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Methimazole as a protectant against cisplatin-induced nephrotoxicity using the dog as a model

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Abstract. The protective effect of methimazole, a commonly used antithyroid drug, on cisplatin-induced nephrotoxicity was studied. Eight dogs received 80 mg/m^2 cisplatin i.v. without saline prehydration. Dogs were randomized into two groups of four dogs each: one group received 40 mg/kg methimazole i.p. at 30 min prior to and 4 h after cisplatin delivery, and the other group received saline placebo i.p. Methimazole protected dogs against the in vivo nephrotoxicity elicited by cisplatin as evidenced by clinicopathologic and histopathologic indices. Protection was not complete, as methimazole-treated animals developed mild histopathologic renal changes. Measures of renal oxidative stress did not differ between the two groups at day 5 following cisplating treatment. No difference was noted for serum thyroxine concentrations before or after therapy in either group; however, serum levels of 3,5,3′-triiodothyronine were significantly higher on day 5 in both groups of dogs receiving cisplatin, regardless of whether they received methimazole or not. Methimazole as used in this study was found to be well tolerated in dogs over the short term, with no significant clinical or clinicopathologic toxicity being observed. The results of this study support the additional evaluation of methimazole as a protectant against cisplatin-induced nephrotoxicity using the dog as a model.

Introduction

Cisplatin (*cis*-diaminedichloroplatinum) is an antineoplastic agent commonly used to treat a variety of malignant tumors in many species. It is the chemotherapeutic of choice for osteosarcoma, the most common bone tumor affecting dogs and humans [14, 16, 31, 36]. Its use in these species is often limited, primarily due to its propensity to cause dose-limiting nephrotoxicosis characterized by decreased glomerular filtration and tubular injury [7, 18, 30]. The mechanism of nephrotoxicity may involve direct interference with tubular or mitochondrial transport processes, covalent modification of cellular constituents, or generation of free radicals [10, 13, 18, 35]. For the latter situations, cytotoxicity is usually observed only after cellular defenses, particularly those involving glutathione (GSH), have been significantly depleted [27]. A large body of evidence supports the concept that oxidative stress may play an important role in the pathophysiology of cisplatin-induced acute renal failure. Selective inhibition of GSH biosynthesis is known to enhance cisplatin nephrotoxicity, whereas in vitro GSH supplementation reduces cisplatin nephrotoxicity [2, 19]. Adequate hydration is mandatory to reduce or prevent the development of cisplatin-induced renal damage, and several administration protocols have been recommended in the dog [7, 20–22]. It is conceivable that if the nephrotoxic qualities of cisplatin could be overcome, more aggressive therapeutic strategies could be employed in tumor-bearing individuals.

Methimazole is an inexpensive thiourylene antithyroid drug commonly used in many species for the treatment of hyperthyroidism [24, 25]. Recently, it has been found that methimazole significantly reduces the renal toxicity of cisplatin and other toxins in rats [29]. In these studies, methimazole administration prevented the elevation of blood urea nitrogen (BUN) and the occurrence of histologic lesions following cisplatin doses known to result in renal tubular damage. The mechanism by which methimazole reduces or eliminates cisplatin-induced renal disease, while not entirely known, is possibly due to its antioxidant effect, resulting in the reduction of cisplatin-induced oxidative stress within the kidneys [3, 9, 18, 29, 37]. Importantly, methimazole concentrates in renal tissue more than would be predicted by the expected renal blood flow [1, 32, 33], giving it qualities that make it attractive for reducing renal oxidative damage from cisplatin.

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This pilot study was designed to determine if the nephroprotectant qualities of methimazole found in the rat would hold true in the dog. Such a dog model is important for a number of reasons. First the dog is an excellent model for the study of osteosarcoma (OSA) and has a proven track record as a model for the same disease in adolescent and preadolescent humans [4, 36]. Second, dogs are susceptible to cisplatin nephrotoxicity that is nearly identical to that observed in humans [18, 30]. Third, the pharmacokinetics of cisplatin has been extensively studied in the dog [12, 17]. Finally, as in humans, cisplatin is the chemotherapeutic drug of choice for OSA [14, 16, 31, 36].

