

The efficacy of diazepam in the treatment of acute iron overload in rats

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Abstract: While conducting studies on the prevention of mortality from acute iron intoxication in rats, diazepam, given to prevent animal suffering, was observed to be associated with reduced mortality in a limited number of animals. The objective was to assess whether diazepam reduces mortality following acute iron intoxication in rats. Survival of rats was compared among groups receiving (i) orally 612 mg/kg iron alone (LD₆₀), (ii) iron with a subcutaneous injection of 2.5 mg/kg diazepam (DZ), or (iii) iron, DZ with 800 mg/kg deferiprone intraperitoneal injections. The administration of DZ decreased mortality from 60 to 16% ($p < 0.001$). The addition of deferiprone to DZ resulted in zero mortality ($p < 0.05$ compared with the DZ group) over the study period. The administration of DZ was not associated with decreased iron absorption or increased urinary iron excretion, whereas the administration of deferiprone did result in urinary iron excretion. Microscopic examination suggests that diazepam administration may be associated with lower intracellular accumulation of iron. In conclusion, diazepam reduces mortality from iron overdose in rats through a yet unidentified mechanism, although the drug does not inhibit iron absorption or enhance urinary iron removal.

Key words: iron overdose, diazepam, rats.

Résumé : Lors d'études sur la prévention de la mortalité due à une intoxication aiguë par le fer chez des rats, le diazépam, administré pour empêcher les animaux de souffrir, a été associé à une diminution de la mortalité chez un nombre limité d'animaux. L'objectif de la présente étude a été d'évaluer si le diazépam réduit la mortalité suite à une intoxication aiguë par le fer chez les rats. La survie des rats a été comparée au sein de groupes recevant : (i) 612 mg/kg de fer uniquement par voie orale (DL₆₀), (ii) du fer accompagné d'une injection sous-cutanée de 2,5 mg/kg de diazépam (DZ) ou (iii) du DZ accompagné d'injections intrapéritonéales de 800 mg/kg de déféripnone. L'administration de DZ a diminué la mortalité de 60 à 16% ($p < 0,001$). La combinaison déféripnone-DZ s'est traduite par une mortalité zéro ($p < 0,05$ comparativement au groupe DZ) pendant toute la durée de l'expérience. L'administration de DZ n'a pas été associée à une absorption réduite de fer ou à une excrétion accrue de fer urinaire, alors que l'administration de déféripnone a induit une excrétion de fer urinaire. L'examen microscopique suggère que l'administration de diazépam pourrait être associée à une plus faible accumulation de fer intracellulaire. Ainsi, chez les rats, le diazépam réduit la mortalité due à une surdose de fer par un mécanisme n'ayant pas encore été identifié, bien que la drogue n'inhibe pas l'absorption de fer et ne stimule pas l'élimination urinaire du fer.

Mots clés : surdose de fer, diazépam, rats.

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Introduction

Acute iron poisoning is a common cause of morbidity and mortality, with thousands of cases reported to the American Association of Poison Control Centers every year (Litovitz et al. 1993). Rapid absorption of high dose

iron from the gut is followed by gastrointestinal damage and cardiovascular collapse (Covey 1964; Banner and Tong 1986). At the present time, gastrointestinal decontamination and parenteral desferrioxamine are the only means proven to be effective for this condition (Engle et al. 1987). During the search for new therapeutic modalities for acute iron

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poisoning we sought ways to prevent animal suffering. Our Animal Care Committee suggested the subcutaneous administration of diazepam (DZ) to rats immediately following gavage with iron, as a mean of eliminating pain and suffering. Preliminary experiments suggested that DZ may affect survival of rats treated with toxic doses of iron.

The objective of the present study was therefore to examine in a meaningful controlled way the potential beneficial effect of DZ in acute iron overload in rats.

