

1 Chronic Exposure to Low Levels of Dibromoacetic Acid, a Water Disinfection By-
2 product, Adversely Affects Reproductive Function in Male Rabbits

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19 Running Title: Effects of Dibromoacetic acid on male reproduction
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22 Key Words: water disinfection by-products, dibromoacetic acid, testis, acrosomal dysgenesis,
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26 Summary Sentence: Chronic developmental exposure via drinking water to low doses of
27 dibromoacetic acid, a water disinfection by-product, impairs sexual function, disrupts sperm
28 acrosomal-nuclear morphogenesis, diminishes sperm membrane fertility protein SP22, and
29 causes infertility in male rabbits.
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47 **ABSTRACT**

48
49 Four groups (minimum of 10/dose group) of male Dutch-Belted rabbits were treated daily to
50 dibromoacetic acid (DBA) via drinking water beginning in utero from gestation day 15 to
51 adulthood; target dosages were 1, 5, and 50 mg DBA /kg body weight. Developmental,
52 prepubertal as well as postpubertal reproductive sequelae were evaluated. One (out of 22), 2
53 (out of 32), and 1 (out of 21) male offspring in 1, 5, and 50 mg DBA/kg groups were unilaterally
54 cryptorchid. There were no significant differences in serum FSH, LH and testosterone (basal
55 concentrations or in response to exogenous GnRH) in both prepubertal and adult rabbits.
56 Chronic exposure to DBA adversely affected mating ability of some rabbits. Number of sperm
57 produced was not affected but spermiogenesis was disrupted resulting in unique sperm
58 acrosomal-nuclear malformations even at 1 mg dose level. Concentrations of SP22, a specific
59 sperm membrane fertility protein, in detergent extracts of ejaculated sperm were significantly
60 lower ($p < 0.05$) in all DBA-treated groups compared to controls. The conception rates
61 following artificial insemination of a constant number of sperm for 1, 5, and 50 mg DBA/kg
62 groups were, 55 (10/18), 65 (13/20), and 55% (9/16), respectively, vs. 85% (17/20) for control
63 group. Histological lesions in testes characterized by spermatogenic arrest predominantly at
64 round spermatid stage, pyknosis of differentiating germ cells, and ultimate degeneration and
65 desquamation leaving focal vacuolation in seminiferous epithelium were evident in DBA-treated
66 groups. Thus, male rabbits exhibit reproductive toxicity with exposure to DBA during
67 reproductive development at dosages as low as 1 mg/kg.

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69

69 INTRODUCTION

70 Haloacetic acids are formed when disinfectants such as chlorine react with organics and
71 bromide in source water. By weight, haloacetic acids are among the most abundant water
72 disinfection by-products and are a concern from a health and regulatory standpoint (Uden and
73 Miller, 1983). Haloacetic acids, including dibromoacetic acid (DBA), have been shown to cause
74 adverse effects on development and reproductive function in some laboratory animal models.
75 Testicular toxicity following acute exposure to DBA (10 to 1250 mg/kg/day via oral gavage for
76 1 to 14 days) has been demonstrated in adult male rats with marked effects on sperm motility
77 and morphological features (Linder et al, 1994a; Linder et al, 1994b). In a subsequent 70 day
78 study a dose-related decrease in the ability of males to sire multiple litters following oral gavage
79 with 10, 50, or 250 mg/kg DBA was observed (Linder et al, 1995). The observed qualitative
80 and quantitative alterations in sperm were later associated with defects in spermatogenesis
81 including delayed spermiation and the formation of atypical residual bodies (Linder et al, 1997).
82 These histological alterations were also observed in P0 and F1 males exposed to 250 and 650
83 ppm DBA (i.e. 56 and 132 mg DBA/kg) in a two-generation study (Christian et al, 2002).

84 A two week exposure of rats to the structurally related haloacid bromochloroacetic acid
85 (BCA) was shown to result in significantly reduced fertility of proximal cauda epididymal sperm
86 upon in utero insemination (Klinefelter et al, 2002). The reductions in fertility were significant
87 at all doses; 8 mg/kg was lowest dose tested. In this study fertility was highly correlated with
88 levels of a specific isoform of a sperm membrane protein in detergent extracts of cauda
89 epididymal sperm previously identified as SP22 (Klinefelter et al, 1997, Welch et al., 1998).
90 More recently, DBA and BCA were shown to be dose- and effect-additive with respect to their
91 ability to decrease both fertilizing ability and the putative SP22 biomarker (Kaydos et al, 2004);
92 low effect levels of 2 and 1.6 mg/kg were observed for DBA and BCA respectively. Finally,

93 exposure was shown to delay puberty in both male and female offspring (Klinefelter et al, 2004).
94 This delay was attributed to continuous exposure throughout reproductive development rather
95 than a specific window of susceptibility.

96 A study in mice demonstrated no significant effects in gametogenic potential following
97 exposure to 5 or 50 mg DBA/kg/day from gestation day 15 throughout life (Weber et al, 2006),
98 but effects have been observed at higher doses in the mouse (Tully et al, 2004) suggesting that
99 the mouse is less sensitive to a haloacid insult than the rat. While the observed differences in
100 sensitivity may be due to species-related differences in haloacid metabolism/disposition,
101 additional research in a nonrodent species seemed indicated.

102 In this study, we sought to determine the effects of chronic exposure via drinking water
103 (to simulate a natural situation) to relatively low doses DBA in the rabbit. We selected a rabbit
104 model because rabbits have a long infantile period before initiation of changes culminating in
105 puberty, mimicking human development, whereas in rodents these changes start at birth
106 (Amann, 1982; Veeramachaneni et al, 2001). Furthermore, use of rabbits (in contrast to
107 rodents) facilitates multiple evaluations of seminal quality and sexual capacity including the first
108 quantitative assessment of the sperm biomarker SP22 in an ejaculate. Herein, we report
109 reproductive sequelae, measured before puberty and also after sexual maturity, in rabbits
110 following chronic prenatal plus postnatal exposure to DBA.

111

112 **MATERIALS AND METHODS**

113 *Rabbit Husbandry*

114 Six-month-old, specific-pathogen-free, Dutch-Belted rabbits were obtained from
115 Myrtle's Rabbitry (Thompson Station, TN) and individually housed in standard stainless steel
116 cages in the university animal care facility accredited by the Association for Assessment and

117 Accreditation of Laboratory Animal Care, International. Animals were treated according to the
118 guidelines stipulated by the Colorado State University's institutional Animal Care and Use
119 Committee. The rooms were maintained at a 12 hr light-darkness cycle at approximately 19-
120 21°C and ~40% humidity. Rabbits were fed certified rabbit ration (#7009, Harlan, Teklad,
121 Madison, WI).

122 After acclimatization for 4-5 wk, 61 rabbit does were artificially inseminated using
123 procedures previously described (Veeramachaneni et al, 2001; 2006). Each rabbit doe was
124 given an intramuscular injection of 10 µg GnRH (Calbiochem, La Jolla, CA) to induce ovulation
125 and inseminated with 20 million spermatozoa, pooled from semen collected from 11 bucks to
126 increase biological heterogeneity of experimental subjects. Rabbits were palpated for pregnancy
127 14 days after insemination and resulting pregnant does (n = 45) were randomly assigned to
128 treatment groups for appropriate exposures (see below). On gestation day 28, nesting boxes
129 were placed in the cages of pregnant does.

130

131 *Dosing Regimen*

132 Four groups (n = minimum of 10/dose group) of pregnant rabbits were treated daily to 0
133 (deionized water; control), and target dosages of 1, 5, or 50 mg DBA/kg body weight via
134 drinking water. Individual water bottles were filled daily. Amber, borosilicate glass water
135 bottles fitted with a fluorocarbon septum containing a stainless steel sipper tube, equipped with
136 balls to minimize water dripping were used. Bottle systems were steam-cleaned twice weekly.

