Comparative Treatment of α-Amanitin Poisoning With *N*-Acetylcysteine, Benzylpenicillin, Cimetidine, Thioctic Acid, and Silybin in a Murine Model

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The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of the Navy, Department of Defense, or the US government.

Study objective: The foraging of wild mushrooms can be complicated by toxicity from several mushroom types. Amatoxin, a peptide contained in several mushroom species, accounts for the majority of severe mushroom poisonings by binding to RNA polymerase II irreversibly, leading to severe hepatonecrosis. There is no effective antidote for severe amatoxin poisoning. We compare the effectiveness of 5 potential antidotal therapies in limiting the degree of hepatonecrosis in a randomized, controlled, murine model of amatoxin-induced hepatotoxicity.

Methods: One hundred eighty male Institute of Cancer Research mice were randomized into 6 equal groups. Within each group, 21 mice were intraperitoneally injected with 0.6 mg/kg of α-amanitin (amatoxin); the remaining 9 were injected with 0.9% normal saline solution. Four hours postinjection, each group of 30 mice was randomized to 1 of 5 intraperitoneal treatments (*N*-acetylcysteine, benzylpenicillin, cimetidine, thioctic acid, or silybin) or normal saline solution. Repeated dosing was administered intraperitoneally every 4 to 6 hours for 48 hours. After 48 hours of treatment, each subject was killed, cardiac blood was aspirated for hepatic aminotransferase measurements (alanine transaminase and aspartate transaminase), and liver specimens were harvested to evaluate the extent of hepatonecrosis. The degree of hepatonecrosis was determined by a pathologist blinded to the treatment group and divided into 5 categories according to percentage of hepatonecrosis.

Results: Amanitin significantly increased aspartate transaminase in treated mice compared with normal saline solution–treated controls (mean [SD] 2,441 [2,818] IU/L versus 310 [252]; $P=.03$). None of the antidotal therapies were found to significantly decrease the increase in aminotransferases compared with controls. Further, none of the antidotal therapies demonstrated an important decrease in hepatonecrosis compared with controls when a histologic grading scale was used.

Conclusion: In this murine model, *N*-acetylcysteine, benzylpenicillin, cimetidine, thioctic acid, and silybin were not effective in limiting hepatic injury after α-amanitin poisoning. Increases of aminotransferases and degrees of histologic hepatonecrosis were not attenuated by these antidotal therapies. [Ann Emerg Med. 2007;50:282-288.]

0196-0644/$-see front matter

doi:10.1016/j.annemergmed.2006.12.015

Editor's Capsule Summary

What is already known on this topic

Poisoning from amanita mushrooms can result in severe liver toxicity and death. Several treatments have been proposed, but effectiveness has not been clearly demonstrated.

What question this study addressed

This randomized, placebo-controlled study examined the efficacy of 5 treatments for α-amanitin poisoning, with 21 poisoned mice per treatment.

What this study adds to our knowledge

None of the 5 treatments (benzylpenicillin, cimetidine, acetylcysteine, thiotic acid, silybin) were effective in preventing or reducing hepatonecrosis compared with saline solution control.

How this might change clinical practice

The lack of an effective antidote reinforces the principle that supportive care is the mainstay of treatment for amanita mushroom poisoning.

INTRODUCTION

Background

The foraging of wild mushrooms can be complicated by toxicity from several mushroom types. Amatoxin, a peptide contained in several mushroom species, accounts for the majority of severe mushroom poisonings by binding to RNA polymerase II irreversibly, leading to severe hepatonecrosis. There is no effective antidote for severe amatoxin poisoning.

Foraging for mushrooms in the wild can be dangerous when various toxic species are mistaken for edible ones. Although the majority of mushroom poisonings are reported in Europe, data have also been published on exposures in South Africa, Malaysia, Chile, Mexico, Australia, and India.[1] In the United States, mushroom exposures remain a persistent complaint to poison control centers. In 2004, 8,601 of these exposures were reported to US poison centers; 5 of these cases resulted in deaths. Of all mushroom poisonings, severe illness and death resulting from exposures to amatoxins remain disproportionately high.[2]

