) using 40% output for 30–40 seconds. After centrifugation (3,000 × *g*, 10 minutes), the supernatant was aspirated through a 6-ml, methanol-activated, Octadecyl C₁₈ (BakerBond; Thomas Scientific, 8281H50). The pellets were re-extracted with 25% methanol, and after centrifugation, this supernatant also was added to the C₁₈ column. After drying the column with *n*-hexane, androgens were eluted with two 4-ml washes of benzene:acetone (4:1, v/v). Recovery of androgens typically exceeded 95%. The organic extract was dried under vacuum, reconstituted with 1 ml of 0.05 M Tris (pH 8.0) with 0.1% gelatin, and diluted 1:10 for T radioimmunoassay (RIA).

Cauda Epididymal Sperm Reserves

After insemination, the caudae epididymides that were used as a source of proximal cauda sperm were frozen at -70°C for

subsequent homogenization and enumeration of sperm as described previously (Robb et al, 1978). The number of sperm liberated from the proximal caudae into the initial 2-ml suspension was determined by hemacytometer counting. To obtain the total number of cauda epididymal sperm, this number was combined with the number of sperm recovered following homogenization of the remaining cauda tissue

Sperm Motility

Immediately after a female was inseminated, an aliquot of the same sperm suspension was removed and videotaped for subsequent sperm motion analysis (Klinefelter et al, 1992, 1994a). Briefly, a 50-μl aliquot of sperm was diluted with 1 ml of medium and the sample was drawn into a 100-μm-deep glass cannula (Vitro Dynamics, Rockaway, New Jersey). The sample was visualized using the Hamilton Thorn integrated visual optical system (IVOS) (Hamilton Thorn Research, Inc., Beverly, Massachusetts) and videotaped on a VCR recorder (JVC, D970U). A sample generating a field containing 15–20 sperm was optimal for the subsequent analysis. The images later were analyzed (30 frames at 60 frames/sec) with the IVOS using the Hamilton Thorn IVOS Toxicology V10 software. A minimum of 200 sperm were analyzed per sample. Sperm were considered motile when average path velocity (VAP) exceeded 25 μm/sec and progressively motile if their straightness (VSL [straightline velocity]/VAP) was greater than 80.

Sperm Morphology

An aliquot of the fixed sperm suspension, i.e., the suspension used for hemacytometer counting, was used for a morphological

Table 2. The effect of various epididymal toxicants on individual sperm morphology parameters

| | VEH-I | VEH-C | CEMS 12.5 mg/kg | CEMS 18.75 mg/kg | EPI 3.0 mg/kg |
|------|-------------------|-------------------|--------------------|---------------------|------------------|
| IH | 3.50 ± 0.99* (6) | 5.60 ± 2.62 (5) | 2.25 ± 1.31 (4) | 2.14 ± 0.67 (7) | 1.00 ± 0.58 (3) |
| MNI | 0.833 ± 0.543 (6) | 0.200 ± 0.200 (5) | 0.500 ± 0.289 (4) | 0.143 ± 0.143 (7) | 0.00 (3) |
| NHDT | 1.33 ± 0.33 (6) | 1.20 ± 0.49 (5) | 0.75 ± 0.48 (4) | 1.43 ± 0.57 (7) | 1.33 ± 0.33 (3) |
| MHTN | 13.2 ± 5.46 (6) | 5.80 ± 2.73 (5) | 7.75 ± 0.63 (4) | 7.43 ± 1.49 (7) | 2.67 ± 0.88 (3) |
| MHTA | 0.833 ± 0.543 (6) | 1.20 ± 0.49 (5) | 0.750 ± 0.479 (4) | 1.14 ± 0.34 (7) | 1.33 ± 0.33 (3) |

Abbreviations: VEH-I, intact vehicles; VEH-C, castrated vehicles; CEMS, chloroethylmethanesulphonate; EPI, epichlorohydrin; EDS, ethane dimethanesulphonate; HFLUT, hydroxyflutamide; IH, isolated, normal head; MHI, misshapen, isolated head, NHDT, normal head, degenerative tail; MHTN, misshapen head, normal tail; and MHTA, misshapen head, abnormal tail.

* Values represent the mean number of sperm ± SEM (standard error of the mean) and in parentheses, *N*.

Table 1. *Extended*

| EPI 3.0 mg/kg | EPI 6.0 mg/kg | EDS 25.0 mg/kg | EDS 50.0 mg/kg | HFLUT 12.5 mg/kg | HFLUT 25.0 mg/kg |
|------------------|------------------|-------------------|-------------------|---------------------|---------------------|
| 1.91 ± 0.17(3) | 21.8 ± 0.7 (7) | 2.03 ± 0.613(3) | 0.070 ± 0.048†(5) | 6.44 ± 4.11(3) | 3.04 ± 0.68(7) |
| 4.14 ± 1.48(3) | 4.99 ± 1.29(7) | 10.2 ± 2.45 (3) | 4.32 ± 1.81 (5) | 4.62 ± 2.27(3) | 3.83 ± 1.33(7) |
| 212 ± 86 (3) | 225 ± 25 (7) | 244 ± 16 (3) | 168 ± 29 (5) | 186 ± 23 (3) | 170 ± 16 (7) |

evaluation as described previously (Linder et al, 1992). Briefly, the aliquot is coverslipped and 200–300 sperm are evaluated using phase-contrast optics at 200× (16× objective, 12.5× eyepiece). Sperm were assigned to one of five categories: isolated, normal head (IH), normal head with degenerative tail (NHDT), misshapen head and normal tail (MHTN), misshapen head and abnormal tail (MHTA), and misshapen isolated head (MHI).

