PREVENTION OF TESTICULAR DAMAGE BY FREE-RADICAL SCAVENGERS
ASHOK AGARWAL, ISAO IKEMOTO, AND KEVIN R. LOUGHLIN

ABSTRACT

**Objectives.** The role of free radicals as mediators of ischemic injury to the testicle has been the subject of much investigation. We studied whether the testicular damage induced in the rat by cadmium chloride (CdCl₂) can be prevented by administration of free-radical scavengers.

**Methods.** Sprague-Dawley rats (n = 45) were divided into 9 groups as follows: a negative control group; two positive control groups, one of which received injection of 1 mg/kg body weight of CdCl₂ and the other 4 mg/kg body weight; and six cotreatment groups, each of which underwent one of these three procedures but was concurrently treated with heparin, oxypurinol, or superoxide dismutase (SOD). The damage was assessed by measurement of testicular weight, lactate dehydrogenase-X (LDH-X) activity, and histology.

**Results.** Testicular weight decreased significantly in the positive control groups (P < 0.05) compared to the negative control group, whereas testicular weight in oxypurinol or SOD cotreatment groups did not decrease significantly with the exception of those rats given the higher dose of CdCl₂. The results were similar with regard to testicular LDH-X activity and histology.

**Conclusions.** These findings suggest that CdCl₂ induces impairment of testicular function and causes a marked reduction in testicular LDH-X activity; that LDH-X activity is a biological marker of testicular damage; and that, except at high doses of CdCl₂, this damage can be prevented by oxypurinol or SOD. UROLOGY 50: 759–763, 1997. © 1997, Elsevier Science Inc. All rights reserved.

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Short-term impairment of testicular blood flow, either by ligation of the testicular artery or by experimental torsion of the spermatic cord followed by reperfusion, results in testicular damage. The degree of damage is related to the duration of ischemia, and spermatogonia and preleptotene spermatocytes are the most sensitive cell types. Lactate dehydrogenase-X (LDH-X), an isozyme of the lactate dehydrogenase system that is present only in primary spermatocytes and spermatids, is the most active form of the enzyme present in mature sperm. The spermatozoa require LDH-X for necessary metabolic activity during passage from the testis to the site of fertilization in the oviduct. The activity of LDH-X in seminal plasma has been regarded as a sign of leakage from the spermatozoa or from their precursor cells. It has been reported that a decrease in LDH-X activity in testicular extract after cadmium chloride (CdCl₂) administration corresponds to the necrotic damage observed histologically.

There is evidence to suggest that the testis is especially susceptible to oxygen toxicity. Hyperoxia is known to affect testicular function. Deficiency of vitamin E, which protects against lipid peroxidation, leads to testicular damage. A recent study indicates that cadmium increases the lipid peroxidation of liver cell membranes. The accumulation of lipid peroxides is toxic to the membrane structure, leading to a change in permeability and probably to disintegration of the cellular organelles. Thus, enzymes scavenging oxygen free radicals, such as superoxide dismutase (SOD) and catalase, have been found to reduce damage in ischemia-reperfusion models in other organs.

This investigation was undertaken to determine whether ischemic injuries induced in the rat testis by CdCl₂ can be ameliorated by administration of free-radical scavengers such as heparin, oxypurin-
nol (xanthine-oxidase blocker), and SOD, and also to ascertain whether LDH-X activity can serve as a biological marker of such testicular damage.

**MATERIAL AND METHODS**

**ANIMALS**

Forty-five sexually mature Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass) weighing 300 to 350 g were used in this experiment. All animals were housed at 21°C with an alternating 12-hour light/12-hour dark cycle. Animals were fed standard rodent laboratory chow. Water was given ad libitum. The animals were randomly divided into nine groups as follows: negative control group, two positive control groups that underwent injection of 1 and 4 mg/kg body weight of CdCl₂ respectively, and six cotreatment groups, each of which underwent one of these three procedures but was concurrently treated with heparin, oxyquinol, or SOD (Table I).

**CHEMICALS**

The following chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo): alpha-ketovaleric acid, nicotinamide adenine dinucleotide (NADH), CdCl₂, oxyquinol, and superoxide dismutase-polyethylene glycol (SOD-PEG). Heparin and sodium pentobarbital were purchased from Elkins-Sinn (Cherry Hill, NJ) and Abbott Laboratories (North Chicago, IL), respectively.

