Protective Effect of ACTIValue N-931 Complex, a Mixture of *Aloe vera* and *Silybum marianum*, on Experimental Acute Liver Injury

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Abstract – The aim of this study was to investigate the hepatoprotective effect of ACTIValue® N-931 complex, a mixture of *Aloe vera* and *Silybum marianum*, against acute liver injuries. Acute liver damages were induced by intraperitoneal injection of galactosamine (GalN, 700 mg/kg), naphthylisothiocyanate (ANIT, 40 mg/kg) and ethionine (500 mg/kg). ACTIValue® N-931 complex (85, 170 and 340) was administered orally 48 h, 24 h, 2 h before and 6 h after the injection of hepatotoxins. At 24 h after GalN treatment the levels of serum aminotransferases and hepatic lipid peroxidation were significantly elevated, whereas hepatic glutathione, serum triglyceride (TG) and total cholesterol were decreased. These changes were attenuated by ACTIValue® N-931 complex. The serum aminotransferase activities and total bilirubin significantly increased at 48 h after ANIT treatment, but were attenuated by ACTIValue® N-931 complex. The bile flow was lower after ANIT treatment, which was restored by ACTIValue® N-931 complex. ACTIValue® N-931 complex reduced the ethionine-induced elevated hepatic TG contents. Histopathological analysis revealed that signs of liver injury were prominent at 24 h as result of ethionine injection, demonstrated by extensive areas of fatty change and microvesicular steatosis were observed around cells. These changes were attenuated by ACTIValue® N-931 complex. Our results suggest that the ACTIValue® N-931 complex has a protective effect on acute liver injury.

Keywords: ANIT, Ethionine, Fatty liver, GalN, Hepatitis

INTRODUCTION

Liver diseases are a major problem throughout the world. Many environmental toxins cause liver injury to human, and despite new advances in hepatology, the treatment for liver diseases does not resolve the problems caused by these toxins. Furthermore despite the increasing need for agents to protect the liver from damage, modern medicine lacks a reliable liver protective drug (Park *et al.*, 2008). Therefore, there has been considerable interest in the role of complementary and alternative medicines for the treatment of liver diseases (Galati *et al.*, 2005). Recently, so-called health foods, including functional foods and dietary supplements, are being increasingly used world-wide on part of complementary and alternative medicine or self-medication

(Onishi and Yokoyama, 2004).

It is well known that the administration of carbon tetrachloride (CCl₄), ethionine and galactosamine (GalN) lead to various forms of liver damage (Hatta *et al.*, 1986). These models are frequently used in prospective studies on liver protecting agents because of experimental ease and their mechanisms of action are well-documented (Nakayama *et al.*, 1989). GalN is a hepatotoxicant, an inducer in hepatic injury models, both *in vivo* and *in vitro*. *In vivo*, GalN causes hepatic damage resembling viral hepatitis and drug-induced hepatitis (MacDonald *et al.*, 1987). The development of cholangiolitic hepatitis in human patients receiving certain drug therapies is a clinical concern (Haibior *et al.*, 1994). In rodents, a similar profile of liver damage can be reproduced by the administration of naphthylisothiocyanate (ANIT), and understanding the mechanisms of ANIT-induced hepatotoxicity may provide clues to the pathogenesis of hepatitis in people (Zimmerman *et al.*, 1978). This damage includes

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decreased bile flow, necrosis of bile duct epithelial cells, injury to periportal hepatic parenchymal cells and a pronounced infiltration of neutrophils into periportal regions (Roth and Dahm, 1997). The administration of ethionine to rats leads to a rapid fall in the concentration of adenosine triphosphate (ATP) in the liver (Bartels *et al.*, 1963). The hepatic ATP deficiency is, in turn, followed by a striking inhibition of ribonucleic acid and protein synthesis. These metabolic alterations appear to be intimately related to the induction of fatty liver by ethionine (Farber *et al.*, 1964).

