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Increased Sensitivity to Testicular Toxicity of Transplacental Benzo[a]pyrene Exposure in Male Glutamate Cysteine Ligase Modifier Subunit Knockout (*Gclm*^{-/-}) Mice

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Polycyclic aromatic hydrocarbons (PAHs), like benzo[a]pyrene (BaP), are ubiquitous environmental pollutants formed by the incomplete combustion of organic materials. The tripeptide glutathione (GSH) is a major antioxidant and is important in detoxification of PAH metabolites. Mice null for the modifier subunit of glutamate cysteine ligase (*Gclm*), the rate-limiting enzyme in GSH synthesis, have decreased GSH concentrations. We investigated the effects of *Gclm* deletion alone on male fertility and spermatogenesis and its effect on the sensitivity of male embryos to the transplacental testicular toxicity of BaP. *Gclm*^{-/-} males had dramatically decreased testicular and epididymal GCL enzymatic activity and total GSH concentrations compared with *Gclm*^{+/+} littermates. Ratios of reduced to oxidized GSH were significantly increased in *Gclm*^{-/-} testes. GSH reductase enzymatic activity was increased in *Gclm*^{-/-} epididymides. We observed no changes in fertility, testicular weights, testicular sperm head counts, or testicular histology and subtle changes in cauda epididymal sperm counts, motility, and morphology in *Gclm*^{-/-} compared with *Gclm*^{+/+} males. Prenatal exposure to BaP from gestational day 7 to 16 was dose dependently associated with significantly decreased testicular and epididymal weights, testicular and epididymal sperm counts, and with vacuolated seminiferous tubules at 10 weeks of age. *Gclm*^{-/-} males exposed prenatally to BaP had greater decreases in testicular weights, testicular sperm head counts, epididymal sperm counts, and epididymal sperm motility than *Gclm*^{+/+} littermates. These results show no effects of *Gclm* deletion alone on male fertility and testicular spermatogenesis and subtle epididymal effects but support increased sensitivity of *Gclm*^{-/-} males to the transplacental testicular toxicity of BaP.

Key Words: glutathione; GCLM; PAH; benzo[a]pyrene; spermatogenesis; developmental toxicity.

Reactive oxygen species (ROS), such as superoxide anion radical, hydrogen peroxide, and hydroxyl radical, are formed as byproducts of the normal cellular processes, oxidative phosphorylation and gonadal and adrenal steroidogenesis (Hanukoglu, 2006). In addition, spermatozoa may contain a membrane-bound NADPH oxidase that generates superoxide anion radical (Banfi *et al.*, 2001; de Lamirande *et al.*, 1997; Drevet, 2006). Low levels of ROS are necessary for sperm capacitation, the process by which sperm become capable of fertilizing an oocyte, and for the acrosome reaction, which enables the sperm to penetrate the zona pellucida of the oocyte and fuse with its plasma membrane (Baker and Aitken, 2005). On the other hand, oxidative stress in spermatozoa is associated with male infertility, due to impairment of sperm motility and, at higher levels, decreased sperm viability (Baker and Aitken, 2005; de Lamirande *et al.*, 1997). Spermatozoa are particularly susceptible to oxidative damage due to large amounts of polyunsaturated fatty acids in their cell membranes, which can be oxidized in the presence of ROS (Baker and Aitken, 2005; de Lamirande *et al.*, 1997; Griveau and Le Lannou, 1997).

Glutathione (GSH), the most abundant intracellular non-protein thiol, is present in cells at millimolar concentrations and is an important intracellular antioxidant. GSH has numerous functions, including reduction of hydrogen peroxide and lipid peroxides as a cofactor for GSH peroxidases, detoxification of electrophilic toxicants as a cofactor for glutathione-S-transferases (GSTs), regulation of protein function, and regulation of nucleotide metabolism (Dalton *et al.*, 2004; Franco and Cidowski, 2009). GSH is synthesized in two ATP-dependent reactions. The first, rate-limiting reaction is catalyzed by GCL, a heterodimer composed of a catalytic (GCLC) and

a modifier (GCLM) subunit. *Gclc* and *Gclm* are ubiquitously expressed, including in the testis and epididymis (Dalton *et al.*, 2004; Giordano *et al.*, 2006; Kendig *et al.*, 2011; McConnachie *et al.*, 2007; Nakamura *et al.*, 2011; Yang *et al.*, 2002). *Gclc* null mice die during embryonic development (Dalton *et al.*, 2000, 2004; Shi *et al.*, 2000). *Gclm* null mice survive and reproduce but have greatly reduced tissue levels of GSH (Dalton *et al.*, 2004; Giordano *et al.*, 2006; McConnachie *et al.*, 2007; Yang *et al.*, 2002). Female *Gclm*^{-/-} mice have greatly decreased fertility due to preimplantation embryonic mortality (Nakamura *et al.*, 2011). Because GSH and GSH-dependent enzymes have been shown to be important in certain aspects of sperm development, such as protamine substitution and midbody structure (Bertelsmann *et al.*, 2007; Kaneko *et al.*, 2002; Li *et al.*, 1989; Maiorino *et al.*, 2005; Schneider *et al.*, 2009), we hypothesized that *Gclm*^{-/-} male mice would have reduced fertility compared with their wild-type littermates.

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants found in air pollution, cooked foods, and mainstream and sidestream tobacco smoke. Human biomonitoring data demonstrate that essentially 100% of the U.S. population is exposed to PAHs (Li *et al.*, 2008). PAHs, including benzo[a]pyrene (BaP), are known testicular toxicants in adults and embryos (Ford and Huggins, 1963; Jensen *et al.*, 1963; MacKenzie and Angevine, 1981). BaP treatment of adult male rats and mice decreased testicular and epididymal weights, testicular DNA synthesis, and sperm counts (Ford and Huggins, 1963; Revel *et al.*, 2001). Treatment of pregnant mice with BaP dose dependently decreased fertility and damaged seminiferous tubules in the adult male offspring (MacKenzie and Angevine, 1981). PAHs in tobacco smoke have been implicated in the decreased fertility and semen abnormalities observed in men who smoke and in men exposed to particulate air pollution (Rubes *et al.*, 1998, 2005, 2007); however, no human studies have examined the effects of transplacental exposure to PAHs on male reproductive function. In addition, no studies have investigated genetic factors that may modulate sensitivity to the transplacental male reproductive toxicity of PAHs.

PAHs require metabolic activation in order to exert toxicity (Xue and Warshawsky, 2005). Prostaglandin-endoperoxide synthases are likely responsible for bioactivation of PAHs in the developing embryo because expression of most cytochrome P450 enzymes is generally low until after birth (Juchau, 1989; Juchau *et al.*, 1998; Rich and Boobis, 1997; Wells *et al.*, 2009). Prostaglandin-endoperoxide synthases oxidize PAHs to free radical intermediates, which can initiate ROS generation (Wells *et al.*, 2009). During mouse development, GCL expression and GSH synthesis by the embryo are present from the blastocyst stage (Stover *et al.*, 2000). GSH-mediated detoxification of ROS produced as a result of PAH metabolism may therefore represent an important line of defense against PAH-induced developmental toxicity.

In view of the importance of GSH in sperm development, in maintaining cellular redox status, and in detoxifying reactive

metabolites of BaP, we hypothesized that gonadal and reproductive tract oxidative stress in male *Gclm* null mice during development and adulthood results in abnormal spermatogenesis and decreased fertility. We further hypothesized that male *Gclm* null embryos are more susceptible to the testicular toxicity of *in utero* BaP exposure than their *Gclm*^{+/+} littermates.

MATERIALS AND METHODS

Animals. *Gclm* null mice were generated by disrupting the *Gclm* gene by replacing exon 1 with a beta-galactosidase/neomycin phosphotransferase fusion minigene (Giordano *et al.*, 2006; McConnachie *et al.*, 2007). The mice have been backcrossed eight times onto a C57BL/6J genetic background (B6.129-*Gclm*^{mtTjka}; hereafter referred to as *Gclm*^{-/-}). Mice for these experiments were generated at the University of California-Irvine or the University of Washington by mating *Gclm*^{+/-} males with *Gclm*^{+/-} females. Offspring were genotyped by PCR using primers for both the native *Gclm* sequence and the β -*Geo* sequence on DNA extracted from tail or toe snips as previously described (Giordano *et al.*, 2006). The mice were group housed with male littermates in American Association for the Accreditation of Laboratory Animal Care-accredited facilities, with free access to deionized water and soy-free laboratory chow, on a 14:10 h light-dark cycle (UC Irvine) or on a 12:12 h light-dark cycle (University of Washington). Temperature was maintained at 21°C–23°C. The experimental protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and were approved by the Institutional Animal Care and Use Committees at the respective institution.

Male fertility assessment. At 8 months of age *Gclm*^{+/+} or *Gclm*^{-/-}, male mice were mated to female C57BL/6Crl mice from Charles River Laboratories (8 weeks old) on the afternoon of proestrus, determined by vaginal cytology. Females were assessed the next morning for vaginal plugs, and the day of plug was designated embryonic day 1 (E1). Pregnancy was further confirmed by at least 7 days of leukocytic vaginal cytology. Each male was initially mated with two females, with at least a 4-day rest between matings. If a female had a nonpregnant uterus despite vaginal cytology consistent with pregnancy, the male was mated with another female. Litter size was assessed at E18. Live and dead fetuses and resorption sites were counted, and fetuses were weighed. All uteri were then stained in 10% ammonium sulfide for 4 h and checked again for additional resorption sites (Narotsky *et al.*, 1997). The means of the numbers of live pups, dead pups, resorption sites, gravid uterine weights, and fetal weights for the two pregnant females per male were calculated and used for statistical analyses and data presentation. Data from a preliminary study of three *Gclm*^{-/-} and three *Gclm*^{+/+} males in which only live and dead fetuses and resorption sites were enumerated were included in the analyses of those variables.

