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## Administration of Hepatic Stimulatory Substance Alone or with Other Liver Growth Factors Does Not Ameliorate Acetaminophen-induced Liver Failure

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### Abstract

Sixty-two beagle dogs were given three doses of acetaminophen over a period of 24 hr in a fulminant liver failure model that is 70% lethal in 72 hr. Treatment of the animals with hepatic stimulatory substance alone or in a mixture with insulin, transforming growth factor- $\alpha$  and insulin-like growth factor II had no effect on mortality. Evidence of maximum regeneration with a mitotic index 20 to 25 times resting was the same in treated and untreated animals. Similarly, the biochemical and hematological indexes of liver injury were unaffected by therapy. These studies illustrate the futility of treating fulminant liver failure with exogenous growth factors that apparently are already present in large amounts in the natural response to liver injury. The results suggest that on-going liver injury by mechanisms other than lack of growth factors is the central problem of fulminant liver failure. If so, provision of regeneration-stimulating substance is an inappropriate therapeutic strategy.

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A commonly proposed strategy for treatment of fulminant liver failure (FLF) has been the administration of factors that can initiate and sustain hepatocyte regeneration (1-4). In the last several years, purified growth factors to confirm or refute this hypothesis have become available (5). We report here the testing of the hepatic stimulatory substance (HSS) that is found in the livers of weanling rats or in the regenerating liver fragments after partial hepatectomy (6). The HSS was infused alone or in combination with other hepatic stimulatory factors into the portal veins of dogs in which FLF was caused by acetaminophen in 80% of untreated animals (7,8).

## MATERIALS AND METHODS

### Chemicals

Acetaminophen and DMSO were purchased from Sigma Chemical Co., St. Louis, MO. Hepatocyte factors that have been shown to be stimulatory in our Eck's fistula model for screening growth factors were transforming growth factor- $\alpha$  (Peninsula Laboratories Inc., Belmont, CA); human regular semisynthetic insulin (E.R. Squibb and Sons Inc., Princeton, NJ); insulin-like growth factor II (Collaborative Research, Inc., Bedford, MA); and HSS, produced in our laboratory with previously described methods (6). The HSS was purified 800,000 times

from the cytosol of weanling rats (9) and had no known relation to hepatocyte growth factor of Zarnegar and Michalopoulos (10) and Nakamura et al. (11). We used doses of the continuously infused growth factors that were twice those shown with earlier testing in an Eck's fistula model to give a maximum response (5). Murine (PCNA) monoclonal antibody (Signet Laboratory, Inc., Dedham, MA) was used to detect the protein cyclin, which appears on the nucleus of dividing cells.

### Animals

Sixty-two beagles (Russel B. Hutton Farms, St. Thomas, P A) were housed in a large animal care facility and maintained at a constant temperature of  $20^{\circ} \pm 1^{\circ}$  C, with a 6 AM to 6 PM light cycle. The animals were quarantined and allowed to acclimatize in the animal facility for a minimum of 1 wk before being used. All dogs were given a standard dry dog chow and water *ad libitum*. Their body weights ranged from 9 to 14 kg.

### Acetaminophen Intoxication

FLF was produced with three subcutaneous injections of acetaminophen in DMSO (7,8) at a concentration of 600 mg/ml. The first dose (750 mg/kg) was at noon, the second (200 mg/kg) was 9 hr later and the third (200 mg/kg) was 24 hr after the initial injection. In the original validation of the methods, it was demonstrated that the methemoglobinemia and nephrotoxicity of the acetaminophen could be separated from the liver injury (7), performing multiple injections of drug. The death of all animals herein reported was caused by liver failure.

### Blood or Serum Determinations

Multiple determinations of the serum or blood before and at various time intervals after acetaminophen administration were obtained. These determinations included levels of serum ALT, ammonia, albumin, bilirubin and cholesterol and the level of blood urea nitrogen. Amino acid profiles were determined with plasma deproteinized with 4% sulfosalicylic acid. The resultant supernatant was applied to an amino acid analyzer (Beckman Instruments, Somerset, NJ), and the levels of free branched chain and aromatic amino acids (including tryptophan) were determined.

