Pergamon

Pharmacology Biochemistry and Behavior, Vol. 52, No. 3, pp. 509–515, 1995

0091-3057/95 $9.50 + .00

0091-3057(95)00118-2

Proline, Ascorbic Acid, or Thioredoxin Affect Jaundice and Mortality in Long Evans Cinnamon Rats

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Received 1 November 1994

HAWKINS, R. L., M. MORI, M. INOUE AND K. TORII. *Proline, ascorbic acid, or thioredoxin affect jaundice and mortality in Long Evans Cinnamon rats.* PHARMACOL BIOCHEM BEHAV 52(3) 509–515, 1995.—The Long Evans Cinnamon (LEC) rat spontaneously develops fulminant hepatitis, which is usually lethal due to excess copper accumulation in the liver and is considered an animal model of Wilson's disease. LEC rats show a strong appetite for proline solution. Daily oral (p.o.) administration of proline resulted in significant delay of mortality. Feeding a copper-deficient diet greatly delayed the onset of jaundice and mortality and voluntary consumption or p.o. administration of proline further delayed jaundice and prevented mortality. LEC rats also consume ascorbic acid solutions, and p.o. administration of ascorbate also results in a significant delay in the appearance of jaundice and mortality. Combined treatment with ascorbic acid and proline is additive to delay further jaundice and mortality. An endogenous antioxidant protein, thioredoxin, when infused by minipump IP, could also inhibit the incidence of jaundice. These results indicate that antioxidant treatment combined with proline may be of benefit in Wilson's disease and possibly other forms of hepatic dysfunction.

Proline Ascorbic acid Thioredoxin Antioxidants Long Evans Cinnamon rat Branched chain amino acids
α-Tocopherol β-Carotene Jaundice Hepatitis Copper Wilson's disease

THE LONG EVANS Cinnamon (LEC) strain of rats has been shown to be a useful model to study genetically transmitted fulminant hepatitis and chronic liver disease (28). The underlying cause is thought to be due to excessive copper accumulation in the liver of LEC rats, thus making this animal a model for Wilson's disease in humans (16). Chelation therapy or feeding a copper-deficient diet can ameliorate the symptoms of LEC rats and Wilson's disease (24). To better understand the mechanism of the toxic effect of excessive copper and to investigate other therapeutic approaches that might also be relevant to other types of hepatitis, we have been examining other agents that would be of benefit to delay the onset of hepatitis and death of LEC rats. In preliminary experiments, we observed that LEC rats given free access to a variety of amino acid solutions would consume disproportionate amounts of proline solution, prompting us to examine further the development of an appetite for proline consumption by LEC rats and whether proline might have a beneficial effect on the development of hepatitis and mortality.

Further work examined whether antioxidant therapy might also be therapeutic, because excessive copper has been reported to promote production of free radicals, which can exert a cytotoxic effect (9,19). LEC rats have altered hepatic activity of enzymes such as glutathione peroxidase, glutathione reductase, Mn-SOD (superoxide dismutase), and Cu,Zn-SOD (22, 23). Therefore, the effect of ascorbic acid, α-tocopherol, β-carotene, or thioredoxin administration on the incidence of jaundice and mortality was examined. Thioredoxin (Trx) is a widely distributed protein found in high concentration in the liver (7), which has potent antioxidant and protein-renaturing

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activities (4,13,17,20). Results indicate that proline, ascorbic acid, and Trx can exert a beneficial effect on the appearance of jaundice and mortality of LEC rats.

METHOD

Long Evans Cinnamon (LEC) rats were obtained from Charles River Japan. Male rats were received at 5 weeks of age and housed either singly or in pairs in a temperature- and humidity-controlled room. Six to 10 animals were used for each group. Lights were on from 0700–1900 h. The rats were initially given standard diet pellets (CRF-1, Charles River, Japan) for 1 week, and then switched to 15% PEP (purified egg protein) powdered diet (25). Other test diets were the same as the 15% PEP diet, with the following modifications: copper-free diet omitted copper sulfate from the mineral mixture, ascorbic acid-supplemented diet added 0.8% w/w ascorbic acid (Wako Chemical, Japan) with an equivalent reduction in corn starch, α-tocopherol-supplemented diet added 0.075% w/w a-tocopherol acetate (Wako Chemical) in addition to the standard concentration of 0.01% tocopherol, and β-carotene-supplemented diet added 0.0125% w/w β-carotene (Wako Chemical). Distilled water was always available ad lib. Additional drinking solutions were also made available ad lib at 6 weeks of age in some experiments. Voluntary drinking solutions were: L-proline (0.2 M in distilled water), BCAA (0.075 M equimolar mix of L-leucine, L-isoleucine, and L-valine), or 3 mg/ml ascorbic acid. Amino acids were pharmaceutical grade from Ajinomoto Co. (Japan). Oral (p.o.) administration of solutions was performed between 0900–1000 h daily. Solutions used were: BCAA (equimolar mix of L-leucine, L-isoleucine, and L-valine, for a total of 233 mg/ml made up in distilled water with 0.3% w/v carboxymethyl cellulose, 1 ml/100 g b.wt./day), L-proline (135–150 mg/day), or ascorbic acid (100 mg/day in distilled water). Control animals received p.o. infusion of distilled water, or in the case of the experiment using BCAA, distilled water with 0.3% carboxymethyl cellulose.