Sperm Protein Analysis

Sperm remaining in the culture dish after insemination and motion analysis were subjected to protein analysis. Sperm ($10\text{--}40 \times 10^6$) were transferred to a microcentrifuge tube and washed twice by centrifugation ($3,000 \times g$, 10 minutes) in Hanks' Balanced Salts Solution buffered with 4.2 g/L HEPES and 0.35 g/L NaHCO₃ and containing 0.9 g/L D-glucose, 0.1 g/L sodium pyruvate, and 0.025 g/L soybean trypsin inhibitor, pH 7.4 with freshly added 0.2-mM phenylmethylsulphonyl fluoride (PMSF; Sigma, P 7626). After the final wash, sperm were extracted for 1 hour at room temperature with 1 ml of 80-mM n-octyl-B-glucopyranoside in 10-mM Tris, pH 7.2 containing freshly-added PMSF. Following a final centrifugation ($10,000 \times g$, 5 minutes), the supernatant was removed and frozen (-70°C).

Prior to 2-D gel electrophoresis, samples were thawed and each extract was concentrated with 1-mM Tris buffer, pH 7.2, by two centrifugations ($3,000 \times g$, 45 minutes, 4°C) in Centri-con-10 units (Amicon, Lexington, Massachusetts). Protein concentration was determined using a Pierce protein assay kit. Sample volumes containing 30 µg protein were lyophilized and protein was solubilized for 30 minutes at room temperature in 45 µl of a sample buffer consisting of 5.7 g urea, 4 ml 10% NP-40, 0.5 ml ampholytes (Serva, Hauppauge, New York; 3–10 only), and 0.1 g dithiothreitol per 10 ml. Isoelectric focusing (750 V, 3.5 hours) was carried out in mini isoelectric focusing

gels consisting of 6.24 g urea, 1.5 g acrylamide (30% acrylamide, 1.2% bisacrylamide), 2.25 ml 10% NP-40, and 0.65 ml ampholytes (3–10 only) per 10 ml. Molecular weight separation was carried out in mini 11% acrylamide gels (200 V, 45 minutes). Gels were soaked in 50% methanol and silver stained using a Daiichi silver staining kit (Integrated Separation Systems, High-park, Massachusetts).

A Kepler 2-D gel analysis system (Large Scale Biology Corp., Rockville, Maryland) 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 and converted to 256 gray levels. Quantitation was done by fitting two-dimensional gaussian distributions to the density distribution of the spot areas following background subtraction. Of the 124 proteins (spots) that were identified in the 50 gel data set, 22 were common to gels representative of sperm extracts of vehicle-treated animals. Of these 22 proteins, only one was affected by all test chemicals in a dose-related fashion. In fact, this was the only one of the 124 that were identified that changed in either a dose or treatment-related fashion. Initial electrophoretic runs suggested that this protein had an apparent molecular weight of 16 kDa. However, after comparing several types of low molecular weight standards, the molecular weight of this protein consistently indicates an apparent molecular weight of 22 kDa.

Statistics

The various data (fertility, motility, morphology, serum T, caput/corpus T, cauda reserves, and sperm protein) were collected over five experimental blocks ($N = 12$ per block; at least one animal per treatment group per block) and analyzed using two-way analysis of variance (SAS Institute, 1985; PROC GLM) for treatment effects. Fertility and motility data were arcsine transformed to

Table 2. *Extended*

| EPI 6.0 mg/kg | EDS 25.0 mg/kg | EDS 50.0 mg/kg | HFLUT 12.5 mg/kg | HFLUT 25.0 mg/kg |
|------------------|-------------------|-------------------|---------------------|---------------------|
| 2.29 ± 0.57 (7) | 2.67 ± 2.19(3) | 3.60 ± 0.81 (5) | 5.33 ± 3.76 (3) | 3.57 ± 0.81 (7) |
| 0.143 ± 0.143(7) | 0.00 (3) | 0.00 (5) | 0.00 (3) | 0.00 (7) |
| 0.86 ± 0.34(7) | 1.33 ± 0.33(3) | 2.00 ± 0.45 (5) | 3.00 ± 1.53 (3) | 1.71 ± 0.29 (7) |
| 5.00 ± 1.60(7) | 8.67 ± 2.03(3) | 5.60 ± 1.75 (5) | 10.7 ± 2.85 (3) | 6.71 ± 1.52 (7) |
| 0.714 ± 0.42(7) | 1.67 ± 1.20(3) | 0.400 ± 0.245(5) | 0.333 ± 0.333(3) | 1.86 ± 0.459 (7) |