Three coagulation parameters were measured: fibrinogen, factor VII and factor X (12-15). In addition, the hematocrit and hemoglobinuria and methemoglobinemia (16) were measured at each time point.

### Histological Conditions

All nonsurviving dogs underwent necropsies that included a full gross anatomic and microscopic examination of the liver, kidneys, lungs and heart. Tissues were fixed overnight in 10% neutral-buffered formalin, dehydrated, embedded in paraffin and cut into 5- $\mu$ m sections. All sections were stained with hematoxylin and eosin for histological examination. Other specimens were used for hepatocyte proliferation study.

### Hepatocyte Proliferation Measures

We determined an index of liver cell proliferation in a nonnecrotic area by counting the number of cyclin-labeled cells. PCNA/cyclin is a 36-kD nuclear protein that increases from the late G<sub>1</sub> phase through the S phase of the cell cycle. The monoclonal antibody against PCNA/cyclin was used with the immunoperoxidase method. The positive-stained cells were counted per 1,000 cells (17-18).

## Study Design

At laparotomy with dogs anesthetized with intravenous sodium pentobarbital, halothane and nitrous oxide, we inserted the tip of a 20-gauge catheter from jejunal vein into the portal vein proximal to its bifurcation into right and left branches. The catheter was externalized by tunneling it through the body wall, and it was connected to a pump that delivered 20 ml saline every 24 hr. Five days later, the dogs were poisoned with acetaminophen by use of the protocol described above. Twenty hours after the acetaminophen injection, the animals were randomly divided into three groups to receive one of three different portal perfusion solutions (Table 1). Blood for analysis was obtained before the first acetaminophen injection at 24, 48 and 60 hr, subsequently. Liver specimens were obtained at necropsy, which was performed immediately after death or after euthanasia of animals that appeared to be dying.

## Statistical Analysis

Survival curves were generated for the three groups with the Kaplan-Meier method. Median survival times were compared with the generalized Wilcoxon (Breslow) test.

## RESULTS

### Clinical Behavior

No animal died in the first 24 hr after acetaminophen administration. Two dogs that died immediately thereafter, without an increase in their serum level of transaminase but with very high levels of methemoglobinemia, were eliminated from the analysis. From 48 through 72 hr a progressive increase in mortality was observed in group 1 control animals, reaching 75% at 72 hr. These results were similar to those reported previously in untreated animals (7,8). The survival rate in animals treated with HSS alone (group 2) or HSS plus a cocktail of other growth factors (group 3) was not significantly different from that in the group 1 controls (Fig. 1) ( $p = 0.934$ ).

All of the animals demonstrated a clinical picture consistent with FLF that paralleled the biochemical and histological data. Two different stages of the clinical course of these animals could be identified. The first stage was characterized by gastrointestinal signs and symptoms such as anorexia, vomiting and diarrhea. The second stage was characterized by the appearance of neurological symptoms that rapidly progressed to coma.

### Biochemical and Hematological Changes

The evolution of biochemical (Table 2) and hematological abnormalities (Table 3) was similar in the control and treated animals of groups 2 and 3.

### Histological Examination and Hepatocyte Proliferation

Histological examination of livers obtained at the time of autopsy in dogs in groups 1, 2 and 3 demonstrated the same severe necrosis involving all of the lobes that we previously reported (7).

The number of cyclin-stained hepatocytes per 1,000 hepatocytes was 38 to 44 in all three groups, with no significant difference. This finding was 20 to 25 times higher than the number of hepatocytes in normal dog livers (1.5/1,000) (Fig. 2), which was approximately the same as the peak response in dogs after 70% hepatectomy (19) and three times greater than the maximal response to stimulation of the Eck's fistula livers (5).