137 DBA (CAS# 631-64-1, lot# 03807JS, purity 97 %) was purchased from Aldrich
138 Chemical (Milwaukee, WI). Stock solutions were prepared by adding DBA to deionized water
139 and bringing the pH to 6.8-7.4 using 1N NaOH and refrigerated. To minimize loss of chemical
140 due to volatility, stock solutions were prepared twice weekly and dosing solutions daily. DBA

141 concentrations in dosing solutions were computed as follows. Water consumption during a 24-
142 hr period was recorded daily from gestation day 15 (when dosing was begun) until 6 wk
143 postpartum (when pups were weaned) in dams and twice weekly from 6 wk to 12 wk of age and
144 once weekly from 12 wk to 24 wk of age in offspring. Body weights were recorded weekly.
145 Dosing solutions were prepared based on the average body weight and average daily water
146 consumption in each treatment group during the previous week such that the animals received 1,
147 5, or 50 mg DBA/kg body weight/day. This strategy ensured continuous delivery of doses very
148 close to those intended.

149 Dosing of dams began on gestation day 15 and continued through parturition and
150 weaning at 6 wk postpartum. After weaning, rabbit pups were housed individually and DBA
151 treatments continued until necropsy at 12 wk (prepuberty; n = 10-22/dose group) or 24 wk
152 (postpuberty; n = 9-10/dose group). Thus, the offspring (experimental units) were exposed to
153 DBA in utero beginning from gestation day 15, throughout nursing via dam's milk, and then in
154 drinking water. Rabbits in each dose group represented at least 6 different litters.

155

156 *Evaluation of Mating Ability, Semen Characteristics, and Fertility*

157 Beginning at 20 wk of age, rabbits were trained for semen collection using an artificial
158 vagina and one of several female rabbits as a teaser. Between 22 and 24 wk of age, mating
159 ability and semen characteristics were evaluated by attempting to collect 6 seminal ejaculates
160 from each rabbit; one ejaculate every third day. All of these procedures are detailed in
161 Veeramachaneni et al (2006).

162 Briefly, one of several female teasers was selected randomly for each attempt at semen
163 collection. For each episode, mating ability was evaluated by monitoring the outcome and
164 recording: 1) sexual interest; 2) status of penile erection; and 3) time from introduction of teaser

165 to ejaculation (reaction time). Once the teaser was introduced, evaluation continued until
166 ejaculation or a maximum period of 180 sec.

167 For each sample, seminal volume (after removing the gel) was recorded (to the nearest
168 0.05 ml), semen was mixed, and a 50 μ l aliquant of semen was fixed in 950 μ l phosphate-
169 buffered formal saline (93 mM NaCl, 35 mM Na₂HPO₄, 19 mM KH₂PO₄; 12.5% v/v formalin;
170 Hancock 1957) and stored at 4°C until evaluation. Sperm concentration (10⁶/ml) was
171 determined by hemocytometer (4 chambers/sample) and total sperm per ejaculate (10⁶)
172 calculated by multiplying volume times sperm concentration. Morphological features of sperm
173 were evaluated in a treatment-blinded manner using a light microscope equipped with
174 differential interference contrast optics. Two hundred sperm were evaluated in wet smears of
175 each ejaculate for abnormalities of the acrosome, head, mid- and principal pieces, retention of
176 cytoplasmic droplet, and presence of residual cytoplasm using criteria previously established for
177 rabbits (Veeramachaneni et al, 2001; 2006).

178 At the end of seminal evaluation period, at 24 wk of age, two additional seminal
179 ejaculates were collected from each rabbit; one was used for quantification of SP22 (see below)
180 and the other for fertility testing. Rabbit does of proven fertility were artificially inseminated
181 with 20 million spermatozoa of each male, 2 does/male. Pregnancy outcome was documented as
182 an indicator of fertility potential.

183

184 ***Quantification of SP22 on Ejaculated Sperm by ELISA***

185 Sperm were prepared for protein analysis as described by Klinefelter et al, (2004). For
186 each semen sample, seminal volume was measured after removal of the gel fraction and the
187 semen was transferred to a 15 ml centrifuge tube and diluted with 10 ml sperm isolation buffer
188 [95 ml/L 10X Hanks Balanced Salts Solution (HBSS), 0.35 g/L NaHCO₃, 4.2 g/L HEPES, 0.9

189 g/L glucose and 10 ml/L 100X Na pyruvate, pH 7.4]. Following centrifugation (3000 g, 10 min)
190 the pelleted sperm were washed twice more and then extracted for 1 hr at room temperature with
191 1 ml of 80 mM n-octyl-β-glucopyranoside (OBG) in 10 mM Tris, pH 7.2 containing freshly-
192 added phenylmethyl sulfonyl fluoride. Following a final centrifugation (10,000 x g, 5 min), the
193 supernatant was removed and frozen (-70°C). Prior to SP22 ELISA sperm extracts were thawed
194 and each extract concentrated with 1 mM Tris buffer, pH 7.2, by two centrifugations (3,000 x g,
195 45 min, 4°C) in Ultrafree-4 centrifugation filter units (Millipore). Protein concentration was
196 determined using a Pierce protein assay kit. Sample volumes equivalent to 10 µg protein were
197 lyophilized.

198 The SP22 ELISA was performed as described recently by Kaydos et al (2004). For this,
199 96-well tissue culture plates (Costar 3595 96-well cell culture; Corning Inc., Corning, NY) were
200 used. A standard curve was generated using serial dilutions of antigen, i.e. full length rat
201 recombinant SP22 (rSP22; Klinefelter et al, 2002); 0, 0.01, 0.05 0.1, 0.5, 1, 5, and 10 ng in 50
202 µl/well; all dilutions were in BupH phosphate buffered saline pH 7.2 (Pierce 28372, Rockford,
203 IL). For each sperm extract, 10 µg of lyophilized protein was diluted with BupH and plated at
204 50 µl/well. Duplicate wells were used for both the SP22 standards and each sperm extract. The
205 plates were stored overnight at 4°C to maximize antigen absorption. The following day,
206 unbound antigen was removed by inverting the plate and shaking gently. A blocking step
207 consisted of addition of milk protein (caseinate or dry milk powder) in DPBS (150 µg/well)
208 followed by incubation for 1 h at 37°C. Sheep anti-rSP22 diluted 1:1000 in DPBS + 1% BSA
209 was added (50 µl/well) and allowed to bind during incubation for 1 h at 37°C. After 3 washes
210 with DPBS + 1% BSA (200ul/well), peroxidase conjugated rabbit anti-sheep antibody (Pierce
211 Immunopure 31480, Rockford, IL) diluted 1:500 in DPBS + 1% BSA was added (50µl /well)
212 and allowed to incubate for 1 h at 37°C. After 4 washes with DPBS w/ 1% BSA, the peroxidase

213 substrate ABTS (Pierce, #37615) was added (100 μ l/well). The reaction was allowed to develop
214 over a 15-20 min period. Absorbance was read using FLUOstar Galaxy software (BMG
215 Labtechnologies Inc., Durham, NC) at 405 nm excitation, no emission and values were
216 expressed as the absorbance or optical density (O.D.). Sample values were always found to fall
217 within the range of the standard curve.

218

219 *Tissue Collection and Processing*

220 During the morning hours of the day before termination of the experiments (at 12 and 24
221 wk), a GnRH challenge test was performed to determine the response of anterior pituitary gland
222 to hypothalamic stimuli. After taking a blood sample by jugular venipuncture, 10 μ g GnRH was
223 injected intramuscularly. Two additional blood samples were collected 30-, and 120-min later.
224 Serum was separated and samples stored at -20°C until assayed for gonadotropins and
225 testosterone.

