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Alcohol-induced hepatic fibrosis in pig

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Abstract

A number of toxicants have been incriminated as a causing hepatic disease. Among many detrimental injury, alcohol has been noted for hepatitis, fatty liver, fibrosis, and hepatic cirrhosis. The purpose of this study was to develop animal model for hepatic fibrosis in pigs fed ethanol, and to search for a new anti-fibrogenic agent via this model. Twelve male Landrace pigs were divided into 3 groups of 4 animals each. Group 1, 2 and 3 were fed with active ceramic water only, ceramic water + liquid diet containing 15% ethanol and normal tap water + liquid diet containing 15% ethanol for 12 weeks, respectively. At week 12, all pigs were immediately sacrificed for collection each tissue and blood. Serologically, serum ALT and AST levels were significantly reversed in group 2, as compared to group 3. They were normal range in pigs of group 1. Microscopically, macrovesicular lipid droplets and moderate hepatocellular necrosis were evident in the tap water + ethanol fed group 3. However, the active ceramic water treated group 1 showed normal architecture. Moreover, in group 2, mild fatty changes and necrosis were observed in hepatocytes. Collagen fibers were increased in spaces surrounding periportal and interlobular connective tissues in the group 3 of tap water + ethanol, but collagen synthesis and its thickness of fibrotic septa connecting portal tracts were markedly reduced in the group 2 of ceramic water + ethanol. Myofibroblasts were detected mainly in the interlobular connective tissues of pig liver of group 3 treated ethanol and tap water. Few to no myofibroblasts were observed in groups 1 and 2. CYP2E1 was not or rarely detected in group 1 fed ceramic water. However, group 2 showed slightly activation of CYP2E1 in the area of pericentral vein, while CYP2E1 was significantly activated in group 3 fed tap water and ethanol.

Based on the above data, we believe that we have developed a unique alcohol induced fibrosis model in pig, which will be useful in developing anti-fibrotic agents and drugs. Furthermore, the active ceramic water used in our study had an inhibitory and may be protective against ethanol induced hepatic toxicity and fibrosis.

Key words : Alcohol, Hepatic fibrosis, Hepatic cirrhosis

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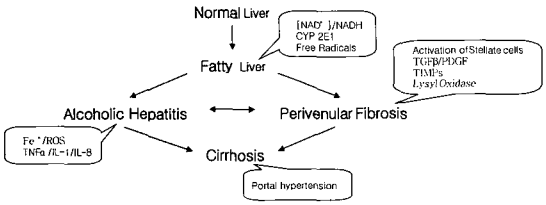
Introduction

Hepatic disease has been noted and as the result of various toxicants such as alcohol, drugs, microbiological agents and other xenobiotics. Alcohol has been noted for causing hepatitis, fatty liver, fibrosis, and hepatic cirrhosis, and also known for producing various free radicals, lipid peroxide and redox, indicating marked increase in [l-Lactate]/[pyruvate] and decreased in the free cytosoplasmic[NAD^+ ; oxidation form of nicotinamide adenine dinucleotide]/[NADH ; reduction form of nicotinamide adenine dinucleotide] ratio, phosphorylation states, which leads to fatal sudden death(Text-Fig 1)[1-3].



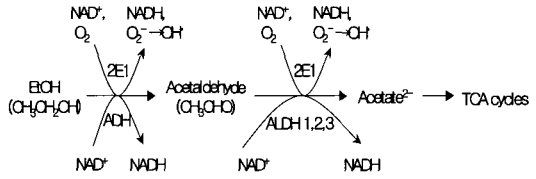
Text-Fig 1. Pathological progress in hepatic cirrhosis

