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CHRONIC CADMIUM EXPOSURE: RELATION TO MALE REPRODUCTIVE TOXICITY AND SUBSEQUENT FETAL OUTCOME

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Acute injections of high doses of Cd induce marked testicular necrosis. However, the effects of low-dose, oral Cd exposure on a chronic basis are not well documented. The present investigation was designed to examine the effects of such exposure as reflected in parameters of spermatotoxicity and histology. Moreover, the impact on fetal outcome was measured by evaluating teratological and postnatal neurobehavior endpoints. Male Long-Evans hooded rats (100 d of age) were exposed to 0, 17.2, 34.4, or 68.8 ppm Cd for 70 d. During this period, the animals were maintained on a semipurified diet to control for the contributions of Zn and other trace elements. Near the end of exposure the males were mated to three female rats. One was sacrificed on d 21 of pregnancy for teratological assessment, including fetal weight, and determination of preimplantation and postimplantation loss. The other two dams were allowed to deliver, and their offspring were tested on tasks of exploratory behavior (d 21) and learning (d 90). Subsequently, the male parent was sacrificed and a variety of measures recorded including weights of testes and caudae epididymides, sperm count and sperm morphology, and Cd content of liver and kidney. One of the testes was also evaluated histologically. No significant effects were observed on any of the parameters of reproductive toxicity or fetal outcome. These findings suggest that, at the doses employed in this study, Cd did not have significant deleterious effects on the male reproductive system. Morever, the traditional view of Cd-related testicular insult, based on acute exposure, injection protocols, needs to be reevaluated in terms of environmental relevance.

INTRODUCTION

The necrosis of testicular tissue following acute injections of high doses of Cd is well documented (e.g., Parizek and Zahor, 1956, Mason et al., 1964). Hypotheses regarding mechanisms of action include either (1) indirect insult resulting from vascular disruption (Gunn and Gould, 1970) or (2) direct injury to spermatogenic cells (Lee and Dixon, 1973).

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These results have been obtained so frequently that Cd-induced gonadal injury has served as a classic example in a number of textbooks. However, the environmental relevance of these findings is questionable in light of the reliance on high doses and parenteral routes of administration.

Moreover, studies in which animals have been subjected to low-level, chronic injections of Cd have failed to reveal histological damage (Piscator and Axelsson, 1970; Nordberg, 1971), even when the Cd levels in the testes are many times higher than those found in the acute studies (Nordberg, 1971). The induction of metallothionein synthesis by low doses and subsequent binding of Cd have been proposed as a mechanism for reducing gonadal toxicity (Nordberg, 1971, 1972).

In most of these chronic studies, Cd was administered by parenteral injection. Only two studies appear to have examined reproductive parameters after oral Cd exposure. The results were equivocal. These two investigations were part of a collaborative U.S.-U.S.S.R. research program sponsored by the National Institute of Environmental Health Sciences (NIEHS).

Dixon et al. (1976) chronically exposed rats to 0.001, 0.01, or 0.1 ppm Cd in drinking water and sacrificed them after 30, 60, and 90 d of exposure. Parameters assessed included weights of the testes, prostate, and seminal vesicle and a number of measures of clinical chemistry and hormone levels [follicle stimulating hormone (FSH) and luteinizing hormone (LH)]. Breeding studies to assess fertility were also conducted, although no direct measures of spermatogenesis (e.g., sperm count, morphology) were recorded. Cd failed to produce significant effects on any of the variables examined. The Russian group (Krasavskii et al., 1976) found numerous gonadotoxic effects at the same Cd levels with exposure continued for 6 mo. However, they also reported elevated blood Cd levels in their control animals (7 μg/dl), suggesting a secondary source of contamination.

None of the chronic studies indicates any attempt to control dietary levels of Zn during Cd treatment. Zn supplementation can prevent Cd-induced gonadal toxicity (Parizek, 1957; Webb, 1972). Moreover, the use of standard laboratory diets in these chronic studies provides the animals with a prophylactic level of Zn.

