A Master's Thesis

The effect of HBx on hepatic fibrosis and hepatoprotective effect of *hippocampus abdominalis* on alcohol intoxicated liver

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The effect of HBx on hepatic fibrosis and hepatoprotective effect of *hippocampus abdominalis* on alcohol intoxicated liver

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LIST OF ABBREVIATIONS

HSCs	Hepatic stellate cells
ECM	Extracellular matrix
TGF-β	Transforming growth factor beta
ROS	Reactive oxygen species
EMT	Epithelial-mesenchymal transition
NOX	NADPH oxidase
HBx	Hepatitis B virus X protein
HBV	Hepatitis B virus
HCV	Hepatitis C virus
α-SMA	Alpha-smooth muscle actin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
RT-PCR	Reverse transcription polymerase chain reaction
GOT	Glutamic oxaloacetic acid transaminase
GPT	Glutamic pyruvate transaminase

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PART I. Introduction

1. Liver

The liver is one of the most vital organs in our body. The liver performs many essential functions related to metabolism, digestion, immunity, and the nutrient storage within the body. Without the liver, human body will not be able to gain energy and crucial nutrients. Thus, liver damage is fatal to human body (Jung and Choi 2014). When liver damage is repeated and chronic, it might develop to liver diseases such as fibrosis, cirrhosis, and hepatocellular carcinoma. Recently, researchers have found that liver disease contributes to high percentage of morbidity and mortality rates worldwide (Lozano et al., 2012; Murray et al., 2012).

2. Hepatic fibrosis

Repeated and chronic liver inflammation causes liver diseases such as hepatic fibrosis. Furthermore, advanced liver fibrosis may lead to cirrhosis, liver failure and hepatocellular carcinoma. Cirrhosis, an end-stage of hepatic fibrosis, mostly develops to liver cancer, which contributes to high morbidity and mortality globally. Hepatic fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) and is observed in most types of chronic liver diseases. Therefore, the development of hepatic fibrosis therapy is very important (Albanis and Friedman, 2001). Hepatic fibrosis results from wound-healing response to repeated liver injury. When the liver is injured, liver cells regenerate and replace the damaged and dead cells as a wound-healing response. This process is associated with a limited deposition of ECM proteins. When the damage is repeated and chronic, ECM production becomes continuous abnormal amounts of collagen fiber deposits in the liver, and ultimately hepatic fibrosis progresses. Hepatic stellate cells (HSCs) mainly produce ECM in the injured liver. HSCs are in quiescent

state in the normal liver. If HSCs are stimulated by liver damage such as alcohol and virus infection (hepatitis B,C), quiescent stellate cells differentiate to activated myofibroblast-like phenotype, resulting in increased ECM synthesis. ECM such as fibronection, collagens, lamin, and elastin increases six-fold in the liver in advanced stage of hepatic fibrosis (De Minicis and Brenner, 2007). Thus, HSCs are the main regulator of this phenomenon. Mediators such as TGF-β and reactive oxygen species (ROS) activate HSCs.

3. Reactive oxygen species (ROS)

ROS play an important role. Antioxidant system removes ROS in normal liver, but chronic liver disease not only decreases the system activity, but also increases ROS production and oxidative stress, resulting in HSCs activation. This trait is commonly observed in most types of experimental liver fibrogenesis and in patients with alcohol use disorder, chronic cholestasis, hepatitis C virus infection and iron overload. During liver damage, NADPH oxidase (NOX) complex is one of molecules involved in ROS production and plays a critical role in ROS production. NOX is a multi-protein enzymatic complex which produces ROS in phagocytic and non-phagocytic cells. Produced ROS are essential in host defense and intracellular signaling regulation. Currently, NOX has been considered as a critical regulator of hepatic fibrosis. NOX induces oxidative stress, activates HSCs, macrophage, and Kupffer cells and mediates liver damage and hepatic fibrosis (De Minicis and Brenner, 2007).

4. Epithelial-mesenchymal transition (EMT)

Epithelial-mesenchymal transition (EMT) is another mechanism inducing hepatic fibrosis. EMT is a process of transdifferentiation of epithelial cells into mesenchymal cells. Epithelial

cells normally possess an apical-basal polarity and interacts with basement membrane via their basal surface. During EMT, epithelial cells lose their characteristics and acquire characteristics of mesenchymal cell which are invasiveness, migratory capacity, increased ECM production and elevated resistance to apoptosis. Mesenchymal cell characteristics include cell-to-cell dissociation, downregulation of epithelial markers such as E-cadherin and ZO-1, up-regulation of mesenchymal markers such as N-cadherin and vimentin, and induction of specific transcription factors such as Snail and Twist. Through EMT, hepatic epithelial cells are regarded to transdifferentiate into HSCs in the damaged liver (Raghu and Robert, 2009).

There are several factors that cause liver damage and liver disease such as HBV, HCV infection, alcohol, drug toxins, obesity, etc. Excessive alcohol consumption and infection by the Hepatitis B virus are other common causes of liver damage.

PART II.

Hepatitis B virus X protein induced fibrosis in Huh7 cells

1. Abstract

Hepatitis B virus infection causes liver fibrosis which leads to cirrhosis and hepatocellular carcinoma. However, the mechanism remains poorly understood. In this study, we found that exogenous Hepatitis B virus X protein (HBx) increased fibrotic markers (vimentin and fibronectin) in Huh7 cells, suggesting that HBx induced fibrosis. To examine whether HBx regulates hepatic fibrosis through Epithelial-to-mesenchymal transition (EMT), we validated the expression of EMT regulator, snail and slug. Slug mRNA and protein levels were significantly increased by HBx transfection. On the other hand, snail protein showed no significant increase while HBx transfection increased snail mRNA significantly. Thus, HBx-induced EMT is regulated by slug rather than snail. Oxidative stress caused by NADPH oxidase (NOX) system in liver has been reported to play a critical role in several liver disease and hepatic fibrosis. The mRNA and protein level of NOX4 was increased by HBx transfection. Since NOX4-induced reactive oxygen species may increase slug and snail, HBx may be a key mediator of hepatic fibrosis development via NOX4 induction.

2. Introduction

Hepatitis B virus (HBV) is one of the main causes of hepatic fibrosis. Annually, two billion people are infected with HBV, 360 million with chronic infection, and 500,000-700,000 people die from HBV-related liver disease or hepatocellular carcinoma. Furthermore, half of HCC and one third of cirrhosis can be attributed to chronic HBV infection (Shepard et al., 2006). The HBV genome is 3.2 kb partial duplex DNA and has four open-reading frames (polymerase gene, P; core gene, preC/C; surface gene, preS1/S2/S; X gene, X) (Kim 2010). X gene encodes hepatitis B virus X protein (HBx). HBx is essential for early stage infection during the viral life cycle. In addition, HBx is critical for viral genome replication, HBV-related disease, and development of HCC. It has been reported that HBx affects differentiation, proliferation, apoptosis, and metastasis in cancer cells via various signaling pathways (Yang et al., 2012). One of the well-known pathways that HBx activates is phosphoinositide 3-kinase (PI3K)/Akt. The pathway is related to cell proliferation and anti-apoptotic activity (Ha and Yu, 2010). HBx downregulates p27 and p21, cell cycle progressive factors, and upregulates PCNA, cyclin E and D, cell cycle progressive factors, and consequently promotes cell cycle progression (Mukherji et al., 2007, Park et al., 2006). It has also been reported that HBx expression is abundant in the livers of patients with chronic liver diseases. However, the mechanism of inducing hepatic fibrosis by HBV remains poorly understood.

3. Materials and Methods

3.1 Cell culture

Huh7, the human hepatoma cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and incubated at 37℃ with 5% CO_2.