Hepatic fibrosis represents a variety of repeated insults, and the recruitment various cells, different factors, such as activation of Kupffer cells, mononuclear cells, and hepatic stellate cells, resulting in the disorganization of hepatic cords, intracellular and extracellular matrix composition. Fibrosis due to chronic ethanol intoxication is always accompanied by excessive oxidation of polyunsaturated membrane lipids and activation of cytochrome P4502E1(CYP2E1)[4]. In the pig liver, the space of Disse lies between the sinusoidal endothelium and the vascular pole of hepatocytes, hepatic stellate cells(HSCs, Ito cells, fat storing cell, lipocyte) are located within the space of Disse. HSCs are play a key role for in the production of matrix in the normal[5] and fibrotic liver[6]. The connective tissue of the external capsule, septa, and perivascular areas and portal tracts of the liver is fibrillar and rich in collagen types I, III, V[7] while collagen types IV, VI, laminin, and fibronectin are major matrix proteins in the space of Disse[8,9]. On the whole, two types of extracellular matrix are present in the normal liver architecture, namely interstitial connective tissue and basement membrane. In the interstitial space, fibroblasts are responsible for maintaining steady state levels of collagen and fibronectin, vitreonectin, while the matrix of the space of Disse is mainly responsibility of stellate cells[10]. In the fibrotic liver, various types of collagen fiber are initially involved and type III[11] collagen is the first present in increased area while later amount of type I is increase mostly within portal tracts, wall of central veins, and around sinusoidal endothelial cells of the vessels and space of Disse[6,12]. Severe deposit in these areas can lead to interference with the uptake of nutrients, and metabolites from the blood. These collagen fibers were mainly produced by activated HSCs. They are activated by many stimulator, such as inflammatory cytokines(interleukin-1 ; IL-1, interleukin-6 ; IL-6, tumor necrosis factor-alpha ; TNF-α), growth factors(fibroblast growth factors ; FGFs, plate derived growth factors ; PDGFs, and transforming growth factor-beta ; TGF-β)[13,14]. Under chronic detrimental condition of liver, TGF-β1 can promote the differentiation of fibroblast of fibrous septa and vessels wall into myofibroblasts, which also producing alpha-smooth muscle actin(α-SMA), indicating activation of fibroblast and critical role for inducing inflammatory status in liver, kidney, central nervous system, skin and etc[15,16]. The simple act of ingestion of ethanol

and its subsequent metabolism by liver profoundly alters a number of the fundamental parameters of hepatocellular function. Among these are immediate lowering of redox potential with a decrease in intracellular [pyruvate] and increase blood marker such as glutamic oxaloacetic transaminase(GOT, aspartate aminotransferase ; AST) and glutamic pyruvic transaminase(GPT, alanine amino-transferase ; ALT), indicating hepatic dysfunction. Chronic ethanol ingestion leads to hepatic fibrosis and these phenomena can be made by generation of free-radicals via microsomal ethanol oxidation system(MeOS). Increased production of free radicals has been noted and implicated with pathogenesis of hepatic damages such as metal storages[17], hepatic steatosis[18], cholestasis[19], drugs with free radical metabolites[20] and ischemia perfusion injury[21,22]. Low plasma levels and hepatic levels of anti-oxidants have been associated with oxidative stress[23,24]. Dietary supplementation with vitamin E[25,26] and N-acetylcysteine[27] has been shown to be reduce hepatic fibrosis in animal models, suggesting involving oxidative stress. Ethanol is normally metabolized via alcohol dehydrogenase (ADH) enzyme and in case of excessive ethanol ingestion, metabolized via MeOS, involving the CYP2E1 gene. Both the ADH and MeOS pathways generate acetaldehyde a toxic metabolite acetaldehyde, which can be make protein adducts and decrease DNA repair, and stimulate antibody production against neoantigens and collagen(Text-Fig. 2). CYP2E1(the ethanol inducible form) and hemoproteins can generate reactive oxygen species(ROS) through the reduction of dioxygen to superoxide anions, which provide to hydrogen peroxide and hydroxyl radical generation[4,28]. Rats with intragastric infusion models(feeding ethanol) have been proved involving induction of CYP2E1 and strong generation of hydroxylethyl free radicals[29]. Another factors, malondialdehyde(MDA) and a major end products of lipid peroxidation and as well as 4-hydroxy-2,3-nonenal(HNE) trigger fibrosis and sclerosis of hepatic tissue and is able to up-regulate collagen synthesis, supporting the theory of a positive correlation between HNE accumulation and the hepatic content of hydroxyproline[30], indicating stimulation for collagen synthesis. Ethanol induced hepatic fibrosis appears through inflammatory and pro-fibrogenic cytokines like TNF-α, TGF-β1 produced by the activation of macrophages and stellate cells, activation of kinases for activator protein 1(AP-1) transcription factors, by HNE, ROS, iron overload, and other hydroxylalkenals[31,32]. Many earlier reports suggest that various chronic ethanol models have been resulted in hepatic fatty change, fibrosis, and finally hepatic cirrhosis through the hypoxia, direct ethanol metabolite accumulation and subsequent DNA modification with acetaldehyde adduct formation aas well as the impairment of nutrient uptake and induction of CYP2E1(Text-Fig 2).