The occurrence of jaundice is easily observable as the time when the ears and tail turn yellow and the urine becomes a bright orange, staining the fur in the lower abdominal region. Usually, the jaundice progressively worsens, ending in death of the animal within about a week.

In experiments using thioredoxin (Trx), human recombinant Trx was obtained from Ajinomoto Co., and administered via minipumps (Alza Corp., #2002) intraperitoneally implanted under diethyl ether inhalation anesthesia. These minipumps deliver 0.5 μl/h of solution continuously for 2 weeks, at which time fresh minipumps were implanted as before.

Statistics were performed using the StatView II software package (Abacus Concepts, Berkeley, CA) for Student's *t*-test, ANOVA, and the Scheffé *F*-test for comparison between means. A $p < 0.05$ level was used as the threshold for significance. All data are expressed as means.

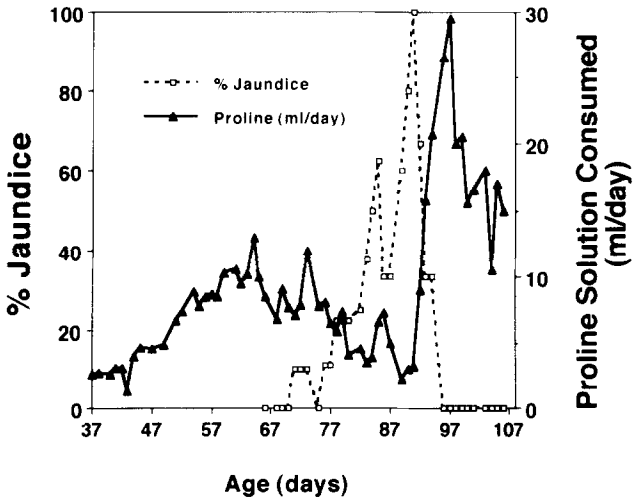

FIG. 1. Comparison of the incidence of jaundice and the increase in ad lib consumption of proline solution with age of LEC rats. The solid line shows the mean daily consumption of 0.2 M L-proline solution and the dotted line shows the mean incidence of jaundice ($n = 10$).

RESULTS

LEC rats show an increasing appetite for ad lib consumption of proline solution with age. Proline consumption starts to increase from the time the proline solution is first made available (about 5 weeks of age) until about the time that jaundice starts appearing. There is some decline in daily consumption during the time the animals are acutely affected with jaundice, but the animals that are recovering from jaundice show a strong appetite for proline solution (Fig. 1). Water intake did not change over the same time period. This appetite for proline may indicate that proline is having some beneficial effect on the symptoms of the disease process of LEC rats, so the next experiment looked at the appearance of jaundice and mortality in LEC rats when proline was administered daily (p.o.) to animals with diets either with (6.2 mg/100 g diet) or without added copper. With added copper, the initial appearance of jaundice was the same in animals with or without proline administration. However, by about 12 days after the first appearance of jaundice, the group given proline ceased to develop fresh cases of jaundiced animals and there were no further deaths over approximately the next 7 weeks (Fig. 2), while the control group continued to increase in the number of jaundiced animals, with 100% mortality by about 13 weeks of age. Animals voluntarily consuming proline solution when fed the copper containing diet were only slightly delayed in the incidence of mortality. Because the proline solution was observed to be mostly consumed during the lights-off period, when most of the feeding occurs, this suggests that proline is more effective when given as a bolus infusion near the start of the lights-on period. When fed the copper deficient diet, there was a 4-week delay in the appearance of jaundice and subsequent mortality, which was significantly better than the protection afforded by proline administration to the animals fed the added copper diet (Fig. 2). However, proline administration, either as voluntary drinking solution or by p.o. infusion, when given the no added copper diet, prevented any deaths over the period studied, until 20 weeks of age.