3.2 Transient transfection

The recombinant pGEM plasmid containing the HBx gene was provided by Dr. Song Byoung-Cheol (Jeju university, Jeju, Korea). We amplified the HBx gene fragment from this recombinant pGEM plasmid using primers containing XhoI or EcoRI restriction sites. HBx was cut out with the restriction enzymes XhoI or EcoRI, and ligated into corresponding restriction sites of the vector pcDNA3. With this vector, we transfected cells using lipofectamin 2000 according to the manufacturer's instructions.

3. 3 RT-PCR

Total RNA was prepared from Huh7 cells using TRIzol (Invitrogen) 48 h after transfection. Then, total RNA was used to synthesize cDNA using a Reverse Transcriptase Kit (Promega). The synthesized cDNA was used for RT-PCR using the G-Taq kit (Cosmo Genetech, Seoul, Korea) according to the manufacturer’s instructions. The results were analyzed using the ImageJ software.

3. 4 Western blot

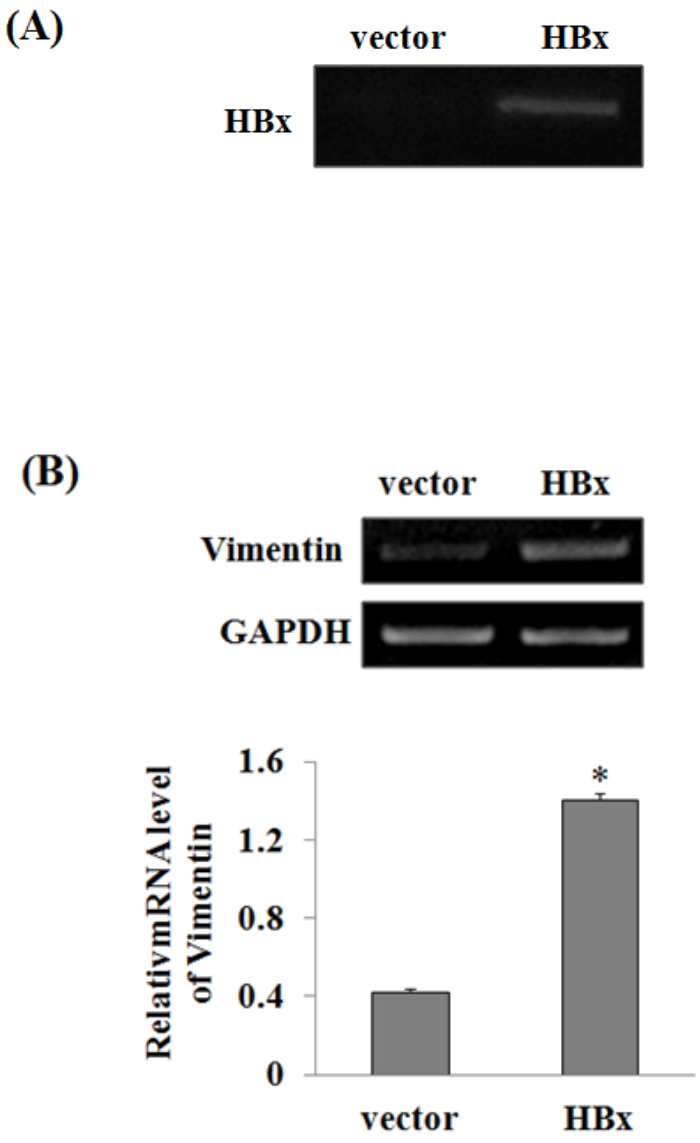
Cells were dissolved in RIPA buffer (50 mM Tris-Hcl of pH 8.1, 150 mM NaCl, 1% v/v NP-40, 0.5% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulphate) for 30 min at 4℃ and

then collected by scraping. After sonication, proteins were isolated by centrifuge. All samples were quantified using bicinchoninic acid assay and electrophoresed through 8-12% SDS-polyacrylamide gels and then transferred to membranes. The membranes were blocked with 5% skim milk, incubated with primary antibodies, washed, and probed with secondary antibodies. Detection was accomplished using ECL detection kit (Biosesang, Seongnam, Korea).

4. Results

4.1 Effects of expression of HBx on hepatic fibrosis in Huh7 cells

HBx plays a critical role in incidence of disease related HBV and hepatocellular carcinoma as well as in genome replication during early step of virus infection. Therefore, to examine if HBx has a role in developing hepatic fibrosis, we transfected control pcDNA or pcDNA-HBx construct to Huh7 cells. HBx expression in Huh7 cells was confirmed by RT-PCR (Fig. 1A). Patients with hepatitis B-induced liver fibrosis were found to have increased fibronectin and vimentin which are representative fibrotic markers (Zhang et al., 2014). Thus, to figure out that this phenomenon is caused by HBx of HBV, we checked mRNA and protein expression level of vimentin and fibronectin after transfection (Fig. 1B, C). Vimentin is a type intermediate filament. It induces adhesion and cell migration and is known to be expressed during HSC activation in liver fibrosis (Vassiliadis et al., 2012). Fibronectin is one of the important ECM in hepatic fibrosis. Compared to control cells, both mRNA and protein of vimentin have increased and protein of fibronectin have also increased. These results suggest that hepatic fibrosis was induced in huh7 cells transfected with HBx.



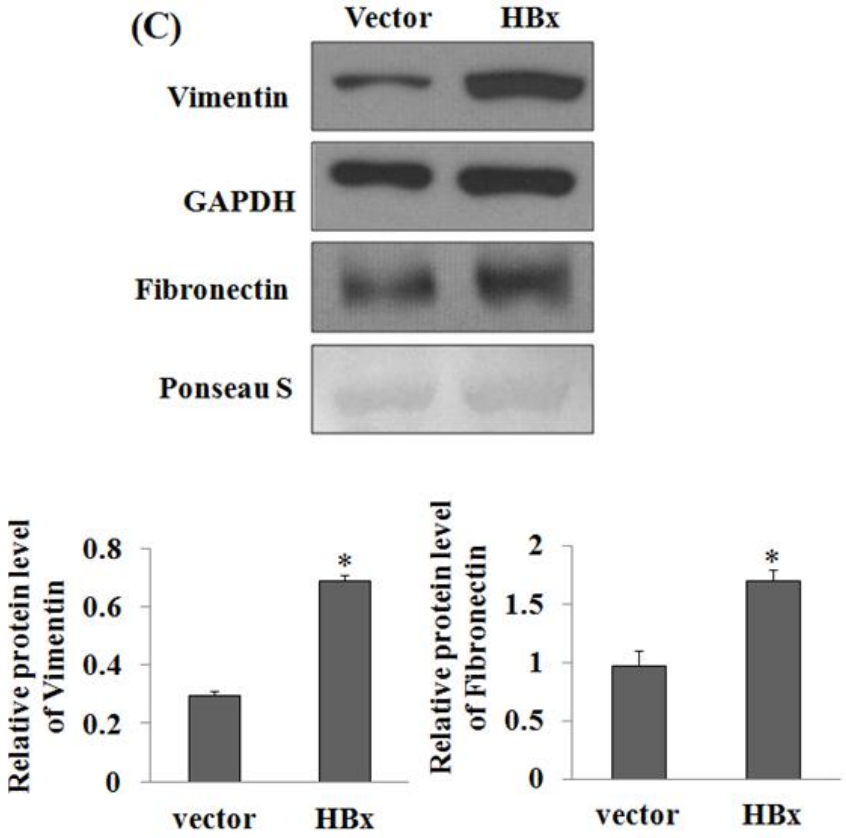


Fig. 1 Effects of HBx expression on hepatic fibrosis in Huh7 cells. Huh7 cells were transfected with pcDNA3.0 empty vector plasmid (10 μg) or pcDNA3.0-HBx plasmid (10 μg) for 48 h. (A) The mRNA level of HBx in Huh7 cells after transfection was measured by RT-PCR. (B) The mRNA level of vimentin in Huh7 cells after transfection was measured by RT-PCR. The relative level of mRNA was obtained by normalization to GAPDH. (C) The protein levels of vimentin and fibronectin in Huh7 cells were measured by western blot. The relative levels of vimentin and fibronectin protein were obtained by normalization to GAPDH and ponseau S staining respectively. Values are mean ± SD. *p <0.5 compared to vector control.