Materials and methods

Protocol

The protocol (1524) was approved by the Hospital for Sick Children (HSC) Animal Care Committee. All of the rats that were used in these experiments were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Rats

Male Wistar rats, with weights ranging from 200 to 300 g were purchased from Charles River Canada Inc. (St-Constant, Que.). Animals were double-housed in plastic shoe box type cages, upon arrival at the HSC Lab Animal Services facility. Till the day of the experiment, animals were allowed to eat standard chow ad libitum and had free access to distilled water. Animals were assigned to one of four groups: group I ($n = 20$) received 612 mg/kg elemental iron by gavage (previously shown by us to be the LD_{50} in the model); group II ($n = 32$) received elemental iron by gavage and a subcutaneous (s.c.) injection of diazepam (DZ); group III rats ($n = 16$) received elemental iron by gavage, a s.c. injection of DZ, followed by four intraperitoneal (i.p.) injections of deferiprone solution; group IV rats were used as controls and were gavaged only with distilled water ($n = 10$). On the evening immediately prior to the experiment, standard rat chow was removed from the cages, and the animals were numbered by a numerical band with a permanent marker across their tails. At approximately 08:00 h the following morning (roughly 14 h of food deprivation), the animals were weighed and appropriate volumes of iron, DZ, and deferiprone solutions were gavaged or injected.

Iron preparation

The iron was prepared 1 h prior to the experiment. Hydrous ferrous sulphate (Fisher Scientific Co., Fair Lawn, N.J.) was dissolved in double-distilled Milli-Q water (Millipore Corp., Bedford, Mass.) for a final concentration of 0.082 g/mL of elemental iron. At the commencement of the experiment all animals were gavaged with a volume of ferrous sulphate solution corresponding to 612 mg/kg of elemental iron.

Drugs

Solutions of diazepam (5 mg/mL) purchased from Sabex Inc., Boucherville, Que.) were used. Deferiprone (L1) was synthesized at the Department of Chemistry, University of Toronto, by Dr. R. McClelland according to previously published methods (Kontoghiorghes and Sheppard 1989). In the afternoon, on the day before the experimental procedure, crystalline deferiprone was dissolved in distilled water for a final concentration of 10 mg/mL. Upon dissolving, the deferiprone solution was sterilized via suction-filtration through a GS 0.22 micron filter (Millipore Corp.) in a laminar flow hood. The filtrate was stored at room temperature in sterile 100-mL injection vials (Bencard Labs, Mississauga, Ont.). The deferiprone solution was always prepared fresh the day before each experimental procedure.

Experimental procedure

Following the administration of the iron solution by gavage to the three animal groups, animals were closely observed. Immediately after the iron gavage, rat feed was returned to each cage. Animals in groups II and III received s.c. injections of 2.5 mg/kg DZ within 5 min of being gavaged. Within 15 min of gavaging, rats in group III were also injected i.p. with an initial loading dose of 400 mg/kg deferiprone solution. Thereafter, group III rats received an additional three i.p. injections of deferiprone solution at 1-h intervals in doses of 200, 100, and 100 mg/kg, respectively. Following the administration of the initial injections, all rats were placed into smaller, clean plastic cages without bedding. Single-time urine specimens were collected from the plastic cage floor, using plastic transfer pipettes (Samco Scientific Inc., Los Angeles, Calif.), and were transferred to labelled Eppendorf tubes (Rose Scientific Ltd., Calgary, Alta.). Animals were then returned to their original cages. Animals that did not die during the 14 h were euthanized at the completion of the observation period by ethrane gas (Anaquest, Ont.) in a gas delivery apparatus (Ohio Medical Products, Wisc.) and dissected rapidly thereafter. At death, cardiac blood samples were drawn from animals, via syringe, and centrifuged at 2500 rpm for 10 min. Serum was collected by plastic transfer pipettes, placed in prelabelled Eppendorf tubes, frozen temporarily at -20°C , and later transferred to a -80°C freezer. Residual bladder urine was also collected from all animals by needle aspiration, placed into separate prelabelled Eppendorf tubes, frozen at -20°C , and then at -80°C .

For ferrokinetic studies, two to six animals were used for each time point.

Pathology studies

Cross-sectional slices of the following organs were obtained for pathology: heart, liver, lung, stomach, duodenum, and kidney. Tissue samples were stored in prelabelled vials containing 10% buffered formalin solution (BDH Chemicals Inc., Mississauga, Ont.). These samples were prepared for hematoxylin-eosin (H/E) and iron staining (Prussian blue reaction). Photographs of the organ slices were taken using 64 ASA film with a 35-mm camera mounted atop a microscope (Leitz, Germany). Slides of iron staining were blindly scored by a pathologist from 0 (no iron) to 4 (maximal iron score) in duplicates (two animals for each state).