Importance

Amatoxins are cyclopeptides contained in several *Amanita* species, which include several members of the *Lepiota* and *Gallerina* genera of mushrooms. The mechanism of action is based on the ability of cyclopeptides to prevent the normal function of RNA polymerase II, thereby inhibiting cellular replication. Organ systems with the highest cell turnover are affected most; these include the gastrointestinal, renal, and hepatic systems. Hepatic vulnerability is exacerbated by the recirculation of amatoxins within the biliary tree and by transporter-mediated cellular uptake of amatoxins into hepatocytes.[3,4] α-Amanitin is the major type of amatoxin found in the *Amanita* species.[5]

Goals of This Investigation

The efficacy of proposed antidotal therapy for severe amatoxin poisoning remains debatable. Therapies that have been postulated have ranged from those that have been directed at decreasing the uptake of amatoxin into hepatocytes to those that decrease the oxidative damage that occurs as a result of severe poisoning. Although various animal studies have suggested benefit in the use of several antidotes, none have been designed to compare simultaneously the effect of several common treatments in limiting hepatic injury (measured by a grading of histologic changes, along with serum markers) in a basic animal model. Of the numerous human studies that have been reported, most are uncontrolled, retrospective, or anecdotal, thereby decreasing our ability to judge benefit from any single therapy.[6-9] Benzylpenicillin and other β-lactam antibiotics, as monotherapy or in combination with other agents, have been the most frequently used drugs in the management of amatoxin poisoning in humans.[5]

We used a murine model previously studied in amatoxin poisoning and used both histologic analysis and serum aminotransferases as objective characteristics to demonstrate benefits of treatments.[10]

MATERIALS AND METHODS

Study Design

The study protocol was approved by the University of California San Diego Animal Care and Use Committee. The care and handling of animals were in accordance with National Institutes of Health guidelines for ethical animal research.

One hundred eighty male Institute of Cancer Research–type mice were purchased commercially (Charles River Laboratories, Inc., Hartford, CT). These laboratory-bred mice (commonly used in experimental research) weighed an average of 42.4 g. After transportation, the mice were allowed to adjust for 72 hours while being fed standard feed and water rations. After delivery to our laboratory, the mice were housed collectively until the beginning of the experiment.

Setting and Selection of Participants

The 180 mice were randomly divided into 6 groups of 30 mice. Nine of the 30 mice in each of the 6 groups were injected with 1 mL intraperitoneal 0.9% normal saline solution as a control; the remaining 21 of the 30 mice were injected intraperitoneally with α-amanitin at 0.6 mg/kg (purified abstract reconstituted in normal saline solution; Roche Diagnostics Corp, Chicago, IL). This amount was chosen because it represents 50% lethal dose value (LD_{50}) of α-amanitin in mice according to several publications and on the material safety data sheets provided by the commercial supplier of our extracted α-amanitin.[11,12] The effectiveness of this

Table. Treatment and control groups.

Poison (or Control)/Treatment (or Control)	Subjects per Group
Amanitin/normal saline solution	21
Amanitin/*N*-acetylcysteine	21
Amanitin/benzylpenicillin	21
Amanitin/cimetidine	21
Amanitin/thioctic acid	21
Amanitin/silybin	21
Normal saline solution/normal saline solution	9
Normal saline solution/*N*-acetylcysteine	9
Normal saline solution/benzylpenicillin	9
Normal saline solution/cimetidine	9
Normal saline solution/thioctic acid	9
Normal saline solution/silybin	9

concentration in inducing hepatonecrosis both histopathologically and by analysis of aminotransferases was verified in a dosing trial we performed before the start of this study.

Each of the 6 groups of 30 mice (9 normal saline solution–injected and 21 amanitin-injected) was then randomized to receive either 1 of 5 antidotes or normal saline solution as a control (Table). The 5 antidotes chosen were *N*-acetylcysteine, benzylpenicillin, cimetidine, thioctic acid, and silybin. Antidotes were injected intraperitoneally 4 hours after initial intraperitoneal injection with α-amanitin or normal saline solution control. The delay interval of 4 hours from the time of initial poisoning was chosen to reflect the reasonable period expected for human victims of amatoxin poisoning to seek medical care. This 4-hour delay to first antidote was also the time chosen in several studies testing the efficacy of antidotal therapies in mice models.[10,12]

Specific antidotes were administered as follows: *N*-acetylcysteine (reconstituted in normal saline solution; Abbott Laboratories, Chicago, IL) was administered at a dosage of 1.2 g/kg and repeated every 4 hours at a dosage of 0.6 g/kg. Benzylpenicillin (reconstituted in normal saline solution; Geneva Pharmaceuticals, Broomfield, CO) was given at a dosage of 1,000,000 U/kg per day, divided into 6 dosages and injected every 4 hours. Cimetidine (reconstituted in normal saline solution; Abbott Laboratories) was dosed at 120 mg/kg (480 mg/kg/day), extracted silybin (reconstituted in normal saline solution; Sigma-Aldrich, St. Louis, MO) at 5 mg/kg (20 mg/kg/day), and thioctic acid (reconstituted in 5% ethanol solution; Sigma-Aldrich) at 37.5 mg/kg (150 mg/kg/day) every 6 hours. One milliliter normal saline solution was injected every 6 hours in mice randomized to the control group.