Glutathione assays. For glutathione assays, testes and epididymides from 2- and 10-month-old males were homogenized in 20mM Tris, 1mM EDTA, 250mM sucrose, 2mM L-serine, 20mM boric acid (TES-SB) (White *et al.*, 2003). After removal of aliquots for protein assay and glutamate cysteine ligase assay, supernatants were acidified with one-quarter volume 5% sulfosalicylic acid for glutathione assays (Tsai-Turton and Luderer, 2005). After incubation on ice and centrifugation at 15,800 × g at 4°C, supernatants were removed and stored at -80°C. Total and oxidized glutathione were measured in testicular and epididymal supernatants using a modification of an enzymatic recycling assay developed by Griffith (Griffith, 1980; Luderer *et al.*, 2001; Tsai-Turton and Luderer, 2005). For measurement of GSSG, reduced GSH was first removed from the sample by conjugation with 2-vinylpyridine, followed by chloroform extraction. For both GSH and GSSG assays, triplicates of samples or standards were combined with 33 μ l metal-free water and incubated for 10 min at 30°C. The samples were then mixed with 140 μ l of 0.3mM NADPH,

20 μ l of 6mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and 2 μ l of 50 U/ml GSH reductase. The rate of thiobis(2-nitrobenzoic acid) (TNB) formation from DTNB is proportional to the total GSH concentration in each sample. TNB formation was monitored by measuring the absorbance at 412 nm for 5 min every 10 s using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The concentrations of total GSH or GSSG in the samples were calculated from a standard curve generated from the slopes of the standards. The concentration of reduced GSH was calculated as [total GSH] - 2 \times [GSSG].

Antioxidant enzyme activities. For GCL, glutathione peroxidase and glutathione reductase enzymatic activity assays, testes, or epididymides from 2- and 10-month-old males were homogenized in 5 μ l TESSB/mg tissue. After centrifugation at 15,800 \times g at 4°C, supernatants were removed and stored at -80°C. The enzymatic activity of GCL was measured in supernatants of testicular and epididymal homogenates according to the method of White and coworkers (Tsai-Turton and Luderer, 2005; White *et al.*, 2003). Briefly, tissue homogenates and standards containing known concentrations of GSH were preincubated with reaction cocktail containing glutamate, adenosine tri-phosphate, boric acid, EDTA, serine, and magnesium chloride. The reaction was started by the addition of cysteine to each tube. After 20 min, the reaction was stopped by acidification with sulfosalicylic acid. After centrifugation, supernatants were pipetted into a 96-well microplate, and naphthalenedicarboxaldehyde was added for fluorescence detection of glutathione using a BioTek fluorescence spectrophotometer microplate reader (Winooski, VT).

The glutathione peroxidase enzymatic activity in sample supernatants was measured using *tert*-butyl hydroperoxide (1.2mM) as a substrate in the presence of 0.25 U/ml glutathione reductase, 0.15mM NADPH, and 1mM GSH by monitoring the rate of oxidation of NADPH spectrophotometrically at 340 nm every 10 s for 5 min using a VersaMax tunable microplate spectrophotometer (Molecular Devices) (Flohé and Günzler, 1984; Vernet *et al.*, 2004). The glutathione reductase enzymatic activity was measured as the rate of oxidation of NADPH as GSSG is reduced by glutathione reductase (Delides *et al.*, 1976; Kaneko *et al.*, 2001). Briefly, homogenates or standards of glutathione reductase from Baker's yeast (Sigma) were pipetted into a 96-well plate. Reaction mixture of NADPH in 100mM potassium phosphate, 1mM EDTA buffer was then added to each well resulting in a final concentration of 0.153mM NADPH. The reaction was initiated by the addition of GSSG in potassium phosphate, EDTA buffer to a final concentration of 1mM GSSG. Negative control wells received buffer without GSSG. Absorbance at 340 nm was monitored as for the glutathione peroxidase assay.

Testosterone assays. Blood was collected by cardiac puncture after carbon dioxide euthanasia. Serum was separated by centrifugation after clotting at room temperature and was stored at -20°C. The Coat-A-Count Total Testosterone radioimmunoassay kit from Siemens Medical Solutions Diagnostics (formerly DPC, Los Angeles, CA) was used to measure testosterone concentrations. Samples were run in duplicate, except in a few cases in which there was insufficient serum. The intraassay coefficients of variation (CVs) were 1.3 and 1.6% and the interassay CV was 1.4%.

Effect of *Gclm* genotype on testicular toxicity of in utero treatment with benzo[a]pyrene. Breeding pairs of *Gclm*^{+/-} females and males were set up in the late afternoon. The day of vaginal plug detection in the female was designated gestational day (GD) 1. Dams were treated by oral gavage with 2 or 10 mg/kg BaP (Sigma-Aldrich Supelco, \geq 99.8% purity) in sesame oil daily from GD7 to GD16. Control animals were gavaged with the same volume of sesame oil alone. The experiment was carried out in two blocks. In the first block, pregnant mice were dosed with 0 or 10 mg/kg BaP (experiment 1), and in the second block, mice were dosed with 0 or 2 mg/kg BaP (experiment 2). The dosing regimens were based on a previous study in CD-1 mice, which showed that male offspring treated from GD7 to GD16 with 10 mg/kg had reduced fertility compared with controls, whereas males treated with higher doses were completely infertile (MacKenzie and Angevine, 1981). The following safety precautions were followed when handling BaP because it is a known carcinogen: BaP dosing solutions were prepared in a designated

chemical fume hood by personnel wearing double gloves, impermeable sleeve covers, safety glasses, and disposable gown. Gavage dosing of animals was performed in a hood by personnel wearing double gloves and disposable gowns. All personal protective equipment, dosing equipment, other supplies potentially contaminated with BaP, and soiled bedding from cages of treated mice were sealed in plastic bags and disposed of as hazardous waste.

Dams were allowed to deliver. Male pups were weaned at 21 days of age and were killed by carbon dioxide asphyxiation at 10 weeks of age. Testes and epididymides were rapidly dissected out and weighed. One testis and epididymis from each animal were used for sperm counts. The other testis and epididymis were processed for histology.

Assessment of sperm counts, motility, and morphology. Testicular sperm head counts, cauda epididymal sperm counts, and sperm motility assessment were performed according to published methods (Blazak *et al.*, 1993; Chapin *et al.*, 1985; Dunnick *et al.*, 1986; Filler, 1993; Revel *et al.*, 2001). The testis and epididymis were dissected out, cleaned of adherent fat, and weighed. The cauda epididymis was dissected from the corpus epididymis, weighed, and then rapidly minced in 500 μ l prewarmed PBS at 37°C. A 10 μ l aliquot was removed and pipetted onto a prewarmed microscope slide for sperm motility assessment. Motile and nonmotile sperm were counted in ten \times 400 microscope fields per sample as rapidly as possible after death of the animal. For experiment 2 of the BaP study and for a subset of males in the fertility study, a 10 μ l aliquot was removed, spread onto a slide, allowed to dry, fixed in 5% acetic acid in ethanol, and stained with 5% eosin Y for sperm morphology. The remaining minced cauda epididymis was incubated for 15 min at 37°C, allowing sperm to swim into the buffer. An equal volume of 4% paraformaldehyde in PBS was then added to fix the sperm, and epididymal sperm counts were enumerated using a hemacytometer. Testes were frozen on dry ice and stored at -80°C. For testicular sperm head counts, testes were thawed on ice, each testis was minced, then homogenized in 8 ml of 0.9% sodium chloride, 0.05% Triton X-100 for 2 min at high speed on ice using an Omni Tissue Homogenizer and allowed to settle for at least 1 min. Sperm heads in the homogenate were counted using a hemacytometer.

Testicular and epididymal histology. Testes and epididymides were fixed in Bouin's fixative at 4°C overnight, followed by washing four times in 50% ethanol, and storage in 70% ethanol until processing for routine histology and staining with Periodic Acid Schiff and hematoxylin (testes) or hematoxylin and eosin (epididymides). Histological sections from each animal were evaluated without knowledge of genotype or treatment group. For quantification of seminiferous tubule defects, images of two testicular cross-sections were captured per animal. The total number of seminiferous tubule cross-sections was counted, and each cross-section was scored for vacuolization by one of the investigators (U.L.). The percentages of tubule cross-sections with vacuolization were calculated and used for data presentation and statistical analyses. The epididymides were evaluated by a board-certified veterinary pathologist (G.W.L.).

