

# A Single Low Cadmium Dose Causes Failure of Spermiation in the Rat

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Ten-week-old male Sprague-Dawley rats were injected ip with cadmium chloride solution in a single dose of 0, 0.5, or 1.0 mg/kg body wt. At 4, 24, 48, and 72 hr after injection, testes of the animals were perfusion fixed, embedded in plastic, and sectioned. Tissue sections were stained and examined under the light microscope. These amounts of cadmium did not result in visible vascular lesion in the testes. However, at a dose of 1 mg/kg, the cadmium treatment resulted in failure of spermiation from stage IX through later stages of spermatogenesis in the seminiferous epithelium. Detailed statistical analysis revealed failed spermiation 24 hr after dosing. As the exposure time increased, failure of spermiation was observed with increasing frequency within an affected stage, and was seen at later stages of spermatogenesis as well. Testes of the rats treated with a cadmium chloride dose of 0.5 mg/kg showed no change in the frequencies of tubules having unreleased spermatids when compared to the controls. There was no difference in the stage frequencies between all the treatment groups and the controls. These results indicate that a single cadmium chloride dose of 1 mg/kg results in failed spermiation in rat seminiferous tubules, without discernible change to the surrounding endothelium. We conclude that cadmium begins to act during early stage VIII of spermatogenesis to induce failure of spermiation, and the action of cadmium is spermatogenic stage-specific. © 1993 Academic Press, Inc.

Cadmium is an important environmental pollutant because it is widely used in industry, and it is present in a number of agricultural products. It is found in pigments, nickel-cadmium rechargeable batteries, plastic, cigarettes, and some fertilizers, as well as food and water via environmental pollution. It is nonbiodegradable and has a long biological half-life. These characteristics have made cadmium a cumulative toxic element which causes tissue damage in humans (for review, see Robards and Worsfold, 1991).

Cadmium damages the testes of many mammals (Parizek and Záhor, 1956; Cameron and Foster, 1963).

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Early studies of cadmium toxicity indicated that cadmium salts produced a purplish discoloration of the testis. Most studies have shown that cadmium treatment results in increased permeability of the testicular endothelium, with subsequent hemorrhage into the interstitial space of the testis (Aoki and Hoffer, 1978; Gouveia, 1988). Many investigators have concluded that damage to the seminiferous tubules in the testis is due to the leaking blood vessels causing an increase in intratesticular pressure which then causes ischemia. While vascular changes and ischemic necrosis are proposed as possible mechanisms of actions of cadmium in the testis (Mason *et al.*, 1964), permeability studies suggest that the permeability barrier of the seminiferous tubules is compromised by cadmium before vascular damage occurs (Setchell and Waites, 1970). Results indicating vascular changes due to cadmium exposure have come from studies using relatively high doses of cadmium, or chronic treatment, or both. A survey of the literature failed to uncover a well documented study of early morphological changes in the testis resulting from treatment of animals with a single low dose of cadmium.

The goal of this investigation was to study the early changes in the seminiferous epithelium of rats treated with a single low dose of cadmium. The hypothesis we wished to test was that cadmium can damage the seminiferous epithelium at a concentration of the metal ion that is lower than that which overtly damages testicular endothelium. Based on observations reported by many workers that a wide range of events in the seminiferous epithelium varies depending on the stage of spermatogenesis, we speculated that threshold levels of cadmium could affect only the most vulnerable events and would exhibit a spermatogenic stage-dependent toxicity. Observations presented here demonstrate that cadmium, administered as a single dose at a concentration that causes no visible change in the integrity of the testicular endothelium, results in a spermatogenic stage-specific lesion, namely failure of spermiation.

## METHODS

**Reagents.** Cadmium chloride (CdCl<sub>2</sub>), heparin, and procaine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Ether and sodium chloride were purchased from Mallinckrodt, Inc. (Paris, KY);

and ethanol (95%, USP) was purchased from Aaper Alcohol & Chemical Co. (Shelbyville, KY). Sodium pentobarbital (injectable, 64.8 mg/ml) was obtained from Anthony Products Co. (Arcadia, CA). Glutaraldehyde (50% EM grade) and 2,4,6-*sym*-collidine buffer kit (EM grade) were purchased from Polysciences, Inc. (Warrington, PA). Glycol methacrylate was obtained as a Bio-Rad plastic embedding media kit from Energy Beam Sciences (Agawam, MA). Hematoxylin was purchased from Fisher Scientific Co. (Springfield, NJ) in the form of Gill's Formulation No. 3. The mounting medium Permount was also obtained from Fisher Scientific Co.