4.2 HBx increases snail and slug expression in Huh7 cells

To examine the mechanisms by which HBx regulates hepatic fibrosis, we validated the expression of epithelial-mesenchymal transition (EMT) related factors. EMT is a process of transdifferentiation of epithelial cells into mesenchymal cells. When a liver is damaged or inflamed, EMT occurs in liver tissue. Hepatocytes differentiate into mesenchymal cell through EMT and then hepatocytes regenerate repair damage. However, if the damage or inflammation is repeated and chronic, liver regeneration fails and excessive ECM is accumulated (Park et al., 2010). Therefore, we examined snail and slug, key regulator of EMT which is the cause of hepatic fibrosis (Fig. 2). Slug was upregulated in presence of HBx both in mRNA and protein level but snail was upregulated compared to control only in mRNA level not in protein level. Several studies reported slug-induced vimentin (Virtakoivu et al., 2015). Therefore, the increase of vimentin by HBx was through slug rather than snail.

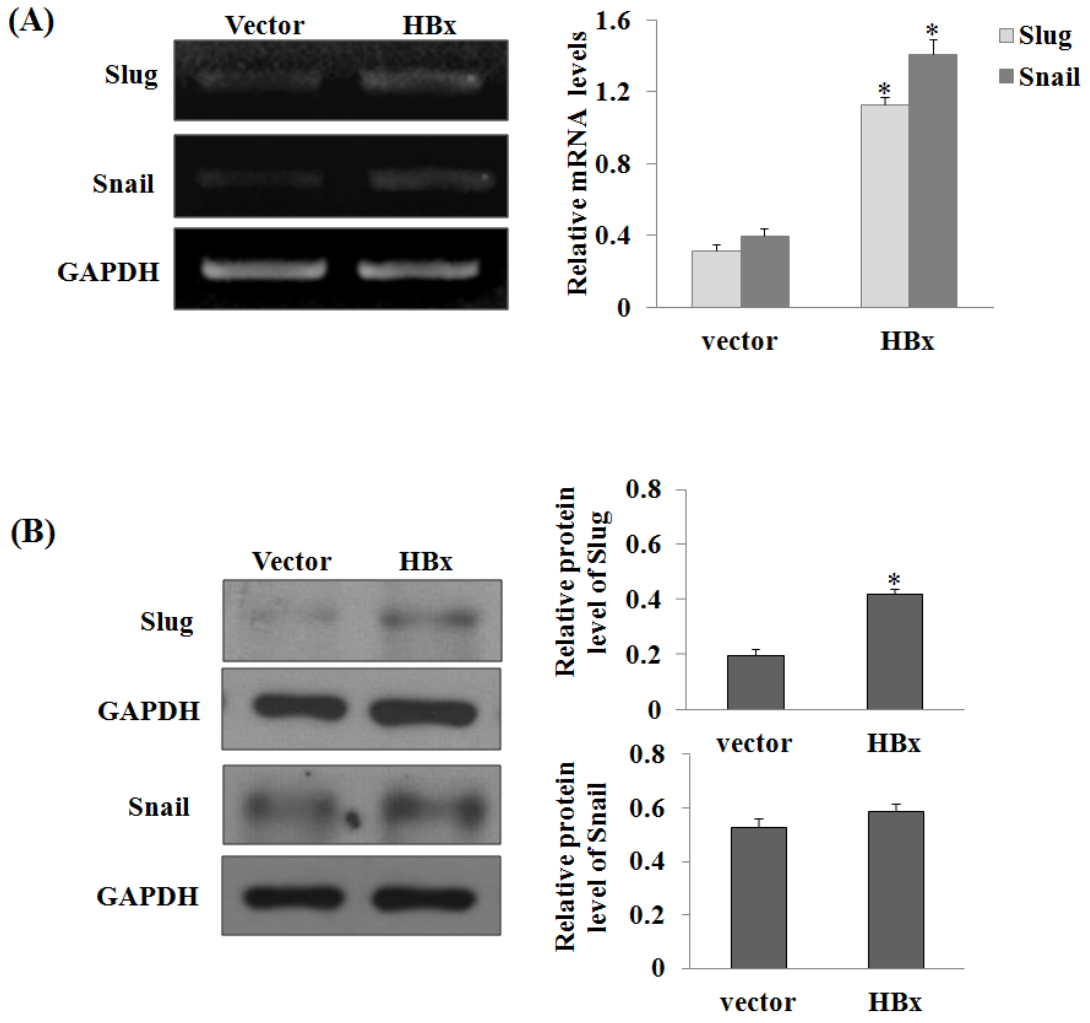


Fig. 2 HBx increases snail and slug expression in Huh7 cells. Huh7 cells were transfected with pcDNA3.0 empty vector plasmid (10 μg) or pcDNA3.0-HBx plasmid (10 μg) for 48 h and analyzed by RT-PCR and western blot. (A) The mRNA levels of snail and slug in Huh7 cells after transfection. The relative levels of mRNA were obtained by normalization to GAPDH. (B) The protein levels of slug and snail after transfection were measured by western blot. The relative levels of proteins were obtained by normalization to GAPDH. Values are mean ± SD. *p <0.5 compared to vector control.

4.3 HBx induces NOX4 expression in Huh7 cells.

Recent studies have shown that reactive oxygen species (ROS) are involved in various cellular processes such as cell proliferation, differentiation and migration (Novo and Parola, 2008). The roles of ROS in fibrosis and cancer are well-known. Moreover, the evidences that ROS are related to EMT have been reported. NADPH oxidase (NOX) is the enzyme generating ROS by various stimulations. Oxidative stress caused by NOX system in liver has a critical role in several liver diseases and hepatic fibrosis (De Minicis and Brenner, 2007). Since NOX4 generates ROS and regulates activation of myofibroblast, it regulates fibrosis in lung and kidney (Jiang et al., 2014). Therefore, we checked NOX expression to examine whether the increase of snail and slug is related to ROS (Fig. 3). The results showed that NOX4 was increased in mRNA and protein level compared to control. However, NOX2 was not increased by HBx. NOX4 usually expresses in hepatocyte, stellate cell, and endothelial cell in liver and it has been shown that NOX4 is upregulated by hepatitis C virus. Serrander et al (2007) have reported that NOX4 generates H_2O_2 and it has been reported that H_2O_2 is generated by HBx by Ha and Yu (2010). These results correspond with our result that NOX4 was increased by HBx. Recent studies have shown that NOX4 has an important role in profibrotic response. When NOX4 was suppressed using siRNA, target genes of TGF-β, namely, fibronectin, collagen I, and α-smooth muscle actin were inhibited in human cardiac fibroblast. Recent study showed that, in mouse cardiac fibroblast, collagen synthesis and myofibroblast differentiation stimulated by TGF-β were abrogated by treatment of EUK-134, scavenger of hydrogen peroxide and superoxide or dominant negative form of NOX4. Such regulation of TGB-β-induced collagen of NOX4 has been observed in kidney fibroblasts, fetal lung mesenchymal cells, and liver stellate cells as well. GKT-136901, inhibitor of NOX1 and NOX4 suppressed hepatic fibrosis by bile duct ligation. Furthermore, it was reported that NOX4 mRNA and protein are upregulated in pulmonary

fibrosis and the expression level of NOX4 correlates with procollagen I and α-SMA expression (Jiang et al., 2014).