In order to see whether this protective effect was specific for proline, we examined the effect of other amino acids, i.e., the branched chain amino acids (BCAA), leucine, isoleucine, and valine. Voluntary intake of BCAA did not have any effect on the appearance of jaundice and had only a slight effect on mortality (Fig. 3). Infusion of BCAA p.o. also had only a small, statistically insignificant effect on the appearance of jaundice and actually produced somewhat of an increase in mortality relative to control animals. The effect of proline was similar to that observed in the previous experiment, in that

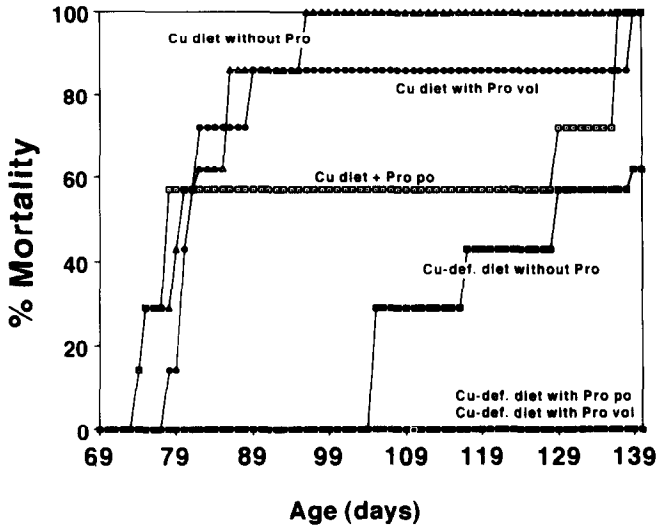

FIG. 2. Mean incidence of mortality with age of LEC rats fed either a normal copper diet (Cu diet) or a copper-deficient diet (Cu-def diet) and either given only distilled water (without Pro), ad lib access to 0.2 M L-proline drinking solution (Pro vol), or daily oral administration of L-proline solution (Pro po). (n = 8 in each group).

voluntary proline consumption was initially without effect and was only slightly protective at later timepoints, possibly when these animals had learned to increase their daily consumption. Proline infusion p.o., again, significantly delayed the appearance of jaundice and mortality as compared to the other groups. Thus, it appears that the effect of proline cannot be replaced nonspecifically by other amino acids.

In addition to the amino acid, proline, and copper, we wanted to see if other dietary factors might also affect the disease progression of LEC rats. Ascorbic acid seemed to be a likely candidate due to its antioxidant properties and effects on bodily copper disposition. Initial screening, to determine whether mature LEC rats that suffer from chronic hepatic disease (28) would consume an ascorbate solution, found that whereas a 5 mg/ml solution was initially heavily consumed, appetite for this solution quickly waned. However, either a 4 mg/ml or 3 mg/ml solution seemed to be the stably consumed preferred concentration (Fig. 4). However, young LEC rats (5 weeks old) initially consumed very little of a 3 mg/ml ascorbate solution, but did tend to increase their consumption over time, in preference to distilled water, especially about the time (8–10 weeks of age) jaundice was becoming evident (Fig. 5). This indicates that ascorbate may help improve the symptoms of hepatitis in LEC rats; however, as was the case for voluntary proline consumption, this appetite developed too late to be of significant effect on mortality. Therefore, ascorbic acid was orally administered daily as a solution (100 mg/day, p.o.). Ascorbate administration was found to be as effective as proline in significantly delaying the appearance of jaundice and was even significantly better than proline to delay the incidence of mortality of LEC rats (Fig. 6).

Because proline and ascorbic acid administered together may have adverse effects on their bioavailability (5), another group of LEC rats was examined to see whether the protective effect of ascorbate and proline administration would be additive. In this experiment, ascorbic acid was added to the diet (0.8% w/w, this concentration did not affect the amount of diet consumed and provided an amount of ascorbic acid equivalent to that given p.o. in the previous experiment) that would be consumed mostly during the lights-off period. Proline was given as a p.o. bolus infusion during the lights-on period, at around 1000 h. In this experiment, as seen previously, proline administration initially did not have a large effect on the appearance of jaundice. Not until about 12 days after jaundice was first noted was there a decrease in the incidence of jaundice observed for the proline group. Both the ascorbic acid-fed group and the ascorbic acid + proline group had a similar delay in the initial rate of appearance of jaundice, but the combined ascorbate and proline treatment group was less severely affected (Fig. 7). Proline or ascorbic acid alone produced similar delays in the incidence of mortal-