**Animals.** Ten-week-old male Sprague-Dawley rats (mean weight  $352 \text{ g} \pm 38 \text{ g SD}$ ) were obtained from the Laboratory Animal Colony of the P30 Center for the Study of Reproduction at the University of Michigan, Ann Arbor. Animals were housed three to five per cage and provided with water and laboratory chow *ad libitum*. The rats were specific pathogen-free and were kept in a controlled environment (14/10-hr light/dark cycle, lights on at 0600 hr; ambient temperature  $22^\circ\text{C}$ ).

**Cadmium exposure.** Male rats were lightly anesthetized with ether, weighed, and injected ip with 0, 0.5, or 1.0 mg  $\text{CdCl}_2/\text{kg}$  body wt (as 0.08%  $\text{CdCl}_2$  solution in deionized water). A preliminary study had determined that at doses of 1.5 mg  $\text{CdCl}_2/\text{kg}$  and above, the testes of most rats showed both vascular and seminiferous epithelial damages after 24 hr of exposure.

**Fixation of testes for microscopic examination.** At 4, 24, 48, and 72 hr after injection of cadmium, each animal was anesthetized with sodium pentobarbital (60 mg/kg, ip), and its testes were fixed by vascular perfusion in a manner similar to the method of Forssmann *et al.* (1977). The abdominal cavity of each rat was exposed, and a loose ligature was placed around the aorta above the left renal artery. Without disturbing the testicular arteries, the descending aorta above the common iliac arteries was cleared of fat and connective tissue. The ligature around the aorta was tightened, and the descending aorta was quickly cannulated in a retrograde fashion with a 21-gauge syringe needle connected to a perfusion pump via polyethylene tubing. The inferior vena cava was cut to provide the outflow of blood and perfusate. Using a perfusion pump (Masterflex, Cole-Parmer Instrument Co.) running at the rate of 10 ml/min, blood was rinsed from the vasculature with 0.9% sodium chloride solution containing 0.5% procaine HCl and 10 USP units heparin/ml. To improve the efficiency of the perfusion, a second ligature was made around the mouth of the syringe needle so that the rinsing solution and fixative only flowed within the vasculature between the two ligatures. After the testes were cleared of blood, the tissues were fixed with 100 ml of 5% glutaraldehyde in 0.1 M *s*-collidine buffer (pH 7.4). The fixed testes were removed, cut into 2-mm-thick transverse slices, and placed in the same fixative for an additional 2 hr at room temperature. The tissue slices were then washed three times (15 min each) in 0.1 M *s*-collidine buffer at room temperature. The washed tissues were stored in this buffer at  $4^\circ\text{C}$  until further processing. The widest tissue slice from each testis was dehydrated through graded alcohols and embedded in glycol methacrylate. Two-micrometer sections were cut with glass knives using a Bio-Rad JB 4A microtome. The sections were mounted on glass slides, stained with periodic acid-Schiff's reaction (PAS), and counterstained with hematoxylin. The stained sections were mounted with Permount and examined under a Bausch & Lomb binocular microscope. The seminiferous epithelium in the sections was staged using the criteria of Leblond and Clermont (1952) with modifications based on Hess (1990) and Russell *et al.* (1990). As recommended by Hess *et al.* (1990), a minimum of 200 tubule cross sections/testis was scored to determine the stage frequencies; 15 rats were used in each treatment group including controls.

**Cadmium analysis.** To analyze the cadmium concentration in the blood, whole blood was drawn from the heart into a syringe just after perfusion began. Samples from three or four animals were taken for cadmium measurement at time points of 24, 48, and 72 hr. The blood was centrifuged immediately in a clinical centrifuge to separate plasma from the formed elements of the blood. Plasma samples (0.5 ml each) were digested using 1 ml of concentrated trace metal grade nitric acid at  $120^\circ\text{C}$

for about 4 hr, followed by 0.4 ml of 30% (v/v)  $\text{H}_2\text{O}_2$  at the same temperature until the sample became dry. The dry residue was dissolved in 1 ml of 1% v/v nitric acid. The cadmium level in this solution was determined by graphite furnace atomic absorption spectrometry (Perkin-Elmer Model 2380 with HGA-400) with gravimetric cadmium standards.