Statistical analyses. The effects of *Gclm* genotype on body and testis weights, testicular morphometry, sperm counts, ROS, antioxidant enzyme activities, and glutathione were analyzed by independent samples *t*-test for equal or unequal variances as appropriate. The effects of *Gclm* genotype and BaP treatment on organ weights and sperm parameters were analyzed using generalized linear models with BaP dose, *Gclm* genotype, BaP \times genotype interaction modeled as fixed effects, and body weight included as a covariate. In order to adjust for litter effects, litter number was entered into the model as a subject effect. If the overall analysis showed statistically significant effects of BaP dose and/or *Gclm* genotype, then intergroup comparisons were conducted using *t*-tests on the litter means. Data expressed as proportions, such as the fraction of motile sperm, were arcsine square root transformed prior to analyses (Pasternack and Shore, 1982). Statistical analyses were performed using SPSS 18.0 for MacIntosh.

TABLE 1
Effects of Lack of *Gclm* on Testicular and Epididymal GCL, GPX, and GR Enzymatic Activities (Mean \pm SEM)

| | Testis | | Epididymis | |
|-------------------------------|----------------------------|------------------------------|----------------------------|------------------------------|
| | <i>Gclm</i> ^{+/+} | <i>Gclm</i> ^{-/-} | <i>Gclm</i> ^{+/+} | <i>Gclm</i> ^{-/-} |
| GCL (nmol GSH/mg protein/min) | 2.94 \pm 0.64 | 0.88 \pm 0.04 ^a | 1.66 \pm 0.11 | 0.95 \pm 0.05 ^b |
| GR (mU/mg protein) | 37.4 \pm 1.3 | 38.5 \pm 1.7 | 45.1 \pm 3.0 | 61.3 \pm 2.6 ^b |
| GPX (mU/mg protein) | 17.6 \pm 1.0 | 16.2 \pm 1.0 | 40.7 \pm 2.3 | 40.3 \pm 4.0 |

Note. $n = 4-7$ per group.

^a $p < 0.05$ versus *Gclm*^{+/+}.

^b $p \leq 0.001$ versus *Gclm*^{+/+}.

RESULTS

Gclm Deletion Results in Greatly Decreased Testicular and Epididymal GCL Enzymatic Activity and GSH Concentrations

As expected, *Gclm*^{-/-} males had significantly decreased GCL enzymatic activity in testes ($p < 0.05$) and epididymides ($p < 0.001$) to 30 and 57% of wild-type levels, respectively (Table 1). Decreased GCL enzymatic activity resulted in greatly diminished testicular and epididymal total GSH and GSSG concentrations in *Gclm*^{-/-} males compared with wild-type littermates. In 2-month-old *Gclm*^{-/-} males, testicular GSH concentrations were 39% of wild-type levels and epididymal GSH concentrations were 19% of wild-type levels ($p < 0.002$ effect of genotype by *t*-test; Fig. 1A). GSSG concentrations were even more decreased than total GSH in 2-month-old *Gclm*^{-/-} testes and epididymides to 25 and 13%, respectively, of wild-type levels ($p < 0.009$ effect of genotype by *t*-test; Fig. 1B). As a result of the relatively greater decrease in GSSG, the ratio of reduced GSH (rGSH) to GSSG was significantly increased in the testes ($p = 0.029$) and nonsignificantly increased in the epididymides ($p = 0.309$) of null males compared with wild-type males, indicating a more reduced state despite the diminished total GSH concentrations (Fig. 1C). In 10-month-old *Gclm*^{-/-} male testes, total GSH concentrations were more decreased relative to wild-type testes than in 2-month-old male testes, to about 16% of wild-type levels (Fig. 1A; $p < 0.001$), and epididymal GSH concentrations were decreased to 20% of wild-type levels ($p < 0.001$). The ratios of rGSH to GSSG were significantly increased in *Gclm*^{-/-} compared with *Gclm*^{+/+} testes and nonsignificantly increased in *Gclm*^{-/-} epididymides at 10 months of age (Fig. 1C).

Effect of *Gclm* Deletion on Testicular and Epididymal Glutathione Peroxidase (GPX) and Glutathione Reductase (GSR) Enzymatic Activities

Because the testicular and epididymal rGSH to GSSG ratios were increased in *Gclm*^{-/-} males relative to wild-type males, we investigated the effects of *Gclm* deletion on the activities of the enzymes involved in glutathione oxidation and reduction,

GPX and GSR. Testicular GSR and GPX activities did not differ significantly between *Gclm*^{-/-} and *Gclm*^{+/+} males (Table 1). Epididymal GSR activity was statistically significantly increased by 1.4-fold in *Gclm*^{-/-} males relative to wild-type males ($p = 0.001$; Table 1). Epididymal GPX activity did not differ by genotype.

Fertility of *Gclm*^{-/-} Males Is Normal

Eight-month-old males were mated to two wild-type C57BL/6 females each (8 weeks old), with at least a 4-day rest between matings. The fraction of matings that resulted in vaginal plugs, the fraction of plugged females with gravid uteri, and the fraction of females that were pregnant by vaginal cytology but had nongravid uteri did not differ by male genotype (Table 2). The numbers of live fetuses, dead fetuses, resorption sites, total implantation sites, and fetal weights per litter also did not differ by male genotype (Table 2).

Serum Testosterone Concentrations Unaffected by *Gclm* Genotype

Serum testosterone concentrations did not differ significantly among 2-month-old *Gclm*^{+/+} (1.92 \pm 1.19 ng/ml; $n = 10$) and *Gclm*^{-/-} (1.68 \pm 1.25 ng/ml; $n = 10$) males or 10-month-old *Gclm*^{+/+} (0.94 \pm 0.49 ng/ml; $n = 6$) and *Gclm*^{-/-} (2.18 \pm 1.43 ng/ml; $n = 8$) males.

Testicular and Epididymal Weights and Sperm Counts in *Gclm*^{-/-} Males

Although *Gclm*^{-/-} males weighed about 1.5 g less on average than their wild-type littermates at 2 months ($p = 0.03$, *t*-test) and 3.6 g less at 10 months ($p = 0.11$), testicular weights did not differ significantly between the genotypes at 2 or 10 months of age (Table 3). Epididymal weights were significantly lower in *Gclm*^{-/-} than in wild-type males at 2 and 10 months, but this difference disappeared when epididymal weights were adjusted for body weights (Table 3). Testicular sperm head counts did not differ significantly by genotype in 2- (Fig. 2, control groups in BaP experiment below) or 10-month-old males (Table 4). Cauda epididymal sperm counts did not differ significantly by genotype in 10-month-old males (Table 4), but cauda epididymal sperm counts were decreased in 2-month-old *Gclm*^{-/-} males compared with *Gclm*^{+/+} males (Fig. 3D, control group in experiment 2). Cauda epididymal sperm morphology did not differ significantly by genotype in 10-month-old males (Table 4). The apparent trend toward *Gclm*^{-/-} males having higher percentages of immature sperm (sperm with cytoplasmic droplets) was not statistically significant ($p = 0.16$).

Gclm^{-/-} Male Conceptuses Are More Sensitive to Disruption of Spermatogenesis by In Utero Exposure to BaP than Are *Gclm*^{+/+} Males

Gclm^{+/-} females were mated with *Gclm*^{+/-} males, and females were treated by oral gavage with 0 or 10 mg/kg BaP