**INDUCTION OF TESTICULAR INJURY**

Injection of CdCl₂. A 1-mL volume of CdCl₂ was dissolved in 0.85% NaCl and administered intraperitoneally. Four groups of animals (groups II through V in Table I) received a dose of 1 mg/kg, while four other groups (groups VI through IX in Table I) received a dose of 4 mg/kg. A dose of 4 mg/kg in Wistar rats has been shown to induce rapid destruction of testicular germ cells 1 day after the injection.¹²

Prevention of Testicular Damage by Treatment with Free-Radical Scavengers. Animals in groups III and VII received 1 mL of heparin subcutaneously at a dose of 200 IU/kg before and 24 hours after CdCl₂ treatment because of heparin's short half-life (1 hour). Animals in groups IV and VIII received 1 mL of oxyquinol in 0.5 M NaOH subcutaneously at a dose of 30 mg/kg because of oxyquinol's long half-life (greater than 16 hours) after ischemia-inducing treatment, and groups V and IX received 1 mL of SOD-PEG in 0.05 M phosphate-buffered saline subcutaneously at a dose of 10,000 IU/kg because of the long half-life of this substance (greater than 30 hours) after ischemia-inducing treatment.

**POSTOPERATIVE FOLLOW-UP**

Fourteen days after initial treatment, all animals were killed by puncture of the abdominal aorta under ether anesthesia. Both testes were resected, cleaned of fat tissue and blood, and weighed on a single-pan electronic balance (Mettler, Highstown, NJ). One testis from each animal was used for the measurement of LDH-X activity. The decapsulated testicular tissue (stored at -70°C) was thawed and homogenized in 0.5 M sucrose solution with an electronic homogenizer (Polytron, Brinkmann Instruments, Westbury, NY). The suspension was centrifuged at 40,000 g for 1 hour at 4°C, and the supernatant was used both for the enzyme assay and for determination of protein concentration. The other testis was placed in Bouin's solution for 1 hour, cut transversely into slices of 3 to 4 mm, fixed overnight in Bouin's solution, and dehydrated in 70% alcohol. The tissues were embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin.

**TABLE I. Classification of experimental groups**

<table>
<thead>
<tr>
<th>Treatment Group*</th>
<th>Description</th>
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<tbody>
<tr>
<td>I ¹</td>
<td>Negative control</td>
</tr>
<tr>
<td>II</td>
<td>CdCl₂ (1 mg/kg)</td>
</tr>
<tr>
<td>III</td>
<td>CdCl₂ positive control</td>
</tr>
<tr>
<td>IV</td>
<td>CdCl₂ + heparin cotreatment</td>
</tr>
<tr>
<td>V</td>
<td>CdCl₂ + oxyquinol cotreatment</td>
</tr>
<tr>
<td>CdCl₂ (4 mg/kg)</td>
<td>CdCl₂ + SOD cotreatment</td>
</tr>
<tr>
<td>VI</td>
<td>CdCl₂ positive control</td>
</tr>
<tr>
<td>VII</td>
<td>CdCl₂ + heparin cotreatment</td>
</tr>
<tr>
<td>VIII</td>
<td>CdCl₂ + oxyquinol cotreatment</td>
</tr>
<tr>
<td>IX</td>
<td>CdCl₂ + SOD cotreatment</td>
</tr>
</tbody>
</table>

Key: SOD = superoxide dismutase.

¹ Nine experimental groups consisting of 5 rats each.

² Rats in group I were injected with 1 mL of 0.9% normal saline intraperitoneally as a negative control for CdCl₂-injected rats.

**DETERMINATION OF LDH-X AND PROTEIN**

LDH-X activity was measured by the method of Itoh.⁶ In brief, the reaction mixture contained 100 μL of testicular supernatant, 0.15 mM alpha-ketovaleric acid, and 0.15 mM NADH in a final volume of 3.0 mL 0.05 M phosphate buffer solution (pH 7.4). LDH-X activity was determined immediately by measurement of the change in absorbance of NADH per minute, at 340 nm with a spectrophotometer (Ultraspex II, LKB-Wallac, Gaithersburg, Md). The reaction was initiated by the addition of the substrate and followed by the decrease in absorbance of NADH at 340 nm. One unit of LDH-X activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADH in 1 minute. Protein was measured by the method of Lowry et al.,¹³ with bovine serum albumin fraction V as a standard.