**Statistical analysis.** The frequency of each spermatogenic stage in each treatment group was determined. If for any time, dose, and stage combination,  $\hat{p}_j$  was the observed proportion of spermatogenic stage  $i$  in a cross section from animal  $j$ , then a simple average of the  $\hat{p}_j$ 's was computed for each stage and treatment group. The standard error of these averages was computed taking due account of the variability both within and between animals. The difference in the averages of each stage between the controls and the cadmium-treated group at each time point was determined by calculating approximate  $z$  scores, where  $z$  has an approximate standard normal distribution under the null hypothesis of no difference between treatment and controls.

The same test was carried out to compare the difference in the mean frequencies of each stage between the cadmium-treated groups (within a single dose) at increasing time of exposure. A comparison was also made regarding the difference in the mean frequencies of each stage between all the control groups. All differences were considered significant at the 0.01 level if  $|z| > 2.58$ .

The abnormality observed in this study, namely failed spermiation, was scored based on whether step 19 spermatids were still present in the seminiferous epithelium of spermatogenic stages later than stage VIII. From the data obtained in this study, it was obvious that there was variability in response from animal to animal. In order to account for the interanimal variability in the response, we applied the beta-binomial model of Crowder (1978). Under this model, the number of responses in animal  $j$  was modeled as a binomial random variable with response probability  $p_j$ . The response probabilities  $p_j$  were themselves random variables, defined on the population of all animals, and were assumed to follow a beta distribution with mean  $p$  and variance  $\sigma^2 p(1-p)$ , where both  $p$  and  $\sigma$  were constrained to lie in the interval between zero and one. Following Crowder (1978), it was possible to obtain maximum likelihood estimates of  $p$  and  $\sigma$  by solving a constrained maximization problem. This was done for all experimental conditions using the computer program NPSOL (Gill *et al.*, 1986). In addition, contour and perspective plots of the likelihood surfaces were produced for a subset of the experimental conditions using the S\* Statistics package (1989).

It was found that the maximum likelihood estimates were consistent but the usual (Fisher Information) estimates of their standard errors were not valid. In order to obtain valid estimates of the variability of the parameter estimates, we performed a Bayesian analysis. Posterior means and variances for  $p$  and  $\sigma$  were calculated using a uniform (flat) prior. This was done by performing a direct numerical integration of the likelihood function and its first and second moments using an adaptive quadrature method (subroutine DTWODQ) from the IMSL numerical analysis library (IMSL, 1989).

Results from the Bayesian analysis were used in the pairwise comparisons and graphical analysis to determine the effects of dose and duration of exposure on sperm release. Since failed spermiation was also seen in control animals, comparison between cadmium-treated groups was made by calculating an approximate posterior distribution of differences between the  $p$  for treated animals and that for control animals in each group. A normal approximation was used. Differences were deemed to be "significant at level 0.01" (see Fig. 2) if the approximate posterior probability that the difference was positive was at least 0.99.

## RESULTS

At a dose of 1 mg/kg, cadmium chloride treatment resulted in failure of sperm release from stage IX through