Recently, many anti-fibrogenic agents such as N-acethylcysteine[27], S-adenosyl methionine (SAMe), polyenylphosphatidyl choline(PPC) and anti-oxidants(silymarin, alpha-tocopherol and selenium)[33] have been noted in alcoholic liver disease. Many therapeutic agents have been developed, however none have proven clinically effective. Here, we hypothesize hepatic model for hepatic injury may be developed using piglets fed ethanol *ad libitium*, and used to discover antifibrogenic agents for treatment of hepatic fibrosis and cirrhosis by using ceramic water.

The purpose of this investigation is to develop an animal model for hepatic fibrosis and to search for new anti-fibrogenic agents.



Text-Fig 2. Signaling pathway of ethanol metabolism

Materials and Methods

Reagents

Normal tap water is passed through bioactive multipurpose magnetized ceramics. The ceramics are composed of Maek-Ban and soft sericite stone with other constituents described below(Table 1 and 2). Antibody for alpha-smooth muscle actin was purchased from Sigma chemicals(St Louis, MO, USA). Cytochrome P4502E1 was purchased from Oxford Biomedical, USA. All other biochemicals, tissue staining chemicals and ethanol(Merck Co) were the highest grade commercially available.

Animals and treatments

Studies were performed on twelve male Landrace pigs weighing around 5 kg. They were divided into 3 groups(n=4) and kept in three different pigpens, with free access to special designed autosupply tap water and the active ceramic water system, which designed with special ceramic stone. This ceramic stone can generated rapidly and is sufficient for the treatment of drinking water. Pigs were fed a freshly made liquid diet containing 15% ethanol for pigs, generally eating three time per day at AM 08:00, PM 12:00, and PM 18:00. Groups were fed with feeding for ceramic water only (G1), ceramic water feeding + liquid diet containing 15% ethanol(G2) and feeding for normal tap water + containing 15% ethanol (G3) for 12 weeks, respectively. At about day of sacrifice at 12 weeks, all pigs were transported to slaughter house from animal holding facility and after transportation, immediately sacrificed for collection each tissue and blood.

Table 1. Composition of active ceramic stone (Maekban portion)

Material	% by weight
Silicic anhydride	69.8
Al_2O_3	14.0
FeO	1.3
FeO_2	1.4
Mg	3.6
CaO	2.0
Na	3.2
K	3.2
Ti	0.3
Phosphoric anhydride	0.3
MnO_2	0.02

Table 2. Composition of active ceramic stone (soft sericite portion is as follows)

Material	% by weight
Silicic anhydride	48.9
Al_2O_3	31.0
FeO/FeO_2	5.0
K_2O	7.3
Na	0.23

Histopathological analysis

Liver and other tissues such as kidney, spleen, stomach, and small intestine from each pig were rapidly removed and fixed in 10% neutral buffered formalin, processed

routinely and embedded in paraffin. Sections were cut to 4㎛ in thickness. Sections were stained with hematoxylin and eosin(H-E) and, for collagen fibers, with Azans-Mallory staining methods for collagen.