The present study was designed to assess the effects of chronic, oral Cd exposure on the male reproductive system, not only at the histological level but also by evaluating a number of spermatogenic parameters. Moreover, constituents in the diet were controlled by maintaining the animals on a semipurified diet with Zn levels adjusted to meet the daily requirements for the rodent. The relation of Cd-induced gonadal toxicity to measures of fertility and pregnancy outcome was examined. In addition, a proportion of the females mated to the exposed males were allowed to litter and a neurobehavioral assessment of the progeny was conducted. Research on another heavy metal (Pb) showed that paternal

exposure alone produces neurobehavioral deficits in the offspring (Brady et al., 1975).

METHODS

Twenty, 100-d-old Sprague-Dawley male rats (Harlan Breeders, Indianapolis, Ind.) of proven fertility were randomly assigned to one of four treatment groups (5 per group) and given 0, 17.2, 34.4, or 68.8 ppm Cd as $CdCl_2$ in distilled water for 70–80 d, corresponding to the time for transition of a spermatogonium to a mature sperm cell.

The rats were allowed to feed *ad libitum* on a semipurified basal diet (HC, Ziegler Brothers, Inc., Gardner, Pa.). To allow more precise control of the Zn content, the diet was formulated with egg white as the source of protein. A supplemental mineral mix was added to produce a diet containing 20 ppm Zn, 2 ppm Cu, 35 ppm Fe, 50 ppm Mn, 0.04 ppm Se, and 0.15 ppm I. By limiting the availability of the essential metals, especially Zn, to the required level, protective effects against Cd toxicity were minimized. Weekly weight and fluid and food consumption were recorded.

At approximately 170 d of age, each male was mated to three female rats, one of which was sacrificed on d 20 of pregnancy for prenatal evaluation. The other two dams were allowed to deliver and their offspring were subjected to postnatal neurobehavioral assessment.

Each male was sacrificed under Nembutal anesthesia 10–14 d after the third mating. Testes and caudae epididymides were removed and prepared for histological or spermatotoxic evaluation. At the time of this experiment, the opportunity arose to have a Cd determination on the kidney and liver. We chose to have such analyses in order to relate the Cd burden in these organs to possible general toxicity.

Spermatotoxic Evaluation

With the animal under anesthesia, an incision was made in the lower abdomen and the left or right testis (based on a predetermined counterbalanced order) and associated cauda were exposed. A ligature was placed around the arterial blood supply and the organs were removed. The animal was then subjected to whole-body, intracardiac perfusion as described in the histology section. Upon dissection, wet weights of caudae epididymides and testes were obtained. The cauda was then minced, transferred to a test tube, and diluted to 6 ml. The suspension was allowed to sit for approximately 10 min and smears were made from a few drops removed from the supernatant. The slides were placed in a fixative containing 50% ethanol and 50% ethyl ether and then stained with 1% eosin Y. The remainder of the solution was diluted to 100 ml and a sample was taken for sperm count determinations with a hemacytometer chamber.

For each animal, 200 sperm per testis were examined at X400

magnification. Since no differences were seen between left and right testes, these values were averaged for each animal. The categories employed for classifying the rat sperm were derived from Wyrobeck and Bruce's (1975) scheme for mice with some modifications. Their amorphous category was eliminated because of the confounding of this category with artifacts of slide preparation. In addition, the frequency of two-tailed sperm was negligible and precluded categorization.

Prenatal/Postnatal Evaluations

At d 20 one of the females was sacrificed under Nembutal anesthesia (50 mg/kg, ip). The female was laparotomized and the ovaries and uterine horns dissected out. Corpora lutea were scored as well as resorption sites, live and dead fetuses, and fetal weight and sex. Fetuses were also examined for external anomalies.

The other two females were allowed to deliver and the pups were subjected to neurobehavioral testing at 21 d of age. Approximately 3 males per litter were tested in photocell activity cages for 1 h.

Beginning at approximately 90 d of age the rats were tested on the acquisition of a simultaneous visual discrimination task. From the original pool of animals, 1 or 2 male rats were selected from each litter, forming a group of 12 for each exposure condition.