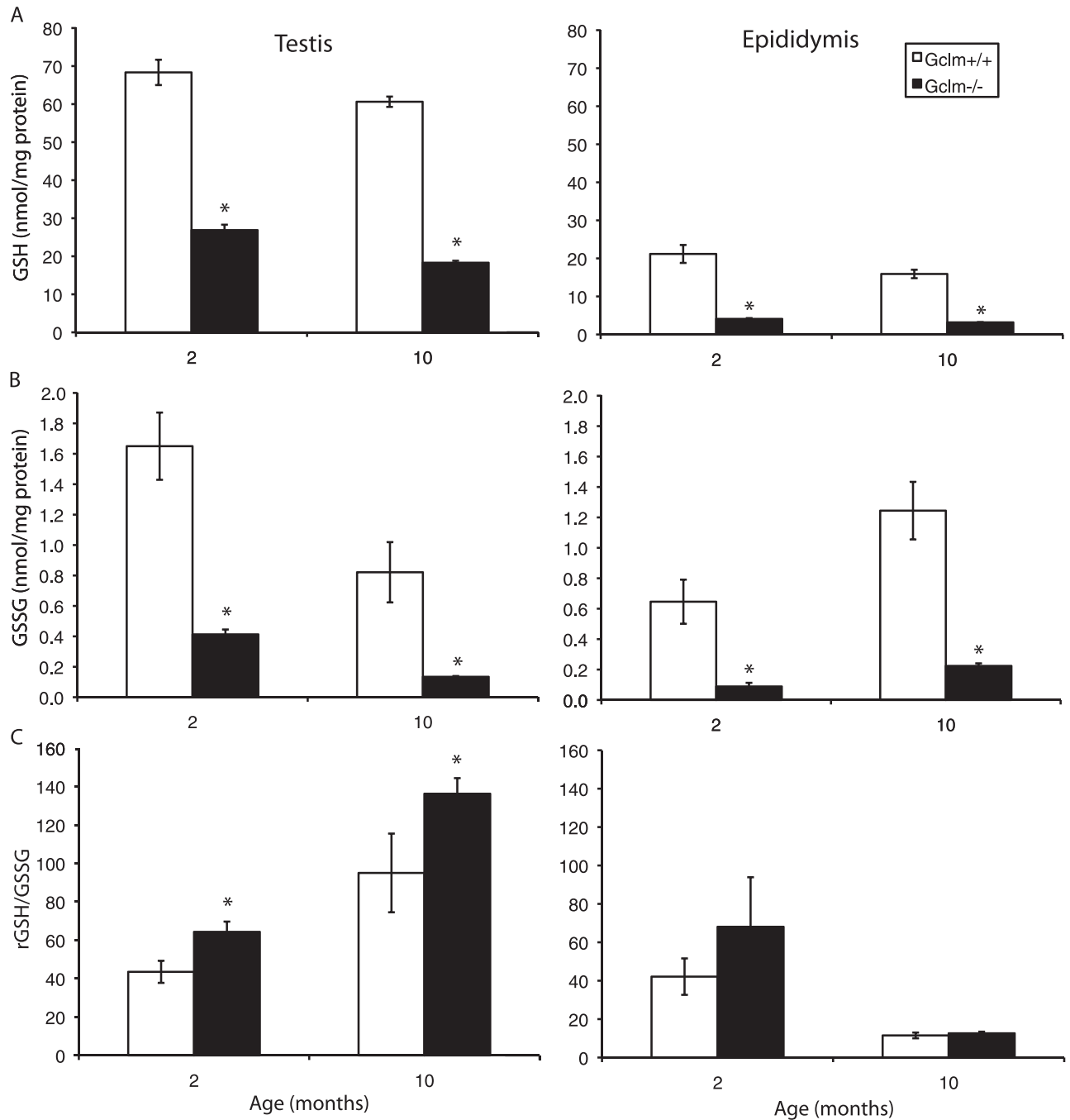


FIG. 1. *Gclm*^{-/-} males have greatly decreased testicular and epididymal concentrations of GSH. Testes and epididymides were collected from 2- and 10-month-old *Gclm*^{-/-} and *Gclm*^{+/+} littermates for measurement of total glutathione and oxidized glutathione (GSSG) as described in the “Materials and Methods” section. (A) Total GSH concentrations were significantly decreased in testes and epididymides of *Gclm*^{-/-} males at both ages (**p* < 0.002 vs. *Gclm*^{+/+} by *t*-test). (B) GSSG concentrations were significantly decreased in testes and epididymides of *Gclm*^{-/-} males at both ages (**p* < 0.009 vs. *Gclm*^{+/+} by *t*-test). (C) The ratio of rGSH:GSSG was significantly increased in testes (**p* = 0.029 vs. *Gclm*^{+/+} by *t*-test) and nonsignificantly increased in epididymides of *Gclm*^{-/-} males at both ages. *n* = 5–8 per group.

from GD7 to GD16 (experiment 1) or with 0 or 2 mg/kg BaP from GD7 to GD16 (experiment 2). The two experiments were conducted 2 years apart. Statistical comparison of various endpoints in the *Gclm*^{+/+} control offspring revealed significantly higher epididymal sperm counts and lower epididymal

sperm motility in the control wild-type males from experiment 2 compared with experiment 1. Therefore, the sperm count data from the two experiments were analyzed separately.

BaP exposure *in utero* did not significantly affect body weight of male offspring in either experiment, but *Gclm*^{-/-}

TABLE 2
Effects of *Gclm* Genotype on Male Fertility

| | <i>Gclm</i> +/+ (mean ± SEM) | <i>n</i> | <i>Gclm</i> -/- (mean ± SEM) | <i>n</i> |
|----------------------------------------------------------------|---------------------------------|----------|---------------------------------|----------|
| Fraction of matings plugged | 0.45 ± 0.14 | 6 | 0.41 ± 0.11 | 8 |
| Fraction plugged pregnant | 0.77 ± 0.12 | 5 | 0.77 ± 0.13 | 8 |
| Fraction nongravid uteri if pregnant by vaginal cytology | 0.11 ± 0.07 | 6 | 0.19 ± 0.07 | 8 |
| Gravid uterine weight (g) | 9.49 ± 1.47 | 6 | 9.67 ± 0.88 | 8 |
| Live fetuses per litter | 7.0 ± 0.8 | 9 | 6.4 ± 0.7 | 11 |
| Dead fetuses per litter | 0.08 | 6 | 0 | 8 |
| Resorption sites per litter | 1.0 ± 0.3 | 9 | 0.8 ± 0.2 | 11 |
| Implantation sites per litter | 8.1 ± 0.6 | 9 | 7.2 ± 0.8 | 11 |
| Mean fetal weight per litter (mg) | 991 ± 65 | 6 | 967 ± 35 | 8 |

males weighed about 2–3 g less than wild-type males ($p < 0.002$, effect of genotype).

There was a striking effect of 10 mg/kg BaP treatment on unadjusted testis weight (data not shown) and testis weight as a fraction of body weight (Table 5) ($p < 0.001$, effect of BaP) and a lesser effect of 2 mg/kg BaP on adjusted testis weight ($p = 0.017$, effect of BaP) but not unadjusted testis weight ($p = 0.166$). In the 10 mg/kg BaP-treated males, but not the vehicle controls, the unadjusted testis weights ($p = 0.019$, intergroup comparison by *t*-test) and adjusted testis weights ($p = 0.058$, intergroup comparison) were lower in the *Gclm*-/- than in the *Gclm*+/+ males, and the interaction between BaP treatment and genotype was statistically significant for adjusted testis weights ($p = 0.005$). This indicates a greater effect of gestational BaP treatment on *Gclm*-/- male testis weights than on *Gclm*+/+ males. Unadjusted (data not shown) and adjusted (Table 5) epididymal weights were also significantly decreased in 10 mg/kg BaP-treated males ($p < 0.001$, effect of treatment) but not in 2 mg/kg BaP-treated males ($p = 0.261$ and $p = 0.050$, respectively). In experiment 2, adjusted and unadjusted (data not shown) epididymal weights were significantly lower in the *Gclm*-/- males ($p < 0.001$, effect of genotype); unadjusted

epididymal weight (data not shown) was also significantly decreased in experiment 1 ($p < 0.001$), but there was no effect of genotype on adjusted epididymal weight in experiment 1 ($p = 0.288$) (Table 5).

Total homogenization resistant sperm heads per testis decreased in 10 mg/kg BaP-treated males ($p < 0.001$, effect of treatment) and in 2 mg/kg BaP-treated males in ($p = 0.022$) (Fig. 2). The effect of genotype on total sperm heads per milligram testis was not statistically significant in either experiment. Intergroup comparisons showed that total sperm heads per testis were fewer in the 10 mg/kg BaP-treated *Gclm*-/- males than in the *Gclm*+/+ males ($p = 0.013$), but there was no significant difference between the *Gclm*-/- and the *Gclm*+/+ 0 mg/kg controls. When testicular sperm counts were expressed per milligram testis, neither the effect of BaP treatment nor the effect of genotype was statistically significant, but the interaction between genotype and BaP dose was statistically significant in experiment 1 ($p = 0.049$), due to a decrease in the *Gclm*-/-, but not in the *Gclm*+/+, males. Together, these results suggest that *Gclm*-/- males are more sensitive to the testicular toxicity of gestational exposure to BaP than are *Gclm*+/+ males.

Cauda epididymal sperm counts were reduced, both as total sperm per cauda epididymis and sperm per milligram cauda epididymis, in 10 mg/kg BaP-treated males ($p < 0.001$, effect of treatment) and in 2 mg/kg BaP-treated males ($p \leq 0.053$) compared with 0 mg/kg controls (Fig. 3). The interaction between BaP dose and genotype was statistically significant in experiment 1 ($p \leq 0.006$); in the 10 mg/kg BaP-treated males in experiment 1, the sperm count per cauda epididymis was significantly lower by 88% in the *Gclm*-/- than in the *Gclm*+/+ males ($p = 0.022$ by *t*-test), whereas the sperm per cauda did not differ by genotype in the vehicle controls. These results are consistent with a greater sensitivity of the *Gclm*-/- males to the effects of gestational BaP treatment on epididymal sperm counts. In contrast, in experiment 2, the numbers of sperm per cauda epididymis were 31% lower in 2 mg/kg BaP-treated *Gclm*-/- males compared with 2 mg/kg BaP-treated *Gclm*+/+ males and 35% lower in 0 mg/kg BaP-treated

TABLE 3
Effects of *Gclm* Genotype on Testicular and Epididymal Weights at 2 and 10 Months of Age

| Genotype | Age (mos) | Paired testis weight, mg (mean ± SEM) | Paired testis weight (% body weight) | Paired epididymis weight, mg (mean ± SEM) | Paired epididymis weight (% body weight) |
|-----------------|--------------|---------------------------------------------|--------------------------------------------|-------------------------------------------------|------------------------------------------------|
| <i>Gclm</i> -/- | 2 | 165.3 ± 2.9 | 0.791 ± 0.019 | 50.4 ± 0.9 | 0.242 ± 0.006 |
| <i>Gclm</i> +/+ | 2 | 171.8 ± 4.5 | 0.767 ± 0.017 | 57.3 ± 1.4 ^a | 0.256 ± 0.007 |
| <i>Gclm</i> -/- | 10 | 197.4 ± 12.0 | 0.711 ± 0.025 | 75.0 ± 1.8 | 0.250 ± 0.010 |
| <i>Gclm</i> +/+ | 10 | 205.1 ± 11.3 | 0.660 ± 0.036 | 83.4 ± 3.1 ^b | 0.249 ± 0.013 |

Note. $n = 6$ –16 per group.

^a $p < 0.001$.

^b $p = 0.030$, by *t*-test versus *Gclm*-/-.