Aloe vera commonly known as Ghee kanwar (Hindi) is a cactus-like plant that grows in hot, dry climates such as dessert. Its survival in such a harsh environment encourages people to believe that *Aloe vera* has wound-healing and antibiotic effects (Reynolds *et al.*, 1999). An *Aloe vera* leaves is used in diseases of eyes and enlargements of spleen and liver (Chandan *et al.*, 2007). And its liver protective function via anti-oxidative and anti-inflammatory effects are well known (Lim *et al.*, 2003; Can *et al.*, 2004). *Silybum marianum* (Milk thistle) is an herbal supplement used to treat liver and biliary disorders (Post-White *et al.*, 2007). It recently has been investigated for use as a cytoprotectant, an anticarcinogen, and a supportive treatment for liver damage from *Amanita phalloides* poisoning. Silymarin, an active ingredient of *Silybum marianum*, is a strong antioxidant that has been proved to promote liver cell regeneration, to reduce blood cholesterol and to help prevent cancer (Vaknin *et al.*, 2007).

This study examined the hepatoprotective properties of ACTIValue® N-931 complex, a mixture of *Aloe vera* and *Silybum marianum*

MATERIALS AND METHODS

Chemicals and reagents

ACTIValue® N-931 complex was provided from Univera, Incorporation. ANIT, ethionine, GalN, glutathione, 2,6-di-tert-butyl-4-methylphenol and 2-thiobarbituric acid were purchased from the Sigma Chemical Co. USA. All other chemicals and reagents used in this study were of reagent grade available.

Animals

Male or female Sprague-Dawley rats weighing 240-260g were obtained from Hyundai-Bio Animal Breeding Company of Korea. All the animals were treated humanely under the Sungkyunkwan University Animal Care Committee Guidelines. Rats were maintained in a

room with controlled temperature and humidity ($25 \pm 1^\circ\text{C}$ and $55 \pm 5\%$, respectively) with a 12 h light-dark cycle.

Acute hepatotoxicity

GalN was dissolved in phosphate buffered saline (PBS) and administered intraperitoneally (700 mg/kg). The male rats were randomly assigned to five groups: i) vehicle-treated control; ii) vehicle-treated GalN; iii) ACTIValue® N-931 complex 85 (a mixture of *Aloe vera* 35 mg/kg and *Silybum marianum* 50 mg/kg)-treated GalN; iv) ACTIValue® N-931 complex 170 (a mixture of *Aloe vera* 70 mg/kg and *Silybum marianum* 100 mg/kg)-treated GalN; v) ACTIValue® N-931 complex 340 (a mixture of *Aloe vera* 140 mg/kg and *Silybum marianum* 200 mg/kg)-treated GalN. ACTIValue® N-931 complex administered orally 48 h, 24 h and 2 h before and 6 h after GalN intraperitoneal injection. The dose and timing of ACTIValue® N-931 complex were selected based on previous reports (Lu *et al.*, 2002; Chandan *et al.*, 2007) and our preliminary studies. Blood samples were taken from the abdominal aorta and the liver was removed 24 h after GalN-treatment. ANIT was dissolved in olive oil and injected intraperitoneally (40 mg/kg). The male rats were randomly assigned to five groups: i) vehicle-treated control; ii) vehicle-treated ANIT; iii) ACTIValue® N-931 complex 85-treated ANIT; iv) ACTIValue® N-931 complex 170-treated ANIT; v) ACTIValue® N-931 complex 340-treated ANIT. ACTIValue® N-931 complex administered orally 48 h, 24 h and 2 h before and 6 h after ANIT intraperitoneal injection. The bile duct was cannulated with a polyethylene tube-10 and the bile flow was measured gravimetrically in prepared tubes. Blood samples were taken from the abdominal aorta and the liver was removed 48 h after ANIT-treatment. Ethionine was dissolved in PBS and injected intraperitoneally (500 mg/kg). The female rats were randomly assigned to five groups: i) vehicle-treated control; ii) vehicle-treated ethionine; iii) ACTIValue® N-931 complex 85-treated ethionine; iv) ACTIValue® N-931 complex 170-treated ethionine; v) ACTIValue® N-931 complex 340-treated ethionine. ACTIValue® N-931 complex administered orally 48 h, 24 h and 2 h before and 6 h after ethionine intraperitoneal injection. Blood samples were taken from the abdominal aorta and the liver was removed 24 h after ethionine treatment.