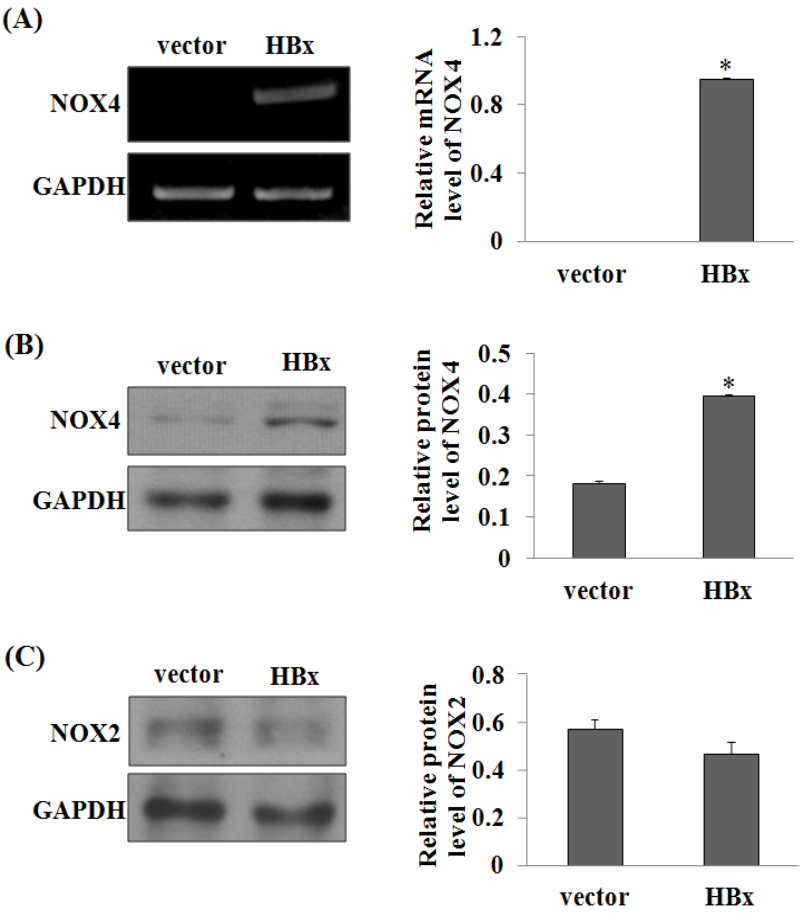


Fig. 3 HBx induces NOX4 expression in Huh7 cells. Huh7 cells were transfected with pcDNA3.0 empty vector plasmid (10 μg) or pcDNA3.0-HBx plasmid (10 μg) for 48 h and analyzed by RT-PCR and western blot. (A) The level of NOX4 mRNA was measured by RT-PCR after transfection with 10 μg DNA plasmid. NOX4 protein (B) and NOX2 (C) were analyzed after transfection with 10 μg DNA plasmid by Western blot. The relative levels of mRNA and proteins were obtained by normalization to GAPDH. Values are mean ± SD. *p <0.5 compared to vector control.

5. Conclusion

In this study, we observed that HBx transfection increased vimentin and fibronectin in Huh7 cells. This result demonstrates that HBx may induce fibrosis as vimentin and fibronectin are fibrotic markers. Also, slug and snail were upregulated by HBx. Slug and snail are well-known for crucial mediators of EMT. It was reported that HBx induced TGF-β, an upstream of snail and slug (Guo et al., 2009). Furthermore, several studies provide evidence that Nox4-mediated TGF-β regulates EMT. Hiraga et al (2013) observed ROS generated by Nox4 modulated TGF-β-mediated EMT in pancreatic cancer and Boudreau et al (2012) also reported such aspects in mammary endothelial cells. Moreover, Nam et al (2010) reported the cell migration correlation between ROS generated by Nox1 and Nox4 and human keratinocyte co-treated with HGF and TGF-β (Nam et al., 2010).

Taken together with our results and the report that ROS-induced snail and slug upregulate vimentin and downregulate E-cadherin (Kim and Cho, 2014), we hypothesize that HBx upregulates Nox4, upregulated Nox4 induces TGF-β, and then snail and slug are induced by TGF-β, finally leading o EMT and hepatic fibrosis.

PART III.

Hepatoprotective effect of

Hippocampus abdominalis hydrolysate

1. Abstract

Recently, liver disease contributes to high percentage of the morbidity and mortality rates worldwide. Excessive intake of alcohol is one of the major causes of liver injury. When liver injury repeats and becomes chronic, it leads to development of fibrosis and cirrhosis. In the liver, TGF-β is a profibrogenic cytokine, which participates in various critical events causing liver fibrosis. Seahorse (*Hippocampus abdominalis*) has been widely used for centuries as a common traditional Chinese medicine. Seahorse is known to have a variety of bioactivities, such as anti-oxidant, anti-fatigue, and anti-tumor. Peptide is one of the main compounds of a seahorse. In this study, we isolated enzymatic hydrolysate from seahorse *H. abdominalis* by alcalase hydrolysis and investigated the effect of the hydrolysate on liver injury. In the present *in vitro* studies, the hydrolysate increases cell viability of Chang cells and protects Huh7 cells from ethanol toxicity. In addition, the hydrolysate inhibits TGF-β-induced responses. *In vivo* studies show that the pretreatment of hydrolysate reduces alcohol-induced increases of serum Glutamic oxaloacetic acid transaminase and Glutamic pyruvate transaminase activities and increases liver weight and body weight. These results suggest that seahorse may have a hepatoprotective effect.

2. Introduction

2.1 Alcoholic liver diseases

Chronic alcohol intake causes health problems in a variety of organs such as brain, kidney and heart. Especially, it can cause serious problem in liver which detoxifies our body. Alcoholic liver disease encompasses liver injury from simple fatty liver, hepatitis, hepatic fibrosis to irreversible cirrhosis which is the last stage of liver diseases (O'Shea et al., 2010). When liver is injured by alcohol, wound-healing response process begins and then ECM accumulation occurs. When the accumulation is repeated and chronic, signaling related to wound-healing response becomes consistently active. This leads to continuous wound-healing response and excessive accumulation of ECM and finally develops hepatic fibrosis and cirrhosis (Parola and Pinzani 2009). Hepatic stellate cell is a crucial mediator of generating ECM. When quiescent stellate cells become active by alcohol, they stimulate collagen, a-SMA, TGF-β and ECM such as matrix metalloproteinase (Wynn 2008). TGF-β is profibrogenic cytokine and involves HSCs activation, hepatic apoptosis or ECM generation. Moreover, TGF-β induces EMT and it induces liver damage and then hepatic fibrosis (Dooley and ten Dijke 2011). However, there are few specific therapies for liver damage induced by alcohol.

2.2 Hippocampus (Seahorse)

Hippocampus, the common name of seahorse, belongs to the order syngnathiformes and family syngnathidae. Hippocampus has been considered as a precious traditional medicine for thousands of years in China (Chen et al, 2015). According to modern pharmacological studies, Hippocampus has various bioactivity such as antitumor (Li et al., 2008), antioxidant (Chen et al., 2011) and antifatigue (Jianying et al., 2000). The main compounds of Hippocampus include peptide, amino acids, steroids, fatty acids, and mineral element etc (Chen et al., 2015). In this

study, we focused only on amino acids and peptide. Amino acids are important in synthesis of hormones and low molecular weight nitrogenous substances. Some of amino acids are cell signaling molecules and regulate gene expression and the protein phosphorylation cascade (Wu 2009). Also, bioactive peptides positively affect human health and eventually improve human health. Until now, 22 kinds of amino acids have been isolated and identified from Hippocampus. However, detail mechanisms related to these remains unstudied (Chen et al., 2015).

3. Materials and Methods

3.1 Cell culture

Both Huh7 and Chang which is normal liver cell line, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and Roswell Park Memorial Institute medium with 10% fetal bovine serum respectively. The cells were incubated at 37℃ with 5% CO_2**.**

3.2 Hydrolysate preparation

Hippocampus was hydrolyzed using alcalase (Novozymes, Denmark), protease enzyme for 24 h at 50℃, pH 8.0. and then, it was centrifuged at 3000 xg for 20 min. Supernant was used as a sample, hydrolysate from *Hippocampus abdominalis*.