Analyses

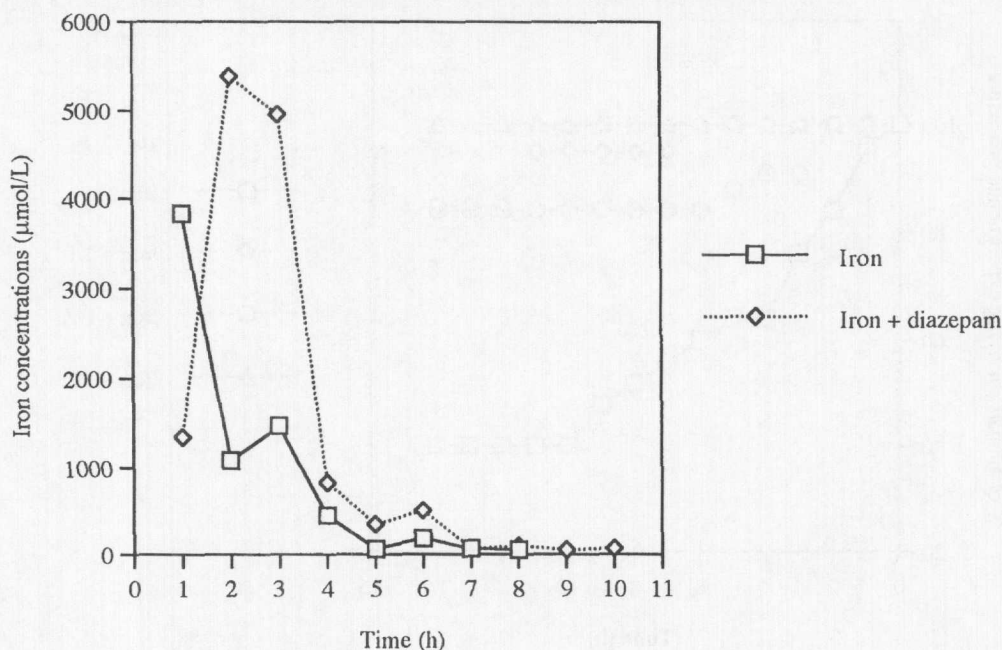
Serum iron samples were analyzed on a Kodak Ektachem 700 analyzer (Rochester, N.Y.) and urine iron samples on a SpectrAA-10 atomic absorption spectrometer (Varian Techtron Pty. Ltd., Australia). Survival (defined as those rats that did not require euthanasia during the 14-h observation period) between groups was compared by the Fisher exact test, and differences in serum or urinary iron excretion were compared by the Mann-Whitney U test. Data are presented as means \pm SD.

Results

Serum iron concentrations

Ferrokinetic (serum iron concentrations vs. time) curves did not appear to differ between animals receiving iron alone versus those receiving iron with DZ as shown in Fig. 1. Since the first concentration time point was at 60 min, it is possible that in the group treated with iron alone, absorption was faster and the peak occurred prior to our initial sampling at 60 min. Iron concentration was significantly lower with diazepam after 1 h ($p < 0.05$), significantly higher with diazepam after 2–3 h ($p < 0.05$), with no differences thereafter. Overall, the area under the concentration-time curve

Fig. 1. Mean serum concentrations of iron in animals receiving LD₆₀ of iron alone, or with diazepam ($n = 2-6$ for each time point). Diazepam did not decrease iron absorption, as a possible mechanism for its protective effect.



(AUC) of iron with DZ was not lower than the AUC of iron without DZ, to explain a potential mechanism of a protective effect.

Urine iron levels

Animals receiving iron with s.c. diazepam had urine iron concentrations of $10.4 \pm 1.13 \mu\text{mol/L}$, which were similar to those urine iron values of control and rats treated with only iron ($p = \text{ns}$). In contrast, rats that received i.p. injections of deferiprone along with s.c. diazepam and iron treatment showed significantly greater urinary iron excretion at 8 h ($1998.8 \pm 1318.7 \mu\text{mol/L}$) ($p < 0.0001$). However, 14 h post-iron administration levels were lower at 456.9 ± 582.1 , ($p = 0.003$), following the administration of deferiprone. The urine of all rats that received deferiprone had a strong red colour, typical of the presence of the deferiprone-iron complex. No such colour was evident in the urine collected from the rats that received only DZ following acute iron intoxication.

Survival

At the conclusion of the 14-h observation period, 60% (12/20) animals receiving iron died, whereas, only 16% (5/32) of the rats that were given iron and DZ ($p < 0.001$) died. In the group receiving both DZ and deferiprone, no animal died or showed any clinical signs of toxicity including withdrawing, tremor, piloerection, gait loss, or collapse (Fig. 2). No control animal, receiving distilled water, died ($p < 0.05$ compared with animals receiving DZ plus iron). For reference, Fig. 2 also presents the survival of animals receiving iron with deferiprone, from a previously published paper (Fassos et al. 1996).