Table 3. The effect of various epididymal toxicants on individual sperm motion parameters

| | VEH-I | VEH-C | CEMS 12.5 mg/kg | CEMS 18.75 mg/kg | EPI 3.0 mg/kg |
|------|------------------|-----------------|--------------------|---------------------|------------------|
| MOT | 84.8 ± 2.17‡ (6) | 68.3 ± 6.85 (5) | 74.6 ± 9.40 (4) | 79.4 ± 2.24 (7) | 77.1 ± 2.57 (3) |
| PMOT | 55.6 ± 2.58 (6) | 54.3 ± 4.79 (5) | 61.7 ± 3.02 (4) | 51.8 ± 1.35 (7) | 67.9 ± 4.89 (3) |
| VSL | 100 ± 5.70 (6) | 104 ± 6.92 (5) | 126 ± 8.91 (4) | 110 ± 5.85 (7) | 111 ± 1.96 (3) |
| VAP | 129 ± 4.30 (6) | 131 ± 5.82 (5) | 154 ± 5.08 (4) | 145 ± 5.23 (7) | 132 ± 10.1 (3) |
| VCL | 259 ± 12.6 (6) | 282 ± 17.4 (5) | 317 ± 23.2 (4) | 292 ± 7.26 (7) | 277 ± 16.8 (3) |

Abbreviations: VEH-I, intact vehicles; VEH-C, castrated vehicles; CEMS, chloroethylmethanesulphonate; EPI, epichlorohydrin; EDS, ethane dithanesulphonate; HFLUT, hydroxyflutamide; MOT, % motile; PMOT, % progressively motile; VSL, straight-line velocity in $\mu\text{m}/\text{second}$; VAP, average path velocity in $\mu\text{m}/\text{second}$; and VCL, curvilinear velocity in $\mu\text{m}/\text{second}$.

* $P < 0.05$.

† $P < 0.01$.

‡ Values represent the mean ± SEM (standard error of the mean) and in parentheses, *N*.

determine treatment-related differences. Data for 50 animals were available for each of the above endpoints; if the inseminated female had no corpora lutea of pregnancy on day 9, i.e., cervical stimulation was insufficient, the male was excluded from analysis. Dunnett's multiple comparison test was used to determine significance ($P < 0.05$). A correlation analysis was performed to determine whether significant ($P < 0.05$) correlations existed between each of the measured endpoints. The background-corrected area of SP22 and fertility data were fitted to a nonlinear function defining a sigmoid curve that approaches a horizontal asymptote as follows:

$$F_{SP22} = F_0 \exp^{A/B(1 - \exp^{-B \cdot SP22})}$$

in which F_{SP22} is the fertility at a given SP22 concentration and F_0 the fertility when SP22 equals 0. A and B are constants, where A is the initial increase in fertility and B is the rate of exponential decay of the increase in fertility. Curve fitting was conducted using a Marquardt curve-fitting algorithm (SAS Institute, 1985; PROC NLIN). This fitting procedure calculated the parameters A and B , adjusting to minimize the simultaneous sum of squares for the model with respect to the data points.

Results

The fertility of proximal cauda epididymal sperm from vehicle-treated controls averaged 68% (intact and castrat-

ed, T-implanted combined), slightly less than the anticipated 75% given the number of sperm inseminated (Klinefelter et al, 1994b). Each of the test chemicals (CEMS, EDS, EPI, and HFLUT) decreased fertility (day 9 implants/corpora lutea of pregnancy) on experimental day 5, 4 days after the onset of exposure (Fig. 1). Specifically, the fertility of sperm from the proximal cauda epididymidis of intact animals that received the higher dose of CEMS, EDS, and EPI was reduced ($P < 0.01$) to 17, 2, and 27%, respectively, compared to 62% for the intact vehicle control. Likewise, the fertility of sperm from castrated, T-implanted animals that received the higher dose of HFLUT was reduced ($P < 0.05$) to 30%, compared to 74% for the castrated, T-implanted vehicle control. Moreover, the lower doses of both EPI and HFLUT also were effective, reducing fertility to 8% ($P < 0.01$) and 21% ($P < 0.05$), respectively, compared to the appropriate vehicles controls.

Both doses of CEMS (12.5 and 18.75 mg/kg) and the higher dose of EDS (50 mg/kg) decreased serum T ($P < 0.01$) compared to values observed for intact controls (Table 1). There were no dose- or treatment-related effects on T concentration in the caput/corpus epididymidis or the number of sperm in the cauda epididymidis (Table 1). Of the numerous parameters associated with morphology (Table 2) or motility (Table 3) of sperm from the proximal cauda epididymidis, few treatment-related effects were observed. However, there were decreases in the percentages of both motile ($P < 0.01$) and progressively motile ($P < 0.05$) proximal cauda epididymal sperm in animals administered 6 mg/kg EPI compared to intact vehicle con-

Table 4. Correlation between endpoints

| Endpoints | Significance | Pearson's correlation |
|-----------------------------------|--------------|-----------------------|
| Fertility vs. SP22 | 0.0001 | 0.76 |
| Caput/corpus T vs. cauda reserves | 0.0001 | 0.63 |
| Motility vs. progressive motility | 0.0007 | 0.46 |
| Serum T vs. caput/corpus T | 0.005 | 0.39 |
| SP22 vs. caput/corpus T | 0.02 | 0.33 |
| Fertility vs. caput/corpus T | 0.02 | 0.32 |