Materials and methods

Dogs. Eight healthy adult (14 months of age) male beagle dogs weighing between 10 and 13 kg were used in this study. All dogs were housed in indoor runs and exposed to a 12-h light schedule (light, 6 a. m. to 6 p. m.). All dogs were fasted for 12 h prior to each study.

Cisplatin and methimazole treatment. The eight dogs were randomized into two groups of four dogs each. Cisplatin (Platinol; Bristol Laboratories, Syracuse, N. Y.) was delivered as an i. v. bolus over 2 min at a dose of 80 mg/m^2 to all dogs in both groups. Cisplatin given in this dose range without prehydration is known to result in measurable and consistent acute renal toxicosis in dogs that peaks on the 5th day following cisplatin delivery [12]. All dogs also received metoclopramide (0.5 mg/kg s. c.) and butorphenol (0.4 mg/kg) 30 min prior to cisplatin to alleviate emesis. One group of dogs (methimazole-treated dogs) received 40 mg/kg methimazole (Aldrich Chemical Company, Milwaukee, Wis.) i. p. at 30 min prior to and 4 h after cisplatin delivery. We chose to deliver methimazole at these two time points so as to have the maximal likelihood of noting a protective effect in dogs. Methimazole was reconstituted in normal saline to a final volume of 10 mg/ml prior to its injection. The other group of dogs (control dogs) received similar volumes of sterile saline placebo i. p. At 4 h after cisplatin delivery, 200 cc of lactated Ringer's solution was given s. c. once to all dogs in both groups to replace gastrointestinal losses due to emesis.

Evaluation of nephrotoxicity. Prestudy (day −1) evaluations included a complete physical examination; a serum biochemistry profile (SBP), including determinations of BUN and serum creatinine levels; a complete blood count (CBC), including a platelet count; urinalysis (UA); and determinations of serum levels of thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3) as well as of exogenous creatinine clearance (CC). An identical evaluation was again performed on day 5 following cisplatin delivery, the known point of maximal cisplatin-induced acute nephrotoxicity in the dog [12]. Daily physical examinations were carried out on the 5 days of the study, and the time and frequency of emesis was recorded. Urine specific gravity, BUN, and serum creatinine values were also measured on day 2 and 4 of the study. Histopathology was performed on renal tissues from all dogs on day 5, and reduced (GSH) and oxidized (GSSG) glutathione and protein free thiol (PT) levels were determined from fresh renal homogenates as a measure of renal oxidative stress.

Thyroid hormone levels. Serum levels of T_4 and T_3 were determined in duplicate using commercially available solid-phase radioimmunoassay kits (Coat-A-Count total T_4 and total T_3; Diagnostic Products Corporation, Los Angeles, Calif.) previously validated in the dog [23].

Exogenous CC. Serum disappearance of creatinine, a simple modified exogenous CC test previously validated in the dog, was used [15]. Simply, 88 mg/kg creatinine is given as an i. v. bolus over 1 min and serum creatinine levels are measured both before and 2 h after creatinine delivery. The difference between the serum creatinine concentrations measured at the two time points is reported.

Histopathology. Immediately after barbiturate euthanasia, sections of renal tissue from all dogs were placed in 10% neutral buffered formalin, fixed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Histologic sections were qualitatively assessed for markers of nephrotoxicity without prior knowledge of the protocol group to which each section belonged and were assigned a necrosis severity grade based on the percentage of renal tubules exhibiting such markers: grade 0, no significant lesions; grade 1, 1%–25% tubular involvement; grade 2, 26%–50% tubular involvement; grade 3, 51%–75% tubular involvement; and grade 4, 76%–100% tubular involvement.