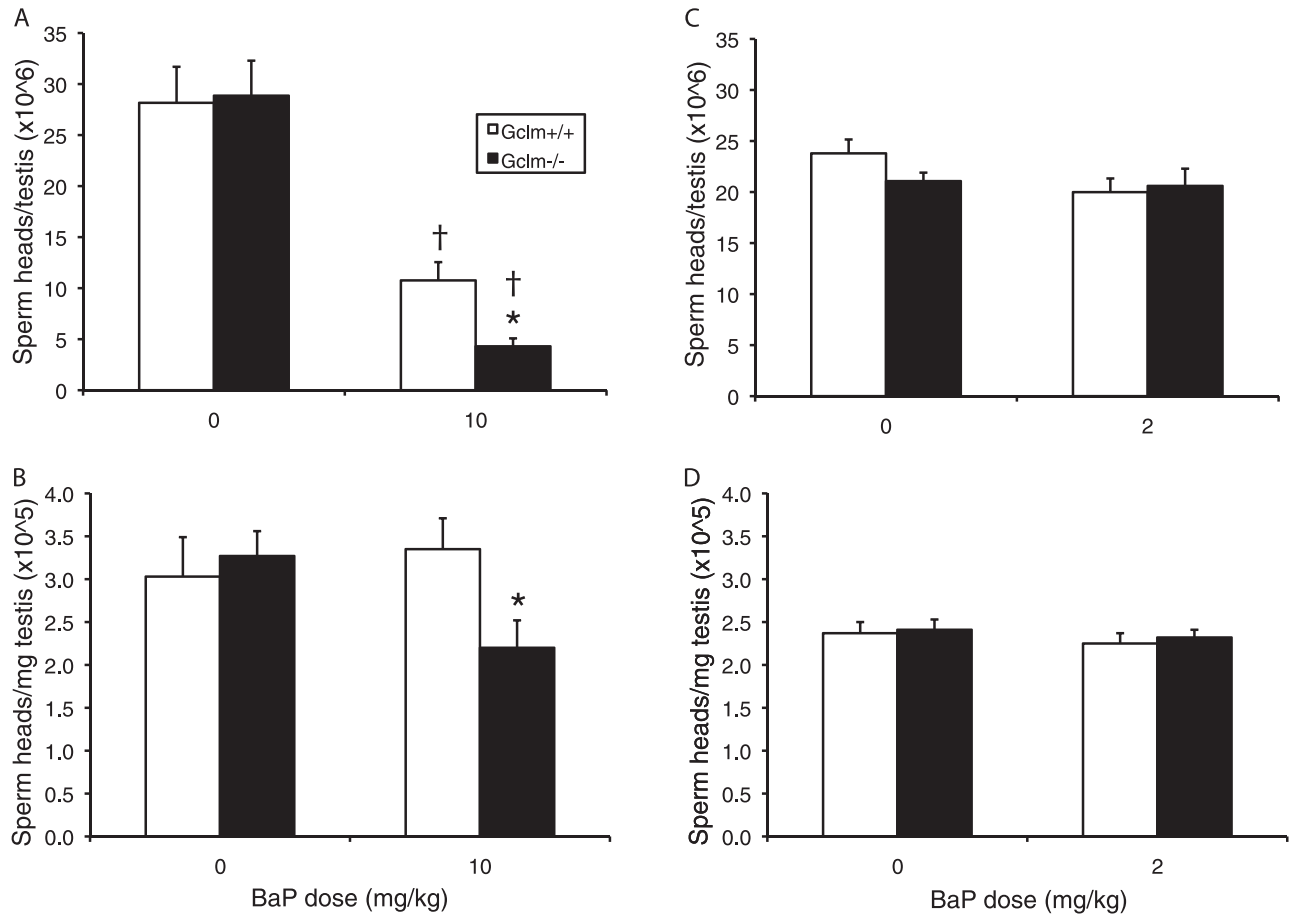


FIG. 2. *In utero* exposure to BaP decreases testicular sperm counts to a greater extent in *Gclm*^{-/-} than in *Gclm*^{+/+} males. The graphs show the mean ± SEM of total homogenization resistant sperm heads per testis (A, C) and sperm heads per milligram testis (B, D) by daily dose of BaP to the mother from GD7 to GD16 in experiments 1 (A, B) and 2 (C, D). (A) $p < 0.001$, effect of BaP dose, the effect of genotype ($p = 0.961$), and the genotype × BaP interaction ($p = 0.250$) were not statistically significant. (B) The genotype × BaP interaction was statistically significant ($p = 0.049$); effects of genotype ($p = 0.741$) and BaP ($p = 0.545$) were not statistically significant. (C) The effect of BaP dose was statistically significant ($p = 0.022$). The effects of genotype and BaP × genotype interaction were not statistically significant. (D) No statistically significant effects were observed. Experiment 1, $n = 6-9$ per group from 4 to 6 litters per group. Experiment 2, $n = 6-14$ per group from 6 to 11 litters per group. *Significantly different from *Gclm*^{+/+} with same BaP dose; †significantly different from 0 mg/kg BaP of same genotype, $p < 0.05$ by *t*-test.

Gclm^{-/-} males than 0 mg/kg BaP-treated *Gclm*^{+/+} males. In addition, experiment 2 showed fewer sperm per cauda epididymis and sperm per milligram cauda in *Gclm*^{-/-} than in *Gclm*^{+/+} males ($p < 0.010$, effect of genotype).

The fraction of motile sperm decreased significantly with 10 mg/kg BaP treatment in experiment 1 ($p = 0.004$, effect of treatment) but not with 2 mg/kg BaP in experiment 2 (Figs. 3A and 3B, lower graphs). In experiment 1, but not experiment 2, there was a statistically significantly lower fraction of motile sperm in the *Gclm*^{-/-} males than in *Gclm*^{+/+} males ($p < 0.001$, effect of genotype).

Cauda epididymal sperm morphology was examined in experiment 2 only. The percentage of sperm with abnormal heads and percentage of sperm with cytoplasmic droplets were significantly decreased in males with *in utero* exposure to BaP ($p \leq 0.006$, effect of BaP), and *Gclm*^{-/-} males had higher percentages of sperm with abnormal heads ($p = 0.003$, effect of

TABLE 4
Effects of *Gclm* Genotype on Testicular Sperm Head Counts and on Cauda Epididymal Sperm Counts and Morphology at 10 Months of Age

| | <i>Gclm</i> ^{+/+} (mean ± SEM) | <i>n</i> | <i>Gclm</i> ^{-/-} (mean ± SEM) | <i>n</i> |
|------------------------------------------------------|-----------------------------------------------|----------|-----------------------------------------------|----------|
| Sperm heads per testis (×10 ⁶) | 28.5 ± 1.6 | 6 | 26.7 ± 1.3 | 8 |
| Sperm heads per milligram testis (×10 ⁵) | 2.6 ± 0.2 | 6 | 2.6 ± 0.1 | 8 |
| Sperm per epididymis (×10 ⁶) | 13.8 ± 1.7 | 6 | 13.3 ± 0.6 | 8 |
| Sperm per milligram epididymis (×10 ⁵) | 11.3 ± 1.3 | 6 | 12.0 ± 0.3 | 8 |
| Percent with abnormal heads | 6.1 ± 2.5 | 3 | 9.1 ± 0.8 | 6 |
| Percent with abnormal tails | 5.4 ± 2.0 | 3 | 6.3 ± 1.6 | 6 |
| Percent with cytoplasmic droplet | 3.6 ± 0.9 | 3 | 14.9 ± 6.9 | 6 |