Table 5. Discriminant analysis based on SP22

| Class | Percentage correctly predicted |
|-------------------|--------------------------------|
| Fertile (>50%) | 90 (17/19) |
| Subfertile (<50%) | 94 (29/31) |

Table 3. Extended

| EPI 6.0 mg/kg | EDS 25.0 mg/kg | EDS 50.0 mg/kg | HFLUT 12.5 mg/kg | HFLUT 25.0 mg/kg |
|------------------|-------------------|-------------------|---------------------|---------------------|
| 59.5 ± 8.39† (7) | 75.2 ± 1.84 (3) | 81.2 ± 1.61 (5) | 66.6 ± 10.3 (3) | 82.5 ± 2.17 (7) |
| 43.2 ± 5.82* (7) | 55.5 ± 3.32 (3) | 59.2 ± 4.12 (5) | 32.4 ± 2.49† (3) | 51.8 ± 1.18 (7) |
| 94.2 ± 11.3 (7) | 102 ± 7.48 (3) | 108 ± 8.01 (5) | 109 ± 4.75 (3) | 112 ± 6.12 (7) |
| 119 ± 6.76 (7) | 139 ± 5.89 (3) | 136 ± 6.68 (5) | 128 ± 10.9 (3) | 146 ± 7.37 (7) |
| 261 ± 17.6 (7) | 294 ± 19.2 (3) | 279 ± 16.0 (5) | 279 ± 28.0 (3) | 297 ± 11.0 (7) |

trols. In addition, there was a decrease in the percentage of progressively motile ($P < 0.01$) sperm in animals treated with 12.5 mg/kg HFLUT compared to castrated, T-implanted vehicle controls.

Figure 2 shows representative silver-stained, 2-dimensional protein profiles for the detergent extracts of proximal cauda sperm from animals receiving the higher dose of each test chemical. An acidic (pI 5.5), 22 kDa protein (SP22) was decreased relative to other sperm proteins by each test chemical. Results from the quantitative analysis of SP22 in 50 gels representing the various treatments are shown in Figure 3. The background-corrected spot area for SP22 was decreased ($P < 0.05$) by the higher dose of CEMS, EDS, EPI, and HFLUT, as well as by the lower dose of EPI and HFLUT.

Of the multiple measures that were evaluated in this study, only two were significantly correlated with fertility: the background-corrected spot area of SP22 and the concentration of T in the caput/corpus epididymidis, with $P < 0.0001$ and $P < 0.02$, respectively (Table 4). In Table 4, the significance of correlation and Pearson correlation coefficients (R) between various endpoints are ranked from highest (most significant, highest R) to lowest. It is clear from this table that the amount of detergent-extracted SP22 in the insemination sample is highly correlated with the fertility of proximal cauda sperm. Although the doses of each chemical were chosen to produce only subtle, if any, effects on sperm motion, the correlation between fertility and the percentage of progressively motile sperm was $P < 0.07$ (not shown).

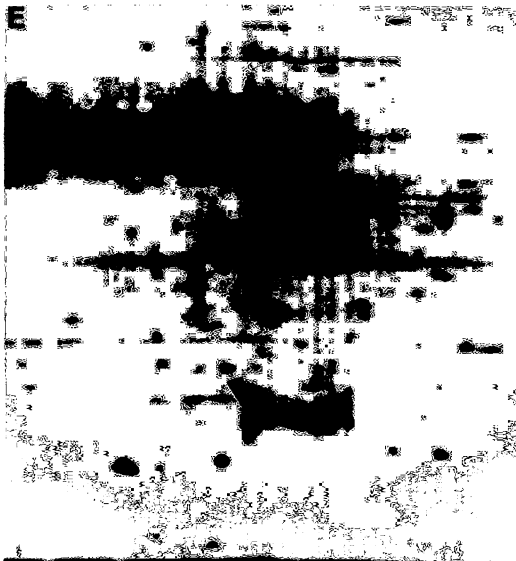
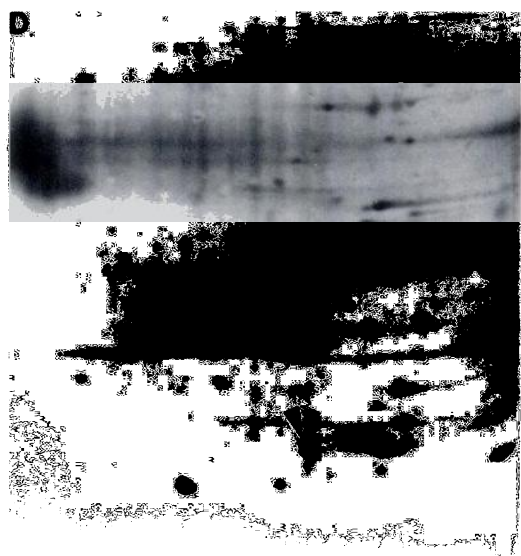
The scatter plot of SP22 and fertility suggested that a threshold amount of SP22 might be required for maximum fertility (Fig. 4). Thus, a non-linear, sigmoid curve model was used with two rate constants, one expressing the increase in fertility with increasing amounts of SP22 and one expressing the decline in this increase after a threshold amount of SP22 is reached. When the background-corrected SP22 area and fertility were fitted *post hoc* to this non-linear equation (Fig. 4), the resultant correlation (r^2) was 0.83. To determine whether the amount of SP22 could be used to predict fertility, a linearized, discriminant analysis program was utilized (SAS, 1985; PROC DISCRIM). For discriminant analysis, the fertility classes were fertile and subfertile, defined by animals