Data Collection and Processing

Treatments were continued at regular intervals (every 4 or 6 hours, depending on the designated treatment) for 48 hours, at which time all subjects were killed sequentially by rapid asphyxiation in a carbon dioxide chamber. Immediate dissection was performed and blood was drawn by direct cardiac stick and aspiration. Blood samples were spun with a centrifuge, and plasma was removed for measurements of aspartate transaminase (AST) and alanine transaminase (ALT).

Subjects dying before the complete 48-hour treatment were not included in the final analysis of AST/ALT levels, nor were their livers harvested for histologic examination.

Liver specimens were immediately harvested, preserved in 10% formalin, and embedded in paraffin. At a later time, 1-mm liver slices were stained with hematoxylin and eosin and reviewed by an independent board-certified pathologist blinded to treatments and controls. The degree of hepatic injury was stratified according to a modification of the Suzuki et al[13] grading scale of hepatic injury. Each specimen was categorized according to the percentage of hepatocytic necrosis observed per field: 0% to 20%, 21% to 40%, 41% to 60%, 61% to 80%, and 81% to 100%. Rater reliability was assessed by a reexamination of a blinded random sample consisting of 15% of all the histology slides reviewed by the pathologist.

Primary Data Analysis

Power analyses were run for AST/ALT as outcome measures to contrast control against toxin and toxin against toxin with treatment. We calculated a power of 0.80 to detect a 33% difference in AST/ALT measurements. Mean values and the SDs were calculated for measures of AST/ALT. Rank sum testing was performed on categories of hepatonecrosis observed on histologic slide analysis.

RESULTS

All mice treated with cimetidine, *N*-acetylcysteine, silybin, and normal saline solution control completed the 48-hour treatment period and survived until being killed. Two of 30 mice treated with benzylpenicillin died during the course of treatment. One mouse in the group treated with thioctic acid died immediately after the fourth treatment injection.

In the analysis of AST and ALT levels, mice poisoned with α-amanitin developed significantly higher aminotransferase levels compared with mice poisoned only with normal saline solution controls (mean [SD], 2,441 [2,818] IU/L versus 310 [252]; $P=.03$). In mice poisoned with α-amanitin and treated with antidotal therapies, AST/ALT levels varied in relationship to controls, but no reductions in levels reached statistical significance (Figure 1). In normal saline solution control mice, no significant increases in aminotransferases resulted from treatment with antidotes compared to treatment with normal saline solution (Figure 1), including treatment with thioctic acid, which was reconstituted in a dilute ethanol solution.

On histopathology examination, mice poisoned with α-amanitin developed higher percentages of hepatonecrosis compared to mice injected with normal saline solution (rank sum test $P=.027$). No statistically significant reductions in hepatonecrosis, as observed on histopathologic examinations, were found among the 5 treatment groups compared with normal saline solution controls (Figure 2).