The rats were reduced to 85% of their body weight when feeding *ad libitum* and were taught to press a lever for liquid reinforcement (0.10 ml condensed milk and water, 1:1). They were maintained at this level by a 23-h water deprivation schedule. All testing was carried out in Coulbourn test chambers (Coulbourn Instruments, Inc., Lehigh Valley, Pa.) in sound-attenuated cubicles. After the rats had learned to press a lever, they were given practice pressing two levers, one on each side of the dipper.

The simultaneous visual discrimination task involved a discrete trial procedure in which the onset of a single cue light (one located above each lever) signaled the current choice. The selection of the lever to be illuminated on each trial was varied randomly. A correction procedure was used; that is, a response on the wrong lever was scored, but did not terminate the trial. After the correct response reinforcement was given and a 20-s intertrial interval ensued. Once the rats had reached criterion (90% correct responses during a daily test session) a reversal procedure was initiated. Fifty trials a day were given, 5 d/wk.

Histological Preparation

Testes were fixed by whole-body intracardiac perfusion with two separate solutions. The first consisted of physiological saline containing 1% procaine hydrochloride and was allowed to perfuse through the animals' circulatory system only long enough to remove most of the blood. The second contained 4% glutaraldehyde and 3% paraformaldehyde buffered to pH 7.4 with 0.2 *N* cacodylate buffer; it was perfused through the animals

for 5–10 min. The hydrostatic pressure of the perfusate was adjusted to match the rats' systolic blood pressure, approximately 125 mmHg.

Following this, the testes were removed and several blocks about 2 mm^3 were taken from the center of each and placed in the final fixing solution for up to 24 h. They were then transferred to cold buffered 1.5% osmium tetroxide for $1\frac{1}{2}$ h, dehydrated in a series of ethyl alcohols, and embedded in Spurr low-viscosity medium. Thin sections were cut on a Reichert OMU-3 ultramicrotome with a diamond knife, stained with 4% uranyl acetate followed by 1% lead citrate, and examined with a Philips 301 transmission electron microscope. For light microscopy, sections were cut from the same blocks at 1.5 μm and stained with toluidine blue.

Tissue Determinations

Tissue Cd concentrations were obtained for the liver and kidney. The methods used for determining tissue concentrations have been published elsewhere (Murthy et al., 1973) and involved atomic absorption spectrophotometry.

RESULTS

A 4 X 11 repeated measures analysis of variance (ANOVA) was run on the food and water consumption data with one between factor (treatment) and one within factor (weeks of exposure). As seen in previous studies (Hastings et al., 1978; Yuhas et al., 1979), water consumption was reduced in groups receiving Cd in the drinking water (Fig. 1). The intake

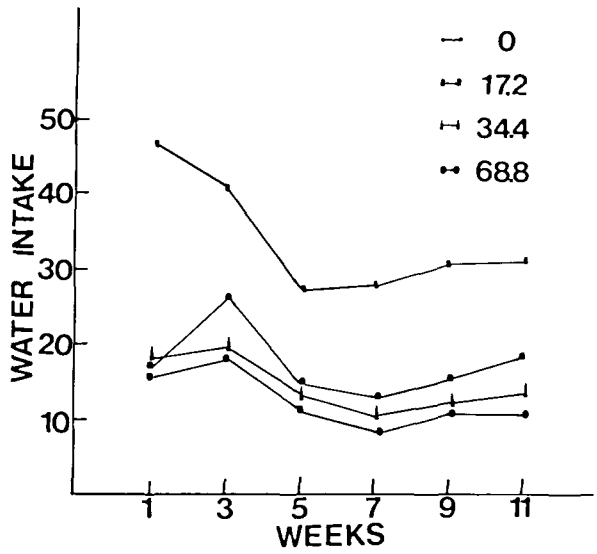

FIGURE 1. Group mean daily Cd-water intake (ml) over the 11 wk of exposure.

of all groups receiving Cd was significantly reduced compared to controls ($F = 26.5$, df = 3, 16; $p < 0.01$). However, the only difference in food intake among the 4 groups was a significant increase in consumption in the 68.8 ppm Cd group with respect to the 34.4 ppm Cd group ($F = 3.17$, df = 3, 16; $p < 0.01$). Finally, the reduction in body weight that usually accompanies decreased water consumption was not observed. Body weights tended to be reduced at the higher level of Cd exposure, but these differences were not significant.