having greater than, or less than, 50% fertility, respectively. Because the mean fertility for vehicle-treated animals (intact and castrated, T-implanted) was 68% with a standard deviation of 18%, 50% fertility (mean - 1 standard deviation) was chosen as the cutoff value. At 50% fertility, the integrated, background-corrected SP22 spot area was ~12,000 integrated optical density (IOD) units. Using data for the integrated, background-corrected SP22 area, discriminant analysis correctly predicted the fertility of 46 of 50 animals (Table 5); 90% (17/19) of the animals with greater than 50% fertility were classified as fertile and 94% (29/31) of the animals with less than 50% fertility were classified as subfertile. When the concentration of T in the caput/corpus epididymidis, also significantly correlated with fertility ($P < 0.02$), was added into the discriminant analysis along with SP22, predictability was not improved (data not shown).

Discussion

A previous *in utero* insemination study established that a single i.p. injection of 50 mg/kg EDS significantly reduced the fertilizing ability of sperm from the proximal cauda epididymidis 4 days later, an effect which was independent of the testis as fertilizing ability was similarly compromised in castrated, T-implanted animals that received EDS (Klinefelter et al, 1994b). Although the amount of an acidic 18 kDa sperm protein and T concentration in the caput/corpus epididymidis both were significantly correlated with fertilizing ability, it seemed unlikely that alterations in these measures alone would be common to other epididymal toxicants that compromise fertility. Thus, a multivariate and discriminant analysis was conducted to determine which, if any, other endpoints were related to and predictive of toxicant-induced alterations in fertility using three additional male reproductive toxicants.

Within 4 days, exposure to EDS, CEMS, EPI, and HFLUT each reduced the fertility of sperm from the proximal cauda epididymidis. Decreased fertility by 4 days of CEMS exposure has not been reported previously. Early studies with CEMS reported that when males received five, daily 50 mg/kg i.p. injections and were allowed to