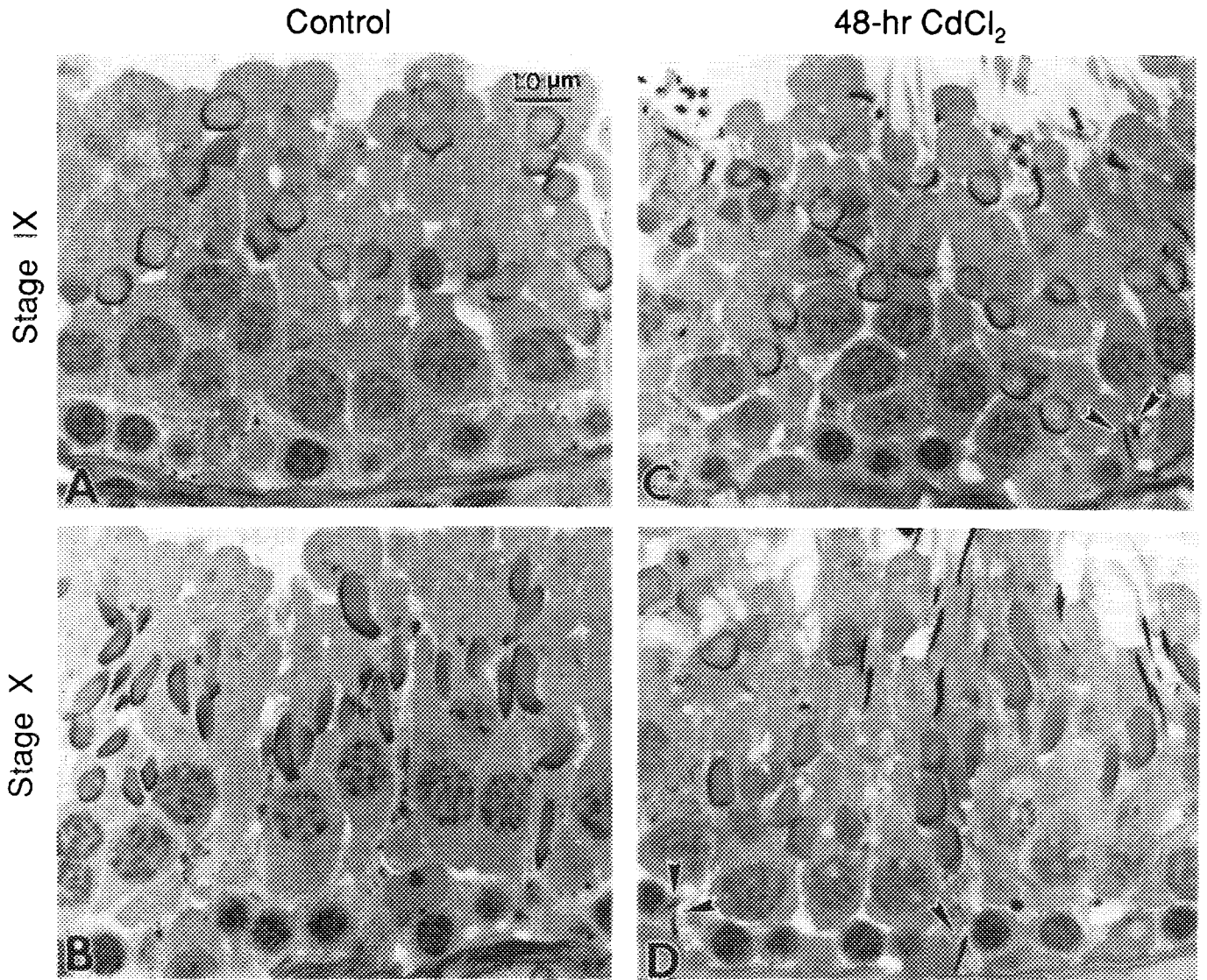


FIG. 1. Cross sections of rat seminiferous tubules. A, B: seminiferous epithelium from control animals; C, D: seminiferous epithelium from rat exposed to  $\text{CdCl}_2$  (1 mg/kg, ip) for 48 hr. A, C: stage IX seminiferous epithelium; B, D: stage X seminiferous epithelium. Note the absence of step 19 spermatids in the control seminiferous epithelium. In the treated animals, groups of these spermatids were still present at the adluminal region of the seminiferous epithelium. Several of the late stage spermatid heads are seen near the basal region (arrowheads).

later stages of spermatogenesis in the rat seminiferous epithelium (Fig. 1). This lesion occurred with increasing frequency as the exposure time increased (Fig. 2). In the control animals, a vast majority of the step 19 spermatids was released from the seminiferous epithelium at the end of stage VIII of spermatogenesis, and spermatids were almost entirely absent in the stage IX through stage XIV tubules (Figs. 1A, 1B). In contrast, in most of the stages IX through XIV tubules from the cadmium-treated testes, groups of step 19 spermatids were still attached to the luminal margin of the Sertoli cells (Figs. 1C, 1D). A few heads of these spermatids were seen near the basal region of Sertoli cells at the tubule periphery where they were probably being digested