226 A day after GnRH challenge tests, rabbits were euthanized by CO₂. Ano-genital distance
227 was measured and visceral and reproductive organs examined. Liver, kidneys, testes,
228 epididymides, and accessory sex glands (prostate, vesicular and bulbourethral glands) were
229 evaluated for any gross abnormalities, removed, and weighed. Testes and epididymides were
230 weighed individually. Left testis and epididymis were processed for light and transmission
231 electron microscopy (Veeramachaneni *et al.* 1986; 1993). The testis was sliced into two pieces;
232 one piece was fixed in Bouin's fixative and the other in 4% (v/v) glutaraldehyde in 0.1 M
233 sodium cacodylate. The epididymis was fixed in Bouin's fluid. Tissues fixed in Bouin's fluid
234 were embedded in paraffin and those fixed in glutaraldehyde were post-fixed in osmium
235 tetroxide and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Thick sections (5
236 μ m) were cut from paraffin-embedded tissues and stained with hematoxylin and eosin (light

237 microscopy) and thin sections (60-80 nm) were cut from Poly/Bed-embedded tissues and stained
238 with uranyl acetate and lead citrate (transmission electron microscopy).

239 The right testis and epididymis were processed for quantification of daily sperm
240 production and epididymal sperm reserves. The epididymis was dissected from the testis, cut
241 into two segments (caput-corporis, cauda), weighed, frozen and stored at -80°C. The testis was
242 decapsulated, weighed, frozen and stored at -80°C.

243

244 *Daily Sperm Production and Epididymal Sperm Reserves*

245 Daily sperm production and epididymal sperm reserves were determined by procedures
246 described for rabbits (Amann and Lambiase, 1969) with minor modifications. Briefly, testicular
247 parenchyma was thawed, minced on a watch glass, and homogenized for 1 min in a semi-micro
248 Waring blender using 50 ml buffer (0.145 M NaCl containing 4 mM NaN₃ and 0.05% (v/v)
249 Triton X-100). The number of homogenization-resistant elongated spermatid nuclei was
250 determined without further dilution using a hemocytometer (4 chambers/sample). Daily sperm
251 production was calculated using a time divisor of 5.35 days (Amann and Lambiase, 1969) and
252 expressed per gram of testicular parenchyma. A caput-corporis or cauda epididymidis was
253 thawed, minced on a watch glass, and homogenized for 3 min using 50 or 125 ml of buffer,
254 respectively. Without further dilution, sperm nuclei were counted using a hemocytometer (4
255 chambers/sample). Epididymal sperm reserve was expressed per epididymal segment.

256

257 *Histopathology of the Testis*

258 Seminiferous epithelium and interstitium were evaluated using a Nikon Microphot-FXA
259 light microscope and a JEOL-1200EX transmission electron microscope. For light microscopy,
260 100 randomly selected, essentially round (major diameter < 1.5X minor diameter) cross sections

261 of seminiferous tubules from each animal were classified into one of eight different grades
262 indicating relative severity of seminiferous epithelial abnormalities (Veeramachaneni et al,
263 1986; 2006). Briefly, grade 0 – normal intact seminiferous epithelium; grade 1 – seminiferous
264 epithelium with pyknotic germ cells and desquamation or focal vacuolation; grade 2 –
265 seminiferous epithelium intermediate between grades 1 and 3; grade 3 – seminiferous
266 epithelium with pre-meiotic germ cells and Sertoli cells; grade 4 – Sertoli cells only; grade 5 –
267 no seminiferous epithelium leaving only the basement membrane; grade 6 – seminiferous tubule
268 with sperm stasis, sperm granuloma, or mineralization; grade 7 – fibrosis of the seminiferous
269 tubule. A weight between 0 and 1, to reflect relative absence of germ cells, was assigned to each
270 grade – 0, 1/4, 2/4, 3/4 to grades 0, 1, 2, 3, respectively, and 4/4 to grades 4 through 7. The
271 degree of germinal epithelial loss (DGEL) was calculated by multiplying the percentage of
272 tubules in each grade by the respective assigned weight and summing products. For electron
273 microscopy, corresponding tissue sections were evaluated for any subtle lesions or abnormal
274 cells.

275

276 *Hormone Assays*

277 Serum concentrations of FSH and LH were determined by validated radioimmunoassays
278 (Pau et al, 1986; Bodensteiner et al, 2004) using homologous rabbit reagents provided by Dr. A.
279 Parlow and the Pituitary Hormone Program, National Institutes of Health. The final dilutions of
280 antisera for FSH and LH were 1:72,000 and 1:2,160,000, respectively. For a given hormone, all
281 samples were assayed in a single assay and the intra-assay coefficients of variation were 11.4%
282 for FSH and 4.9% for LH. Testosterone was extracted from each serum sample and assayed

283 using a validated radioimmunoassay (Berndtson et al, 1974). All samples were assayed
284 concurrently and intra-assay coefficient of variation was 4%.

285

286 *Statistical Analyses*

287 Differences in parameters were analyzed by one-way ANOVA using the GLM procedure
288 of SAS (Statview, version 5.0, SAS Institute Inc., Cary, NC). If a significant difference ($p <$
289 0.05) was indicated, Tukey/Kramer posthoc test was used as an indicator of differences among
290 means. For fertility and morphological data the percentage values were transformed using
291 arcsine of the square root of the percentage/100 to minimize inequality in variance and Fisher's
292 protected LSD posthoc test was used to detect differences among means.

293

294 **RESULTS**

295 *Delivered Doses*

296 The calculated average (\pm SE) daily delivered doses for the post-weaning rabbits were 0,
297 1.02 ± 0.03 , 5.8 ± 0.55 , and 61.08 ± 4.64 for the 12 wk group and 0, 0.99 ± 0.02 , 5.2 ± 0.23 and
298 55.57 ± 1.95 mg/kg body wt for the 24 wk group (Table 1). These doses were very close to the
299 targeted dosages, viz., 0, 1, 5 and 50 mg DBA/kg body wt. Water consumption in the high dose
300 exposure (50 mg/kg) was tested in a preliminary study and did not differ significantly from that
301 of control in the definitive study (Table 1).

302

303 *General Parameters*

304 DBA did not cause any overt toxic effects in the pregnant dams. The average body
305 weights for 0, 1, 5, and 50 mg/kg dose groups on gestation days 22 and 29 were 2.50 and 2.46
306 kg; 2.56 and 2.50 kg; 2.45 and 2.39 kg; and 2.54 and 2.42 kg, respectively.

307 DBA had no effect on growth rate or total body weight in either age group (Table 2).
308 Testicular descent was impaired in DBA-treated rabbits; 1 (out of 22), 2 (out of 32), and 1 (out
309 of 21) in 1, 5, and 50 mg DBA/kg groups were unilaterally cryptorchid; in each instance the
310 retained testis was abdominal and was associated with a poorly developed gubernaculum. No
311 other gross abnormalities of the reproductive tract were observed. Ano-genital distances were
312 similar between control and treated rabbits in both age groups. Except for an increased ($p <$
313 0.05) liver weight in 50 mg/kg group of adult (24 wk) rabbits, none of the organ weights
314 differed from those of controls. No gross abnormalities of the viscera were observed.

315

316 *Hypothalamo-Pituitary-Testicular Axis*

317 In both prepubertal and adult animals, serum FSH and LH peaked at 30 min and declined
318 by 120 min post-GnRH; there were no significant differences in basal concentrations or in
319 response to exogenous GnRH (Table 3). Similarly, except for a significant increase ($p < 0.05$) in
320 testosterone by 30 min post-GnRH in 50 mg DBA/kg group, there were no differences in basal
321 or post-GnRH concentrations of testosterone.