Determination of nonprotein and protein thiols. Renal tissue (1 g) was homogenized in a 10-ml solution (pH 4.3) of KCl (0.15 m*M*) and ethylenediaminetetraacetic acid (EDTA, 30 m*M*). To 2 ml of homogenate, 3 ml of a solution containing NaCl (0.3 g/ml), metaphosphoric acid (0.017 g/ml), and EDTA (0.002 g/ml) was added and the solution was centrifuged for 20 min at 3,000 rpm using a Beckman TJ-6 table-top centrifuge. The supernatants were used for nonprotein thiol (NPT) determinations as previously described [28]. When analyzed by this method, the recovery of known concentrations of either oxidized (GSSG) or reduced (GSH) glutathione was greater than 95%. Protein thiols (PTs) were determined as GSH equivalents by a modified assay based on previously published methods [5, 8]. Briefly, the pellets obtained after the above-described centrifugation step were washed twice by resuspension in 5 ml 1 *N* $HClO_4$ and centrifugation for 10 min at 3,000 rpm. The supernatant was discarded and the resulting pellet was resuspended in 9.9 ml TRIS-HCl (0.05 *M*) containing 5 *M* urea (final pH, 8.8 unadjusted) and 0.1 ml 10% Triton X-100. The resuspended pellet solution (0.5 ml) was added to tubes containing 2 ml Na_2HPO_4 followed by the addition of 0.5 ml 0.04% 5,5′-dithiobis-(2-nitrobenzoic acid) in 10% sodium citrate. The solution was vortexed and the absorbance at 412 nm was determined immediately. Data were expressed as micromoles of PT per gram of protein as calculated on the basis of a GSH standard curve.

Statistical analysis. Differences between the treatment groups with respect to renal and thyroid function as well as measures of oxidative stress were compared using the Wilcoxon rank-sum test for two groups (a nonparametric independent *t*-test). The Friedman nonparametric repeated-measures test was used to determine if overall differences existed within a group over time; if such a difference was identified, it was subsequently located using the Wilcoxon signed-rank test (a nonparametric paired *t*-test). When $P < 0.05$ was present, data were considered to be significantly different.

Results

Clinical effects

All dogs receiving i.p. methimazole subjectively experienced transient i.p. discomfort immediately following injection, characterized by rubbing of the ventral abdomen on the floor of the run and occasional scratching at the ventral abdomen with the hind legs. Activity normalized within 2 min of injection. Dogs in both groups experienced emesis, which developed at between 1 and 2 h after cisplatin injection. The frequency of vomiting did not vary between the two groups, with the mean and median number of events being 3.2 and 3, respectively. All vomiting had ceased by 3 h postinjection.

Clinicopathologic data

Clinical data showing protection from renal injury are presented in Fig. 1. BUN and serum creatinine (SC) values did