**HISTOLOGIC EVALUATION**

Testicular histology was evaluated by an observer who was unaware of the experimental groups. The histologic parameters studied included rupture of tubules, degeneration of germ cells, germ cell disarray, loss of sperm/spermatids, edema, hemorrhage, and fibrosis/granuloma.¹⁴ Each testis was scored on a scale of 0 to 4 for each parameter, with the highest score indicating the most widely distributed pathology for that parameter. For quantitative estimation of the overall histologic changes in the testes after various treatments, the total histologic score was assigned by adding the scores of all parameters studied for each testis from all the rats in a given group.

**STATISTICAL ANALYSIS**

A nonparametric Kruskal-Wallis test was used for each type of damage. A P value less than 0.05 was considered significant. For any significant Kruskal-Wallis test, pairwise comparisons were made for all possible pairs using the Wilcoxon rank sum test. Results were analyzed by the SAS statistical software package (SAS Institute Inc., Cary, NC 1992).

**RESULTS**

**TESTICULAR WEIGHT**

Testicular weight was severely reduced in CdCl₂ positive control animals (groups II and VI). This
Figure 1. Rat testicular weight changes after CdCl₂ injury. Animals in all groups except those injected with free-radical scavengers oxypurinol or SOD along with CdCl₂ treatment (1 mg/kg) (groups IV and V) showed significant decrease in testicular weight from that in the negative controls (*P < 0.001; †P < 0.01). In contrast, coadministration of heparin did not prevent such a loss.

Figure 2. Rat testicular LDH-X activity after CdCl₂ injury. Administration of heparin, oxypurinol, or SOD (groups III, IV, and V) along with CdCl₂ (1 mg/kg) prevented a reduction of testicular LDH-X activity. In animals injected with 4 mg CdCl₂/kg, injection of free-radical scavengers did not prevent a significant decrease in testicular LDH-X activity from negative control values (*P < 0.001).

The administration of free-radical scavengers did not prevent a decrease in testicular weight.

Activity of Testicular LDH-X
Testicular LDH-X activity was severely reduced in positive controls (groups II and VI) compared to that in negative controls (Fig. 2). These findings correlated with testicular weight. In animals injected with 1 mg CdCl₂/kg plus heparin, oxypurinol, or SOD (groups III, IV, and V), testicular LDH-X activity did not differ significantly from that in negative control animals. Thus, the use of free-radical scavengers in these groups prevented a reduction in testicular LDH-X activity. Animals injected with 4 mg CdCl₂/kg (groups VI through IX) showed significant reduction of testicular LDH-X activity from the level in negative controls (P < 0.05). Injection of free-radical scavengers did not prevent a decrease in testicular LDH-X activity at this dose of CdCl₂.

Testicular Histology
A high degree of histologic damage was seen in one positive control group (group II, Fig. 3). Rats given 4 mg CdCl₂/kg plus heparin (group III) exhibited the most severe damage. In contrast, the germinal epithelium of negative controls showed full spermatogenesis. Animals injected with 1 mg CdCl₂/kg plus oxypurinol (group IV, Fig. 4) or SOD (group V). However, animals injected with 4 mg CdCl₂/kg plus heparin (group VII), oxypurinol (group VIII), or SOD (group IX) incurred a high level of testicular damage similar to that in the positive control injected with 4 mg CdCl₂/kg (group VI).
over, the majority of patients diagnosed with clinical torsion are adolescent men.\textsuperscript{18} Therefore, studies devoted to the prevention of this problem are very relevant. There are conflicting articles regarding the prevention of ischemia-reperfusion testicular injury. According to Bergh et al.,\textsuperscript{19} testicular damage induced in the rat by 60 or 100 minutes of ischemia could not be prevented by cotreatment with yeast CuZn superoxide dismutase and bovine catalase. On the contrary, Akhter et al.\textsuperscript{20} showed that testicular damage induced in the rat by 1- or 4-hour surgical ischemia could be prevented by SOD-PEG and heparin. Both articles evaluated the testicular damage by using morphologic changes. The present results are in agreement with the results of Akhter et al.\textsuperscript{20} The results of our study were supported by evaluation of testicular LDH-X activity as well as testicular morphology. In general, a good correlation to testicular weight and morphologic scores in LDH-X activity was seen among experimental groups. Testicular LDH-X activity in this study was found to be a good indicator of testicular damage induced by low doses of CdCl\textsubscript{2}.