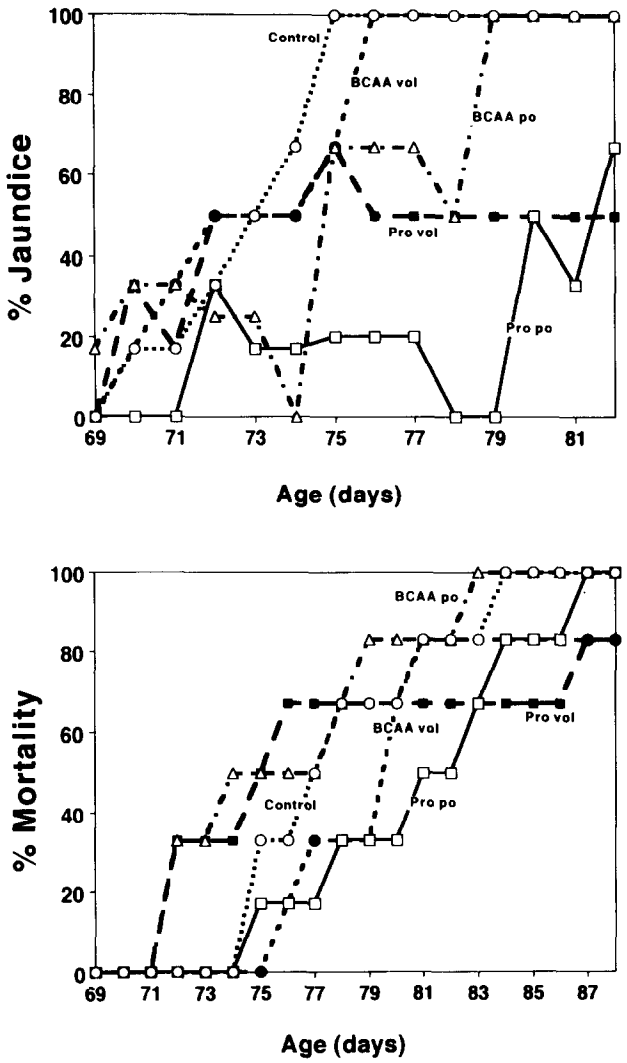

FIG. 3. Mean incidence of jaundice (upper panel) and mortality (lower panel) with age, effect of proline, or BCAA. LEC rats were given only distilled water (control), ad lib access to BCAA solution (0.075 M L-leucine, L-isoleucine, and L-valine) to drink (BCAA vol), daily oral administration of 2.33 mg/100 g b.wt. BCAA (BCAA po), ad lib access to 0.2 M L-proline drinking solution (Pro vol), or daily oral administration of 135 mg/ml proline (Pro po). (n = 6 in each group).