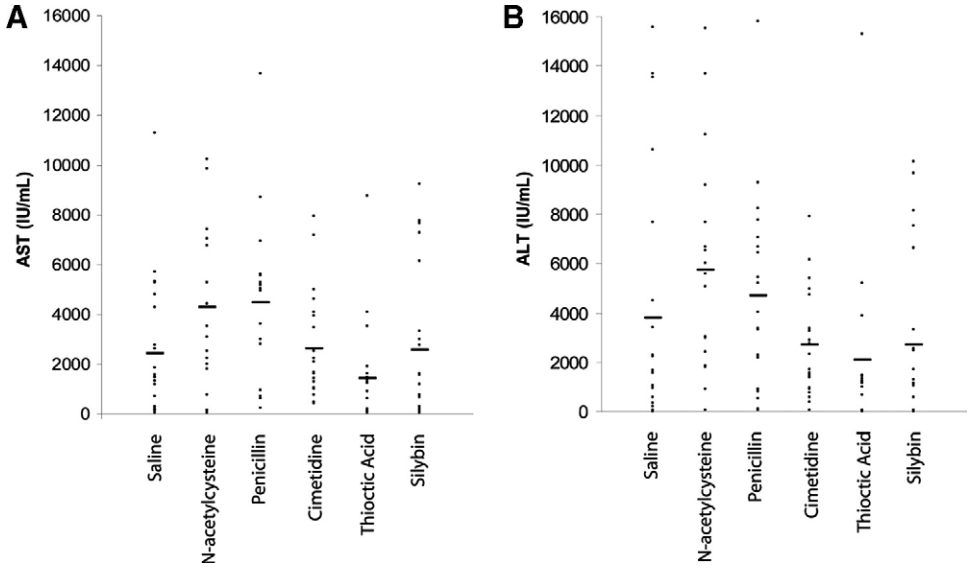

Figure 1. Serum AST and ALT (IU/mL) measurements after 48 hours of treatment with antidotes or normal saline solution after initial poisoning with α-amanitin. For all treated controls injected initially with normal saline solution, mean AST/ALT=204/41 and maximum AST/ALT=699/172. The horizontal bar represents the group mean. There were 21 mice in each group.

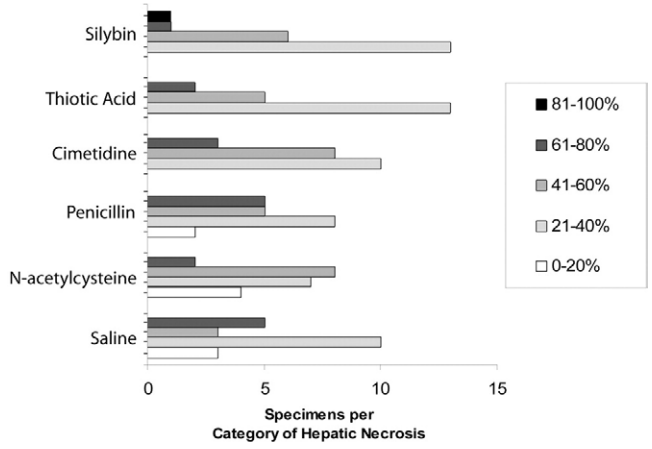

Figure 2. Liver specimens categorized by percentage of hepatic necrosis (eg, 0%-20%) after 48 hours of treatment with antidotes or normal saline solution after initial poisoning with α-amanitin. There were 21 mice in each group.

When a 15% random sample of the total histopathology slides was reread by the blinded pathologist, 93.3% agreement was found between the initial and the repeated readings.

LIMITATIONS

There are multiple limitations to an animal study. The first and foremost limitation is that an animal model may not reproduce the toxicity or benefits of treatment observed in human beings. Differences in the metabolism of amanitin in humans compared to mice may result in a larger treatment window and an improved response to therapies. With this in mind, we chose a murine model because it has been used in previous investigations of amanitin poisoning and because its response to hepatic toxins is considered closely analogous to that of humans.[14] Another limitation is the amount of amanitin used. We used 0.6 mg/kg of amanitin injected intraperitoneally according to previous studies. Although this amount has previously been shown to represent the LD_{50} for this species, it does not actually estimate the absorption and bioavailability of amanitin in a human ingestion. We chose this method according to previous studies, but we realize that a model of oral ingestion with delayed treatment would more closely model human exposure.

There are several methodologic limitations to our study as well. One such limitation was that animals that died were excluded from further analysis. However, this amounted to only a few animals (2 in the benzylpenicillin group and 1 in the thioctic acid group) and was unlikely to change the results. Another limitation was the 4-hour delay to treatment time. Previous studies indicated that treatments may have an effect if they are given before or immediately after the intraperitoneal injection or ingestion of amanitin.[11] In choosing a delay, we tried to recreate an actual scenario in which patients may not seek immediate care and treatment. Although 4 hours is a relatively short time to presentation, we thought that it would more closely resemble the treatment offered humans after a real ingestion. Certainly, had this period been shortened or had our subjects been pretreated, effects on hepatic injury may have been attenuated. In this model, we chose intraperitoneal poisoning

rather than oral poisoning to ensure a more predictable delivery of amanitin and to allow comparisons to previous studies that have used the intraperitoneal delivery method. Because of differences in distribution kinetics (an intraperitoneal dose of amanitin would be expected to act more quickly than an oral dose), this may have exaggerated the delay to treatment time and may have diminished any potential benefits of early treatment. In addition to the route of amanitin delivery, variations in the route of antidote delivery in an actual scenario of poisoning may also affect outcome. Antidotes would be given intravenously rather than intraperitoneally in an actual human case of poisoning, and variations in absorption and distribution may alter effectiveness.