322

323 *Mating Ability*

324 Chronic exposure to DBA adversely affected mating ability of some rabbits. On at least 1
325 occasion out of the 6 episodes, 1 rabbit in each of the 1 and 5 mg DBA/kg groups, and 2 rabbits
326 in the 50 mg DBA/kg group failed to accomplish ejaculation (Table 4). One rabbit in 1mg
327 DBA/kg group never evinced sexual interest in a female teaser and failed to achieve penile
328 erection or ejaculation in all 6 episodes. Serum concentration of testosterone in this rabbit was
329 not substantially different from the values of other rabbits in this group. Excluding episodes
330 culminating in failure, the interval between placement of a teaser into the male's cage and

331 ejaculation averaged 15 sec for control males, but averaged 29 sec for 50 mg DBA/kg males
332 (Table 1); the averages for 1 mg DBA/kg and 5 mg DBA/kg groups were not significantly
333 different ($p > 0.1$) from that of control group (Table 4).

334

335 *Seminal Parameters and Spermatogenesis*

336 Chronic exposure to DBA at the dose levels tested did not affect number of sperm
337 produced but impaired spermiogenesis resulting in morphologically defective sperm (Table 5).
338 Daily sperm production per gram of testis, sperm reserves in the epididymis, and total number of
339 sperm per ejaculate for DBA-treated rabbits were not different ($p > 0.1$) from those of controls.
340 However, the volume of the ejaculate in each of the DBA groups was higher ($p < 0.05$) than that
341 of the control resulting in lower ($p < 0.05$) sperm concentration in DBA treatment groups.

342 The percentage of morphologically normal spermatozoa was reduced ($p < 0.05$) in all
343 DBA groups compared to the control group (Table 5). Predominant sperm morphological
344 defects in DBA-treated rabbits involved acrosome and nucleus. Nine to 18% of sperm were
345 affected by one or both defects in DBA-treated rabbits vs. ~2% in control rabbits.

346 These defects included abnormal acrosomes characterized by cystic or knobbed
347 appearance (Figures 1a, 2a and 2b). These dysplastic acrosomes occasionally spread around two
348 or more spermatids in a cohort (Figure 1b) resulting in conjoined heads sharing a common
349 acrosome (Figure 1b) and causing nuclear aberrations (Figure 2c). Occasionally, clusters of
350 such abnormal sperm had been exfoliated along with Sertoli cell remnants (Figure 3a). At an
351 electron microscopic level, it was evident that these cells were from a cohort of spermatids
352 whose nuclear division was impaired during spermiogenesis (Figure 3b).

353

354 ***SP22 and Fertility***

355 Concentrations of SP22, a specific sperm membrane fertility protein, in detergent
356 extracts of ejaculated sperm of DBA-treated groups were significantly lower ($p < 0.05$) than that
357 in control group (Figure 4). The conception rates for 1, 5, and 50 mg DBA/kg groups were, 55
358 (10/18), 65 (13/20), and 55% (9/16), respectively, vs. 85% (17/20) for control group (Table 4).
359 One male from 5 mg DBA/kg group and 2 males from 50 mg DBA/kg group failed to
360 impregnate either test female.

361

362 ***Histopathology of Testis***

363 Histopathological changes in testes representing grades 1 and 2 were found in all groups
364 of rabbits (Table 6), including controls; the incidence was higher ($p < 0.05$) for grade 1 lesions
365 in 5 and 50 mg DBA/kg groups and for grade 2 lesions in 1 and 50 mg DBA/kg groups. The
366 higher incidence of these lesions in DBA-treated rabbits was mirrored by fewer ($p < 0.05$) normal
367 (grade 0) seminiferous tubules (Figure 5) and higher DGEL (Table 6). Lesions in seminiferous
368 epithelium were characterized by pyknosis of differentiating germ cells, formation of
369 “multinucleated giant cells” (a syncytium of spherical spermatids), and ultimate degeneration
370 and desquamation leaving focal vacuolation in seminiferous epithelium (Figure 5b). Exfoliated
371 premature germ cells were evident in the excurrent ducts (Figures 5e and 5d) and seminal
372 ejaculates. Spermiogenesis was arrested largely at round spermatid stage in 1 of the 50 mg
373 DBA/kg rabbits (Figures 5c and 5f).

374

375 **DISCUSSION**

376 Chronic exposure of male rabbits to DBA beginning at differentiation of fetal
377 reproductive system impaired testicular descent, spermiogenesis, and sexual function as adults

378 in some animals. There was no change in ano-genital distance or in the weights of the testis,
379 epididymis, or accessory sex glands. This is consistent with a general lack of observed effects
380 on serum FSH, LH, or testosterone either before or after GnRH stimulation. These findings also
381 are consistent with the results of previous studies on haloacid exposures in rodents. Daily
382 administration of 270 mg DBA/kg failed to alter serum testosterone (Linder et al, 1994b). More
383 recent studies with DBA (Klinefelter et al, 2004) or BCA (Sloan et al, 2005) demonstrating
384 significant body weight-independent delays in attainment of puberty (as indicated by preputial
385 separation) did not detect any hormonal deficiency in either serum or testicular interstitial fluid.
386 While it is commonly accepted that a delay in puberty (i.e., delay in preputial separation) would
387 be prompted by androgen insufficiency during early reproductive development this may not
388 always be the case. Notably, the definitive indicator of the onset puberty in the male, viz., the
389 first presence of sperm in the epididymis or ejaculate, has rarely been determined in studies
390 involving laboratory animals.

391 DBA exposure compromised mating behavior significantly. Rabbits exposed to 50 mg
392 DBA/kg required twice the time for ejaculation than control males. While all rabbits in the
393 control group ejaculated at each opportunity, 10% of the males exposed to 1 or 5 mg DBA/kg
394 and 22% of the males exposed to 50 mg DBA/kg failed at least one attempt. The observed
395 deficit in mating behavior is consistent with an earlier rat study on DBA (Linder et al, 1995) in
396 which 40, 60, 80, and 90 % of males exposed to 2, 10, 50, and 250 mg DBA/kg daily for 70
397 days failed to successfully sire two females during one week of cohabitation. One of the rabbits
398 exposed to 1 mg DBA/kg in the present study never ejaculated and in fact displayed no sexual
399 interest and was unable to achieve an erection. Thus, it would seem some underlying
400 neurological deficit following developmental exposure to DBA may account for the observed
401 sexual dysfunction. Further support that early development is when the effect on sexual

402 behavior is manifested comes from our observations that male rabbits exposed developmentally
403 to chemical pollutants common in drinking water (Veeramachaneni et al, 2001) or pesticides
404 such as vinclozolin (Veeramachaneni et al, 2006) manifest aberrant sexual behavior and
405 function. That some of these chemicals indeed affect normal differentiation of sexually
406 dimorphic areas of brain important for regulating male sexual behavior is evident from our
407 recent studies with vinclozolin (Bisenius et al, 2006).

408 Haloacid exposure consistently has been found associated with deficits in sperm quality.
409 Toth et al, (1992) reported a decline in motility and morphology of epididymal sperm following
410 exposure of rats to dichloroacetic acid (DCA). Subsequently, Linder et al (1994a and b, 1995)
411 reported similar findings in rats exposed to DBA. More recently bromochloroacetic acid (BCA)
412 has been shown to produce compromise in both epididymal sperm motility and morphology
413 (Klinefelter et al, 2002). In the present study, the percentage of morphologically normal sperm
414 was decreased significantly at each exposure level. Moreover, the magnitude of the changes in
415 morphology were consistent across exposures, with 18, 13, and 16 % of the sperm displaying
416 acrosomal defects in rabbits exposed to 1, 5, and 50 mg DBA/kg compared to 2 % in control
417 males. Similarly, 13, 9, and 16 % of the sperm had nuclear defects compared to 2.5 % for sperm
418 in control males.