Analytical procedures

The levels of serum alanine aminotransferases (ALT), aspartate aminotransferase (AST) total cholesterol (TC) and triglyceride (TG) were determined by standard spectrophotometric procedures using the ChemiLab ALT, AST,

TC and TG assay kit (IVDLab Co.), respectively. The total glutathione level was measured spectrophotometrically at a wavelength of 412nm using yeast glutathione reductase and 5, 5'-dithio-bis (2-nitrobenzoic acid), as described by Tietze (1969). The oxidized glutathione (GSSG) level was measured using the same method but in the presence of 2-vinylpyridine (Griffith, 1980). The level of reduced glutathione (GSH) was determined by the difference between the total glutathione and GSSG levels. The steady-state level of malondialdehyde (MDA), which is the end product of lipid peroxidation, in the liver was determined by measuring the level of thiobarbituric acid-reactive substances spectro photometrically at 535 nm using the method described by Buege and Aust (1978). Bile flow was calculated from the volume of bile collected, time of collection, and liver wet weight and indicated as ml /min /g liver.

Histopathological analysis

The liver slices were made from a portion of left lobe, and fixed immediately in a 10 % formalin buffered phosphate solution, embedded in paraffin and sectioned at 5mm thickness. The serial sections were stained with hematoxylin and eosin (H&E) to evaluate the portal inflammation, hepatocellular necrosis, inflammatory cell infiltration, macro- and micro-vesicular steatosis. The sections were examined in a blind manner under an Olympus CKX 41 microscope (Olympus optical Co. Ltd., Tokyo, Japan) (karaa *et al.*, 2005).

Statistical analysis

All the results are presented as mean $\pm$ S.E.M. One-way analysis of the variance (ANOVA) followed by a Dunnett's test was used to determine the significance of the differences between the experimental groups. A p value <0.05 was considered significant.

RESULTS

Effect of ACTIValue® N-931 complex on GalN-induced hepatitis

The serum ALT activity in the control group was very low. The serum ALT activities increased markedly 24 h after the GalN injection. These increases were attenuated by the ACTIValue® N-931 complex 170. Similar to ALT activity, the serum AST activities increased significantly 24 h after GalN injection and these increases were attenuated by ACTIValue® N-931 complex 85, 170 and 340. The MDA level increased significantly in the GalN-injected rats. ACTIValue® N-931 complex did not affect the level of MDA. GSH in the GalN-injected rats decreased to 77 % of the control and this decrease was slightly attenuated by ACTIValue® N-931 complex 170 and 340 (Table I). As shown in Fig. 1, the serum concen-

Fig. 1. Effect of ACTIValue® N-931 complex on serum triglyceride and total cholesterol levels in GalN-induced hepatitis. The values are represented as the mean $\pm$ SEM for 7-8 rats per group. *,**Significantly different ($P<0.05$, $P<0.01$) from control group. #Significantly different ($P<0.05$) from GalN group. GalN, D-galactosamine; N-931C, ACTIValue® N-931 complex.

Table I. Effect of ACTIValue® N-931 complex on the serum aminotransferase activity, hepatic lipid peroxidation and glutathione content in the GalN-induced rats

Treatment	ALT (IU/L)	AST (IU/L)	MDA (nmol/mg protein)	GSH (mmol/g liver)
Control	30.8 $\pm$ 0.4	110.0 $\pm$ 3.2	0.34 $\pm$ 0.02	8.5 $\pm$ 0.4
Vehicle + GalN	160.6 $\pm$ 11.3**	444.1 $\pm$ 8.4**	0.51 $\pm$ 0.05**	6.5 $\pm$ 0.2**
N-931 C85 + GalN	126.7 $\pm$ 6.0**	358.8 $\pm$ 10.1**, #	0.46 $\pm$ 0.03**	6.6 $\pm$ 0.4*
N-931 C170 + GalN	94.4 $\pm$ 11.0*, ##	271.7 $\pm$ 36.4*, ##	0.48 $\pm$ 0.02**	7.2 $\pm$ 0.4
N-931 C340 + GalN	109.5 $\pm$ 4.7*	271.7 $\pm$ 36.4**, ##	0.52 $\pm$ 0.04**	7.6 $\pm$ 0.4