## DISCUSSION

It has been estimated that recovery from FLF can be expected with 12% to 15% of the normal liver mass. The use of putative hepatic growth factors to achieve this critical functional level has been discouraging. After the demonstration that insulin is critical to a full expression of regeneration (20-23), this hormone alone (or usually with glucagon) has been tested for the treatment of FLF in mice (24), rats (25,26) and human beings (27,28). The animal studies, particularly in mice, have been encouraging, but the human trials have not shown a benefit from such treatment. The efficacy of noninsulin growth factors also has been controversial (29-30).

Although the number of putative growth factors has greatly increased in the last decade (5, 31,32), evaluation of their therapeutic effectiveness for FLF has been limited by the excessive cost of testing them and the lack of discriminating models that are severe enough to preclude spontaneous recovery but not so severe that the liver injury produced cannot be reversed by any means.

The results described in this report could be an example of this quandary. In these experiments four of the most powerful known *in vivo* stimulators of hepatocyte proliferation were given in a mixture after liver injury by acetaminophen: HSS, transforming growth factor- $\alpha$ , insulin and insulin-like growth factor II. The rate of liver cell regeneration with this mixture, or when HSS was given as a single agent, was no greater than in the untreated animals; therefore the residual surviving cells were already maximally proliferating. Furthermore, the biochemical and hematological manifestations of liver injury were not ameliorated in either of the treatment groups nor was the mortality decreased.

The inability to demonstrate therapeutic efficacy of the growth factors could mean that their use is incorrectly conceived and therefore futile. A high rate of mitosis similar to that in our animals (33,34) has been reported in human beings with FLF. Evidence in animals subjected to carbon tetrachloride injury (35) and in human beings with acute hepatitis (36-37) suggests that at least one growth promoter, hepatic growth factor, increases so much that it is difficult to imagine a benefit from exogenous supplementation of this and presumably other substances that modulate regeneration. Alternatively, our failure to see a therapeutic effect with a growth factor cocktail could mean that the proportions of the constituents were inappropriate.

However, it has been suggested recently that in FLF the inability of the liver to recover from injury may be a result of microcirculatory failure and consequential perpetuation of parenchymal injury, rather than a result of the inability of the hepatocytes to regenerate. This pathogenesis has been particularly evident in the ischemic injury of livers during preservation for use in transplantation (38). The primary cause of failure of these organs is damage to the sinusoidal endothelium that leads to capillary leak, complex coagulopathies and secondary damage to the hepatocytes by hypoperfusion (39). Protection of the microcirculation with prostaglandins (40) or measures that prevent diffuse intravascular coagulation (41) may be more appropriate than the provision of growth factors that already are present in excess.