Biochemical analysis

Blood from each pig was collected at the time of sacrifice and centrifuged. Collected serum were immediately frozen at -80℃ until analysis. Serum ALT and AST, concentration of total protein, albumin, cholesterol and triglyceride(TG) were determined with an automated analyzer (Hitachi 736-10, Hitachi Co, Ltd). Mean values of these parameters were subjected to statistical analysis by ANOVA test.

Immunohistochemistry

Sections of liver were deparaffinized in xylene, dehydrated in graded alcohol series, and incubated in a solution of 3 % hydrogen peroxide(H_2O_2) in methanol for 10 min. Tissue sections were washed with PBS containing 0.03 % nonfat milk and 0.01 % Tween 20, and then immunostained with primary antibodies for α-SMA and CYP2E1. The antigen-antibody complex was visualized by the labeled streptavidin-biotin method using a Histostatin-plus bulk kit (Zymed Laboratories Inc, San Francisco, CA, USA) with 3,3-diamino benzidine(Zymed Laboratories Inc, San Francisco, CA, USA). Tissue sections were then rinsed in distilled water and counter stained with Mayer's hematoxylin. Non-immunized goat sera, which were used instead of the primary antibody, served as the negative control. The primary antibodies used were : monoclonal anti α-SMA at a dilution of 1:800(clone 1A4, Sigma Co, Saint Louis, MO, USA) and CYP2E1 at a dilution of 1:200(Oxford Biomedical, USA).

Degree of fibrogenic activity

Collagen was identified in liver using Azans-Mallory staining method. Liver sections were deparaffinized and stained in Azocarmine G for 1hr at 56℃ and then differentiated in aniline alcohol, treated with acid alcohol for 1min at room temperature. Liver sections transferred to phosphotungstic acid for 3hrs. Rinse in distilled water and stained in aniline blue. After rinsing in distilled water, sections were treated with phosphotungstic acid and then rinsed in distilled water and acidulated water. Finally, after rinsing in 70% alcohol, sections were dehydrated, cleared and mounted. By this method, collagen stained distinctly blue and noncollagenous proteins stained red and yellow.

Statistical analysis

Data were expressed as mean ± SEM. For statistical analysis, ANOVA test was employed. Values of $p<0.05$ were considered significant.

Results

Serological results

Serum ALT(GPT) and AST(GOT) levels were significantly reversed in the active ceramic water treated group, comparing to ethanol plus tap water treated group, indicating hepatic damage(Text-Fig 3). Also capability of serum total protein and albumin synthesis, there were also significantly differences between them, suggesting hepatic fibrosis progressing(Text-Fig 4). Pig fed active ceramic water were successfully protected and shown normal hepatic function

from ethanol induced hepatic dysfunction. Another criteria liver function, total cholesterol was also reversed compared with that of ethanol + tap water(Text-Fig 5). All these data can be explained for hepatic protective effects of active ceramic feeding.

Histopathological observation

Ethanol induced hepatic fatty change and fibrosis were successfully induced and identified as a useful drug screening animal model for anti-fibrogenic development. As expected, only ceramic fed group shown normal hepatic architecture(Fig 1, 2), and while mild little fatty change and necrosis was demonstrated in the ceramic + ethanol fed animals(Fig 3, 4). Macrovesicular lipid droplets and moderate necrosis were evident in the tap water + ethanol fed group(Fig 5, 6) with numerous vacuoles and necrosis surrounding interlobular connective tissue and pericentral veins, respectively. These results indicate the hepatoprotective effects of feeding active ceramic water.