3.3 Analysis of cell viability

The effects of the sample on the viability of Chang cells and the protective effect from ethanol in Huh7 cells were determined by MTT colorimetric assay. The cells were seeded in 96 well culture plates. After 24 h incubation, Chang cells were treated with various concentrations of the seahorse hydrolysate for 24 h; Huh7 cells were treated ethanol for 24 h after 2 h pretreatment of seahorse hydrolysate. Then, MTT solution was added to each well, and the cells were incubated at 37℃ for 4 h. The growth medium was carefully removed and 150 μL DMSO was added to each well. The intensity of produced formazan was measured immediately at 570 nm using a microplate reader. The percentage of cell viability was calculated as the percentage reduction in absorbance.

3.4 RT-PCR

After pretreatment of seahorse hydrolysate 0.2, 0.5 mg/mL for 2 h, 10 ng/mL TGF-β was treated to Chang cells. After 24 h, total RNA from Chang cells was extracted from cells with TRIzol. Then, total RNA was used to synthesize cDNA synthesis using a Reverse Transcriptase Kit (Promega). The resulting cDNA was used for RT-PCR using the G-Taq kit (Cosmo Genetech, Seoul, Korea) according to the manufacturer's instructions. The results were analyzed using the ImageJ software.

3.5 Experimental animals

ICR mice were purchased from Orientbio (Korea) at 5 weeks of age. During experiment, mice were raised under standard laboratory conditions (24℃, 50% relative humidity, with 12 h/12 h light-dark cycles).

3.6 Experimental design

3.6.1 Induction of acute alcohol intoxication

Mice were randomly selected to three groups and treated as follows:

Group-I: Control group: 5 mice were orally administered with PBS and then after 30 min, mice underwent oral administration with PBS.

Group-II: Alcohol group: 10 mice were orally administered with PBS and then after 30 min, mice underwent oral administration with Ethanol (2 g/kg body weight).

Group-III: Seahorse hydrolysate group: 10 mice were orally administered with Seahorse hydrolysate (50 mg/kg body weight) and then after 30 min, mice underwent oral administration with Ethanol (2 g/kg body weight).

Treatment were conducted once a day for 1 week.

3.6.2 Induction of chronic alcohol intoxication

Mice were randomly selected to three groups consisting 10 in each and treated as the same way of acute alcohol intoxication except experiment period and dosage. 0.9 g/kg body weight of ethanol was treated and 25 mg/kg body weight of Seahorse hydrolysate was treated once a day for 4 weeks. Every 7 days, we measured weight and food intake of mice.

3.7 Measurement of liver weight

Liver was harvested after blood-collecting. Liver was washed with PBS, dried by blotting paper and weighted.

3.8 Estimation of serum level Glutamic oxaloacetic acid transaminase (GOT), Glutamic pyruvate transaminase (GTP)

Blood was collected by cardiac puncture and centrifuged at 3000 rpm for 30 min. Serum GOT and GPT levels were determined with GOT-GPT assay Kit (Asan, Korea).

3.9 Hepatic histological analysis

Liver were fixed in 10 % formalin and processed for preparation of 5 μm-thick paraffin section. These sections were sequentially stained with hematoxylin and Eosin (H & E).

4. Results

4.1 Effects of Seahorse hydrolysate in Chang cells and alcohol-damaged Huh7 cell.

Chang cells were treated with various concentration of Seahorse hydrolysate for 24h and cell viability was measured by MTT assay (Fig. 1A). We couldn't observe any cytotoxicity of Seahorse hydrolysate and cell viability was increased in dose-dependent manner. Then, we examined the effect of Seahorse hydrolysate in alcohol-induced toxicity in Huh7 cells. After pretreatment with Seahorse hydrolysate for 1 h and treatment with 800 mM ethanol, we measured the cell viability by MTT assay (Fig. 1B). Ethanol alone showed significant decrease in cell viability while Seahorse hydrolysate pretreatment suppressed the damage by ethanol. At the highest concentration of 1 mg/mL, cell viability was rescued to level of control (no ethanol treatment).

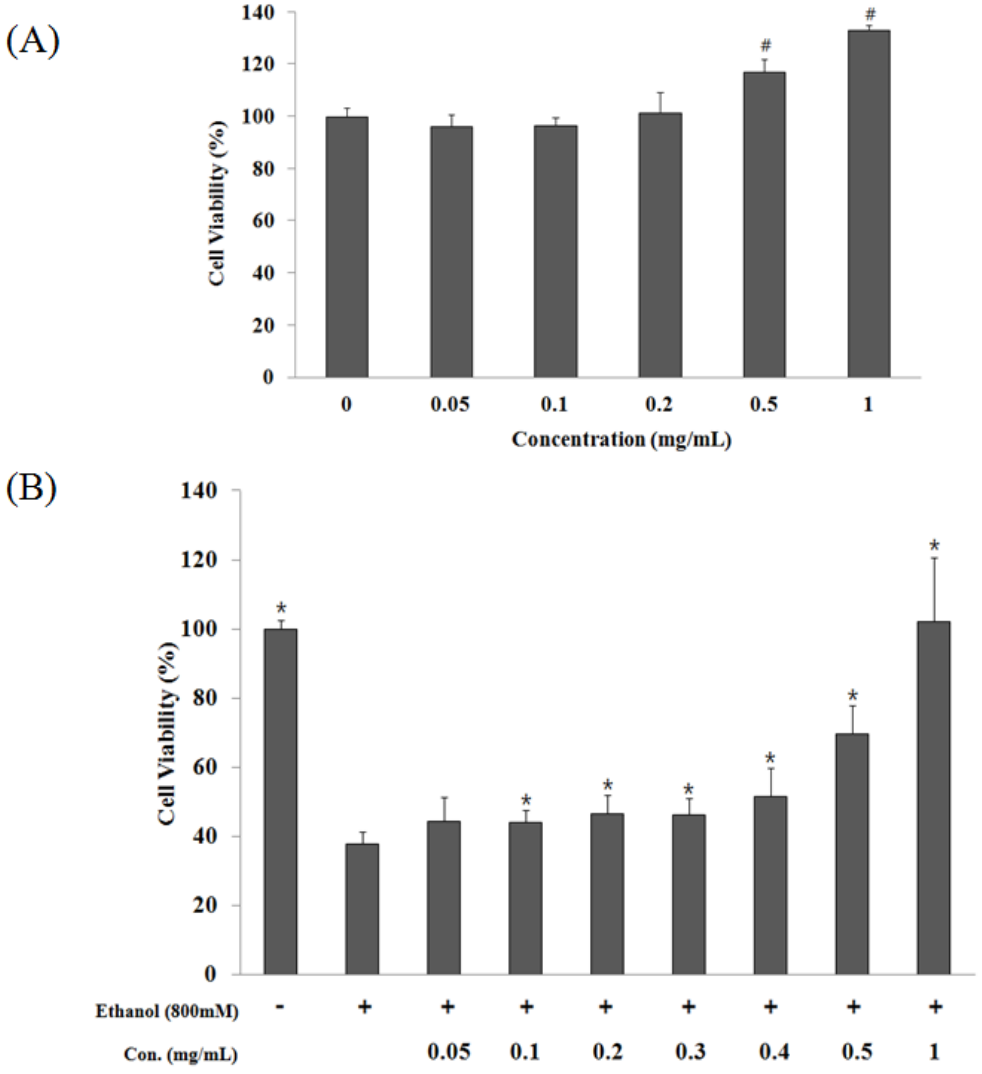


Fig. 1 Effects of Seahorse hydrolysate in Chang cells and alcohol-damaged Huh7 cell. (A) Cell viability of Chang cells treated with Seahorse hydrolysate for 24 h by MTT assay. (B) Cell viability of Huh7 cells pretreated with increasing concentrations of Seahorse hydrolysate for 24 h and cytotoxitciy was induced by ethanol for 24 h. Values are expressed as the mean ± SD (n =6). $^{\#}p < 0.05$ vs. control, $^{*}p < 0.05$ vs. ethanol