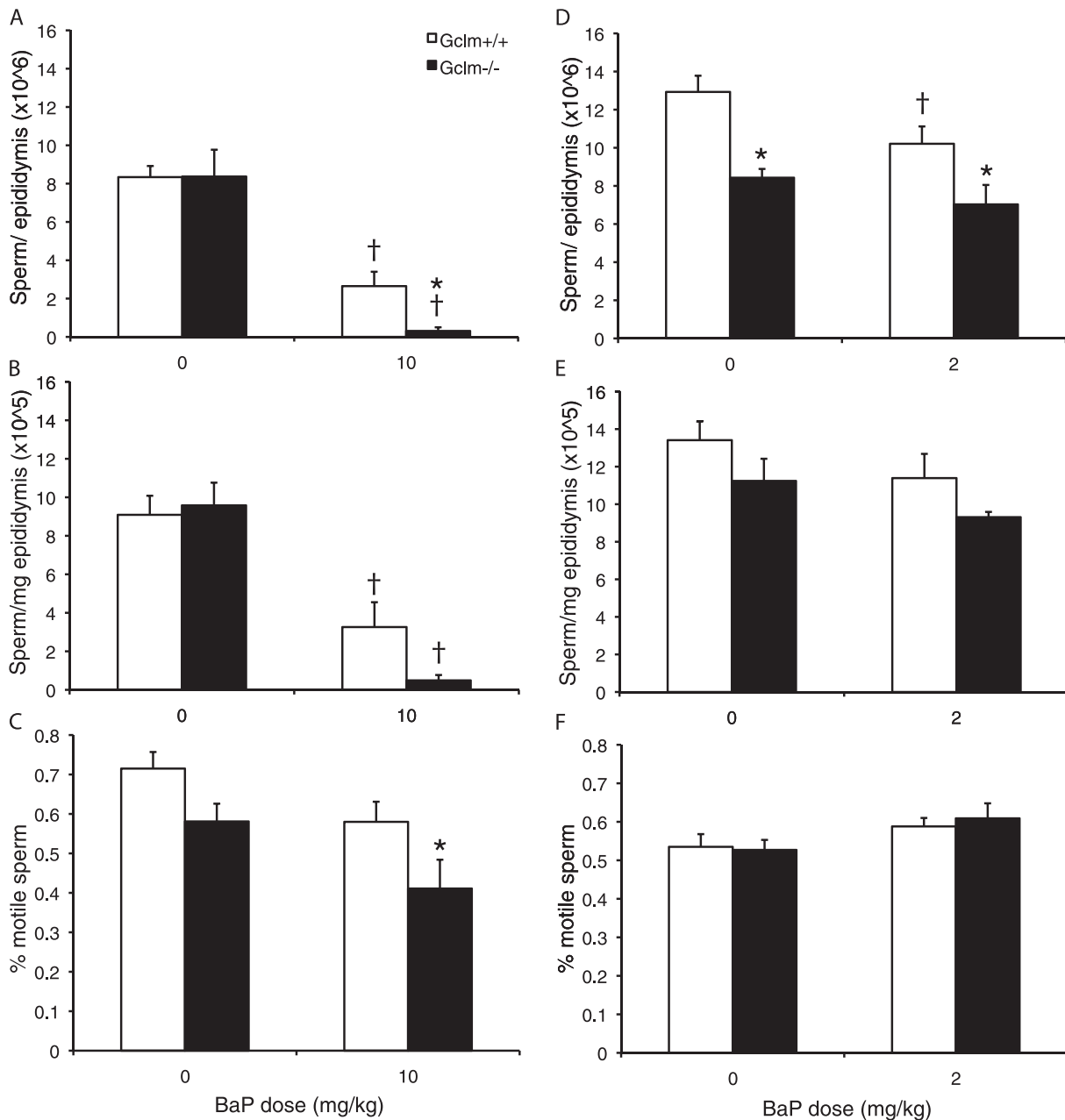


FIG. 3. *In utero* exposure to BaP decreases epididymal sperm counts to a greater extent in *Gclm*^{-/-} than in *Gclm*^{+/+} males. The graphs show the mean \pm SEM of sperm per cauda epididymis (A, D), sperm per milligram cauda epididymis (B, E), and fraction motile cauda epididymal sperm (C, F) by daily dose of BaP to the mother from GD7 to GD16. (A) The effects of BaP dose ($p < 0.001$) and genotype \times BaP interaction ($p = 0.006$) were statistically significant; the effect of genotype ($p = 0.397$) was not statistically significant. (B) The effects of BaP dose ($p < 0.001$) and genotype \times BaP interaction ($p = 0.001$) were statistically significant; the effect of genotype ($p = 0.361$) was not statistically significant. (C) $p = 0.004$, effect of BaP dose; $p < 0.001$, effect of genotype. The BaP \times genotype interaction was not statistically significant ($p = 0.978$). (D) $p = 0.035$, effect of BaP dose; $p \leq 0.001$, effect of genotype; the effect of genotype \times BaP interaction ($p = 0.482$) was not statistically significant. (E) $p = 0.053$, effect of BaP dose; $p = 0.009$, effect of genotype; the genotype \times BaP interaction ($p = 0.716$) was not statistically significant. (F) $p = 0.008$, effect of BaP dose; the effects of genotype ($p = 0.079$) and BaP \times genotype interaction ($p = 0.413$) were not statistically significant. Experiment 1, $n = 6-9$ per group from 4 to 6 litters per group. Experiment 2, $n = 6-14$ per group from 6 to 11 litters per group. *Significantly different from *Gclm*^{+/+} with same BaP dose; †significantly different from 0 mg/kg BaP of same genotype, $p < 0.05$ by *t*-test.

genotype) than *Gclm*^{+/+} males (Table 6). The results showed no statistically significant effects of BaP or genotype on percentage of sperm with abnormal tails (Table 6).

Examination of testicular histology revealed normal spermatogenesis in oil-treated *Gclm*^{-/-} and *Gclm*^{+/+} males (Figs. 4A and 4B; Table 7). Four of four testes of 10 mg/kg

TABLE 5
Effects of *In Utero* Treatment With BaP From GD7 to GD16 and *Gclm* Genotype on Testicular and Epididymal Weights as Percentages of Body Weight at 10 Weeks of Age

| | <i>Gclm</i> ^{+/+} (mean ± SEM) | | <i>Gclm</i> ^{-/-} (mean ± SEM) | |
|------------------------------------------|-----------------------------------------|---------------|-----------------------------------------|---------------|
| | Oil | BaP | Oil | BaP |
| Paired testis weight | | | | |
| Experiment 1 (10 mg/kg BaP) ^a | 0.736 ± 0.033 | 0.262 ± 0.032 | 0.762 ± 0.024 | 0.183 ± 0.012 |
| Experiment 2 (2 mg/kg BaP) ^b | 0.767 ± 0.023 | 0.702 ± 0.034 | 0.734 ± 0.019 | 0.671 ± 0.039 |
| Paired epididymal weight | | | | |
| Experiment 1 (10 mg/kg BaP) ^c | 0.237 ± 0.015 | 0.179 ± 0.014 | 0.225 ± 0.016 | 0.171 ± 0.019 |
| Experiment 2 (2 mg/kg BaP) ^d | 0.256 ± 0.009 | 0.239 ± 0.010 | 0.219 ± 0.006 | 0.206 ± 0.006 |

Note. *n* = 6–9 per group from 4 to 6 litters per group, experiment 1. *n* = 6–14 per group from 6 to 11 litters per group, experiment 2.

^a*p* < 0.001, effect of BaP dose; *p* = 0.602, effect of genotype; *p* = 0.005, BaP × genotype interaction.

^b*p* = 0.017, effect of BaP; *p* = 0.257, effect of genotype; *p* = 0.422, BaP × genotype.

^c*p* < 0.001, effect of BaP dose; *p* = 0.288, effect of genotype; *p* = 0.956, BaP × genotype.

^d*p* = 0.050, effect of BaP dose; *p* < 0.001, effect of genotype; *p* = 0.454, BaP × genotype.

BaP-treated *Gclm*^{-/-} males had severe vacuolization of the seminiferous tubules with only Sertoli cells apparent in most tubule cross-sections (Fig. 4F; Table 8). Five of five testes of 10 mg/kg BaP-treated *Gclm*^{+/+} males also had many affected tubules (Fig. 4E; Table 8). However, in contrast to the *Gclm*^{-/-} testes, spermatids and meiotic stages appeared to be present in many affected tubules. The testes of the 2 mg/kg BaP-treated *Gclm*^{-/-} males had a similar percentage of vacuolated seminiferous tubules as the testes of the 2 mg/kg BaP-treated *Gclm*^{+/+} males (Figs. 4C and 4D; Table 7). The effect of BaP dose was statistically significant (*p* < 0.001 by two-way ANOVA). Intergroup comparisons showed significantly higher percentages of vacuolated seminiferous tubules in the 10 mg/kg BaP-treated *Gclm*^{-/-} and *Gclm*^{+/+} males compared with 0 or 2 mg/kg BaP-treated males of the same genotype. The number of interstitial cells also appeared to be increased in testes of males of both genotypes treated with 10 mg/kg BaP *in utero* (Figs. 4E and 4F). However, Leydig cell steroidogenesis was not affected by prenatal BaP exposure. There was a nonsignificant trend toward decreased serum testosterone

concentrations in the *Gclm*^{-/-} males (*p* = 0.088, effect of genotype; Table 8).

Epididymal histology was not affected by *Gclm* genotype (Fig. 5) or prenatal exposure to 2 mg/kg BaP (data not shown).

DISCUSSION

Our results show that decreased testicular and epididymal enzymatic activity of GCL, the rate-limiting enzyme in GSH synthesis, and GSH concentrations in *Gclm*^{-/-} male mice did not affect fertility, testicular sperm counts, or testicular histology compared with *Gclm*^{+/+} males. *Gclm* deletion was associated with decreased cauda epididymal sperm counts and motility and with increased abnormal sperm morphology, but these effects were not observed in all experiments. In addition, *Gclm*^{-/-} male embryos were more sensitive to the testicular toxicity of gestational treatment with the PAH BaP. *Gclm*^{-/-} males exposed to 10 mg/kg/day BaP from GD7 to GD16 had lower testicular and epididymal weights, testicular and

TABLE 6
Effects of Exposure to 2 mg/kg BaP From GD7 to GD16 and *Gclm* Genotype on Cauda Epididymal Sperm Morphology

| | <i>Gclm</i> ^{+/+} (mean ± SEM) | | <i>Gclm</i> ^{-/-} (mean ± SEM) | |
|-----------------------------------------------------|-----------------------------------------|------------|-----------------------------------------|------------|
| | Oil | BaP | Oil | BaP |
| Percent sperm with cytoplasmic droplet ^a | 23.5 ± 3.1 | 19.1 ± 2.7 | 25.4 ± 6.5 | 15.7 ± 5.4 |
| Percent sperm with abnormal heads ^b | 10.6 ± 1.4 | 8.3 ± 1.0 | 14.7 ± 2.0 | 11.6 ± 2.8 |
| Percent sperm with abnormal tails ^c | 19.4 ± 1.9 | 20.2 ± 3.5 | 17.7 ± 2.8 | 17.8 ± 1.5 |

Note. *n* = 6–9 per group from 5 to 7 litters per group.