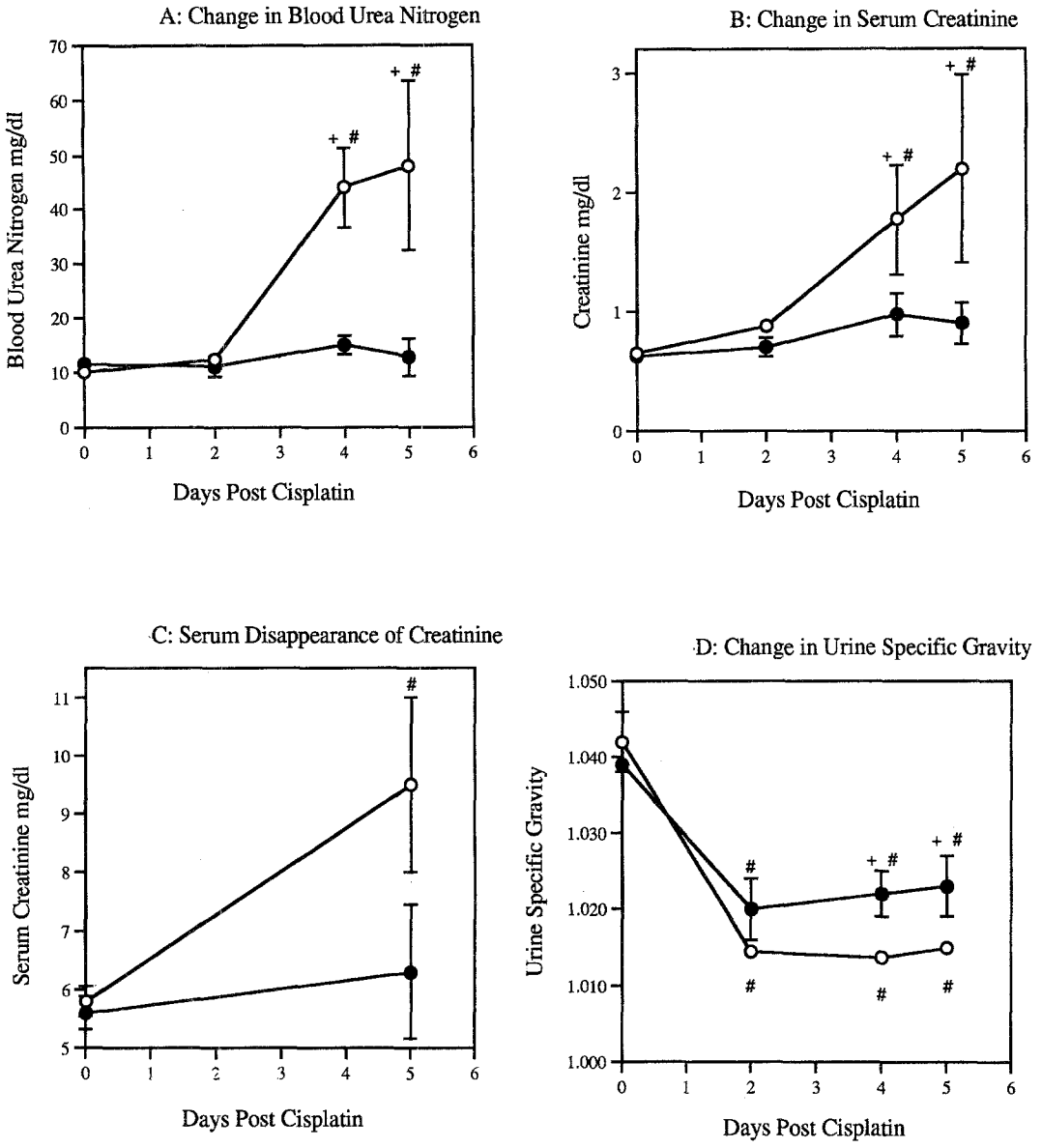

Fig. 1 A–D. Changes in **A** blood urea nitrogen, **B** serum creatinine, **C** serum disappearance of creatinine, and **D** urine specific gravity over time following cisplatin delivery in dogs receiving methimazole protection (●) or saline placebo (○). + Values obtained for methimazole-treated dogs differed significantly ($P < 0.05$) from those obtained for saline placebo-treated control dogs the same time point. # Values differed significantly ($P < 0.05$) from the day 0 baseline values within the same treatment group. Error bars indicate 1 SD

not vary over time in the methimazole-protected animals, nor did the serum disappearance of creatine (SDC) differ between day 0 and day 5 in this group. However, a significant rise in BUN ($P = 0.019$) and SC ($P = 0.009$) values occurred over time in the group of dogs receiving cisplatin alone. This rise in BUN and SC levels occurred at day 4 ($P = 0.046$) and day 5 ($P = 0.046$), respectively, following cisplatin delivery. The SDC value was also significantly different ($P = 0.046$) between day 0 and day 5 in the unprotected group. Differences in BUN and SC values were found between methimazole-treated and control dogs at day 4 ($P = 0.02$ and 0.046, respectively) and day 5 ($P = 0.042$ and 0.046, respectively). A trend toward significance between methimazole-treated dogs and controls was noted for SDC values at day 5 ($P = 0.059$). Urine specific gravity decreased significantly over time in both methimazole-treated ($P = 0.043$) and control ($P = 0.042$) dogs; however, the drop was significantly less dramatic in methimazole-protected dogs on days 4 ($P = 0.038$) and 5 ($P = 0.042$) than in the control dogs.