Testicular damage after ischemia is thought to be caused by the formation of toxic oxygen free radicals when oxygen returns to the previously ischemic tissue.\textsuperscript{10} In this study, the injection of free-radical scavengers—either oxypurinol or SOD—along with CdCl\textsubscript{2} at a dose of 1 mg/kg protected the rat testis from the deleterious effects of cadmium. In these rats, the CdCl\textsubscript{2} injection failed to cause a reduction in testicular weight or LDH-X level, and failed to induce histologic damage. However, these free-radical scavengers were not effective in preventing testicular damage at a dose of 4 mg CdCl\textsubscript{2}/kg. It appears that a high dose of CdCl\textsubscript{2} causes extensive testicular damage that cannot be blocked by free-radical scavengers at the doses used in this study. A number of hypotheses have been put forward to explain cadmium-induced testicular damage, including hemorrhagic necrosis, increased vascular permeability, and a direct effect on seminiferous tubules.\textsuperscript{21} Localization of cadmium within the endothelial walls of testicular capillaries and subsequent tubules.\textsuperscript{21} There are no reports in the literature describing prevention of cadmium-induced testicular damage by oxypurinol and SOD. However, it is well known that testicular damage induced by CdCl\textsubscript{2} can be prevented by coadministration of metals such as zinc\textsuperscript{22} and selenium.\textsuperscript{23} CdCl\textsubscript{2} damages the testis by increasing the lipid peroxidation of the cell membranes, and selenium (a free-radical scavenger) protected the testis against lipid peroxidation from CdCl\textsubscript{2}.\textsuperscript{24} We suggest that oxypurinol and SOD-

![Figure 4](image)

**Figure 4.** Rats given oxypurinol with 1 mg CdCl\textsubscript{2}/kg (group IV) showed full preservation of seminiferous epithelium (original magnification, ×40).

![Figure 5](image)

**Figure 5.** Histologic changes in the rat testis after CdCl\textsubscript{2} injury. Animals in all groups except those injected with the free-radical scavenger oxypurinol or SOD plus 1 mg CdCl\textsubscript{2}/kg (groups IV and V) showed significant decrease in histologic scores from those in the negative controls (*P <0.001; †P <0.01).

Evaluation of testicular histology scores after various treatments (Fig. 5) supported the histologic results just mentioned. Rats injected with 1 mg CdCl\textsubscript{2}/kg and given oxypurinol or SOD (groups IV and V) had testicular histology scores in the same range as those of negative controls.

**COMMENT**

The effects of interrupting the blood flow to the testes have been investigated previously.\textsuperscript{15–17} Interruption of the spermatic blood flow leads to morphologically demonstrable injuries of the parenchyma and hence to an impairment of male fertility. It is well known that ischemic injury causes serious lesions to the testis that affect the germinal epithelium, Sertoli cells, tunica propria, interstitial tissue, and Leydig cells.\textsuperscript{15,16} Short-term testicular ischemia following clinical torsion is known to cause testicular damage in adolescent men. More-
PEG used in this experiment may prevent testicular damage by blocking excessive CdCl₂-induced lipid peroxidation in rat testes. Other work in our laboratory (not described here) suggests that free-radical scavengers (antioxidants) may ameliorate the effects of testicular torsion on the testicle. In summary, our results demonstrated a correlation between a decrease in testicular weight with histologic damage and a decrease of LDH-X activity. Free-radical scavengers, oxypurinol, and SOD, with longer half-lives than heparin, were found to protect against the deleterious effects of the lower dose of CdCl₂ (1 mg/kg). We suggest that further studies be conducted on the clinical application of antioxidant treatment for torsion of the testicle. An antioxidant may be administered after a diagnosis of torsion is made and as the patient is being prepared for surgery.

REFERENCES