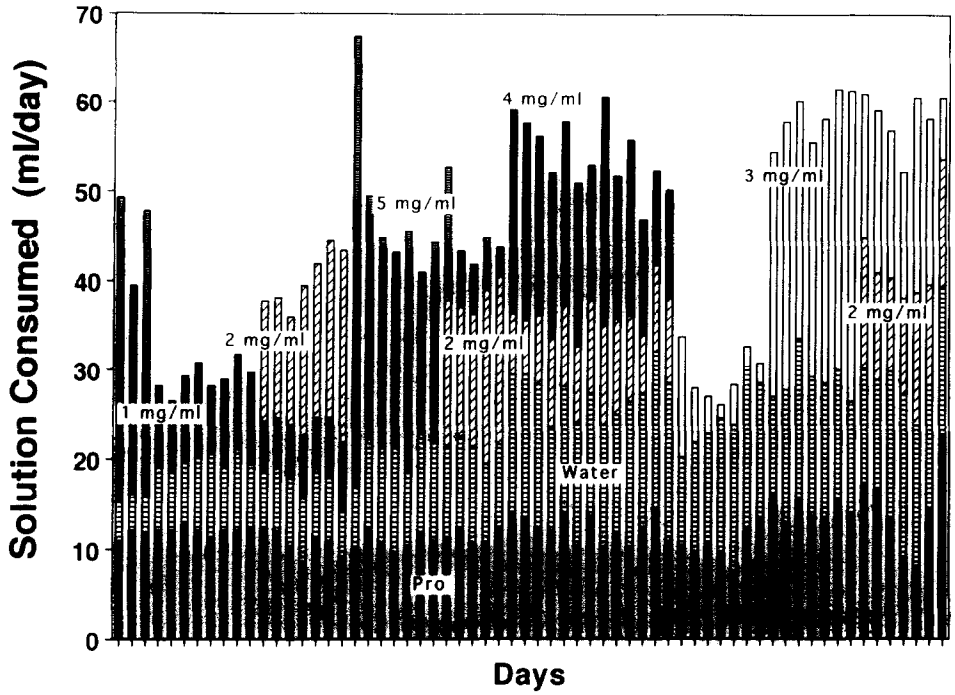

FIG. 4. Mean amount of various concentrations of ascorbic acid solution consumed daily by LEC rats about 6 months of age that had successfully recovered from hepatitis. Distilled water and 0.2 M L-proline were continuously available. Additional ad lib access to solutions of 1, 2, 3, 4, and 5 mg/ml ascorbate was also provided. (n = 11).

ity, with ascorbic acid being slightly more effective, confirming the results of the previous experiment (compare Figs. 6 and 7, bottom panels). Combined treatment with both proline and ascorbic acid led to a further significant delay in mortality, suggesting that the protective effects of these two agents are additive.

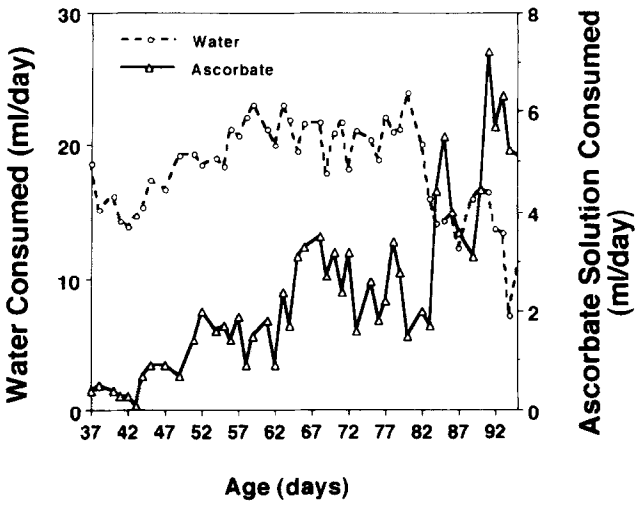

FIG. 5. Increase in the amount of ascorbic acid solution consumed with age of LEC rats. Mean daily consumption of distilled water or 3 mg/ml ascorbate solution. (n = 10 in each group).

The effect of other antioxidants added to the diet was also examined. α-Tocopherol acetate (750 mg/kg diet) or β-carotene (125 mg/kg diet) were added either alone or in combination with each other and ascorbic acid and were found to be without effect on the incidence of jaundice and mortality in LEC rats; in fact, the groups given α-tocopherol or β-carotene alone tended to show earlier mortality (data not shown). This could indicate that copper-mediated free radical damage to the liver is primarily found in the soluble cytosolic compartment, where ascorbate would tend to be localized rather than to membrane lipids and proteins where α-tocopherol and β-carotene are primarily effective (11). Alternatively, it could be that the effect of ascorbic acid is mediated at least in part by its direct chemical interaction with copper in addition to its antioxidant properties.

Therefore, we examined the effect of a different antioxidant agent on LEC rats. Thioredoxin (Trx) has very potent reducing and protein refolding activities and, thus, might be a good candidate for protection against intracellular oxidative damage due to excess copper accumulation. Trx was continuously administered via intraperitoneally implanted osmotic minipumps for a total of 4 weeks. There was only a small dose-dependent effect to delay the appearance of jaundice during the first 2-week period of infusion. However, during the second 2 weeks, the highest dose of Trx (36 μg/h) had a clearly protective effect to reduce the incidence of jaundice, which then disappeared after the minipumps were removed (Fig. 8). However, there was no effect of Trx on the incidence of mortality, suggesting that Trx was only protective against the initial oxidative damage but could not reverse the cumulative hepatotoxicity. Proline consumption was found to be additive with Trx to delay the onset of jaundice (Fig. 9), because