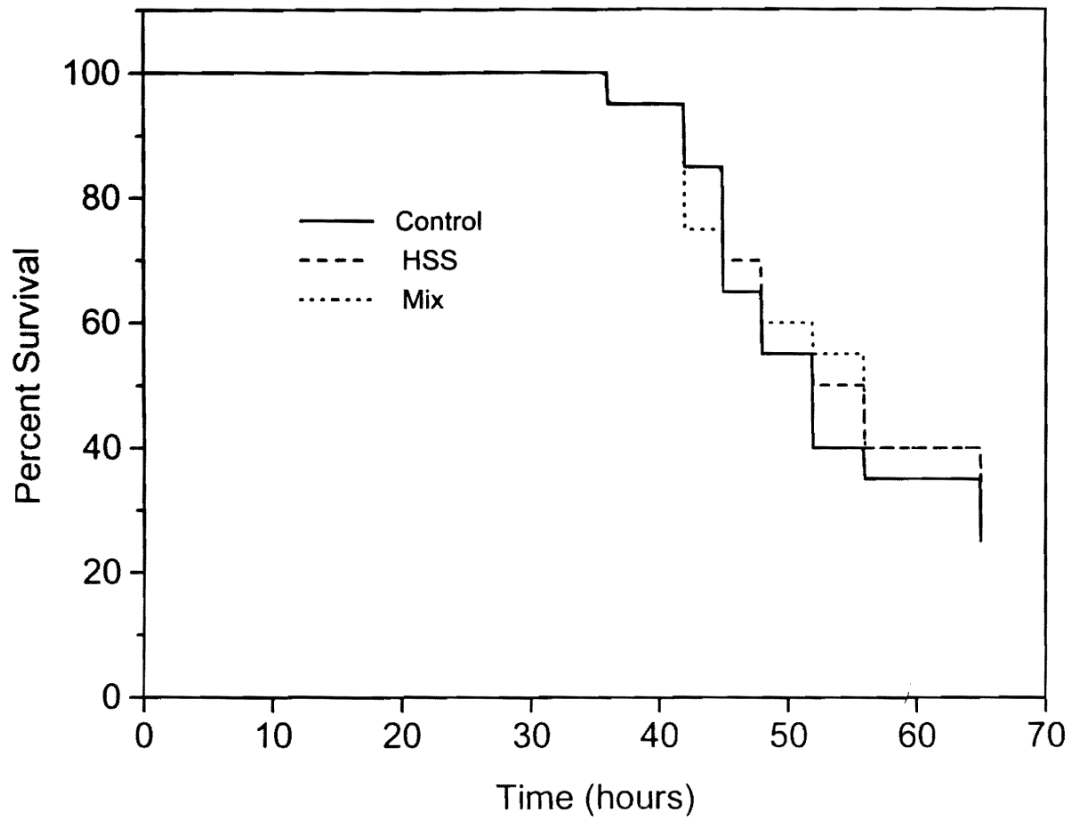
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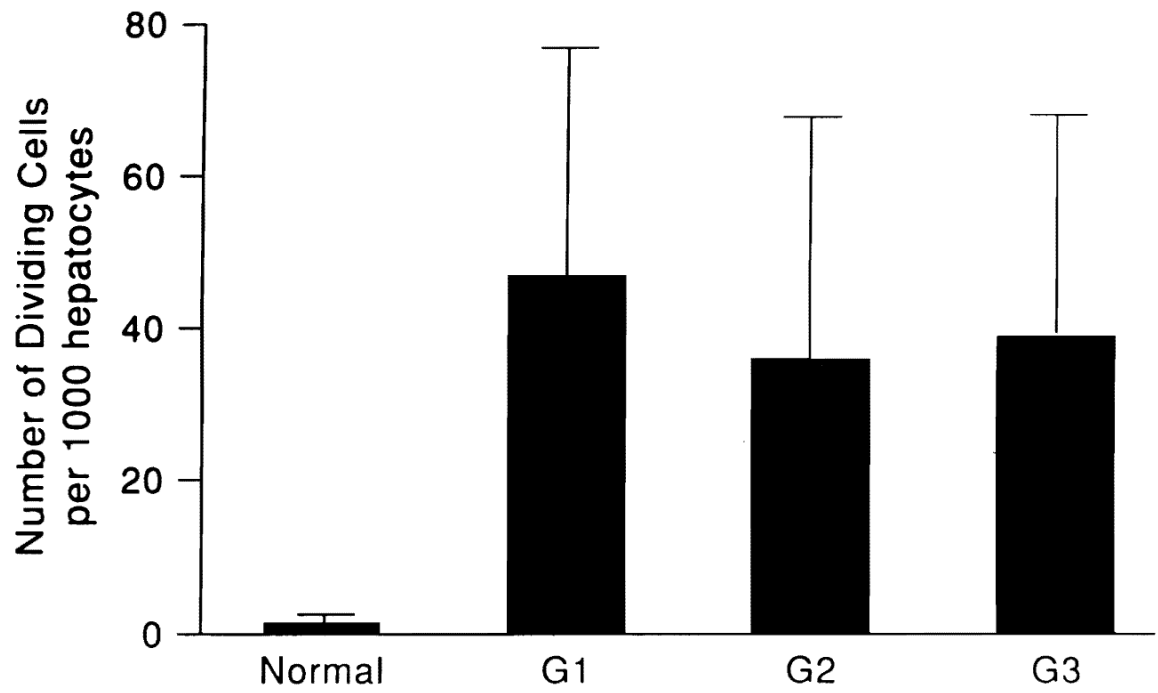
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**Fig. 1.** Survival rates of groups 1, 2 and 3 expressed in percentages.