Microscopic tissue analysis

Table 1 compares the iron deposition in various organs in the three groups. The lung, liver, kidney, and heart tissues of

animals treated with DZ and deferiprone did not appear to have any iron deposits. Pancreatic iron-staining scores also decreased with DZ and deferiprone treatment.

With iron and DZ treatment, iron crystals were dispersed throughout the renal tubules. Animals that were treated with DZ and deferiprone showed removal of iron crystals from the tubules. Unlike those animals receiving iron alone, pancreatic examination did not show any iron deposition in the endothelium and serosa in the presence of DZ, with or without the administration of deferiprone. Iron virtually disappeared from the circulation when deferiprone was administered.

Heavy iron deposition could be demonstrated in the gastrointestinal tract of animals receiving iron alone. In the stomach tissue of those rats that were given iron with DZ, iron deposition in the serosa and between the stomach cells continued to be heavy. Rats that received only iron had abundant amounts of intracellular and nuclear deposits of iron, whereas rats treated with DZ showed an absence of iron in their nuclei and a substantial decrease in cytoplasmic iron content. The coadministration of deferiprone and DZ to animals resulted in the reduction of iron-staining intensity within the serosa and an absence of iron within the cells and nuclei.

Iron staining of the duodenum showed a similar pattern to that of the stomach. Iron adhered to the villous surface and was absent in the nuclei, cytoplasm, serosa, and smooth muscle layers in those rats treated with iron and diazepam. Yet in those rats that were injected with diazepam and deferiprone solution, there was virtually no iron either within the cells or within the circulatory vessels; iron appeared to be completely absent from the nuclei.

Discussion

As part of our ongoing interest in developing optimal drug

Fig. 2. Survival curve of rats receiving LD₆₀ with different other drugs. For reference, L1 (deferiprone) effect by itself are cited from Fassos et al. (1996). In the group of rats that received only distilled water, no deaths occurred. For clarity this group is not shown.

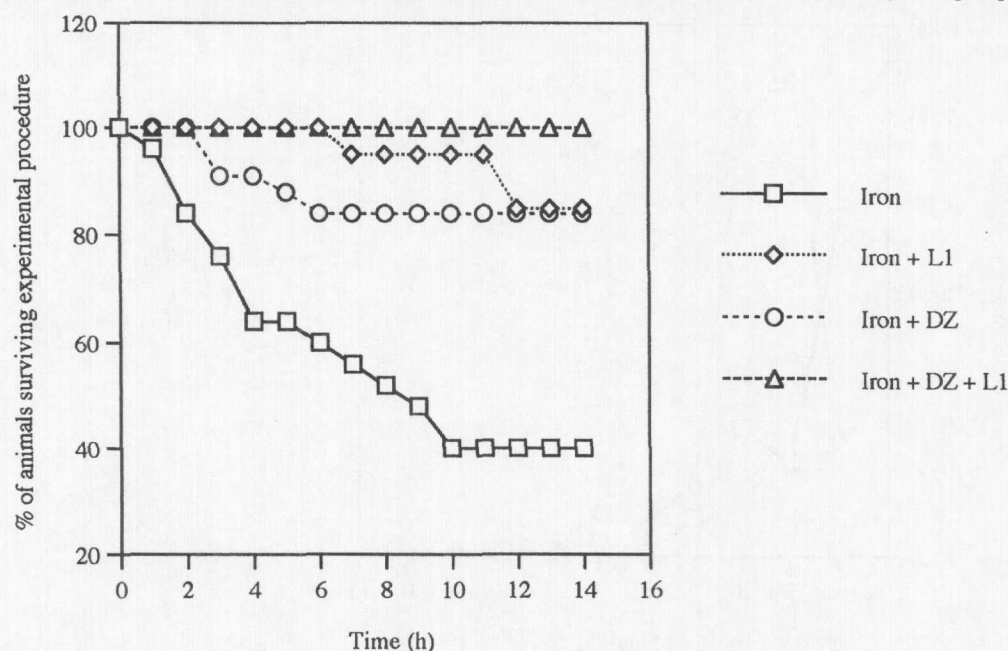


Table 1. Tissue iron scores (0 = no iron, 4 = maximal iron score).