by the Sertoli cells (Figs. 1C, 1D). The presence of these spermatids near the basal region of the Sertoli cells was observed with increasing frequency at 48 and 72 hr of exposure. In many tubular sections that showed failed spermiation, especially those that had step 19 spermatids attached to the luminal margin of the Sertoli cells, the lesion was usually most obvious in one area of the seminiferous epithelium. In any affected tubule, the younger generation of spermatids (steps 9–14) in the region of the tubule showing the lesion frequently appeared to be at a developmental stage slightly earlier than that of the spermatids in other areas of the same tubule cross section. The stage designation for a tubule cross section was determined in such cases

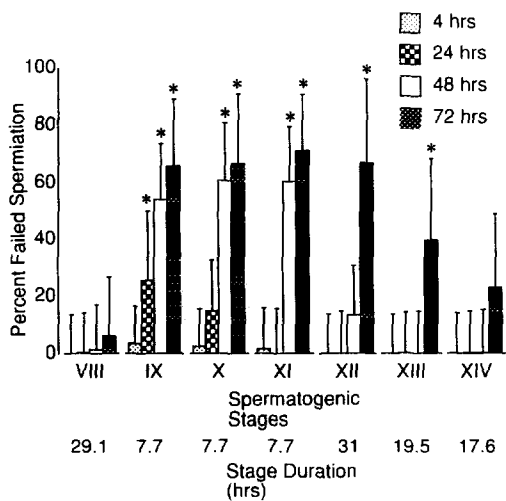


FIG. 2. Effect of a single dose of  $\text{CdCl}_2$  (1 mg/kg, ip) on spermiation in the rat at increasing time of exposure. All values for percentage failure of spermiation are corrected for controls. The vertical line on each bar denotes the upper Bayesian "confidence" intervals (99%). \*Indicates that percentage failed spermiation in the particular stage is significantly different from the control ( $p < 0.01$ ). See the last paragraph of the Statistical Analysis under Methods for further detail.

by the younger spermatids occupying the majority of the seminiferous epithelium in the cross section. In the control animals, such a cellular arrangement was never observed in a cross section, even in tubules showing retained step 19 spermatids. A few control animals showed retained step 19 spermatids in a few stage IX through stage XI seminiferous tubules. In these animals, isolated spermatids (usually fewer than three) were seen either at the luminal or basal region of the seminiferous epithelium. The frequency of failed spermiation in these control rats was much lower than that seen in the cadmium-treated animals. Failed spermiation appeared to be the most conspicuous lesion observed as a result of the cadmium treatment. With the exception of occasional pyknotic germ cell nuclei in some seminiferous tubules of the exposed rats, the seminiferous epithelium of the remaining tubules, as well as the blood vessels in the exposed testes, did not show any discernible change under the light microscope. The absence of vascular lesion in the testes was further indicated by the absence of testicular edema in all but a very few of the testes studied.

While the testis sections from most rats in this study showed readily identifiable spermatogenic stages, testis sections from some cadmium-treated rats (1 of 15 in the 24-hr group, 2 of 15 in the 48-hr group, and 7 of 15 in the 72-hr group) showed massive cell death in the seminiferous epithelium. In the testes of these affected animals, the interstitial space was greatly widened and was filled with blood cells and fibrous material. It was evident that the endothelial junctions had opened and allowed blood to enter the testicular interstitium. These changes were similar to those

seen in testes exposed to higher doses of cadmium, although with higher doses of cadmium such changes occur within hours, not days. Since the aim of this investigation was to identify early changes, if any, in intact seminiferous epithelium after cadmium treatment, testes showing severe damage such as that described above were not included in our analysis. Moreover, the disintegrating seminiferous epithelium in such severely affected testes made determination of spermatogenic stage virtually impossible.

**Cadmium analysis.** Rats receiving a cadmium chloride dose of 1 mg/kg had a mean cadmium concentration of  $3.6 \mu\text{g/liter}$  ( $\pm 0.9 \mu\text{g/liter SD}$ ) in their plasma at 24 hr after the ip injection. This level remained unchanged at 48 or 72 hr after the cadmium treatment, at which times cadmium levels were  $3.6 \mu\text{g/liter}$  ( $\pm 1.1 \mu\text{g/liter SD}$ ) and  $3.5 \mu\text{g/liter}$  ( $\pm 1.1 \mu\text{g/liter SD}$ ), respectively.