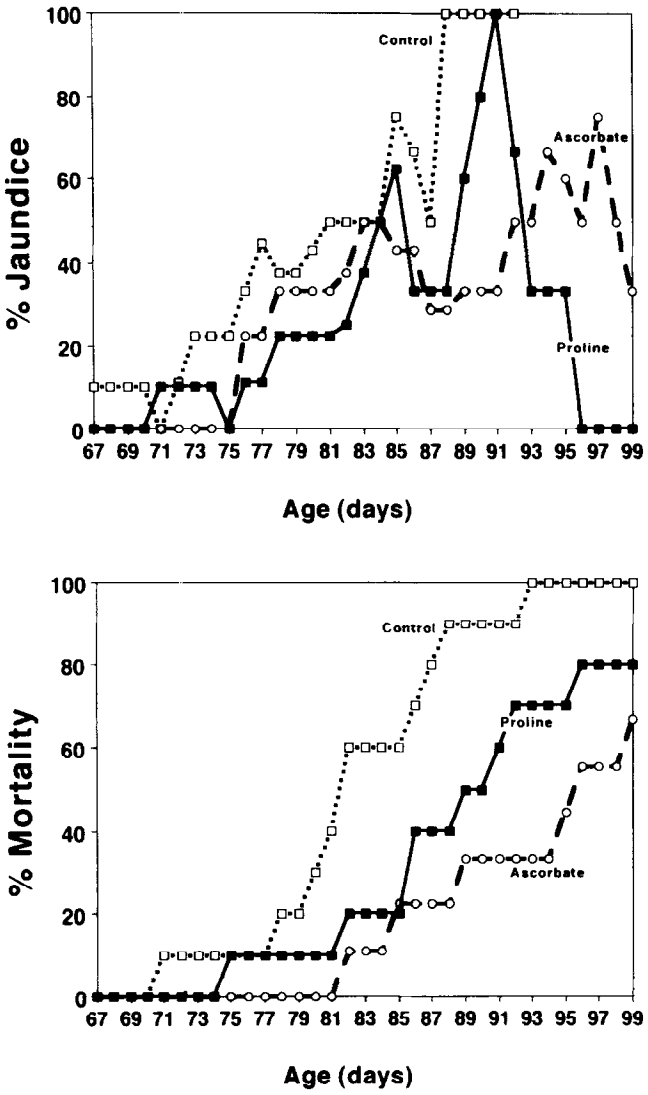

FIG. 6. Mean incidence of jaundice (upper panel) and mortality (lower panel) with age, effect of proline or ascorbate. LEC rats were given daily oral administration of either distilled water (control), 150 mg/day L-proline solution (Proline), or 100 mg/day ascorbic acid solution (Ascorbate). (n = 10 in each group).

the combination of the two agents produced a significantly later appearance of jaundice than either agent alone. Preliminary data for assay of Trx activity in the liver found that LEC rats have significantly increased Trx concentrations at the onset and during the maximal incidence of jaundice but no change prior to or after recovery from jaundice (not shown), suggesting that this endogenous antioxidant system is upregulated to help cope with acute oxidative crisis in LEC rats during the appearance of hepatitis.

DISCUSSION

This work has shown that proline and ascorbic acid have a protective effect to delay the onset of jaundice and eventual mortality in LEC rats. Although excess copper accumulation in the liver seems to be the major pathologic defect as evidenced by the largest protective effect having been observed during feeding with the copper-deficient diet, proline and ascorbic acid might be inexpensive and conveniently useful adjunct therapies in cases of Wilson's disease, and possibly other hepatic disorders, because proline administration has also been found to blunt increases in plasma GOT and GPT levels and to prevent mortality in Fisher rats treated with galactosamine (Mori, unpublished data).

BCAA have been reported to be beneficial in the treatment of chronic liver failure and hepatic encephalopathy (3) and to promote liver regeneration in partially hepatectomized rats