^a*p* < 0.001, effect of BaP; *p* = 0.164, effect of genotype; *p* = 0.180, BaP × genotype.

^b*p* = 0.006, effect of BaP; *p* = 0.003, effect of genotype; *p* = 0.654, BaP × genotype.

^c*p* = 0.895, effect of BaP; *p* = 0.705, effect of genotype; *p* = 0.308, BaP × genotype.

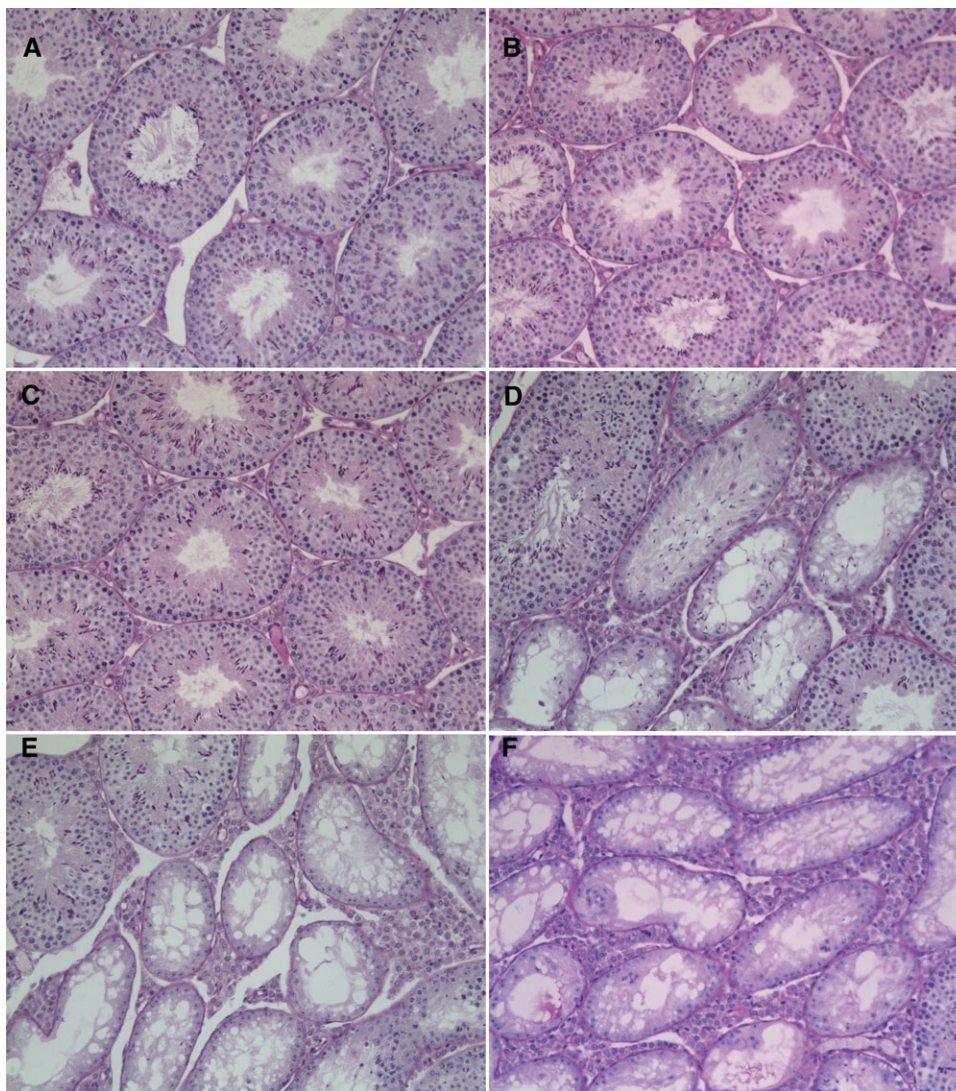


FIG. 4. Dose-dependent seminiferous tubule damage after *in utero* exposure to BaP in *Gclm*^{-/-} and *Gclm*^{+/+} males. Images show Periodic Acid Schiff and Hematoxylin-stained cross-sections of testes from 10-week-old *Gclm*^{-/-} and *Gclm*^{+/+} male mice exposed to BaP or oil vehicle *in utero* as in Figure 2. (A) *Gclm*^{+/+} male exposed to 0 mg/kg/day BaP. (B) *Gclm*^{-/-} male exposed to 0 mg/kg/day BaP. (C) *Gclm*^{+/+} male exposed to 2 mg/kg/day BaP. (D) *Gclm*^{-/-} male exposed to 2 mg/kg/day BaP. (E) *Gclm*^{+/+} male exposed to 10 mg/kg/day BaP. (F) *Gclm*^{-/-} male exposed to 10 mg/kg/day BaP. Original magnification for all images, $\times 200$.

epididymal sperm counts, and epididymal sperm motility than their wild-type, 10mg/kg BaP-exposed littermates.

Gclm deletion decreased testicular and epididymal GSSG concentrations to a greater extent than total GSH concentrations, resulting in significantly higher (more reduced) rGSH to GSSG ratios in the testis. Although epididymal enzymatic activity of GSR was significantly increased, the increased rGSH to GSSG ratio in the testis remains unexplained, as testicular GSR and GPX activities were not altered. This contrasts with previous observations that the rGSH to GSSG ratios in liver, kidney, pancreas, and erythrocytes did not differ between *Gclm*^{-/-} and *Gclm*^{+/+} mice (Yang *et al.*, 2002) or were decreased in plasma and livers of *Gclm*^{-/-} mice (Kendig *et al.*, 2011) and suggests that there may be compensatory

protective mechanisms in the testis that protect against potential adverse effects of low GSH concentrations on spermatogenesis.

Deletion of *Gclm* alone did not affect male fertility or testicular spermatogenesis. In contrast, deletion of the transcription factor *Nrf2*, which regulates the transcription of numerous antioxidant genes, including *Gclm*, *Gclc*, and several GSTs, adversely affected spermatogenesis (Nakamura *et al.*, 2010). *Nrf2* null mice had normal testicular sperm counts and histology as young adults but developed an age-associated decline in spermatogenesis that was already apparent by 4 months of age (Nakamura *et al.*, 2010). In the present study, we observed no difference in sperm parameters or fertility between 8- and 10-month-old *Gclm*^{-/-} and *Gclm*^{+/+} males.

TABLE 7
Effects of Exposure to BaP From GD7 to GD16 and *Gclm* Genotype on Testicular Histology at 10 Weeks of Age

| BaP dose (mg/kg/day) | Percent vacuolated seminiferous tubules ± SEM | |
|----------------------|-----------------------------------------------|----------------------------|
| | <i>Gclm</i> ^{+/+} | <i>Gclm</i> ^{-/-} |
| 0 | 0.8 ± 0.2 | 0.2 ± 0.2 |
| 2 | 7.5 ± 5.1 | 7.6 ± 3.8 |
| 10 | 65.3 ± 8.8 ^{a,b} | 81.4 ± 7.5 ^{a,b} |

Note. *n* = 4–7 per group; *p* < 0.001, effect of BaP dose; *p* = 0.452, effect of genotype, on arcsine square root transformed data by two-way ANOVA.

^aSignificantly different from 0 mg/kg group of same genotype by post hoc Dunnett's T3 test.

^bSignificantly different from 2 mg/kg group of same genotype by post hoc Dunnett's T3 test.

Although *Nrf2*^{-/-} males had modestly decreased testicular and epididymal GSH concentrations (Nakamura *et al.*, 2010), the lack of a similar age-associated decline in spermatogenesis in *Gclm*^{-/-} mice suggests that the testicular phenotype of the *Nrf2* null mice is not primarily caused by the effects of *Nrf2* deletion on GSH synthesis. Mice null for γ -glutamyl transpeptidase 1 (*Ggt1*) displayed a shortened lifespan, stunted growth, and a severe male reproductive phenotype, with complete infertility, decreased testis size with reduced seminiferous tubule diameter despite normal development of somatic and spermatogenic cells, severely decreased epididymal sperm counts and motility, and low FSH and testosterone levels (Kumar *et al.*, 2000; Lieberman *et al.*, 1996; Will *et al.*, 2000). GGT catalyzes the cleavage of GSH into cysteinyl glycine and γ -glutamate, an essential step in the γ -glutamyl cycle that helps to maintain cellular levels of cysteine and GSH. *Ggt1* null mice had decreased testicular cysteine concentrations, but normal testicular GSH concentrations, compared with wild-type controls, and the male reproductive phenotype was completely rescued by cysteine replacement (Kumar *et al.*, 2000). These findings together with the results of the present study, which showed minimal effects of pronounced GSH depletion on male reproduction, provide strong

TABLE 8
Effects of Exposure to BaP From GD7 to GD16 and *Gclm* Genotype on Serum Testosterone Concentrations at 10 Weeks of Age

| BaP dose (mg/kg/day) | Testosterone, ng/ml (<i>n</i>) (mean ± SEM) | |
|----------------------|-----------------------------------------------|----------------------------|
| | <i>Gclm</i> ^{+/+} | <i>Gclm</i> ^{-/-} |
| 0 | 4.25 ± 1.56 (12) | 2.24 ± 1.28 (11) |
| 2 | 4.18 ± 1.99 (10) | 2.17 ± 1.71 (6) |
| 10 | 4.27 ± 2.75 (6) | 0.20 ± 0.06 (5) |

evidence that the male reproductive effects of *Ggt1* deletion are caused by cysteine deficiency and not by GSH deficiency.