419 An interesting new effect described in the present rabbit study is the DBA-induced
420 increase in volume of the seminal ejaculate resulting in an apparent decrease in sperm
421 concentration (23-29%) as total sperm per ejaculate in these animals was not different from that
422 of controls. The accessory sex glands were heavier, but not significantly, in DBA-treated
423 rabbits; perhaps this reflects increased secretion and/or biochemically altered constitution of
424 seminal plasma components. Although it is not possible to delineate, under the experimental
425 conditions, if it is the impaired secretion of seminal plasma or morphologically defective sperm

426 that contributed to impaired fertility (see below), the value of an animal model such as rabbit
427 which enables evaluation of a seminal ejaculate is obvious.

428 Using a very limited number of test females (n=2)/male, insemination of a constant
429 number of ejaculated sperm from DBA-treated rabbits resulted in a reduced conception rate (55-
430 65% across treatment groups vs. 85% in controls). Assuming that these conception rates hold
431 with larger number of test females, the reduced conception rate for ejaculated sperm from male
432 rabbits exposed to 1 mg DBA/kg is consistent with the observed reduction in the fertility of
433 cauda epididymal rat sperm following a two week exposure to 2 mg DBA/kg (Kaydos et al,
434 2004).

435
436 One aspect of sperm quality that now has been repeatedly associated with fertilizing
437 ability is the protein SP22 and its level of expression on the sperm membrane (Klinefelter et al,
438 1997). It is well established that a unique testis-specific mRNA transcript for SP22 is expressed
439 in pachytene spermatocytes and subsequent germ cells (Klinefelter, 2003). SP22 is quantified in
440 sperm extracts either by quantitative 2D SDS-PAGE or by enzyme linked immunosorbant assay
441 (ELISA). In the present rabbit study SP22 was quantified for the first time in extracts of
442 ejaculated sperm by ELISA. Levels of SP22 were diminished significantly in each treatment
443 group. It is likely that the reduced expression of SP22 together with the decreased number of
444 morphologically normal sperm (discussed below) account, in part, for the reduced conception
445 rate of male rabbits exposed to DBA. Haloacids have been shown to diminish SP22 in rodent
446 epididymal sperm; diminished levels of SP22 were highly correlated with observed declines in
447 fertility of rats exposed to BCA (Klinefelter et al, 2002) or a binary mixture of DBA and BCA
448 (Kaydos et al, 2004).

449 Haloacid-induced deficits in sperm quality (i.e., fertility, SP22, motility and morphology)
450 likely result from an initial toxic insult on spermatogenesis. Over the years, consistent
451 histological findings in testes have been noted in rodents exposed to haloacids. Delayed
452 spermiation and/or retention of atypical residual bodies have been reported following exposures
453 to DCA (Toth et al, 1992) and DBA (Linder et al, 1994a and b). Linder et al (1997) described a
454 sequential appearance of lesions in germinal epithelium following 2 to 79 day exposure to 250
455 mg DBA/kg; delayed spermiation, atypical residual bodies and spermatid fusion during the first
456 two weeks were followed by misshapen spermatid nuclei, vesiculated acrosomes, and Sertoli cell
457 vacuolation culminating in marked atrophy of seminiferous tubules at 6 months (after 42 doses).
458 The low effect level in this study was 10 mg DBA/kg based on an increased incidence of
459 spermatid retention. In a two-generation drinking water exposure study of DBA (Christian et al,
460 2002) comparable alterations in spermatogenesis were seen again; atypical residual bodies and
461 delayed spermiation were reported at 12 mg DBA/kg.

462 The present rabbit study is the first to characterize haloacid-induced alterations in
463 spermatogenesis in a species other than the rat. Strikingly, at an exposure level as low as 1mg
464 DBA/kg, impaired spermiogenesis (acrosomal-nuclear dysgenesis) and an increased incidence of
465 germinal epithelial loss were observed. In the adult rat study by Linder et al (1997) no
466 abnormalities were detected at 2 mg DBA/kg.

467 Unique acrosomal-nuclear defects, characterized by sharing of an acrosome by 2 or more
468 spermatids, acrosomal dysplasia, and nuclear malformations, observed in this study have also
469 been found following shorter, developmental exposures to common chemical contaminants in
470 drinking water (Veeramachaneni et al, 2001) and fungicide vinclozolin (Veeramachaneni et al,
471 2006). In these studies, which were also performed in rabbits, acrosomal-nuclear defects
472 continued to manifest long after cessation of exposure, suggesting that a permanent epigenetic

473 change had been induced in stem-spermatogonia and/or Sertoli cells. It is not discernible if
474 spermatogenic arrest and karyorrhectic or pyknotic changes in germ cell nuclei and consequent
475 cell death are a direct effect of DBA on differentiating germ cells or an indirect effect via actions
476 on Sertoli cells.

477 In summary, at the 3 dose levels studied in the present study, a no-effect-level was not
478 observed. In fact, the low-effect-level of 1 mg DBA/kg was established on effects observed in
479 multiple endpoints. Male rabbits exposed throughout reproductive development to 1 mg
480 DBA/kg were found to have: deficits in mating behavior, reduced conception rate, decreased
481 concentration of sperm in ejaculates, increased numbers of morphologically abnormal sperm in
482 ejaculates, diminished SP22 levels on ejaculated sperm, and an increased incidence of germ cell
483 dysplasia and desquamation. It is reasonable to attempt to put these data in some context to
484 human risk relevance by estimating a margin of exposure (MOE). A MOE simply attempts to
485 relate an experimental exposure where no observed adverse effect occurs to an estimated
486 human exposure, i.e., no-effect-level / consumption. When a no-effect-level is not established
487 experimentally, it is computed by dividing the observed low-effect-level by 10; yielding 0.1
488 mg/kg in this case. For adults, consumption is estimated based on the assumption that a 60 kg
489 male consumes 2 liters of water per day containing 20 µg DBA/L (10 times the average DBA
490 level); $[0.1\text{mg/kg} / (0.02\text{mg/L} \times 2\text{L}/60\text{kg}) = 0.1 / 0.0007 = 142]$. Likewise, for a 3 kg infant
491 drinking 1 liter per day ; $[0.1\text{mg/kg} / (0.02\text{mg/L} \times 1\text{L}/3\text{kg}) = 0.1/0.0067 = 14.9]$. Thus, the
492 estimated no-observed effect exposure in this study is 142 times the comparable exposure in
493 men (driven by deficits in mating, sperm quality, etc.) and 15 times for children (driven by
494 compromised testicular descent). While this in itself may not be alarming, it also is not very
495 comforting in view of the knowledge that all haloacids in drinking water appear to act similarly
496 in the testis. Indeed, if the 9 prevalent haloacids in drinking water are considered collectively,

497 one could essentially divide the above MOE's by a factor of 10. Moreover, it needs to be
498 recognized that one's 'cumulative' exposure to testicular toxicants occur via myriad other
499 sources (e.g., pharmaceuticals, pesticide residues, alcohol etc.).

500

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TABLE 1
Average daily consumption of water and doses of DBA delivered

	Treatment Group (mg DBA/kg/day)			
	0	1	5	50
Water Consumption (ml/d)				
Dams (GD15-PNW6)	278.8 ± 42.7	312.7 ± 68.1	319.0 ± 54.7	239.6 ± 37.7
Male Offspring (PNW6-12)	204.4 ± 10.1	236.3 ± 14.0	193.0 ± 8.7	200.9 ± 7.6
Male Offspring (PNW6-24)	218.8 ± 8.6	236.1 ± 7.6	196.5 ± 4.6	212.6 ± 4.8
DBA Delivered (mg/kg/d)				
Dams (GD15-PNW6)	---	1.25 ± 0.18	6.68 ± 0.42	58.44 ± 4.46
Male Offspring (PNW6-12)	---	1.02 ± 0.03	5.80 ± 0.55	61.08 ± 4.64
Male Offspring (PNW6-24)	---	0.99 ± 0.02	5.20 ± 0.23	55.57 ± 1.95

Values represent mean ± SEM. GD=gestation day, PNW=post-natal week.