Serum thyroid hormone levels

Figure 2 presents the serum T_4 and T_3 levels measured in both groups of dogs before and 5 days after cisplatin injection. No difference was noted in the serum T_3 levels determined before or after therapy in either group; however, serum levels of T_4 were significantly higher on day 5 in both groups of dogs receiving cisplatin, regardless of whether they received methimazole ($P = 0.025$) or not ($P = 0.046$).

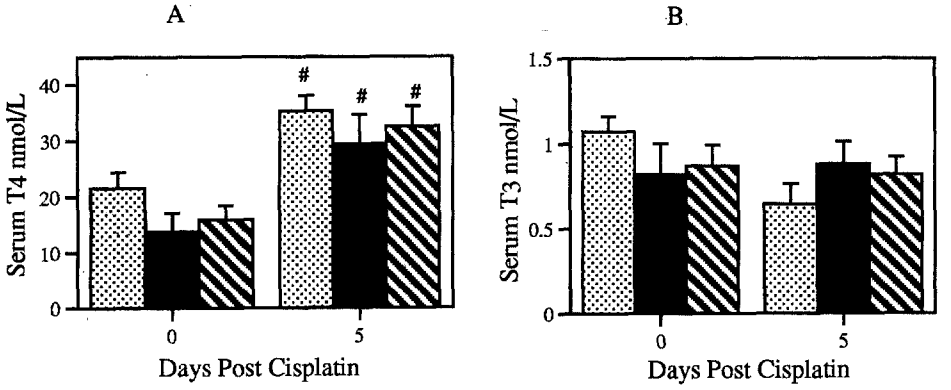

Fig. 2 A, B. Changes in **A** serum T_4 and **B** serum T_3 levels as measured before and 5 days following cisplatin delivery in dogs receiving saline placebo (▨), those receiving methimazole (■), or a combination of both groups (▧). # Values differed significantly ($P < 0.05$) from the day 0 baseline values within the same treatment group. Error bars indicate 1 SE