Analysis of variance in tissue concentrations of Cd revealed dose-related effects in the liver ($F = 60.51$, df = 3, 14; $p \leqslant 0.001$) and kidney ($F = 13.29$, df = 3, 14; $p \leqslant 0.001$). The Cd concentration increased significantly with increasing level of exposure as compared to controls (Neuman-Keuls, $p \leqslant 0.05$). These values are presented in Table 1. Comparable results have been reported elsewhere (Hastings et al., 1978; Yuhas et al., 1979). Finally, even though the level of Cd increased greatly in the kidney, it was still below that (> 200 ppm, wet weight) necessary to produce detectable renal dysfunction.

Light microscopic examination showed that the testes from the Cd-treated animals appeared normal in all respects compared to those of the controls. For this reason, only samples from the high-dose group (68.8 ppm) and the controls were examined further with the electron microscope. Particular attention was paid to early or subtle vascular changes since testicular vessels may represent a primary site of action in acute exposures to Cd. No differences in spermatogenesis, Leydig cell morphology, or testicular vessels could be detected ultrastructurally between testes of animals fed the highest dose of Cd and those of controls (Figs. 2 and 3). Comparable findings were recently reported in rats at a comparable age (Zielinska-Psuja et al., 1979).

Analysis of variance in the parameters of spermatotoxicity revealed no significant differences among the treatment groups in terms of testes or cauda weight or in terms of sperm morphology or count (Table 2). The

TABLE 1. Cadmium Concentration in Liver and Kidney following 70–80 d of Exposure

Treatment (ppm Cd)	Cd concentration (μg/g, dry weight)[a]	
	Liver	Kidney
0	1.39 ± 0.20	1.00 ± 0.08
17.2	17.90 ± 2.02[b]	51.83 ± 3.51[b]
34.4	33.91 ± 5.69[b]	111.72 ± 9.46[b]
68.8	61.43 ± 5.10[b]	124.44 ± 24.25[b]

[a]Mean ± SE.
[b]Significantly different from controls, $p < 0.05$.

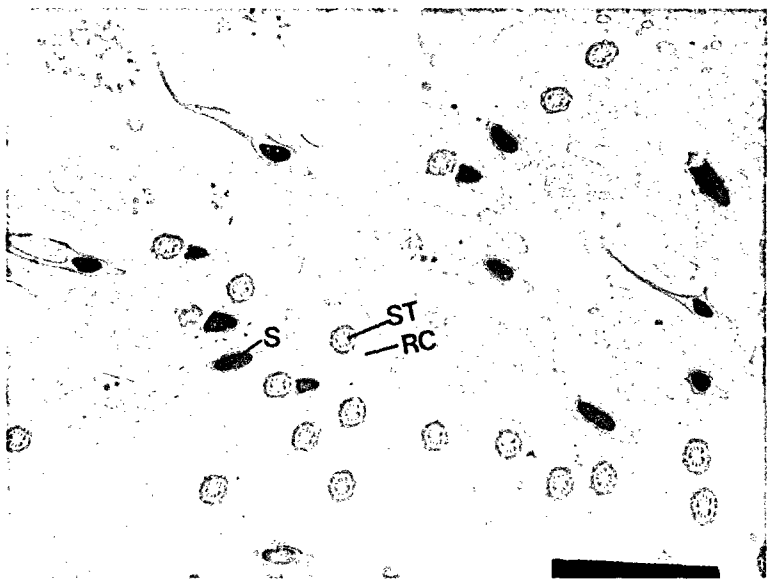

FIGURE 2. Electron micrograph from the seminiferous tubule or a rat administered 68.8 ppm $CdCl_2$ in the drinking water for 70 d. Developing spermatozoa are numerous and their component parts appear normal, which indicates that spermatogenesis and spermiogenesis were proceeding normally. Abbreviations: S, sperm head with acrosome; ST, cross section through middle of a sperm tail surrounded by residual cytoplasm (RC).