Because there is no single dosage that has been proven to be consistently effective for most of the tested antidotes, differences in dosing could alter results. In general, our choices on antidote dosages were based on previous animal models that suggested benefit at that dosage (eg, cimetidine) or we chose the higher dosage of a generally accepted range of treatment dosages (eg, benzylpenicillin). In our study, treatment was limited to 48 hours. It could be argued that continued treatment for a longer duration may have resulted in different outcomes.

A final limitation of our study is in our established outcome measures, which were measurements of aminotransferases and histologic evidence of hepatic necrosis at 48 hours. Other characteristics, such measuring survival, might have been more useful in gauging benefit. Unfortunately, our need to kill our subjects to determine liver pathology and our limited ability to gather only a small amount of blood through cardiac aspiration made this alternative unachievable. Future studies examining survival rates after 48 hours may be helpful in correlating degrees of hepatic injury with clinical outcome.

DISCUSSION

The exact mechanism of amatoxin poisoning has yet to be definitively elucidated. However, experimental evidence suggests that amanitin inhibits RNA polymerase II by enzymatic inhibition. Transport of amanitin into hepatocytes likely occurs through a Na^+-mediated transporter.[3,4] The majority of pharmacologic treatments for a severe amatoxin poisoning have been directed at the transport process into hepatocytes and the oxidant effects accompanying hepatic necrosis.

In this experiment, we validated the use of a murine model to study hepatic injury from α-amanitin (measured objectively by increases in aminotransferases and histologic examinations of hepatocytic necrosis). The degree of injury we observed was consistent with what has been previously reported when a dose of amanitin in the range of the LD_{50} was used for this species.[10,12] This murine model of amanita poisoning was developed originally on the basis of landmark work by Piperno and Berssenbruegge[15] on the effectiveness of *N*-acetylcysteine in treating acetaminophen poisoning in mice.

With regard to any direct evidence of hepatonecrosis on pathology examination or to the surrogate markers of aminotransferases, we found no improvement after 48 hours of treatment with *N*-acetylcysteine, benzylpenicillin, cimetidine, thioctic acid, or silybin. None of the treatments used in our study improved the course of poisoning from α-amanitin compared to controls (Figure 1).

Because of differences in methodology, it is difficult to compare fully these results to those in previous studies. *N*-acetylcysteine functions as a reducing agent "scavenger" of free radicals and as a glutathione precursor when endogenous stores are depleted. Schneider and Michelson[12] had previously failed to demonstrate benefit from *N*-acetylcysteine treatment in a similar murine model but had only used a single dose during a 48-hour period. Given that the plasma half-life of intraperitoneal L-cysteine has been shown to be less than 2 hours,[16] we addressed this limitation by administering multiple doses of *N*-acetylcysteine over time but still found no benefit.

Benzylpenicillin and other β-lactam antibiotics have been the most frequently used drugs in the management of amatoxin poisoning as monotherapy or in combination with other agents.[5] Although it has been classically theorized that β-lactam antibiotics interfere with amanitin uptake, it is more likely that if hepatoprotection does occur, it is through an intracellular mechanism through effects on eukaryotic DNA replication.[17] In a previous study, Floersheim et al[18] found that benzylpenicillin injected intravenously in dogs poisoned with amatoxin prevented the increase of hepatic aminotransferases. Different from our study, however, was that these subjects were treated almost immediately after being given a sublethal dose of *Amanita* extracts. We chose to begin treatment in a delayed fashion to reflect an expected delayed time to treatment after poisoning. In other animal studies, benzylpenicillin was used experimentally to protect mice and rats against lethal doses of α-amanitin, but no correlation was made to pathology examination.[19,20]

Like *N*-acetylcysteine, cimetidine has been used for its potential antioxidant benefits and impedance of lipid peroxidation. As a cytochrome P450 inhibitor, it was originally used in amatoxin exposures because of the clinical similarity of amatoxin poisoning to other hepatic toxins affecting cytochrome P450. Schneider[11] demonstrated the value of cimetidine in limiting hepatic steatosis observed on photomicrographs. Although a significant finding, prophylactic treatment with cimetidine used in one arm of that study limits its applicability to a realistic scenario. We thought that delayed treatment was necessary to offer external validity to any significant findings that might be observed. In addition, we chose to use a more standardized method of pathologic analysis and to correlate those findings with objective laboratory analysis of hepatic aminotransferases.