4.2 Effect of Seahorse hydrolysate on TGF-β induced changes in Chang cells.

Effects of Seahorse hydrolysate on TGF-β induced changes in Chang cells. TGF-β activates hepatic stellate cell and induces generation of ECM and finally EMT. It also induces liver injury and could develop liver fibrosis. Fibrosis is defined by the overgrowth, hardening, and/or scarring of various tissues. It is also attributed to excess deposition of extracellular matrix components including collagen. To examine whether Seahorse hydrolysate influence TGF-β-induced liver fibrosis, we analyzed its effect on Chang cell. After pretreatment with Seahorse hydrolysate for 2 h, 10 ng/mL TGF-β was treated for 24 h. Then we measured mRNA expression level of α-SMA, vimentin, and slug by RT-PCR (Fig. 2). α-SMA and Vimentin are known to express when hepatic stellate cells are activated (Friedman 2008). Slug is one of key mediators of EMT (Kim et al., 2010). By treatment of TGF-β, α-SMA, vimentin, and slug were increased and the increase was suppressed by Seahorse hydrolysate treatment. Therefore, we believe that Seahorse hydrolysate has an inhibiting effect on TGF-β -induced liver damage.

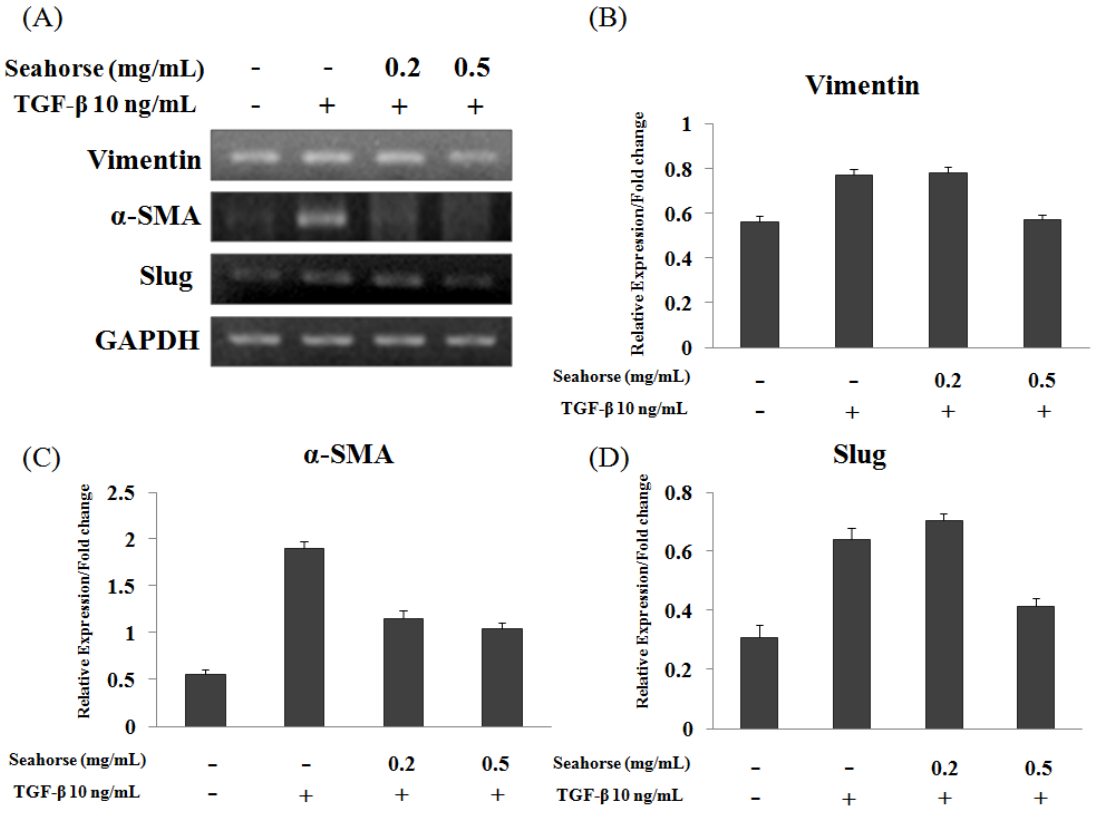
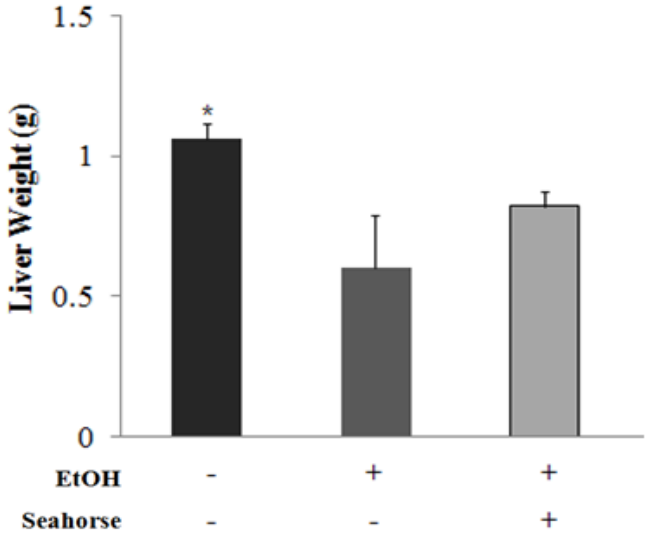


Fig. 2 Effect of seahorse hydrolysate on TGF-β induced changes in Chang cells. Chang cells were pretreated with seahorse hydrolysate for 2 h, then treated with TGF-β for 24 h. (A) The effect of seahorse hydrolysate on the mRNA level of vimentin (B), α-SMA (C), slug (D) and GAPDH was measured by RT-PCR. Relative levels of mRNA were normalized to the GAPDH.

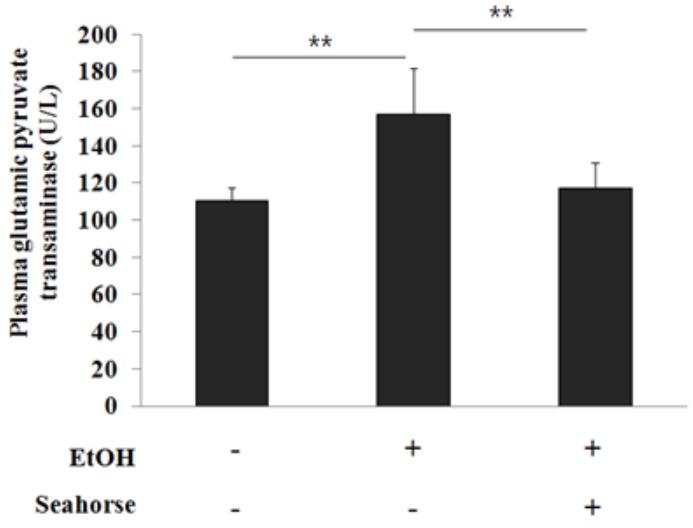
4.3 Seahorse hydrolysate ameliorates alcohol induced acute liver injury in mice.

We measured weight of livers (Fig. 3A). Ethanol alone treatment significantly decreased liver weight of mice compared to the control group. Liver weight of seahorse hydrolysate diet group was increased compared to the ethanol group but not significantly. GPT and GOT are enzymes which mainly exist in the liver cells. If the liver tissue is damaged, the liver cells spill these enzymes into blood, raising levels of enzyme (Coudray et al.,1993). Thus, serum GPT and GOT levels are used in the diagnosis of liver disease. We measured serum GPT and GOT (Fig. 3B, C). Both GPT and GOT in ethanol diet group were increased compared to the control group, demonstrating that liver injury was caused by ethanol treatment. GPT activity of seahorse hydrolysate group significantly increased compared to ethanol group. GOT activity also increased in seahorse hydrolysate group compared to ethanol group but not significantly. However, in consideration of all results, seahorse hydrolysate has a hepatoprotective activity.