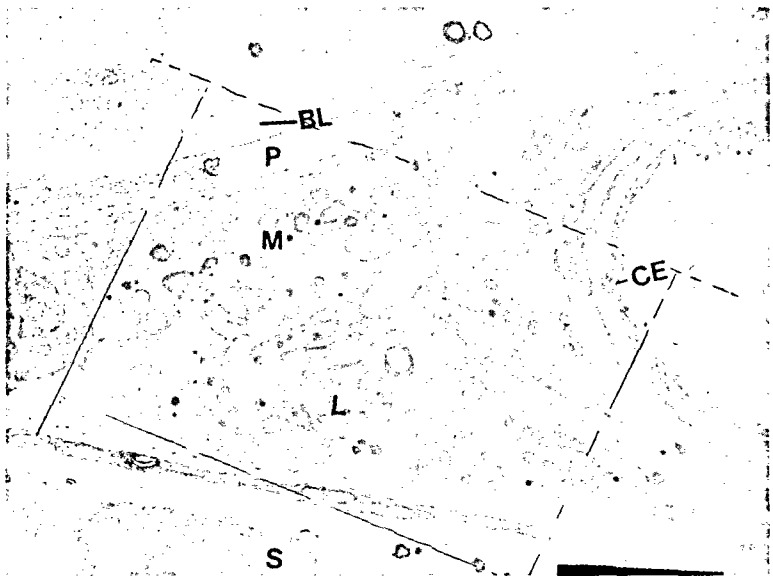

FIGURE 3. Electron micrograph from another animal in the same group as that in Fig. 2, showing a representative area of normal Leydig cells (L), capillary endothelium (CE), macrophage (M), peritubular myeloid cell (P), basal lamina (BL), and Sertoli cell cytoplasm (S).

TABLE 2. Effect of Treatment on Spermatogenic Indices[a]

Treatment[b] (ppm Cd)	Body weight (g)	Testes weight (g)	Cauda weight (g)	Sperm count ($\times 10^8$)	No. of normal sperm/200
0	446 ± 30	1.92 ± 0.19	0.143 ± 0.03	7.69 ± 2.27	169 ± 18
17.2	446 ± 45	1.88 ± 0.18	0.138 ± 0.03	7.82 ± 3.01	176 ± 5
34.4	430 ± 34	1.86 ± 0.11	0.142 ± 0.03	8.14 ± 1.63	173 ± 14
68.8	431 ± 24.8	1.81 ± 0.18	0.140 ± 0.02	7.63 ± 4.41	171 ± 18

[a]Values are means ± SD.
[b]Sample size was 5 males per treatment group exposed to Cd for 70–80 d.

baseline percentage of spontaneous abnormal sperm was approximately 15% across groups. This is slightly higher than the 10–12% routinely observed for this strain in our laboratory.

All males successfully impregnated their three females within a comparable number of days. Thus male fertility was not compromised by the Cd treatment. An analysis of covariance run on pup weights at the time of sacrifice (d 20), adjusted for litter size, revealed no significant differences across treatment groups. Also, there were no differences in the number of implantations, resorptions, or corpora lutea expressed in conventional ratios (Table 3).

An analysis of covariance was run on the birth weights of dams allowed to litter as well as the litter weights at d 7, 14, and 21. Although there appeared to be a trend toward decreased weight in the 68.8 ppm group, the analyses revealed no significant treatment effects (Table 4).

Finally, postnatal behavioral evaluations revealed no treatment effects in terms of measures of exploratory activity recorded at 21 d of age or the acquisition and subsequent reversal of the simultaneous discrimination learning task conducted at 90 d of age.