**Statistical analysis.** Our statistical analysis was based on the examinations of testicular cross sections from 140 animals. A total of 35,496 tubular cross sections (mean No. tubules counted/testis cross section:  $254 \pm 31 \text{ SD}$ ) were scored to determine the stage frequencies of individual stages of spermatogenesis, as well as the frequency of failed spermiation within each stage. An examination of the stage frequencies of all 14 stages of spermatogenesis in each treatment group revealed that there was no difference in the stage frequencies of all the stages examined in the treatment groups.

In the cadmium-treated rats which were exposed for 48 or 72 hr, some late stage spermatids were seen at the basal region of the stage VIII seminiferous epithelium. Since almost all of the late stage spermatids were aligned close to the lumen at this stage in the control animals, the presence of these spermatids in the basal region at stage VIII was considered indicative of failed spermiation. We decided to include these data in our failed spermiation analysis.

Figure 2 shows the time course and the percentage of tubules from stages VIII through XIV displaying failed spermiation in rats exposed to a cadmium chloride dose of 1 mg/kg. The percentage of failed spermiation shown in Fig. 2 was corrected for controls at each time point by subtracting the percentage failed spermiation in the control from that in the treatment group. Within a particular stage, the percentage of tubules showing failed spermiation increased with longer exposure time. In addition, with a longer exposure time, the unreleased step 19 spermatids were seen at later stages of spermatogenesis in the cadmium-treated animals. Our statistical analysis indicated that 4 hr of cadmium treatment had no effect on spermiation in all stages of spermatogenesis. At 24 hr of exposure, a significant increase in failed spermiation was observed in stage IX tubules compared to that in the controls. After 48 hr of exposure, a significant increase of the lesion was observed in stages IX through XI. At 72 hr, increased failure

of spermiation was detected in stages IX through XIII. While failed spermiation was observed in some stage VIII tubules, our analysis did not indicate a significant difference from the controls. Using knowledge of the estimated duration of individual stages of spermatogenesis in the rat (Russell *et al.*, 1990), we conclude that at a single ip dose of 1 mg/kg, cadmium chloride most probably acts during early stage VIII of the seminiferous epithelium to induce failure of spermiation.

In the testes of rats treated with a cadmium chloride dose of 0.5 mg/kg, there was no difference in the percentage of tubules showing unreleased spermatids between the controls and the treated groups. Similarly, the stage frequencies of the tubules in these cadmium treated groups did not show any significant difference from those in the control animals at periods of time of up to 72 hr.

### DISCUSSION

In the present study we have demonstrated that a single ip cadmium chloride dose of 1 mg/kg resulted in failure of sperm release from the seminiferous epithelium in the rat. The amount of cadmium used in this study was lower than that used in previously reported studies by other investigators. Other studies of the effects of ip administered cadmium have reported that vascular damage is the primary or initial lesion in the testes (Aoki and Hoffer, 1978 (3.4 mg CdCl<sub>2</sub>/kg); Gázdik and Kaminski, 1984 (1.5 mg CdCl<sub>2</sub>/kg)). In the present study, 1 mg/kg cadmium chloride treatment caused no apparent vascular lesion or testicular edema in most animals. Furthermore, because we excluded from our analysis the tissue from animals sensitive to the low cadmium dose we employed, the results demonstrate that the seminiferous epithelium is affected by cadmium at a concentration lower than that which causes visible vascular damage. This suggests that the cells of the seminiferous epithelium, probably Sertoli cells, are more sensitive than testicular endothelial cells to the effect of cadmium.

Our study also examined the time course of cadmium-induced damage. At increasing times after treatment, later stages of spermatogenesis were seen to contain retained mature spermatids. At 24 hr, failure of spermiation was seen in stage IX tubules; at 48 hr failure of spermiation was seen in stage XI tubules; and at 72 hr the lesion was observed in stage XIII tubules. Considering that stages VIII, IX, X, XI, XII, and XIII of spermatogenesis have been estimated to endure for 29.1, 7.7, 7.7, 7.7, 31, and 17.6 hr, respectively (Russell *et al.*, 1990) (see Fig. 2), we conclude that the adverse effect of cadmium on spermiation probably begins during early to midstage VIII of spermatogenesis.