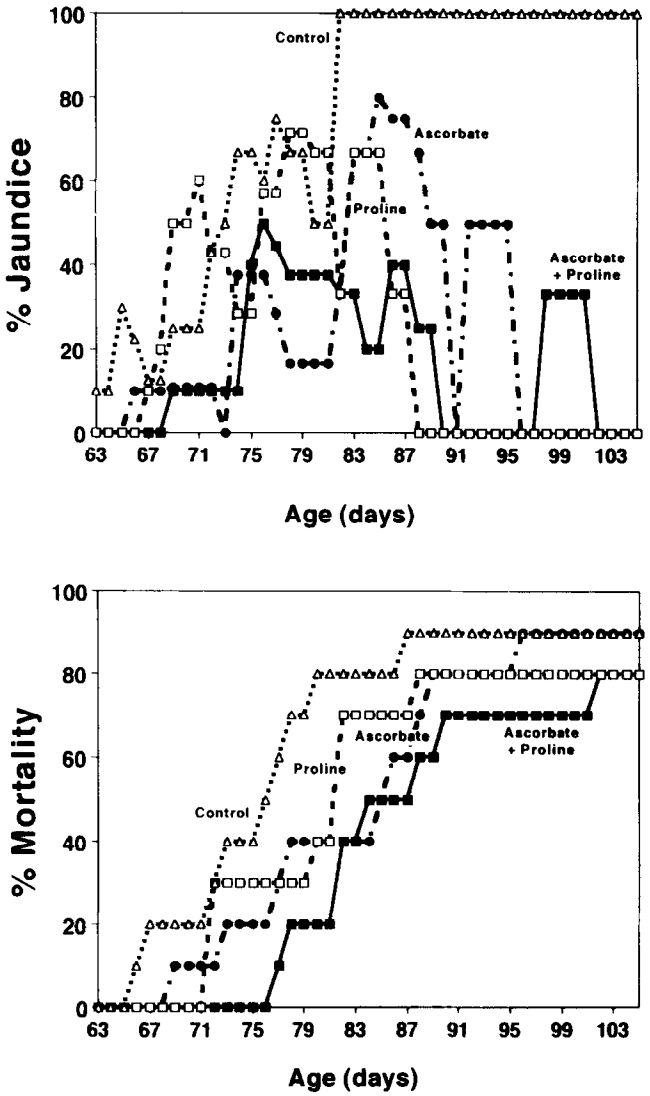

FIG. 7. Mean incidence of jaundice (upper panel) and mortality (lower panel) with age, effect of combined proline and ascorbate. LEC rats were given daily oral administration of distilled water (control and ascorbate groups) or 150 mg/day p.o. L-proline solution (proline and ascorbate + proline groups. Diets were either standard 15% PEP diet (control and proline groups) or 15% PEP with 0.8% ascorbic acid added (ascorbate and ascorbate + proline groups). (n = 10 in each group).

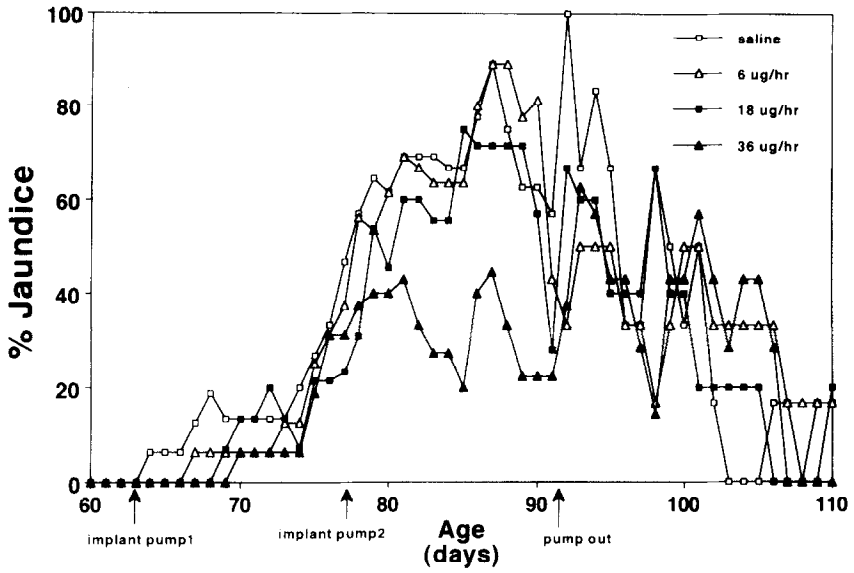

FIG. 8. Mean incidence of jaundice with age, effect of thioredoxin (Trx) administration. Osmotic minipumps were implanted IP to deliver 0.5 μl/h saline, 6 μg/h Trx, 18 μg/h Trx, or 36 mg/h Trx continuously for 2 weeks. After 2 weeks, fresh minipumps were implanted as before and then removed after an additional 2 weeks. (n = 8 in each group).