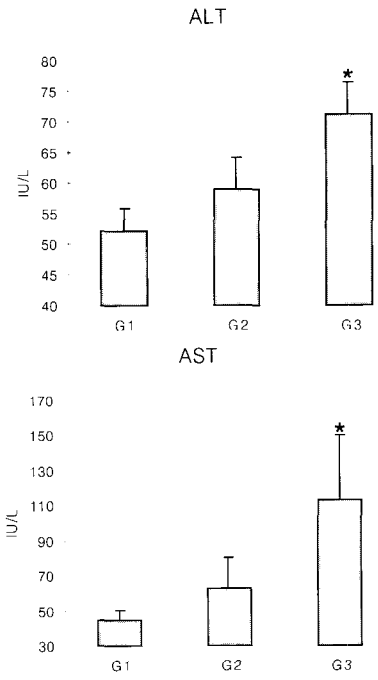
Collagen synthesis

To determine the extent of fibrosis, Azan-Mallory staining method for collagen synthesis was carried out. Total collagen was measured under ×33 magnification fields by surrounding periportal areas and interlobular areas in the group of tap water + ethanol (Fig 11), but in the group of active ceramic water + ethanol fed animals, collagen synthesis and its thickness of fibrotic septa connecting portal tracts was markedly reduced(Fig 9), showing inhibitory effect of collagen synthesis by feeding active ceramic water. In the group of active ceramic water fed animals, collagen fibers were detected as normal(Fig 7).

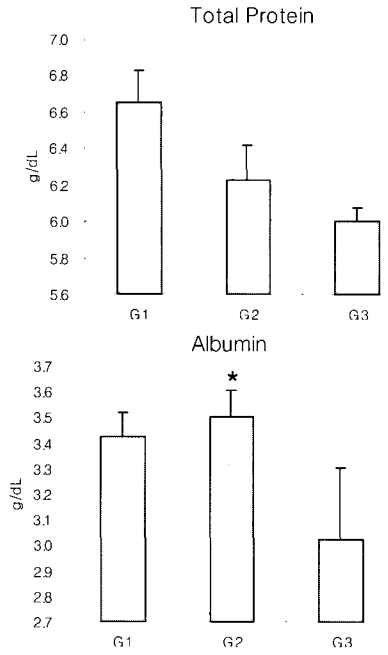
Immunohistochemical results

1) Activation of myofibroblast(hepatic stellate cells)

Hepatic stellate cells have been identified as being responsible for the pathogenesis of cirrhosis. In the normal liver, HSCs are quiescent. However, after liver injury by various stimulants, HSCs transform into myofibroblasts-like cells[34], showing intense cytoplasmic α-SMA activity. Expression of α-SMA can be used as a identifier for activated HSCs. In acute liver injury, α-SMA-positive HSCs may not persist for long enough time to induce fibrosis, but in chronic cases, α-SMA-positive HSCs are consistently present, and maintain active chronic liver diseases. HSCs are originally located at the space of Disse in hepatic tissue. Activated myofibroblasts in the major proportion of connective tissue septa(interface myofibroblast,

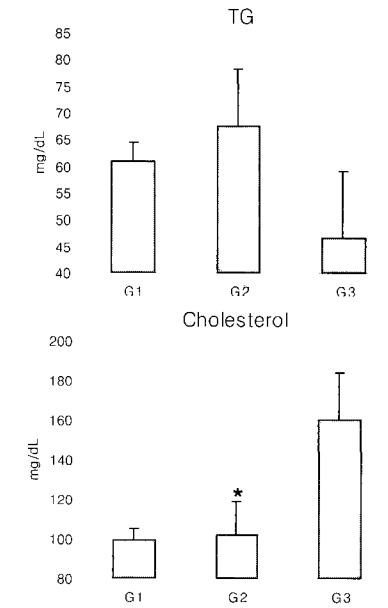


Text-Fig 3. Serum ALT and AST values
* : P < 0.05, significant difference from the value for G2



Text-Fig 4. Serum total protein and albumin values

* : P < 0.05, significant difference from the value for G3.



Text-Fig 5. Serum Triglycerides and total cholesterol values.

* : P < 0.05, significant difference from the value for G3.