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TABLE 2
Incidence of cryptorchidism, ano-genital distance and organ weights at necropsy in 12-wk and 24-wk-old rabbits exposed to 0, 1, 5, or 50 mg DBA/kg/day

Treatment Group	12 wk				24wk			
	0 mg	1 mg	5 mg	50 mg	0 mg	1mg	5 mg	50 mg
n	10	12	22	11	10	10	10	10
Cryptorchidism	0	0	1 unilateral	0	0	1 unilateral	1 unilateral	1 unilateral
Ano-genital distance (mm)	7.1 ± 0.26	7.3 ± 0.28	7.5 ± 0.18	7.1 ± 0.20	7.9 ± 0.42	7.5 ± 0.31	7.2 ± 0.42	7.4 ± 0.40
Organ Weight (g)								
Body	1532 ± 28	1533 ± 24	1527 ± 26	1535 ± 38	1961 ± 21	2046 ± 61	2017 ± 54	2080 ± 68
Scrotal Testis	0.72 ± 0.04	0.78 ± 0.04	0.80 ± 0.05	0.85 ± 0.06	1.98 ± 0.09	2.01 ± 0.14	2.19 ± 0.16	2.09 ± 0.08
Scrotal Epididymis	0.34 ± 0.02	0.34 ± 0.01	0.30 ± 0.02	0.29 ± 0.02	0.67 ± 0.03	0.71 ± 0.03	0.69 ± 0.04	0.75 ± 0.04
Accessory Sex Glands	1.13 ± 0.09	1.05 ± 0.06	1.06 ± 0.05	1.03 ± 0.06	3.10 ± 0.15	3.66 ± 0.23	3.57 ± 0.32	3.52 ± 0.19
Liver	56.02 ± 2.6	55.35 ± 1.6	56.89 ± 1.6	64.35 ± 3.0	57.76 ± 2.5	65.97 ± 3.4	62.33 ± 2.7	81.86 ± 4.6*
Paired Kidneys	11.90 ± 0.3	11.87 ± 0.4	12.21 ± 0.3	12.75 ± 0.4	11.96 ± 0.4	12.90 ± 0.5	12.56 ± 0.4	13.58 ± 0.7

Values represent mean ± SEM. Weights of left testis and epididymis are presented unless left was cryptorchid.
 Values with an asterisk within a row in an age group are different from control ($p < 0.05$).

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TABLE 3
Reproductive Hormones in 12-wk and 24-wk-old male rabbits exposed to 0, 1, 5, or 50 mg DBA/kg/day

Treatment Group	12 wk					24 wk		
	0 mg	1 mg	5 mg	50 mg	0 mg	1 mg	5 mg	50 mg
n	10	12	21	10	10	10	10	9
Serum FSH (ng/ml)								
Pre-GnRH	17.70 ± 1.74	13.90 ± 1.83	23.17 ± 4.05	18.66 ± 1.45	10.59 ± 1.17	13.37 ± 1.90	11.20 ± 1.22	11.42 ± 1.52
30 min post-GnRH	24.04 ± 3.00	27.38 ± 3.58	33.08 ± 2.46	33.00 ± 2.52	18.96 ± 1.97	24.79 ± 7.07	18.52 ± 2.06	16.41 ± 1.99
Change	6.34 ± 2.53	13.48 ± 2.34	9.91 ± 4.51	14.34 ± 1.90	8.37 ± 1.36	11.42 ± 5.46	7.32 ± 1.61	4.98 ± 1.39
120 min post-GnRH	21.61 ± 1.82	17.54 ± 1.74	23.31 ± 1.53	24.42 ± 1.54	14.63 ± 1.30	17.92 ± 3.45	15.03 ± 0.93	13.97 ± 1.91
Change	3.91 ± 2.25	3.64 ± 0.76	0.14 ± 4.21	5.76 ± 1.03	4.04 ± 0.85	4.55 ± 1.91	3.83 ± 0.87	2.55 ± 1.09
Serum LH (ng/ml)								
Pre-GnRH	1.05 ± 0.77	0.14 ± 0.10	0.67 ± 0.25	0.09 ± 0.02	0.11 ± 0.08	0.41 ± 0.20	0.03 ± 0.01	0.23 ± 0.14
30 min post-GnRH	4.74 ± 0.95	5.27 ± 0.49	6.21 ± 0.51	4.74 ± 0.52	7.13 ± 0.76	6.90 ± 1.07	6.30 ± 0.88	4.63 ± 0.76
Change	3.69 ± 1.39	5.13 ± 0.51	5.54 ± 0.57	4.65 ± 0.51	7.02 ± 0.77	6.49 ± 1.05	6.27 ± 0.88	4.41 ± 0.78
120 min post-GnRH	0.41 ± 0.12	0.22 ± 0.03	0.28 ± 0.05	0.24 ± 0.05	0.87 ± 0.19	0.82 ± 0.22	0.56 ± 0.12	0.40 ± 0.13
Change	-0.64 ± 0.76	0.08 ± 0.12	-0.39 ± 0.25	0.16 ± 0.05	0.76 ± 0.22	0.41 ± 0.24	0.53 ± 0.12	0.17 ± 0.20
Serum Testosterone (ng/ml)								
Pre-GnRH	5.68 ± 1.95	5.84 ± 1.35	9.32 ± 1.58	6.24 ± 2.03	4.94 ± 1.42	3.35 ± 0.62	3.27 ± 0.85	6.08 ± 2.26
30 min post-GnRH	16.23 ± 1.57	19.55 ± 1.34	19.69 ± 1.02	22.02 ± 1.5*	18.45 ± 1.02	21.55 ± 1.97	19.65 ± 1.12	22.11 ± 1.39
Change	10.55 ± 1.95	13.71 ± 2.01	10.37 ± 1.86	15.96 ± 1.95	13.51 ± 1.56	18.19 ± 2.21	16.38 ± 1.10	16.02 ± 2.34
120 min post-GnRH	15.99 ± 0.83	19.48 ± 1.07	20.34 ± 1.15	21.73 ± 3.50	13.70 ± 1.40	15.21 ± 1.04	16.63 ± 2.05	18.13 ± 1.63
Change	10.31 ± 2.18	13.64 ± 1.71	11.02 ± 2.18	15.49 ± 4.18	8.76 ± 1.76	11.86 ± 1.27	13.35 ± 2.52	12.05 ± 2.85

Values represent mean ± SEM.

Values with an asterisk within a row in an age group are different from control (p<.05).

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TABLE 4
Mating Ability and Fertility of 24-wk-old rabbits exposed to 0, 1, 5, or 50 mg DBA/kg/day

Treatment Group	n	Average time for ejaculation (sec) [†]	No. of males failed to ejaculate at least once	No. of males never ejaculated	Percent conception (after AI)	Failed to fertilize both test females
0 mg	10	14.71 ± 1.35 (59)	0	0	85 (17/20)	0/10
1 mg	10	12.76 ± 0.90 (53)	1	1	55 * (10/18)	0/9 [‡]
5 mg	10	19.61 ± 2.88 (57)	1	0	65 (13/20)	1/10
50 mg	9	28.96 ± 4.43* (45)	2	0	55 * (9/16)	2/8 [‡]

[†] Excludes time for episodes which culminated in failure to ejaculate.

[‡] One male failed to ejaculate for fertility test.

Values represent mean ± SEM. Numbers in parentheses indicate number of ejaculates.
 Values with an asterisk within a column are different from control (p < 0.05).