Although the exact mechanisms of action are unclear, several theories exist on the mechanisms by which silybin

and thioctic acid are purported to exert their effects. Silybin is the major compound isolated from the seeds of the Mediterranean milk thistle, *Silybum marianum*. The main benefit of silybin has been in its purported hindrance of amanitin penetration into the hepatocyte by competing with amanitin for transport systems in the cell membrane. Hahn et al[21] demonstrated improvement in death rate and survival times in mice after poisoning with α-amanitin. However, measures of aminotransferases and pathologic examinations were not performed. Vogel et al[22] found a marked increase in the survival of intraperitoneally poisoned dogs after 2 doses of silymarin. This decrease was also accompanied by decreased aminotransferase levels and less bloody necrosis in liver specimens. In our murine model, we were unable to reproduce these beneficial findings.

The potential benefit of thioctic acid in amatoxin poisoning lies in its antioxidant effects and its purported ability to impede lipid peroxidation after oxidative damage.[23] It was introduced in the treatment of amatoxin poisoning in Europe in the 1960s, with apparent beneficial results, although multiple subsequent evaluations of its benefit have failed to prove a direct causal effect.[24,25] When thioctic acid was used to treat amatoxin-poisoned mice and dogs, gross metabolic imbalances[26] or a lack of clinical benefit in improving survival[27] was found. Our study used aminotransferase measurements and histopathology analysis as outcome measures, but in this regard, we similarly found no benefit in its use.

We chose our dosing regimens according to previous experimental models. *N*-acetylcysteine was dosed at 1.2 g/kg, the same dose used by Schneider and Michelson[12] in their single-treatment study and by Piperno and Berssenbruegge[15] in their experiments with *N*-acetylcysteine after acetaminophen poisoning. *N*-acetylcysteine was injected every 4 hours at 0.6 g/kg after the initial 1.2 g/kg dose, mimicking the treatment regimen that would be given in humans. Choices in benzylpenicillin dosing both in animal models and in actual human poisoning have greatly varied. In adults and children, frequently suggested doses are 40,000,000 and 1,000,000 total U/day, respectively.[5] We chose the dose of 1,000,000 U/kg per day because it is similar to the doses that have been used in previous murine models. Because of the short plasma half-life of benzylpenicillin and its metabolites, we chose to dose it every 4 hours. Cimetidine was dosed at the 120 mg/kg per day, the same dose that was demonstrated by Schneider[11] to show histologic improvement in mice poisoned with amanitin and by Mitchell et al[28] to demonstrate cimetidine's effects in acetaminophen toxicity. Cimetidine was redosed every 6 hours. The dose of silybin, 5 mg/kg every 6 hours (20 mg/kg/day), was based on several recommended dosing regimens for human patients and the half-life of intraperitoneal injections in animal models.[29] Thiotic acid was dosed 37.5 mg/kg (150 mg/kg/day) according to a similar combination of previous suggested human dosing and intraperitoneal half-life.[5]

In conclusion, in this murine model, *N*-acetylcysteine, benzylpenicillin, cimetidine, thioctic acid, and silybin were not found to be effective treatments in limiting hepatic injury after α-amanitin poisoning. Increases of aminotransferases and degrees of histologic hepatonecrosis were not attenuated by these antidotal therapies.

Supervising editor: E. Martin Caravati, MD, MPH

Author contributions: TCT conceived the study design, obtained funding, and conducted the study. RFC assisted in conceiving the study design and assisted in obtaining funding. WHR, DPB, MH, DAT, and MF assisted in conducting the study. DAT assisted in conceiving the study design and the statistical analysis. RHR performed the statistical analyses. TCT takes responsibility for the paper as a whole.

Funding and support: By *Annals* policy, all authors are required to disclose any and all commercial, financial, and other relationships in any way related to the subject of this article, that may create any potential conflict of interest. See the Manuscript Submission Agreement in this issue for examples of specific conflicts covered by this statement. Funding was provided by a research grant through the Wilderness Medical Society.

Publication dates: Received for publication July 10, 2006. Revisions received November 3, 2006, and December 11, 2006. Accepted for publication December 20, 2006. Available online June 7, 2007.

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