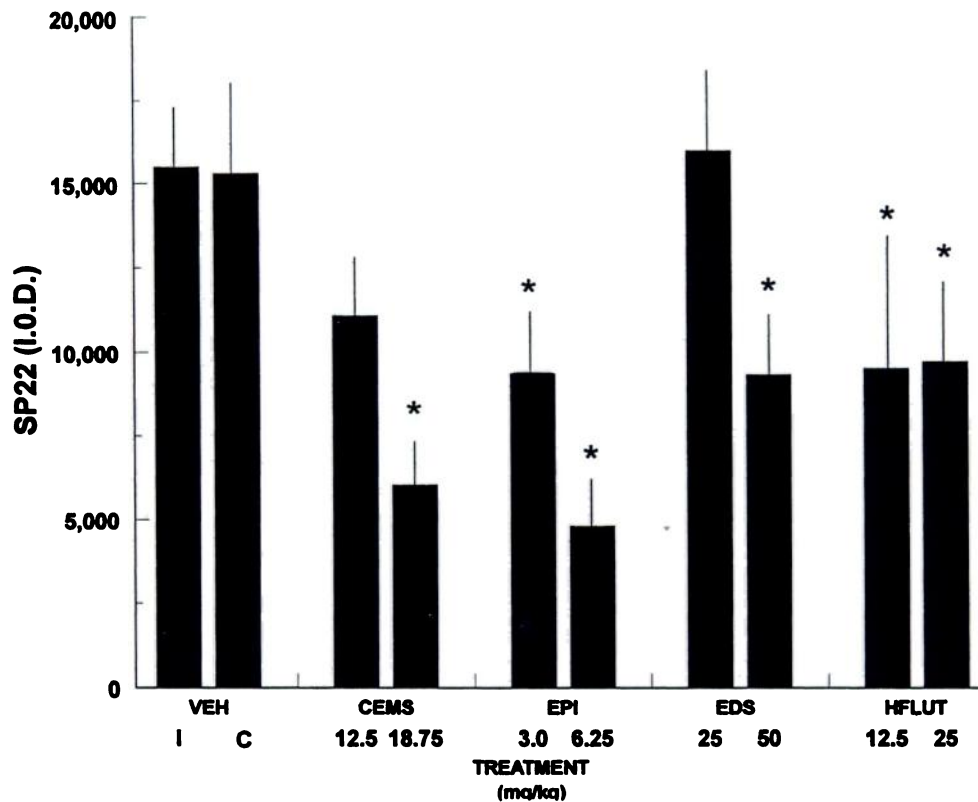


FIG. 3. Graph showing the treatment-related changes in integrated, background-corrected spot area (IOD or integrated optical density units) of an acidic 22-kDa, detergent-extracted proximal cauda sperm protein (SP22) isolated by 2-D gel electrophoresis under denaturing conditions. Vehicle controls (intact [I] [$N = 6$] and castrated with T implant [C] [$N = 5$]), chloroethylmethanesulphonate (CEMS; 12.5 mg/kg [$N = 4$] and 18.75 [$N = 7$]), epichlorohydrin (EPI; 3 mg/kg [$N = 3$] and 6 mg/kg [$N = 7$]), ethane dimethanesulphonate (EDS; 25 mg/kg [$N = 3$] and 50 mg/kg [$N = 5$]), hydroxyflutamide (HFLUT; 12.5 mg/kg [$N = 3$] and 25 mg/kg [$N = 7$]). EPI, CEMS, and EDS were compared with intact vehicles (VEH-I) and HFLUT was compared with castrated vehicles (VEH-C). Significant reduction in SP22 compared to appropriate controls is indicated * ($P < 0.05$).

mate naturally with untreated females, fertility was reduced 2 to 3 weeks later (Jackson et al, 1961), results that are suggestive, but not indicative, of epididymal toxicity. Our earlier study of CEMS (Klinefelter et al, 1994a) demonstrated that four daily 12.5 mg/kg doses alter both the structure and function of the epididymis even when the CEMS-induced reduction in serum T is prevented by T-implantation. These earlier findings, together with the results of the present study, support the notion that CEMS alters the fertility of epididymal sperm by perturbing sperm maturation within the epididymis.

Fertility was reduced significantly by the lower 3-mg/kg dose of EPI. Previously, the lowest dose of EPI shown to decrease fertility was 50 mg/kg (Jones et al, 1969; Cooper et al, 1974; Toth et al, 1989). In the study by Cooper et al, 50 mg/kg EPI was administered daily for 5 days and fertility of the naturally mated females was

significantly reduced at 1 week, suggesting that infertility might be the result of compromised epididymal sperm maturation.

The reduced fertility resulting from exposure to HFLUT also has not been reported. Because the epididymis is an androgen-dependent organ, antiandrogens such as flutamide or its metabolite HFLUT have been used frequently to elucidate androgen-dependent processes, but most studies have not determined whether these nonsteroidal antiandrogens can compromise fertility (Peets et al, 1974; Dhar et al, 1982; Poyet and Labrie, 1985; de las Heras et al, 1988). However, Dhar and Setty (1976) administered 25 mg/kg flutamide to intact males orally for 30 days and failed to observe any effect on the fertility of naturally mated males. Because HFLUT animals were castrated in the present study, the data strongly suggest that fertility was compromised by direct action on the

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FIG. 2. Sperm proteins of detergent-extracted proximal cauda sperm separated by 2-D gel electrophoresis under denaturing conditions. Representative protein profiles are shown for (A) vehicle (intact or castrated), (B) 18.75 mg/kg CEMS (chloroethylmethanesulphonate), (C) 6 mg/kg EPI (epichlorohydrin), (D) 50 mg/kg EDS (ethanedimethanesulphonate), and (E) 25 mg/kg HFLUT (hydroxyflutamide). The pI range and mol weights are indicated in A. The diminution of a 22 kDa protein, pI = 5.5 (SP22), is indicated by the arrowhead.

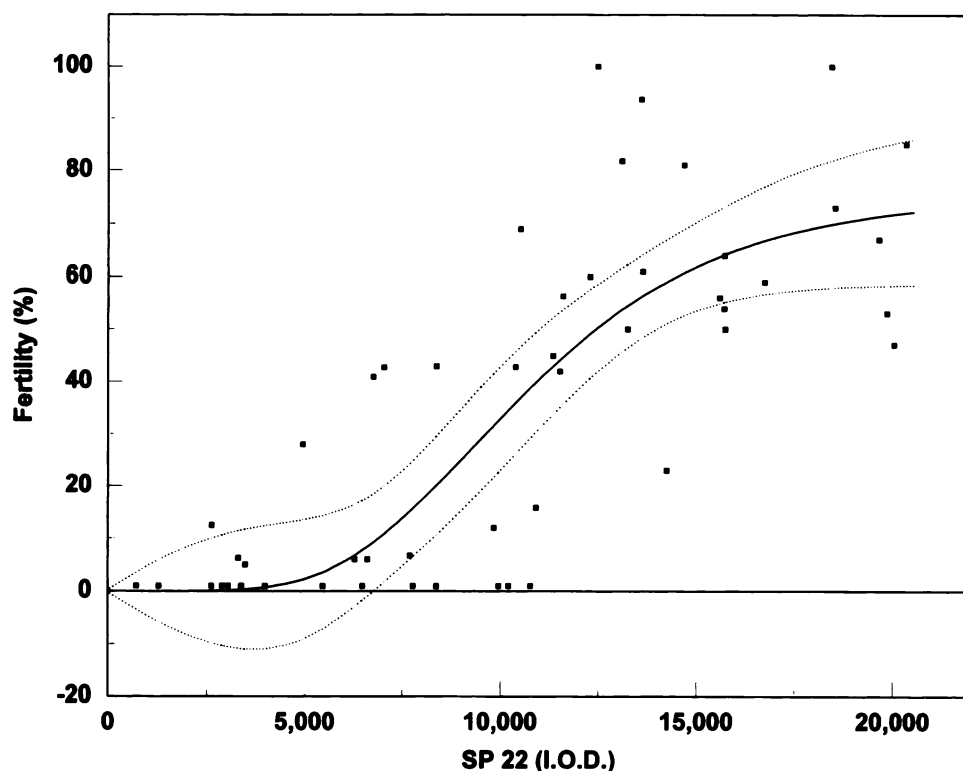


FIG. 4. Data ($N = 50$) for fertility and the integrated, background-corrected area (IOD) of SP22 (an acidic 22-kDa detergent-extracted sperm protein) were fitted to the following equation:

$$F_{SP22} = F_0 \exp^{A/B(1 - \exp^{-B \cdot SP22})}$$

in which F_{SP22} is the fertility at protein concentration SP22 and F_0 is the fertility at 0 protein concentration. A and B are constants (A is the initial increase in fertility and B is the rate of exponential decay of the increase in fertility). The dotted lines represent the 95% confidence limits around the fitted line. Fitted parameters are $F_0 = 2.810^{-5} \pm 3.567^{-6}$, $A = 4.283^{-3} \pm 3.965^{-5}$, and $B = 2.894^{-4} \pm 1.140^{-1}$ with $r^2 = 0.83$.

epididymis. Moreover, because the one alteration that was coincident with decreased fertility in the HFLUT animals, and all other treatment groups, was the diminution in a 22 kDa sperm protein, changes in the expression of this protein in a sperm sample may be the consequence of direct toxicant action on the epididymis. If so, each of the test chemicals were acting directly on epididymal sperm maturation.

Exposure to both EDS and CEMS produced the anticipated reductions in serum T but failed to alter T concentration in the caput/corpus epididymidis. While these chemicals failed to alter the T concentration in the caput/corpus epididymidis, there was a significant correlation between caput/corpus T concentration and serum T. Interestingly, T concentration in the caput/corpus was not only correlated significantly with serum T, but also with fertility, the amount of SP22 in the insemination sample, and the number of sperm stored within the cauda epididymidis. Although we failed to observe statistical significance for the treatment-related decreases in cauda epididymal sperm in the present study, results of other studies were significant (Klinefelter et al, 1994a,b).

The fact that none of the epididymal toxicants altered

the morphology of sperm from the proximal cauda epididymidis is not surprising; parameters of sperm morphology would undoubtedly be more useful if a similar study were conducted using various testicular toxicants that have greater opportunity to elicit morphological defects during spermatogenesis and spermiogenesis.

The selection of doses of each chemical that would exert modest, if any, effects on sperm motion parameters not only ensured that equal numbers of progressively motile sperm would be inseminated into each recipient female, regardless of the treatment the male received, but also that other endpoints that play a role in the fertility of epididymal sperm might become evident. With the exception of the higher dose of EPI and the lower dose of HFLUT, equivalent numbers of progressively motile proximal cauda sperm were inseminated into each recipient female. The observed decreases in the percentages of motile and progressively motile proximal cauda sperm from animals treated with EPI are consistent with effects on sperm motion parameters observed previously with higher doses of EPI (Toth et al, 1989; Slott et al, 1990). That the progressive motility of proximal cauda sperm was decreased in animals exposed to the lower dose of

HFLUT seems consistent with the fact that fertility was reduced to a greater extent in animals that received 12.5 mg/kg HFLUT than those that received 25 mg/kg and with the fact that the overall correlation between progressive motility and fertility was $P < 0.07$.

As indicated above, the significant treatment-related decreases in the amount of SP22 in the insemination sample were completely coincident with the significant treatment-related decreases in the fertility of the sample. The correlation between the amount of background-corrected SP22 in a silver-stained, 2-D gel and fertility was highly significant ($P < 0.0001$). Because detergent extraction and 2-D gel conditions have changed since our previous *in utero* insemination study testing the effects of EDS (Klinefelter et al, 1994b), we do not know whether there is any similarity between SP22 and the 18 kDa sperm protein that was significantly correlated with fertilizing ability in the former study.

When SP22 and fertility data are fit to a nonlinear mathematical model, a correlation coefficient (r^2) of 0.83 is achieved. A nonlinear fit of the data was indicated since a threshold of 10,000 units of SP22 appeared necessary before the first animal exceeded 50% fertility. From the fitted curve, 50% fertility was achieved when SP22 reached ~12,000 units. When SP22 was 10,000 units or greater, the percentage of animals classified as fertile was 74%; when SP22 exceeded 12,000 units, the percentage of animals classified as fertile was 90%. The concept that thresholds of specific male reproductive endpoints might be pivotal to successful fertilization is not new (Amann and Hammerstedt, 1993; Amann et al, 1993). Indeed, these authors hypothesized that threshold levels of multiple factors act in concert to confer and maintain fertility of epididymal and ejaculated sperm. Because no other endpoint(s) measured in this study improved the results of discriminant analysis (data not shown), other factors needed for optimal fertility of proximal cauda epididymal sperm remain elusive. Once these factors are identified, much of the remaining variability (17%) between SP22 and fertility could be accounted for. However, it is important to recognize that the amount of SP22 in the insemination sample alone enabled correct identification of 46 of the 50 experimental animals using discriminant analysis and cutoff of 50% fertility to distinguish between fertile and subfertile animals.