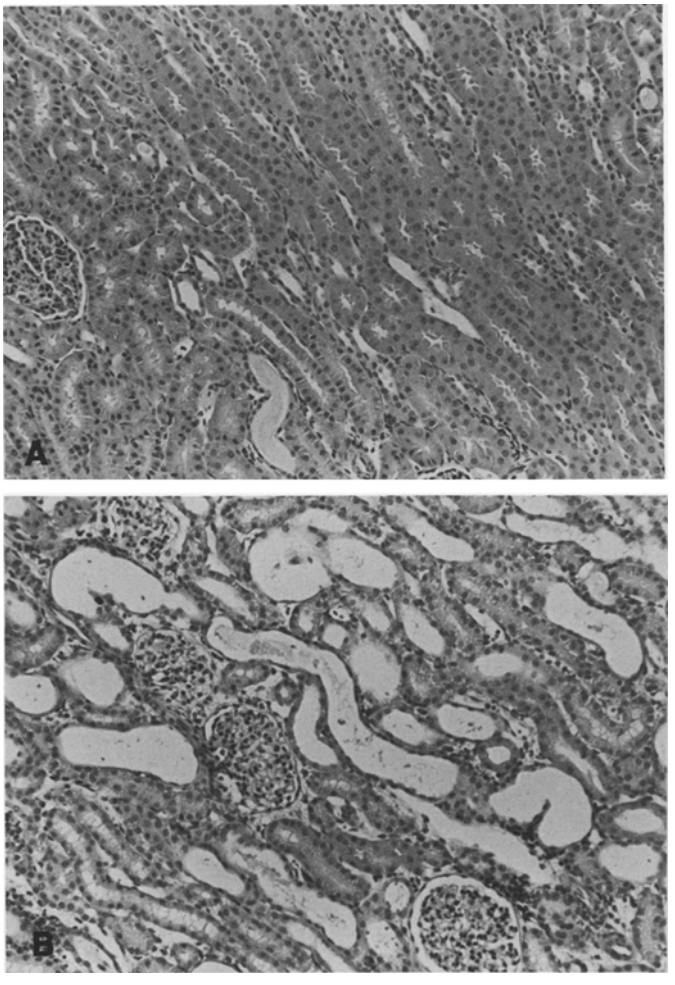

Fig. 3 A, B. Representative views of the corticomedullary junction of renal sections taken from **A** a dog euthanized 5 days after treatment with cisplatin only (80 mg/m²) or **B** a dog given methimazole (40 mg/kg) 30 min prior to and 4 h after cisplatin. ×100

Histopathologic assessment of renal tissue

In the methimazole-treated dogs, a tubular necrosis severity grade of 1 was present in three dogs and that of grade 2, in one dog. In contrast, three of the control dogs had a tubular necrosis severity grade of 4 and one had a grade of 3. The mean grade of 1.25 determined for the methimazole-treated group was significantly lower than the 3.75 mean grade found for the control group ($P = 0.022$). A representative view of renal sections comparing a methimazole-treated dog and a control dog is presented in Fig. 3. Necrosis was evidenced by disruption of the tubular epithelium, nuclear pyknosis, cytoplasmic granularity, and desquamation of rounded up necrotic cells into the tubular lumina; many tubules contained amorphous debris in addition to sloughed epithelial cells.

Measures of renal oxidative stress

Figure 4 illustrates the renal levels of GSSG, reduced GSH, and PTs determined in fresh renal homogenates on day 5 following cisplatin injection. No significant difference was found at this time point.

Discussion

The present study showed that methimazole protected dogs against the in vivo nephrotoxicity elicited by cisplatin (Figs. 1, 3). Such protection was not complete as evidenced by mild histopathologic changes occurring in methimazole-protected dogs; however, such changes were significantly less dramatic than those observed in unprotected dogs. Both groups of dogs had declines in urine specific gravity following cisplatin treatment. Whereas these values reached isosthenuric levels (i.e., indicative of a loss of renal concentrating ability) in unprotected control dogs, methimazole-protected dogs enjoyed a significantly less pronounced decline in specific gravity and levels did not reach the isosthenuric range. Because water intake was not monitored in this study, it is possible that the drop in urine specific gravity observed in methimazole-protected dogs resulted secondarily from polydipsia associated with drug treatment, as the degree of renal disease necessary to elicit an inability to concentrate urine, while present in unprotected dogs, was not observed histologically in methimazole-treated dogs. Indeed, methimazole treatment is known to increase water intake secondary to an increase in urinary output in the rat [11].

The most likely mechanism for the renal protection conferred by methimazole relates to its structure as a sulfur-containing nucleophile. It therefore can act as an an-

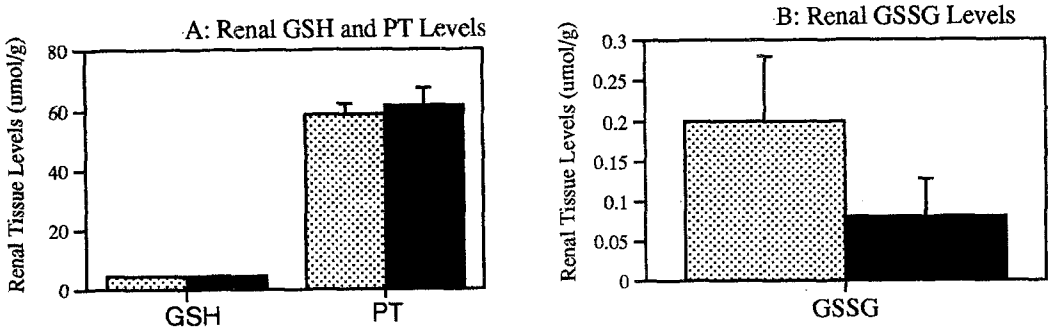

Fig. 4 A, B. Renal tissue levels of **A** reduced glutathione (GSH), protein thiols (PT), and **B** oxidized glutathione (GSSG) as measured 5 days following cisplatin delivery in dogs receiving saline placebo (▨) or methimazole (■). Error bars indicate 1 SE