The values are the mean $\pm$ S.E.M. of 8 rats per group. *,**Significantly different ($p<0.05$, $P<0.01$) from the control group. #,##Significantly different ($p<0.05$, 0.01) from the vehicle-treated GalN group. GalN, D-galactosamine N-931C, ACTIValue® N-931 complex.

Fig. 2. Histological analysis of the livers after GalN administration. (A) Control, normal architecture of liver. (B) GalN, inflammation of lymphocytes and Kupffer cells and hyaline degeneration around the portal vein area. (C) ACTIVAlone® N-931 complex+ GalN, minimal hepatocellular inflammatory cell infiltration and mild portal inflammation. All the photographs are at $\times 100$ magnification.

tration of TG in the control was 74.5 ± 1.84 mg/dl. The serum TG concentrations significantly decreased after the GalN-injected rats. These decreases were attenuated by the ACTIVAlone® N-931 complex 170. The serum TC levels in the GalN-injected group significantly decreased. ACTIVAlone® N-931 complex did not affect the level of serum TC. Histopathological analysis revealed that signs of liver injury were prominent at 24 h as a result of GalN injection, demonstrated by severe hepatocyte necrosis, inflammatory cells infiltration. ACTIVAlone® N-931 complex significantly attenuated these pathological alteration in the liver of GalN-treated rats (Fig. 2).

Effect of ACTIVAlone® N-931 complex on ANIT-induced cholestasis

The serum levels of ALT and AST in the control group were 30.8 ± 0.40 IU/l and 100 ± 10.0 IU/l, respectively. 48 h after ANIT injection, the serum ALT and AST levels increased to approximately 39.0 and 20.4 times those in the control group, respectively. Treatment of ACTIVAlone® N-931 complex 170 significantly reduced these increases (Fig. 3). Bile flow in the control group was 1.26 ± 0.02 μ l/min/g liver. In the vehicle-treated ANIT group, the level of bile flow decreased to 0.17 ± 0.04 μ l/min/g liver. The decrease in bile flow was markedly restored by ACTIVAlone® N-931 complex 170. In contrast, the serum TB level increased significantly 48 h after ANIT injection, which was attenuated by ACTIVAlone® N-931 complex 170 (Table II).

Effect of ACTIVAlone® N-931 complex on ethionine-induced fatty liver

As shown in Table III, the level of hepatic TG was 3.3 ± 0.3 mg/g liver in the control group. On the other hand, in the vehicle-treated ethionine group, the level of hepatic TG increased to 2 times that of the control group. This increase was significantly attenuated by treatment with ACTIVAlone® N-931 complex 170 and 340. The level of

Fig. 3. Effect of ACTIVAlone® N-931 complex on serum ALT and AST activities in ANIT-induced cholestasis. The values are represented as the mean $\pm$ SEM for 7-8 rats per group. **Significantly different ($P < 0.01$) from control group. #Significantly different ($P < 0.05$) from ANIT group. ANIT, α -naphthylisothiocyanate; N-931C, ACTIVAlone® N-931 complex.