We have successfully incorporated several epididymal toxicants, *in utero* insemination, multiple endpoint evaluation, and discriminant analysis to demonstrate: 1) that EDS, CEMS, EPI, and HFLUT are each capable of compromising the fertility of sperm from the proximal cauda epididymidis, presumably by directly disrupting one or more processes of epididymal sperm maturation, and 2) the amount of an acidic, 22 kDa sperm protein (SP22) is not only highly correlated with the fertility of proximal

cauda epididymal sperm, but also is highly predictive of the fertility of these sperm. Studies now are underway to characterize SP22 and determine whether the toxicant-induced diminutions in SP22 on proximal cauda sperm reflect decreased incorporation of this protein by the sperm or the modification of incorporated protein.

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References

- Amann RP. The use of animal models for detecting specific alterations in reproduction. *Fundam Appl Toxicol* 1986;2:13–26.
- Amann RP, Hammerstedt RH. *In vitro* evaluation of sperm quality: an opinion. *J Androl* 1993;14:397–406.
- Amann RP, Hammerstedt RH, Veeramachaneni DNR. The epididymis and sperm maturation: a perspective. *Reprod Fertil Dev* 1993;5:361–381.
- Cooper ERA, Jones AR, Jackson H. Effects of α -chlorohydrin and related compounds on the reproductive organs and fertility of the male rat. *J Reprod Fertil* 1974;38:379–386.
- de las Heras MA, Suescun MO, Calandra RS. Ornithine decarboxylase activity as a marker of androgen and antiandrogen action in the rat epididymis. *J Reprod Fertil* 1988;83:177–183.
- Dhar JD, Setty BS. Studies on the physiology and biochemistry of mammalian epididymis: effect of flutamide, a nonsteroidal antiandrogen, on the epididymis of the rat. *Fertil Steril* 1976;27:566–576.
- Dhar JD, Srivastava SR, Setty BS. Flutamide as an androgen antagonist on epididymal function in the rat. *Andrologia* 1982;14:55–61.
- Dyson AL, Orgebin-Crist M-C. Effect of hypophysectomy, castration, and androgen replacement upon the fertilizing ability of rat epididymal spermatozoa. *Endocrinology* 1973;93:391–402.
- Ewing LL, Desjardins C, Irby DC, Robaire B. Synergistic interaction of T and oestradiol inhibits spermatogenesis in rats. *Nature* 1977;269:409–411.
- Jackson H, Fox BW, Craig AW. Antifertility substances and their assessment in the male rodent. *J Reprod Fertil* 1961;2:447–465.
- Jones AR, Davies P, Edwards K, Jackson H. Antifertility effects and metabolism of alpha- and epichlorohydrin in the rat. *Nature* 1969;224:83.
- Klinefelter GR, Gray, LE, Suarez, JD. The method of sperm collection significantly influences sperm motion parameters following ethane dimethanesulphonate administration in the rat. *Reprod Toxicol* 1990;5:39–44.
- Klinefelter GR, Laskey JW, Kelce WR, Ferrell J, Roberts NL, Suarez JD, Slott V. Chloroethylmethanesulphonate-induced effects on the epididymis seem unrelated to altered Leydig cell function. *Biol Reprod* 1994a;51:82–91.
- Klinefelter GR, Laskey JW, Perreault SD, Ferrel J, Jeffay S, Suarez J, Roberts N. The ethane dimethanesulphonate-induced decrease in the fertilizing ability of cauda epididymal sperm is independent of the testis. *J Androl* 1994b;15:318–327.
- Klinefelter GR, Roberts NL, Suarez J. Direct effects of ethane dimethanesulphonate on epididymal function in adult rats. *J Androl* 1992;13:409–421.
- Linder RE, Strader LF, Slott VL, Suarez JD. Endpoints of spermatotoxicity in the rat after short duration exposures to fourteen reproductive toxicants. *Reprod Toxicol* 1992;6:491–505.
- Peets EA, Henson MF, Neri R. On the mechanism of the antiandrogenic

- action of flutamide (α - α -trifluoro-2-methyl-4-nitro-m-propionotoluidide) in the rat. *Endocrinology* 1974;94:532-540.
- Poyet P, Labrie F. Comparison of the antiandrogenic/androgenic activities of flutamide, cyproterone acetate and megestrol acetate. *Mol Cell Endocrinol* 1985;42:283-288.
- 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.
- SAS Institute. *Statistical Analysis Systems User's Guide: Basics, Version 6*. Cary, North Carolina: SAS Institute, Inc; 1985.
- Slot VL, Suarez JD, Simmons JE, Perreault SD. Acute inhalation exposure to epichlorohydrin transiently decreases rat sperm velocity. *Fundam Appl Toxicol* 1990;15:597-606.
- Toth GP, Zenick H, Smith MK. Effects of epichlorohydrin on male and female reproduction in Long-Evans rats. *Fundam Appl Toxicol* 1989;13:16-25.