(6). However, in the present study, there was only a slight nonsignificant effect of BCAA on the appearance of jaundice and mortality of LEC rats. Parenteral supplementation with a mixture of amino acids did not affect mortality of patients with alcoholic hepatitis, but did improve biochemical and metabolic liver function (12). This effect is most likely due to the specific effect of L-alanine and L-glutamine, which have been shown to reverse the metabolic imbalance leading to liver damage consequent to ethanol oxidation in the liver (21). Analysis of the effect on plasma amino acid levels of mixed amino acid infusions to partially hepatectomized rats has found that, in addition to large increases in BCAA, there is also a large elevation in proline levels that accompanies the accelerated rate of liver regeneration (18). In a comparison of amino acids, proline has been reported to be a very strong and the fastest acting amino acid to alter hepatocyte enzymatic activity involved in glycogen synthesis, lipogenesis, and ketogenesis (1). In addition to such metabolic effects, proline has also been shown to be necessary for hepatocyte replication by synergism with epidermal growth factor stimulation of DNA synthesis (8), and also to be necessary for hepatocyte growth and function by virtue of its vital role in collagen synthesis (10,14).

The present results indicate that proline does not seem to be primarily effective during the initial events leading to damage in the jaundice phase in LEC rats, but rather, once liver damage has occurred, on later actions such as growth factor activity or collagen synthesis to promote liver regeneration and, hence, delay death. Because Trx was only protective during the jaundice phase, this suggests that antioxidants can inhibit the primary degenerative events (i.e., copper-catalyzed free radical damage) but do not promote gross hepatic tissue regenerative mechanisms. Trx is able to reactivate enzymatic activity damaged by H_2O_2 oxidation (4,20) and to promote correct protein refolding of scrambled (i.e., mispaired reoxidized disulfides) proteins (13,17), thereby reversing free radical damage. Ascorbic acid seems to be beneficial both to delay jaundice, possibly due to its antioxidant properties, and to delay mortality, possibly a combination of its effects as a cofactor for collagen synthesis and in alteration of copper disposition. Collagen synthesis has been shown to be a vital determinant of the survival of hepatocytes in culture (2,10,14). Furthermore, copper has been shown to catalyze the H_2O_2-mediated oxidative degradation of collagen, primarily by fragmentation at proline residues (9), presumably due to its

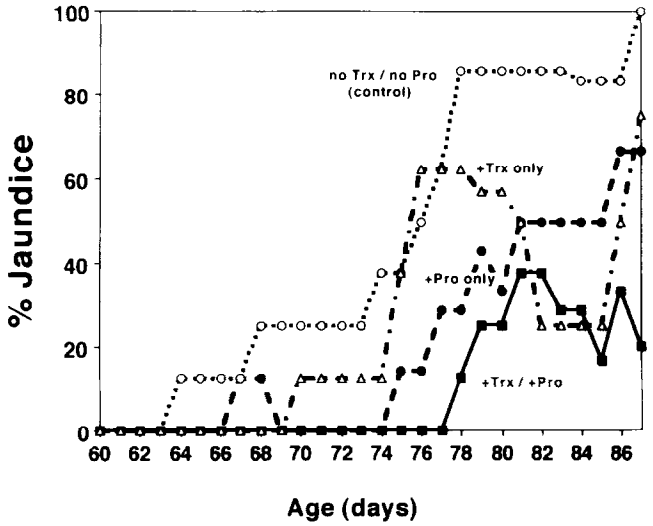

FIG. 9. Mean incidence of jaundice with age, effect of thioredoxin (Trx) and/or proline. Osmotic minipumps were implanted IP to deliver 36 μg/h Trx (+Trx groups) or saline (no Trx groups). Distilled water was available ad lib. Proline solution (0.2 M L-proline) was also available ad lib to +Pro groups. (n = 8 in each group).

promotion of protein damage by generation of superoxide radicals from H_2O_2 (19), which are readily scavenged by ascorbate (15). Additionally, ascorbic acid supplementation to the diets of Wistar rats, at levels similar to that used in the present experiment, reduced plasma and tissue (including liver) concentrations of copper (26). This effect was attributed mainly to reduced intestinal absorption of copper, but there was also a significant effect to stimulate transit through the liver with increased biliary excretion of copper.

Although α-tocopherol has been reported (27) to delay the onset of jaundice and mortality in LEC rats, the concentrations of α-tocopherol used (from 2 to 58.5%, w/w of total diet) were impractically high compared to the supplemented diet tried in the present study (0.075% w/w in addition to the standard PEP diet concentration of 0.01% used in all experiments here). This lower level of tocopherol supplementation was found to be without effect, as was β-carotene supplementation.

This work indicates that proline and ascorbic acid are effective to delay the onset of jaundice and mortality in LEC rats and should be considered as additional approaches to chelation therapy in patients with Wilson's disease. The effect of proline and ascorbic acid on other forms of hepatic dysfunction should also be investigated because preliminary data also have shown a beneficial effect in rats treated with galactosamine.

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