Table II. Effect of ACTIVAlone® N-931 complex on the bile flow and serum total bilirubin in the ANIT-induced rats

Treatment	Bile flow (μ l/min/g liver)	Serum total bilirubin (mg/dl)
Control	1.26 ± 0.02	0.01 ± 0.01
Vehicle + ANIT	$0.17 \pm 0.04^{**}$	$0.70 \pm 0.05^{**}$
N-931 C85 + ANIT	$0.24 \pm 0.02^{**}$	0.57 ± 0.06
N-931 C170 + ANIT	$0.42 \pm 0.08^{**}, \#$	$0.37 \pm 0.05^{\#}$
N-931 C340 + ANIT	$0.38 \pm 0.07^{**}$	0.48 ± 0.07

The values are the mean $\pm$ S.E.M. of 8 rats per group. **Significantly different ($p < 0.01$) from the control group. #Significantly different ($p < 0.05$) from the vehicle-treated ANIT group. ANIT, α -naphthylisothiocyanate; N-931C, ACTIVAlone® N-931 complex.

serum TC in control group was 55.9 ± 2.06 mg/dl. In the ethionine-treated group, the serum TC level significantly decreased to 12.8 ± 3.09 mg/dl. The ACTIVAlone® N-931

Fig. 4. Histological analysis of the liver after ethionine administration. (A) Control, normal hepatic parenchyma. (B) ethionine, extensive areas of fatty change and microvesicular steatosis were observed around cells. (C) ACTIValue® N-931 complex + ethionine, minimal fatty change and mild microvesicular steatosis. All the photographs are at x400 magnification.

Table III. Effect of ACTIValue® N-931 complex on the liver triglyceride and serum total cholesterol in the ethionine - induced rats

Treatment	Liver triglyceride (mg/ g liver)	Serum total cholesterol (mg/dl)
Control	3.38±0.33	55.9±2.06
Vehicle + Ethionine	6.87±0.31**	12.8±3.09**
N-931 C85 + Ethionine	5.84±0.95*	11.4±1.14**
N-931 C170 + Ethionine	3.70±0.44 ###	11.9±1.32**
N-931 C340 + Ethionine	3.83±0.59##	12.9±1.68**

The values are the mean ± S.E.M. of 8 rats per group. *, ** Significantly different ($p < 0.05$, $P < 0.01$) from the control group. ###Significantly different ($p < 0.01$) from the vehicle-treated ethionine group. N-931C, ACTIValue® N-931 complex.

complex did not affect the level of serum TC. Histopathological analysis revealed that signs of liver injury were prominent at 24 h as a result of ethionone injection, demonstrated by extensive areas of fatty change and microvesicular steatosis were observed around cells. These histopathological changes were ameliorated by ACTIValue® N-931 complex (Fig. 4).

DISCUSSION

Drug/chemical-induced liver injury has been used as a model for screening hepatoprotective agents. Among these toxins, GalN is known as specific hepatotoxic agent. Its hepatotoxicity has much clinical importance because GalN-induced liver injury shows many metabolic and morphological aberrations in the liver of experimental animals, which is pathologically similar to those observed in human viral hepatitis (Nakama *et al.*, 2001; Morikawa *et al.*, 1996). The detail mechanism of GalN-induced hepatotoxicity has not been clearly established

yet, but recent studies suggest that GalN induces the depletion of uracil nucleotides, resulting in inhibition of RNA and protein synthesis in liver and leading to damage of hepatocytes eventually (Kaplowitz *et al.*, 2002; Jaeschke, 2002). Koff *et al.* (1971) observed marked accumulation of hepatic triglyceride, concluding that the cause of GalN-induced fatty liver was inhibition of synthesis of the protein moiety of lipoprotein.

In the vehicle-treated GalN group, the ALT and AST levels increased dramatically compared with the control group, indicating severe hepatocellular damage. In contrast, a treatment with ACTIValue® N-931 complex markedly attenuated the release of ALT and AST. In our preliminary studies, *Aloe vera* or *Silybum marianum* alone suppressed the increase in the serum ALT and AST activities in GalN-induced rats. Combination of *Aloe vera* and *Silybum marianum* appeared to have a further synergistic effect in suppressed the increases in the serum aminotransferase activities (data not shown). The histo-pathological observations of liver sample strongly support the release of aminotransferase by the damaged hepatocytes as well as the protective effect of ACTIValue® N-931 complex. GalN caused various histopathological changes to the liver, including cell necrosis, cell inflammation and ballooning degeneration. These alterations were significantly attenuated by ACTIValue® N-931 complex. Furthermore, ACTIValue® N-931 complex attenuated the decrease in TG concentration induced by GalN. These results indicate that ACTIValue® N-931 complex may enhance lipoprotein synthesis and prevent the accumulation of lipid in liver.