**Fig. 2.** Number of dividing cells determined by immunocytochemical staining of PCNA/cyclin in the liver of the three groups of dogs and in the liver of normal dogs.



**Table 1**

## Experimental groups and solutions infused

Group	No. of animals	Treatment
1	20	Vehicle <sup>a</sup>
2	20	HSS 100 ng/kg/day
3	20	HSS 100 ng/kg/day, insulin 0.4 U/kg/day, insulin-like growth factor II 100 ng/kg/day, transforming growth factor- $\alpha$ 100 ng/kg/day

<sup>a</sup>Saline modified by the addition of ammonium acetate 5 mmol/L and BSA 5 mg/L.

**Table 2**

Biochemical features in nonsurviving dogs in groups 1, 2 and 3

Groups	0 hr	24 hr	48 hr	60 hr
<b>1</b>				
ALT (U/L)	48 ± 5	105 ± 10	9,206 ± 3,100	24,218 ± 3,783
Bilirubin (mg/100 ml)	1.2 ± 0.1	0.9 ± 0.2	1.8 ± 0.3	2.6 ± 0.8
Albumin (gm/100 ml)	3.2 ± 0.4	2.6 ± 0.3	1.9 ± 0.1	1.8 ± 0.22
Ammonia (μmol/L)	92 ± 15	310 ± 120	304 ± 60	625 ± 250
BCAA/AAA (μmol/L)	3.14			0.88
<b>2</b>				
ALT (U/L)	45 ± 4	65 ± 6	8,140 ± 9,810	21,112 ± 6,250
Bilirubin (mg/100 ml)	1.0 ± 0.1	0.8 ± 0.7	1.2 ± 0.12	2.0 ± 0.3
Albumin (gm/100 ml)	3.0 ± 0.35	2.5 ± 0.3	2.0 ± 0.4	1.8 ± 0.18
Ammonia (μmol/L)	86 ± 26	197 ± 2.8	270 ± 34	498 ± 121
BCAA/AAA (μmol/L)	3.0 ± 0.45			0.94 ± 0.04
<b>3</b>				
ALT (U/L)	50 ± 5	78 ± 7	8,115 ± 3,325	20,083 ± 5,352
Bilirubin (mg/100 ml)	0.9 ± 0.2	1.0 ± 0.2	1.1 ± 0.1	2.2 ± 0.25
Albumin (gm/100 ml)	3.2 ± 0.5	2.8 ± 0.35	2.1 ± 0.35	1.9 ± 0.19
Ammonia (μmol/L)	79 ± 23	186 ± 38	254 ± 62	465 ± 145
BCAA/AAA (μmol/L)	2.9 ± 0.2	-	-	0.96 ± 0.07

BCAA = branched chain amino acid; AAA = aromatic amino acid.

**Table 3**

Coagulation profile in nonsurviving dogs of groups 1, 2 and 3

Groups	0 hr	24 hr	48 hr	60 hr
1				
Fibrinogen mg/100 ml	294 ± 42	140 ± 32	45 ± 18	20 ± 3
Factor VII U/ml	50.68 ± 0.9	0.80 ± 0.06	0.39 ± 0.02	0.20 ± 0.03
Factor X U/ml	3.99 ± 0.3	0.74 ± 0.08	0.20 ± 0.02	0.19 ± 0.01
2				
Fibrinogen mg/100 ml	300 ± 54	155 ± 28	60 ± 10	37 ± 6
Factor VII U/ml	4.55 ± 0.9	0.89 ± 0.08	0.40 ± 0.06	0.20 ± 0.03
Factor X U/ml	2.99 ± 0.8	0.90 ± 0.07	0.30 ± 0.04	0.21 ± 0.04
3				
Fibrinogen mg/100 ml	320 ± 62	188 ± 30	60 ± 11	29 ± 8
Factor VII U/ml	4.00 ± 0.7	0.90 ± 0.08	0.30 ± 0.04	0.28 ± 0.05
Factor X U/ml	3.81 ± 0.2	0.96 ± 0.07	0.20 ± 0.03	0.25 ± 0.04