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TABLE 5
Semen parameters measured in 24-wk-old rabbits exposed to 0, 1, 5, or 50 mg DBA/kg/day

Treatment Group	0 mg	1 mg	5 mg	50 mg
n	10	9	9	9
Tissue Homogenates				
Daily sperm production (X 10 ⁶ /g testis)	23.54 ± 1.07	25.98 ± 1.18	22.82 ± 0.74	22.02 ± 1.29
Caput epididymal sperm reserve (X 10 ⁶)	113.45 ± 9.87	121.30 ± 15.50	162.70 ± 10.91	107.67 ± 9.76
Cauda epididymal sperm reserve (X 10 ⁶)	513.70 ± 49.22	464.04 ± 43.93	537.53 ± 61.31	452.74 ± 43.59
Semen Parameters				
Ejaculate volume (ml)	0.25 ± 0.01 (55)	0.36 ± 0.02* (52)	0.32 ± 0.02* (56)	0.30 ± 0.03 * (42)
Sperm concentration (X 10 ⁶ /ml)	594.03 ± 45.70	457.57 ± 25.47*	423.99 ± 27.40*	438.16 ± 46.14*
Total sperm/ejaculate (X 10 ⁶)	145.08 ± 11.84	164.95 ± 11.09	140.88 ± 10.80	150.83 ± 22.57
Sperm Morphology				
Morphologically normal sperm (%)	86.56 ± 0.60	63.22 ± 4.91*	71.04 ± 3.63*	61.18 ± 2.31*
Acrosomal defects (%)	2.11 ± 0.36	18.33 ± 2.84*	13.38 ± 3.94*	15.95 ± 1.40*
Nuclear defects (%)	2.51 ± 0.17	13.67 ± 1.45*	9.18 ± 2.02*	16.26 ± 1.35*

Values represent mean ± SEM. Values do not include cryptorchid animals and episodes which culminated in failure to ejaculate. Values with an asterisk within a row are different from control (p < 0.05). Numbers in parentheses are total number of samples counted.

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TABLE 6
Histopathological changes in the seminiferous epithelium of 24-wk-old rabbits exposed to 0, 1, 5, or 50 mg DBA/kg/day

Treatment Group	n	Percentage of seminiferous tubules graded as:								Degree of germinal epithelial loss
		0	1	2	3	4	5	6	7	
0 mg	9	91.1 ± 0.8	7.7 ± 0.8	1.1 ± 0.6	0.1 ± 0.1	---	---	---	---	2.6 ± 0.3
1 mg	10	77.9 ± 2.7*	13.0 ± 1.7	6.7 ± 1.5*	2.7 ± 1.4*	---	---	---	---	8.6 ± 1.5*
5 mg	10	79.5 ± 3.1*	16.6 ± 2.5*	3.9 ± 1.3	---	---	---	---	---	6.1 ± 1.0
50 mg	8 [†]	76.0 ± 2.8*	17.9 ± 2.2*	5.1 ± 1.2*	0.5 ± 0.3	0.4 ± 0.4	0.1 ± 0.1	---	---	7.9 ± 0.9*

Values represent mean ± SEM. Values with an asterisk within a column are different from control (p<0.05).

[†]Excludes animal with spermatogenic arrest.

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642 **Figure Legends**

643

644 **Figure 1.** Acrosomal dysgenesis: shared acrosome. (A) Differential interference contrast
645 micrograph of ejaculated sperm of a 24-wk-old rabbit exposed to 5 mg DBA. Note the
646 dysplastic acrosome (arrow) shared between two sperm heads resulting in conjoined sperm.
647 Inset light micrograph shows two fused spermatid nuclei sharing a common acrosomic vesicle
648 (arrow). (B) Transmission electron micrograph of a testicular section depicting morphogenesis
649 of conjoined sperm sharing a common vesiculated acrosome. Scale bars = A: 10 μm , B: 1 μm .

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651 **Figure 2.** Acrosomal dysgenesis: vesiculation and dysplasia. (A) Differential interference
652 contrast micrograph of ejaculated sperm of a 24-wk-old rabbit exposed to 5 mg DBA. Note
653 presence of knobbed/cystic acrosomes (arrows). Dysplastic acrosomal membranes manifest as
654 craters and protrusions on the sperm head (left). (B) Transmission electron micrograph of a
655 testicular section depicting acrosomes enveloping elongating spermatid nuclei. Note excessive
656 acrosomal matrix and vesiculation of the spermatid on the left. The cytoplasmic inclusions of
657 the acrosomal cyst (arrow) resemble that of Sertoli cell. (C) Transmission electron micrograph
658 of a testicular section depicting acrosomal/nuclear dysplasia. Note aberrant membranes (arrows)
659 and irregular, fragmented nuclear chromatin (asterisks). Scale bars = A: 10 μm , B: 0.5 μm , C: 1
660 μm .

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662 **Figure 3.** Nuclear dysgenesis in spermatid cohorts: incomplete nuclear division. (A)
663 Differential interference contrast micrograph of ejaculated sperm of a 24-wk-old rabbit exposed
664 to 5 mg DBA. Note a cluster of sperm (arrow) embedded in a denuded seminiferous epithelial

665 fragment. That these sperm are a cohort that failed to segregate during spermiogenesis is
666 evident upon ultrastructural examination. (B) Light (inset) and transmission electron
667 micrographs of a testicular section depicting impaired spermiogenesis. Note the connection
668 (arrow) between two spermatid nuclei extending through the intracellular bridge of a cohort. As
669 they condense and spermiate, these sperm cells appear to exfoliate still embedded in fragments
670 of Sertoli cells as seen in panel A. Scale bars = A: 10 μm , B: 1 μm .

671
672 **Figure 4.** Histogram depicting concentrations of SP22 measured by ELISA in detergent extracts
673 of ejaculated rabbit spermatozoa. Values are expressed as the mean integrated optical density
674 (IOD). IOD is decreased significantly in each DBA treatment group; asterisk denotes significant
675 difference from control ($p < 0.05$). N=8 samples/group.

676
677 **Figure 5.** Photomicrographs of testicular and epididymal sections from 24-wk-old control and
678 DBA-treated rabbits. (A) Seminiferous tubules in a control testis showing normal progression of
679 spermatogenesis. (B) Seminiferous tubules in a 50 mg DBA rabbit showing extensive
680 vacuolation in seminiferous epithelium resulting from exfoliation of germ cells. Multinucleated
681 giant cells (spherical spermatid syncytia; arrows), which eventually get denuded, leaving
682 vacuoles in seminiferous epithelium are seen. (C) Seminiferous tubules in a 50 mg DBA rabbit
683 showing impaired spermiogenesis. Spermiogenesis is largely arrested at round spermatid stage.
684 Exfoliation of syncytia of round spermatids (arrows) is seen. (D) Caput epididymidis of a
685 control rabbit showing normal luminal contents. (E) Caput epididymidis of the 50 mg DBA
686 rabbit (same as in panel B) with luminal sperm containing aggregates of exfoliated immature
687 germ cells. (F) Caput epididymidis of the 50 mg DBA rabbit (same as in panel C) showing
688 exfoliated premature germ cells in the lumina; no sperm are evident. Hematoxylin and eosin

689 staining. Scale bars = 100 μm .