As the time after treatment increased, the percentage of tubules within a stage showing failed spermiation also increased. In addition, not only were the unreleased late stage spermatids present at later stages of spermatogenesis as

time after treatment increased but also failure of spermiation continued to occur. For example, at 72 hr after treatment, retained spermatids were seen in stage XIII tubules as well as all other stages between VIII and XIII. These results indicate that the single cadmium treatment may have a persistent harmful effect on spermiation.

Our measurements showed cadmium to be at a low but constant concentration in blood at 24, 48, and 72 hr. As discussed above, at 72 hr after treatment failure of spermiation was seen in almost all tubules at stages IX–XII of spermatogenesis, indicating that a persistent effect occurred. Possibly the low level of cadmium in the blood continues to exert an effect on the seminiferous epithelium after this length of time. However, we did not measure cadmium in blood at shorter times after the injection, and measurement of total blood cadmium could not reveal how much cadmium was bound to plasma proteins and how much, if any, of the cadmium might be available to affect the seminiferous tubules. Thus, we cannot conclude whether the apparently enduring nature of the lesion resulted from the continued presence of a low level of cadmium in the blood or from an initial and permanent damage to tubules caused by an initially possibly high cadmium concentration.

Failure of sperm release has been reported by other investigators to result from using a variety of agents, including fungizone (Swierstra *et al.*, 1964), the antitumor drug procarbazine (Parvinen, 1979), dibutyl cyclic AMP (Gravis, 1980), vitamin A deficiency (Huang and Marshall, 1983), dimethyl methylphosphonate (Chapin *et al.*, 1984a), methyl chloride (Chapin *et al.*, 1984b), 1,3-dinitrobenzene (Hess *et al.*, 1988), ethanol (Anderson *et al.*, 1989), boric acid (Linder *et al.*, 1990; Treinen and Chapin, 1991), vitamin B<sub>6</sub> (Kaido *et al.*, 1991) and several chemotherapeutic agents including 5-fluorouracil, *cis*-platinum, doxorubicin, and amethopterin (Russell and Russell, 1991). The wide spectrum of agents causing this same lesion, namely failure of spermiation, indicates that this lesion may be a common phenomenon, and suggests that a common mechanism of action may be involved. However, in none of the studies previously reported has a time course study been employed together with a large sample size to allow for analysis of the point during spermatogenesis at which the lesion is most likely to occur.

Spermiation is a complex series of events during which sperm are separated from the seminiferous epithelium and released into the seminiferous tubule lumen (for review, see Russell, 1984). The participation of the Sertoli cells in events such as the dissolution of the ectoplasmic specializations facing the late stage spermatids (Fawcett and Phillips, 1969) and the elimination of spermatid cytoplasm by the Sertoli cells via the tubulobulbar complexes prior to sperm release (Russell, 1979), suggests that the Sertoli cells are actively involved in this process. Cadmium treatment affects Sertoli cell tight junctions *in vitro* (Janecki *et al.*,

1992). Other agents which induce failed spermiation also affect the Sertoli cells. These agents include dibutyl cyclic AMP (Welsh *et al.*, 1980), *cis*-platinum (Pogach *et al.*, 1989), and 2,5-hexanedione (Hall *et al.*, 1991). These observations suggest that cadmium as well as the other agents may affect sperm release by acting on the Sertoli cells.

In conclusion, our study indicates that a single cadmium chloride dose of 1 mg/kg results in failure of sperm release from the rat seminiferous epithelium. This dose of cadmium is without visible change to the testicular endothelium. The effect of this single cadmium treatment is spermatogenic stage-specific. A relatively large sample size (140 animals, 35,496 tubular cross sections examined) used in this study, together with a detailed statistical analysis, allowed us to determine that stage VIII of the spermatogenic cycle is sensitive to the action of cadmium. The experimental approach used in our study can be applied to investigate the short-term morphological response of the seminiferous epithelium to a variety of toxicants. This approach will enable investigators to determine the stage or stages of the spermatogenic cycle which are sensitive to these toxicants, and possibly predict the mechanism of action of these agents.

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