tioxidant and maintain reduced GSH levels, restoring cellular defense and blocking lipid peroxidation. In an attempt to show such an effect in our study, renal tissue levels of reduced GSH, GSSG, and PTs were determined 5 days following cisplatin delivery. Decreased oxidative stress on renal tissues would be expected to result in maintenance of reduced GSH and PT in renal tissues without producing increased levels of GSSG indicative of oxidative stress. Results of this type have been reported in the rat model during methimazole protection from cephaloridine [29]; however, no difference was noted in methimazole versus placebo-grouped dogs in our study. Our inability to demonstrate a difference was likely due to timing, as the changes in oxidative stress demonstrated in rats were determined during exposure to the nephrotoxic agents, whereas our study compared the values obtained 5 days following the insult. Alternatively, the cisplatin-induced changes in thiol status may be localized only in certain regions of the kidney or in certain cellular components (e.g., mitochondria). Whereas methimazole's effects on cisplatin disposition were not investigated, the finding that rats given methimazole 4 h after cisplatin were protected against nephrotoxicity [29] suggests that methimazole's mechanism of protection is not likely to involve modification of cisplatin disposition. Further evidence for this hypothesis is provided by work with cephaloridine, whose nephrotoxicity can also be blocked by methimazole in rats. Uptake by rat kidney cortical slices in vitro was not affected by methimazole, and in vivo methimazole treatment did not alter the renal disposition of cephaloridine [29].

Other sulfur-containing nucleophiles have been reported to confer protection against cisplatin nephrotoxicity, including diethyldithiocarbamate, thiourea, and methionine. However, these chemicals have toxic side effects that may make their therapeutic use questionable [3, 26]. Methimazole, unlike these other agents, has been used safely for decades in both human and veterinary practice, and the dose shown to be protective against nephrotoxicity does not cause hepatotoxicity. Another potential advantage of this compound over other sulfur-containing nucleophiles arises from the observation that in the rat, methimazole blocks cisplatin-induced nephrotoxicity when given up to 4 h after cisplatin [29]. Protection in rats up to 4 h after cisplatin administration may be important, as treatment at this time is less likely to alter cisplatin's therapeutic effects since the latter drug would be distributed to target organs prior to methimazole delivery. The only significant clinical or clinicopathologic side effect of methimazole observed in our study was mild, transient i.p. discomfort following i.p. administration. The i.p. route was chosen to follow more closely the rat model of previous studies; however, a safe i.v. methimazole preparation has been formulated for veterinary use by dissolving the compound in 0.5 *M* TRIS buffer (pH 7.4) [34].

No decline in serum T_4 or T_3 levels was noted on the 5th day following exposure to cisplatin and methimazole. Interestingly, serum concentrations of T_4 had actually risen significantly by day 5 in both groups of dogs, implying that cisplatin administration either directly or indirectly resulted in such an increase. Although a number of reasons may be postulated for such a rise, diminished clearance of T_4, as has previously been reported in patients with mild illness, is most likely [6].

In conclusion, methimazole given at the dose and timing used in this study was found to protect dogs from the nephrotoxic effects of cisplatin. No significant short-term (i.e., over 5 days) toxicity associated with methimazole was ascertained. Methimazole may have advantages over other sulfur-containing nucleophiles with respect to nephroprotection, as it is readily available, inexpensive, and safe over the short term and may prove to be effective when given several hours after cisplatin. Future investigations related to the dose, timing, and effect of methimazole, if any, on the tumorcidal activity of cisplatin are necessary to evaluate further its potential use.

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