In contrast to the lack of an effect of *Gclm* deletion on the testis, epididymal function appeared to be modestly affected by lack of *Gclm*. Unadjusted epididymal weights were consistently significantly lower in *Gclm*^{-/-} males than in *Gclm*^{+/+} males, but adjustment for body weight attenuated or abolished this effect. Although no epididymal histological abnormalities were noted, decreased cauda epididymal sperm counts and motility and increased abnormal sperm morphology were inconsistently observed among experiments in *Gclm*^{-/-} males. A number of redox-sensitive processes are important for sperm activation and acquisition of forward motility. Activation of spermatogenic cell-specific Type 1 hexokinase (HK1S) is necessary for the acquisition of motility and is mediated by cleavage of disulfide-linked HK1S dimers to active monomers (Nakamura *et al.*, 2008). In contrast, compensatory antioxidant mechanisms in the *Gclm*^{-/-} sperm may disrupt proper oxidative events necessary for motility. For example, normal levels of the mitochondrial form of *Gpx4* are critical for male fertility. GPX4 stabilizes the mitochondrial capsule of the sperm midpiece by oxidation of protein thiols, where low GSH concentrations are normal and permissive of oxidation (Schneider *et al.*, 2009). The impact of *Gclm* deficiency on these processes is beyond the scope of this study; however, the inconsistency of the observed effects on sperm motility and morphology suggests a complex mechanism of regulation.

Despite the lack of effects of *Gclm* genotype alone on testicular spermatogenesis, the *Gclm*^{-/-} male conceptuses were more susceptible to the testicular toxicity of *in utero* exposure to the PAH BaP. *Gclm*^{-/-} males exposed prenatally to 10 mg/kg/day BaP had significantly lower testicular sperm head counts, epididymal sperm counts, and testicular and epididymal weights than their wild-type littermates. Testicular histology of the 10 mg/kg/day BaP-exposed *Gclm*^{-/-} males showed that 81% of seminiferous tubules were vacuolated compared with 65% of tubules in *Gclm*^{+/+} males. Most of these tubules were essentially aspermic. Testicular histological changes observed in 10 mg/kg BaP-treated males were more severe than those reported previously for CD-1 males treated with 10 mg/kg BaP during the same gestational interval, resembling those observed in CD-1 males exposed to 40 mg/kg (MacKenzie and Angevine, 1981) and suggesting that the C57BL/6J strain is more sensitive to the transplacental testicular toxicity of BaP than the CD-1 strain. C57BL/6 female mice have been reported to be more sensitive than other strains to PAH ovarian toxicity (Takizawa *et al.*, 1984). An alternative explanation for the increased sensitivity of male embryos to the transplacental testicular toxicity of BaP in the current study is that haploinsufficiency of the *Gclm*^{+/-} dams may modify the effects of BaP in the offspring. This could be tested in the future by transferring fertilized eggs from *Gclm*^{+/-} × *Gclm*^{+/-} matings into C57BL/6J pseudopregnant

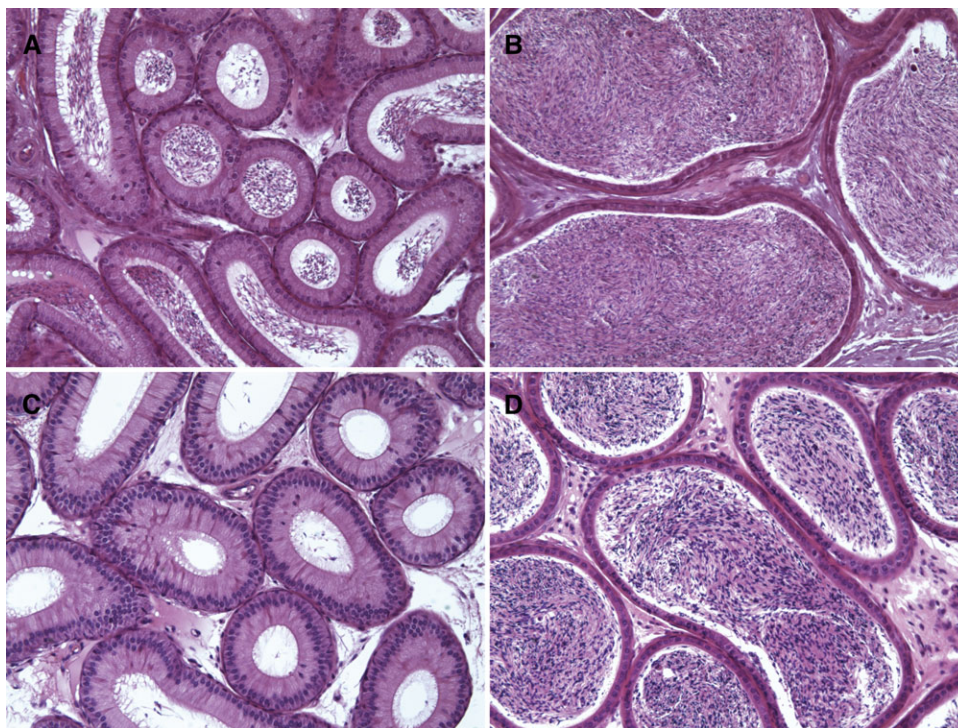


FIG. 5. Normal epididymal histology in *Gclm*^{-/-} and *Gclm*^{+/+} males. (A) Caput epididymis of *Gclm*^{+/+} male. (B) Cauda epididymis of *Gclm*^{+/+} male. (C) Caput epididymis of *Gclm*^{-/-} male. (D) Cauda epididymis of *Gclm*^{-/-} male. Original magnification of all images $\times 200$.

females, followed by BaP treatment and assessment of the offspring. Our findings are relevant to humans. The PAH 9,10-dimethyl-1,2-benzanthracene has previously been shown to induce apoptosis of germ cells in cultured human fetal testis via an arylhydrocarbon receptor-dependent pathway (Coutts *et al.*, 2007). In addition, polymorphisms in *Gclm* and *Gclc*, which affect GSH synthesis, exist in humans (Le *et al.*, 2010; Nakamura *et al.*, 2002, 2003; Nichenametla *et al.*, 2008; Walsh *et al.*, 2001; Willis *et al.*, 2003). Our data lead us to hypothesize that these polymorphisms may modulate the effects of prenatal exposure to PAHs on male offspring.

The increased sensitivity of the *Gclm*^{-/-} embryonic testis to BaP may be due to decreased ability to detoxify reactive metabolites of BaP and/or ROS produced as a result of BaP metabolism. GSH conjugation is an important phase II detoxification mechanism for the diol epoxide metabolites (Jernström *et al.*, 1996; Romert *et al.*, 1989; Seidel *et al.*, 1998) and for the arene oxide and quinone metabolites of PAHs (Agency for Toxic Substances and Disease Registry, 1995). Cytochromes P450 1A1, 1A2, and 1B1 are the major P450 enzymes involved in the metabolic activation of BaP (Kleiner *et al.*, 2004; Xue and Warshawsky, 2005). Expression of most cytochrome P450 metabolizing enzymes remains low until after birth, with the exception of induction of embryonic *Cyp1a1* expression in the liver and lung by exposure to PAHs as early as GD12.5 in the mouse (Dey *et al.*, 1989; Rich and Boobis, 1997). Dey *et al.* (1989) did not observe constitutive or

inducible *Cyp1a1* expression in other organs, presumably including the developing testis. Therefore, electrophilic reactive intermediates of BaP likely play a limited role in the testicular developmental toxicity of BaP. Previous work by Wells *et al.* (2005, 2009) has shown that embryos constitutively express prostaglandin-endoperoxide synthase 1 and 2, that prostaglandin-endoperoxide synthases can bioactivate BaP to free radical intermediates that initiate ROS formation, that gestational treatment with BaP increases embryonic oxidative protein and DNA damage, and that antioxidants are protective against teratogenesis caused by BaP (Parman and Wells, 2002; Winn and Wells, 1997). Although Wells and coworkers did not examine developmental toxicity to the reproductive system, our findings of increased sensitivity of GSH-deficient male embryos to *in utero* testicular toxicity of BaP provide further support for a role for oxidative stress in the developmental toxicity of BaP.

In conclusion, although deletion of *Gclm* greatly decreased GCL enzymatic activity and GSH concentrations in testes and epididymides, the GSH redox state was more reduced in *Gclm*^{-/-} testes. Deletion of *Gclm* alone did not affect male fertility, testicular histology, testicular weights, or testicular sperm head counts. We observed some evidence of an effect of *Gclm* deletion alone on epididymal function, with inconsistently decreased cauda epididymal sperm counts and motility and increased abnormal sperm morphology among experiments. *Gclm*^{-/-} male embryos were more sensitive to the

transplacental testicular toxicity of BaP, with greater decreases in testicular and epididymal weights and testicular and epididymal sperm counts than *Gclm*^{+/+} littermates. These data demonstrate an important role of embryonic GSH in protecting the developing male reproductive system against PAH-induced toxicity. Future studies should investigate the potential for polymorphisms in human *GCLC* and *GCLM* to modulate sensitivity to PAH developmental toxicity.

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