(A)



(B)



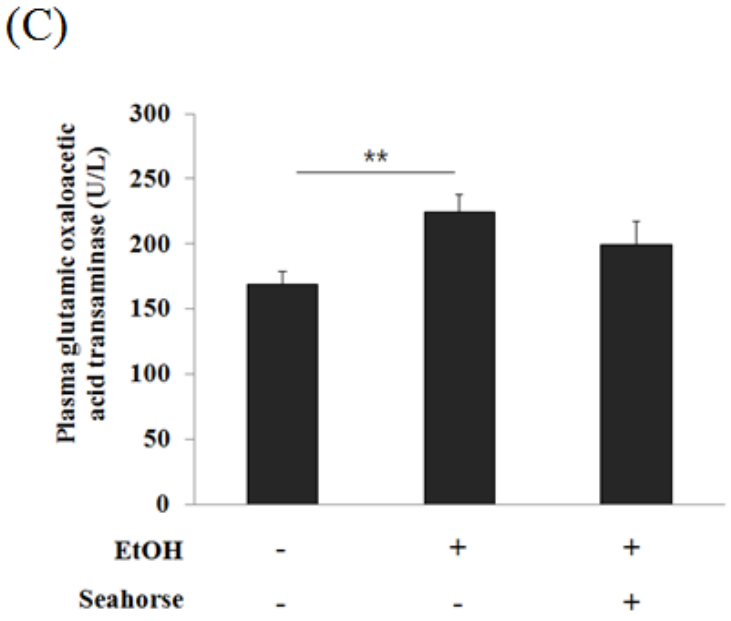
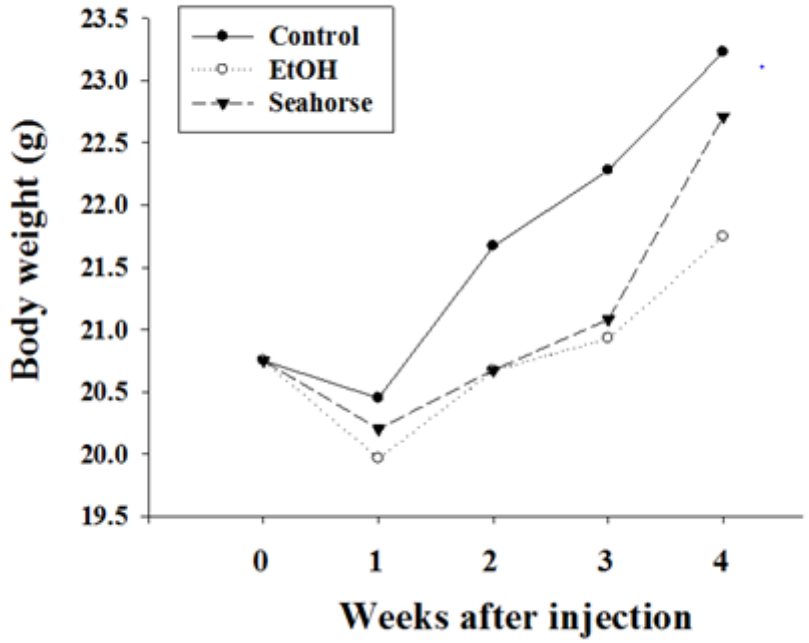


Fig. 3 Seahorse hydrolysate ameliorates alcohol induced acute liver injury in mice. (A) Liver weight of mice after 1-week treatment. (B) Change of serum GPT activity after 1-week treatment. (C) Change of serum GOT activity after 1-week treatment. Values are expressed as the mean ± SD. *p <0.05 and **p <0.001 vs. ethanol

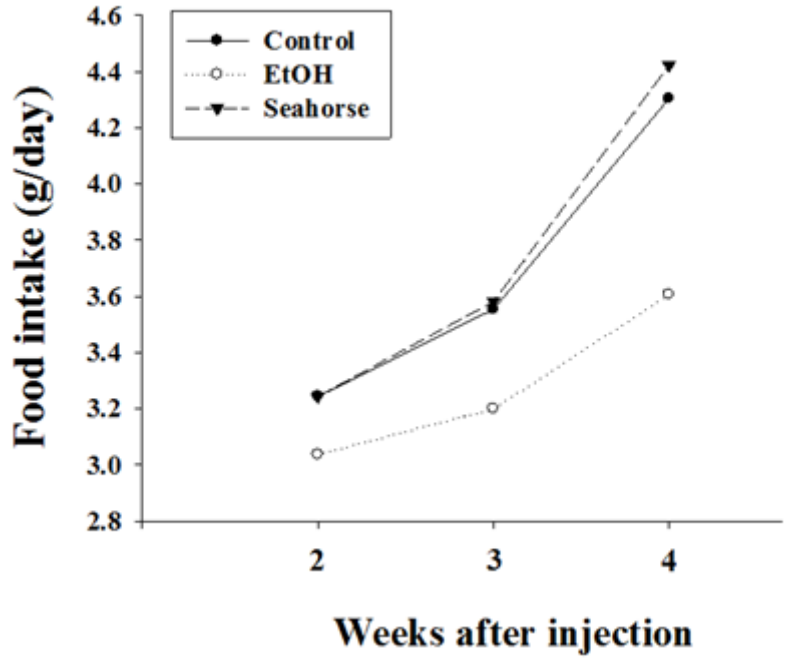
4.4 Seahorse hydrolysate ameliorates alcohol induced chronic liver injury in mice

It has been reported that alcoholism showed decreased dietary intake, caused malabsorption in the metabolism of nutrients, and consequently led to weight loss (Mezey 1980; Scheig 1970). Moreover, alcohol abuse produces heat in the human body which causes energy consumption leading weight loss (Pikaar et al., 1987). Therefore, we measured weight and food intake of mice every 7 days for experiment period, 4 weeks (Fig. 4A, B). Control group gained 2.5 g/per mouse after 4 weeks but ethanol group gained 1.0 g/per mouse. Seahorse hydrolysate group gained 2.0 g/per mouse which is similar to the change of control group. Also, food intake showed analogous tendency. Food intake of control group (1.1 g/day) or Seahorse hydrolysate (1.2 g/day) were approximately twice that of ethanol group (0.6 g/day). These results correspond with the study (Scheig, 1970) that weight and food intake were reduced by alcohol. Taken together, Seahorse hydrolysate treatment inhibited chronic-alcohol intoxication. Consistent with previous data, liver weight results also showed hepatoprotective ability of Seahorse hydrolysate (Fig. 4C). Chronic ethanol treatment decreased liver weight compared to that of control group and seahorse hydrolysate treatment. Liver weight of Seahorse hydrolysate treatment was significantly higher than that of ethanol group demonstrating that Seahorse hydrolysate protects liver from alcohol. Serum GPT and GOT were diminished by ethanol treatment. Compared to seahorse hydrolysate and ethanol, we did not observe any decrease by seahorse hydrolysate in GPT, but GOT was decreased by seahorse hydrolysate treatment (Fig. 4D, E). Histopathological investigation of liver tissue after 4-week experiment showed no significant differences among control, seahorse hydrolysate, and ethanol group (Fig. 4F). Although there was no effect on GPT activity and no significant difference in GOT activity, others, such as body weight, food intake, and liver weight suggest that seahorse hydrolysate has a protective effect from alcohol intoxication