Organ	Location	Iron	Iron + DZ score	Iron + DZ + L1 score
Liver	Kupffer cells	<1	0	0
	Vein endothelium (periportal)	2	0	0
	Hepatocytes	0	2	0
Kidney	Vascular endothelium	2	0	0
	Crystals in tubules	4	4	1
	Capsule	4	4	0
Heart	Capillary endothelium	1	0	0
	Myocardium	0	0	0
Lung	Pleural lining	2-3	1	0
	Alveolar lining	0	0	0
Stomach	Superficial mucosal lumen	4	4	4
	Vascular endothelium	4	1	1
	Muscle layers	4	1	3
	Serosa	4	3	4
Pancreas	Vascular endothelium	4	0	0
	Serosa	4	2	2
	Islets	0	0	0
Duodenum	Serosa	4	1	2
	Superficial mucosal lumen	4	4	0
	Crypts	1	3	1
	Vascular endothelium	4	1	0
	Muscle layers	4	0	1

therapy for acute iron intoxication, we have had preliminary evidence that diazepam may reduce mortality following the administration of a lethal dose of iron. To date all attempts at decreasing morbidity and mortality from acute iron overdose have focused on gut decontamination and systemic chelation of the metal (Tenenbein 1985; Proudfoot et al. 1986). The benzodiazepines have never been either proposed or tested in this condition, as there was no theoretical or hypothetical framework to rationalize such an experiment. If

diazepam, given to alleviate animal suffering, also affects the survival of the rats following iron intoxication, then the administration of diazepam is unsuitable for future experimental work, which measures survival as an end point, because it will affect survival data.

Our experiment shows a statistically and clinically significant effect of diazepam on the survival of rats receiving iron at an LD₆₀ dose. The serum ferrokinetics do not appear to differ in a major fashion between the animals that received

iron and those that received iron with diazepam. Although there was a trend towards slower absorption of iron by diazepam, the extent of absorption did not appear to differ, indicating that diazepam did not cause the measurable effects. No excess iron was measured in the urine of those rats treated with diazepam and iron. These observations suggest that diazepam does not affect the extent of either absorption or excretion of iron.

Our pathological examination provides preliminary evidence that diazepam may change intranuclear distribution of iron in several organs. Of particular interest is the effect that diazepam has on the gastrointestinal tract, a system that is subjected to serious damage during acute iron overdose, which may be life threatening.

The possibility that diazepam may interfere with iron transport into cells is supported by the findings of Hill (1985) who found that the administration of benzodiazepines decreased intracellular iron concentration in GABAergic nerves. Alternatively, it is possible that diazepam may decrease morbidity by inhibiting a secondary or even tertiary event initiated by the iron overdose, rather than by decreasing intracellular iron accumulation.

A review of the toxicological literature reveals several instances where diazepam has reduced morbidity and mortality following serious cases of acute intoxication. For example, the coadministration of diazepam with atropine and oxime inhibited the development of lung lesions in rats treated with aerosolized sarin (Pant et al. 1993), prevented soman-associated neurotoxicity in animals exposed to organophosphates (Martin et al. 1985; Nordgren et al. 1992; Clement and Broxup 1993), and malathion toxicity in rats (Hussain and Ansari 1990). It has been suggested that the ability of diazepam to lower the rate of synthesis of acetylcholine may contribute to its role in reducing organophosphate poisoning. Diazepam has also been shown to have an antidotal effect in cases of severe chloroquine poisoning (Rajah 1990; Liu et al. 1991).

Our study documented a potentially additive protective effect of DZ and the oral chelator deferiprone (Fig. 2). Both diazepam, in the present study, and deferiprone (Fassos et al. 1996) decreased mortality from 60 to 16%; following a combination of deferiprone and diazepam, none of the animals receiving an LD₆₀ dose of iron died.

The abundant presence of specific receptors for benzodiazepine in many organs has not been linked to any known physiologic or pathologic process (Mestre et al. 1984; Richards et al. 1982). The binding of diazepam to peripheral benzodiazepine receptors may in fact play an important role in protecting organs from the toxic effects of iron.

More work is needed at the molecular and cellular levels to elucidate the potential role of diazepam in acute iron poisoning. Meanwhile, due to the very large numbers of cases of acute iron toxicity and its high toxic potential, the therapeutic role of diazepam in acute iron poisoning should be further evaluated.

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