690

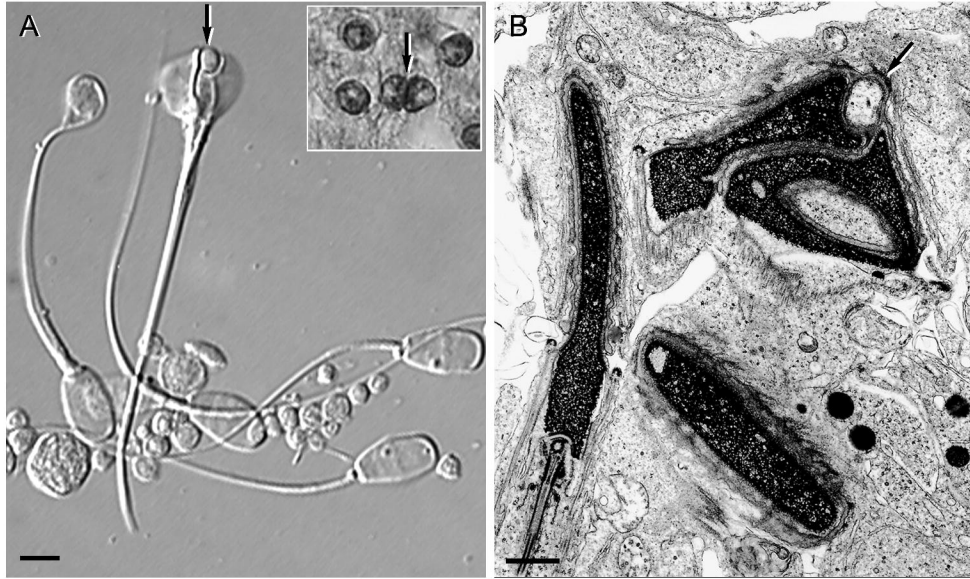


Figure 1

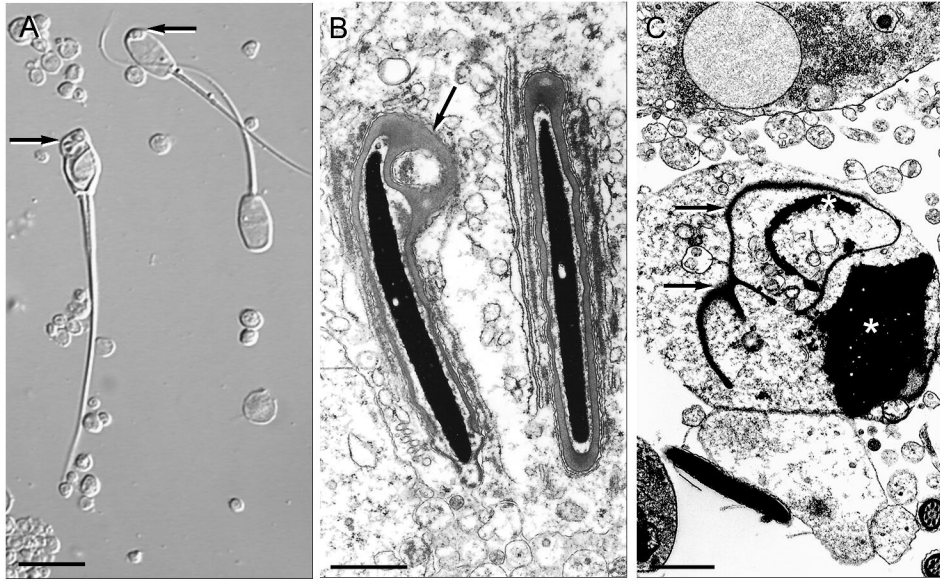


Figure 2

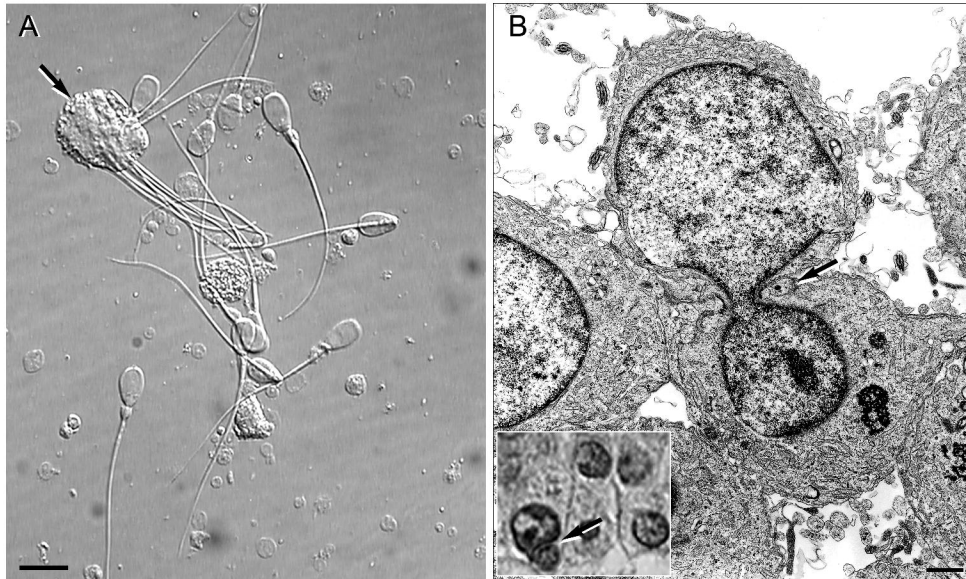


Figure 3

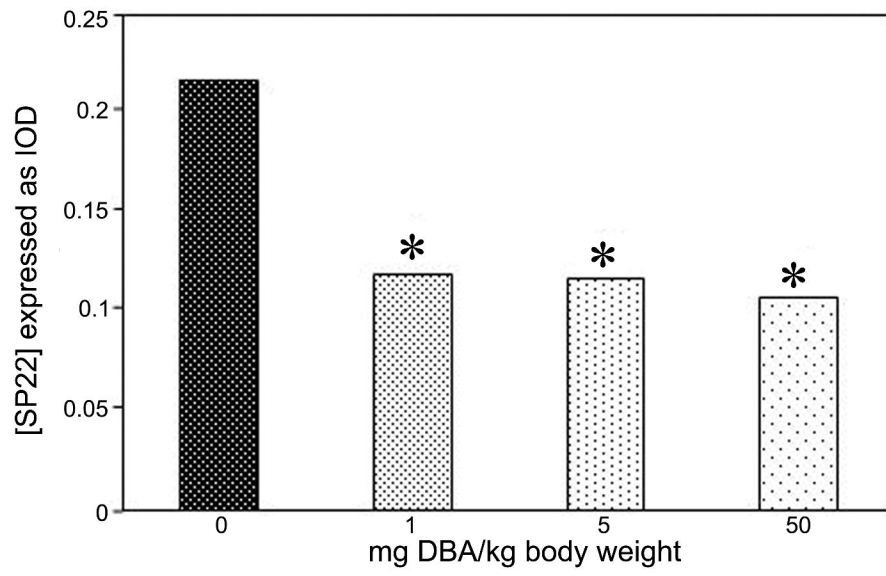


Figure 4

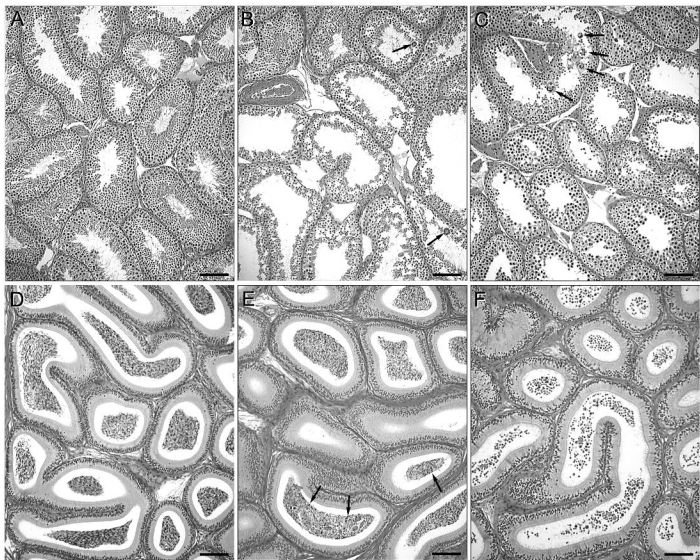


Figure 5