The induction of cell death by GalN is associated with the generation of reactive oxygen species (ROS) in cultured hepatocytes (González *et al.*, 2007). The presence of oxidative stress is caused by either an increase in ROS generation or a depletion of antioxidants. Different antioxidant strategies have shown to be useful to reduce

oxidative stress and cell death in hepatocyte. GSH constitutes the first line of defense against free radicals and is a critical determinant of the tissue susceptibility to oxidative stress (Park *et al.*, 2008). GalN significantly lowered the hepatic GSH level compared to control group. In contrast, the hepatic MDA level was higher than the control group. These results indicate ROS produced in the vehicle-treated GalN groups cause cell damage directly through GSH depletion and the subsequent lipid peroxidation. The treatment of ACTIValue® N-931 complex slightly attenuated the decrease in hepatic GSH content but did not affect to the lipid peroxidation, which suggests that ACTIValue® N-931 complex increases the hepatic pool of GSH and reduces oxidative stress.

ANIT is a toxicant that targets the bile duct and causes an intrahepatic cholestasis which is commonly used as model for human chronic cholangitic diseases (Plaa and Priestly, 1976). ANIT is thought to be bioactivated in the liver by cytochrome P450s. Normally bioactivated ANIT is detoxified in hepatocytes by conjugating with glutathione and catalysed by glutathione S-transferases. ANIT–glutathione complexes are transported into bile, then ANIT–conjugate damages bile-duct epithelial cells, causing cholangiolitis that leads to intrahepatic biliary obstruction and increases accumulation of toxic bile acids in the liver. Thus, ANIT induces hepatotoxicity which involves impair of xenobiotic metabolism and transport leading to cholestasis, inflammation and finally hepatocellular death (Roth *et al.*, 1997). Accordingly total bilirubin concentration and bile flow rate are used as markers of ANIT-induced hepatotoxicity in addition to ALT and AST activities. In our study, ALT and AST levels significantly increased in ANIT-induced rats. However, the increased ALT and AST levels markedly attenuated when ACTIValue® N-931 complex were treated. Also, ANIT causes a significant increase in serum total bilirubin and decrease in bile flow. These changes were reversed by the treatment of ACTIValue® N-931 complex. These results suggest that administration of ACTIValue® N-931 complex enhances bile flow and attenuates hyperbilirubinaemia, promoting recovery from hepatocellular injury.

Fatty liver results from the abnormal accumulation of triglyceride within the cytoplasm of hepatocyte and is a common histopathological finding in human liver biopsies (Ijaz *et al.*, 2003). The reported worldwide prevalence of fatty livers ranges from 16 % to 26 %. Ethionine, causes steatosis in experimental animals of various species (Waterfield *et al.*, 1998). Fat accumulation in the liver can be seen under the electron microscope as lipid droplets form 3-4 h after the administration of large doses of

ethionine (100-1000 mg/kg) to rats (Farber *et al.*, 1964). It has been suggested that the ethionine-induced accumulation of triglycerides in the liver is related to ATP deficiency, since ATP may participate in releasing hepatic triglycerides into the plasma (Farber *et al.*, 1964). Ethionine induces cholesterol accumulation in liver via inhibition of lipoprotein synthesis and accordingly, serum total cholesterol and neutral lipid levels were decreased. In present study, the liver triglyceride contents of ethionine-treated group significantly increased compared with the control. This change was attenuated by ACTIValue® N-931 complex 170 and 340. Furthermore, the histopathological observation of liver strongly shows that ethionine induces extensive areas of fatty change and microvesicular steatosis around hepatocytes and these change were significantly attenuated by ACTIValue® N-931 complex.

Overall, it appears that ACTIValue® N-931 complex protects hepatocytes from hepatotoxicity caused by GalN, ANIT and ethionine. This study provides evidence that ACTIValue® N-931 complex may offer an alternative for the prevention of acute hepatic injury.

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