DISCUSSION

No adverse effects were noted in the general health status of the males receiving chronic Cd exposure. The consumption of the semipurified diet

TABLE 3. Effect of Treatment on Fetal Outcome Measures[a]

Treatment[b] (ppm Cd)	Litter size	Mean pup weight per litter (g)	No. of implantations	Implants, corpora lutea
0	10 ± 3	5.6 ± 0.10	10 ± 3	0.83 ± 0.19
17.2	8 ± 3	5.6 ± 0.13	8 ± 3	0.88 ± 0.09
34.4	10 ± 2	5.6 ± 0.18	10 ± 3	0.91 ± 0.07
68.8	11 ± 1	5.0 ± 1.0	11 ± 2	0.93 ± 0.10

[a]Values are means ± SD.
[b]Sample size was 5 litters per treatment group.

TABLE 4. Birth and Postpartum Litter Weights following Exposure of Male Parent to Cd

Treatment[b] (ppm Cd)	Litter (g) weight[b]			
	Birth	d 7	d 14	d 21
0	7.5 ± 1.0	19.4 ± 3.0	31.0 ± 4.2	49.4 ± 9.5
17.2	7.4 ± 1.3	17.5 ± 2.4	30.9 ± 6.5	46.6 ± 11.0
34.4	7.9 ± 2.0	17.2 ± 1.3	31.1 ± 5.6	49.5 ± 9.5
68.8	6.8 ± 1.1	16.8 ± 3.1	29.5 ± 5.4	47.7 ± 7.7

[a]Values are means ± SD.
[b]Sample size was 10 litters per treatment group.

may have prevented a significant reduction in body weight in the presence of a sharp decrease in water consumption by males receiving Cd. The diet has a high fat content (9%) compared to the normal laboratory diet as well as a low fiber content (3%). Thus the Cd-exposed rats could reject the drinking water and still obtain adequate moisture by increasing food consumption and/or resorption. This is supported by the fact that the controls also initially decreased their water consumption, yet showed no weight loss.

No treatment effects were observed in terms of fertility or adverse fetal outcome. Results were comparable to those reported by Dixon et al. (1976); in that study, neither acute nor chronic oral administration of Cd resulted in decreased fertility as measured by number of resorptions or number of viable fetuses. Moreover, the absence of histological changes in the testes is in agreement with results obtained after low-dose, repeated injections of Cd (Piscator and Axelsson, 1970; Nordberg, 1971; Der et al., 1976). This absence of effect was also reflected in previously unexamined measures of spermatotoxicity, namely, sperm count and sperm morphology.

Our overall conclusion is that Cd, orally administered in water at concentrations up to 68.8 ppm, is without significant reproductive toxicity even with a diet controlling for protective effects by other trace elements. Similar conclusions were reached by Dixon et al., but for markedly lower Cd concentrations (0.001, 0.01, and 0.1 ppm) and a standard laboratory diet with elevated levels of Zn.

We did not analyze various biochemical and hormonal parameters of reproductive function; thus the Cd may have exerted a more subtle influence than is revealed by morphological evaluation. However, if such effects exist, they do not appear to compromise fertility or fetal outcome.

Finally, our results do not eliminate the possiblity that Cd exerts a gonadal effect with longer exposure at higher doses. A report by Zielinska-Psuja et al. (1979), published after we completed this paper, supports such

a hypothesis. These authors found consistent histological and hormonal alterations in rats chronically exposed to $CdCl_2$ (88 mg/kg·d) in the diet for up to 15 mo. This dosage resulted in an intake approximately 16 times higher than the highest dose employed in the present study. Moreover, the changes were seen primarily after 12 and 15 mo of exposure, at which times the animals' body weights were reduced to 50–70% of those of the control groups. Finally, the Cd levels in kidney and liver in the present study suggest that continued exposure would have produced renal or hepatic damage before interfering with reproductive function. These findings suggest the need to reevaluate the classic view of Cd-related testicular injury based on acute exposure, injection protocols in terms of relevance to the actual environmental situation.

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