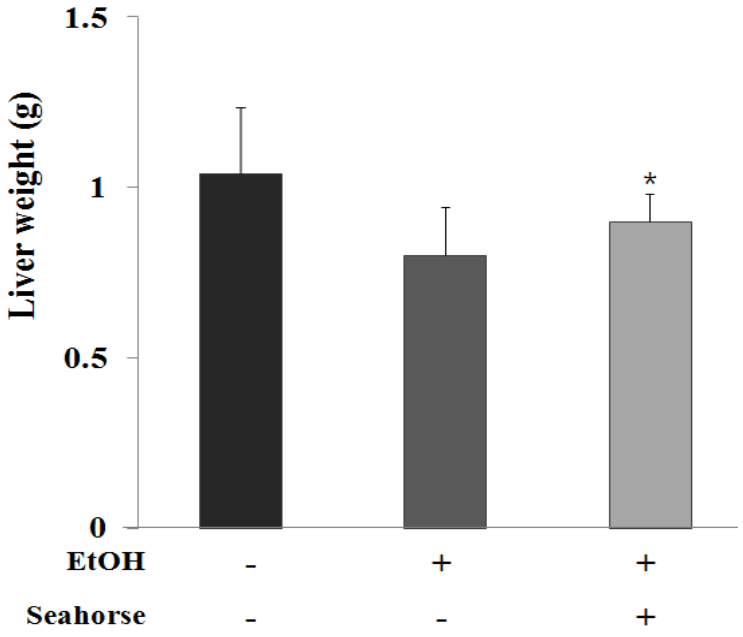
(A)



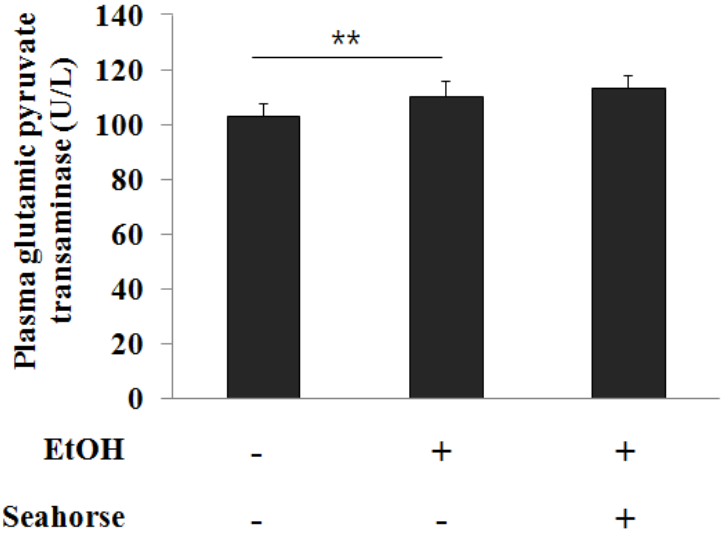
(B)

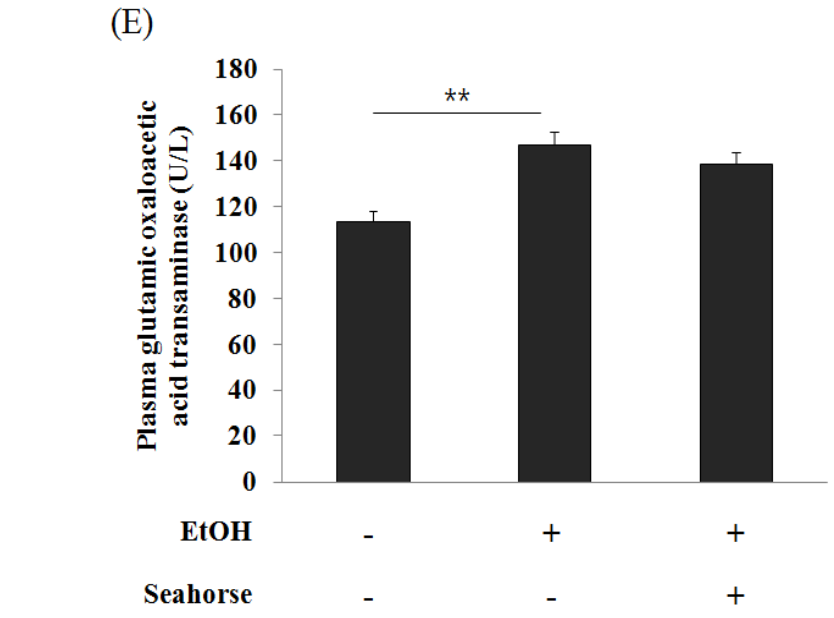


(C)



(D)





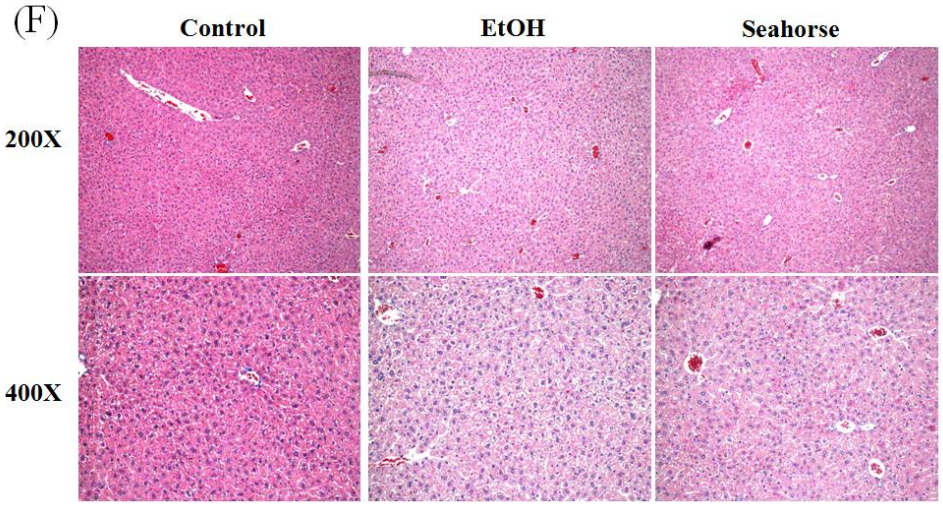


Fig. 4 Seahorse hydrolysate ameliorates alcohol induced chronic liver injury in mice. (A) Change of body weights of mice during treatment. (B) Change of food intake of mice during treatment. (C) Liver weight of mice after 4-week treatment. (D) Change of serum GPT activity after 4-week treatment. (E) Change of serum GOT activity after 4-week treatment. (F) Hematoxylin and eosin (H&E) staining of mouse liver tissue. Values are expressed as the mean ± SD. *p <0.05 and **p <0.001 vs. ethanol

4. Conclusion

Alcohol intake is one of the major causes of liver injury. When alcohol-induced liver injury becomes chronic, it drives to development of fibrosis and cirrhosis. *Hippocampus abdominalis* has been used as medicine for inflammation, immune system, and disease of liver and kidney etc. in Asia, especially in China. In the current study, we demonstrated that seahorse hydrolysate has hepatoprotective activity. *In vitro,* the hydrolysate increased cell viability of normal liver Chang cells and protected Huh7 cells from ethanol toxicity. In addition, the hydrolysate inhibited TGF-β-induced fibrotic responses. Pretreatment of hydrolysate before TGF-β treatment reduced expression of vimentin, α-SMA and slug mRNA.

In vivo studies show that the pre-diet of hydrolysate group showed lower decrease of liver weight compared to ethanol-diet group in both acute and chronic ethanol intoxication experiments. Serum GPT was significantly increased in pre-diet of hydrolysate group compared to ethanol-diet group, while GOT was increased but no significant decrease in acute alcohol intoxication experiment. In chronic alcohol intoxication, GOT activity was reduced more than GPT activity. Same results were confirmed in Soh et al's study (2003) and Bae et al's study (2009). Furthermore, pre-diet of hydrolysate group showed similar increase of mouse weight and food intake after 4 weeks with control group, which were higher than ethanol-diet group.

Overall, the results of the present study demonstrate that seahorse has a hepatoprotective effect and may be a potential material of functional food against liver damage.

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