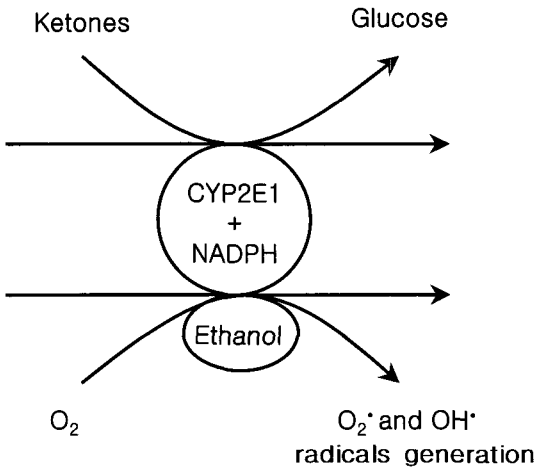
septal myofibroblast and portal myofibroblast) were successfully identified by α-SMA-positive staining, appearing as spindle shaped cell bodies with long processes in the ethanol and tap water treated group(Fig 12). The control group and active ceramic water + ethanol fed groups contained quiescent hepatic stellate cells, and little fibrogenesis-dependent α-SMA expression(Fig 8, 10), indicating inhibition of hepatic stellate cells and fibrogenic cells like myofibroblasts in these animals.

2) Localization of CYP2E1 protein in pig liver

CYP2E1 was observed via immuno-histochemical staining reactions on liver sections from all experimental groups. Virtually constitutive immunoreactive CYP2E1 was observed by immunohistochemically from ceramic water(Fig 13, 14). However, CYP2E1 immunopositivity was slightly activated on the area of pericentral veins in pig livers fed with active ceramic water + ethanol(Fig 15, 16), while tap water + iso-amount of ethanol fed group had significantly increased, indicating the protective effect of active ceramic water(Fig 17, 18). CYP2E1 genes play a critical role in ethanol metabolism in the liver. Chronic ethanol intake changes numerous cellular functions, leading to parenchymal damage. Ethanol is primarily metabolized via cytosolic alcohol dehydrogenase to acetaldehyde, which is continuously metabolized to acetate in the mitochondria. In the mitochondria, redox reactions convert NAD^+ to NADH, and the resulting redox status causes fatty changes, fibrosis and cirrhosis in the liver. Severe ethanol intoxication also triggers the MeOS system for ethanol metabolism in liver. It is considered that CYP2E1 is directly responsible for the formation of ROS such as hydroxyl

radicals and other free radicals by using co-factors NADPH a major producer from glucose-6-phosphate dehydrogenase(G6PD) and $NADP^+$-dependent cytosolic isocitrate dehydrogenase(IDPc), which interacts with cellular macromolecules(Text-Fig 6). Inhibition of mechanism for CYP2E1 is critically important for protection against ethanol-induced hepatoxicity. In this experiment, we demonstrated a lower acitivty level, CYP2E1 in the active ceramic water fed group, while ethanol + tap water fed group shown strong positive immune reaction at this experiment, suggesting CYP2E1 dependent protecting mechanism.

According to the data above, active ceramic water fed pigs showed significant protection patterns from ethanol-induced hapatoxicity, suggesting this may be a development of valuable hepatic fibrosis model and useful for development of antihepatic therapeutic agents(Table 3).



Text-Fig 6. Ethanol metabolizing pathway via cytochrome P4502E1

Discussion

Extracellular matrix constituents in the liver are synthesized by hepatocytes, bile duct epithelial cells and endothelial cells, under the influence of mesenchymal myofibroblast-like cells of liver[5,35-37]. In the space of Disse, typical HSCs, transitional cells(intermediate between HSCs and myofibroblasts) and myofibroblasts have been described and identified with immunohistochemical stains for α-SMA. Another mesenchymal(myo) fibroblast-like cell type in the liver is the portal (myo)fibroblast[38], located around centrilobular veins[18].

Table 3. Comparison data for histopathological changes : fibrosis, α-SMA and CYP2E1 in each group

Group	H&E[a]	Azan[b]	α-SMA[c]	CYP2E1[d]
Group 1 (n=4)	Normal and Hyperemia	Normal	Normal	Normal
Group 2 (n=4)	Normal~Centrilobular necrosis	Normal~Collagen (+)	Normal~Myofibroblast (++)	Mild (+)
Group 3 (n=4)	Centrilobular necrosis~ Mild fatty change	Collagen (+~++)	Myofibroblast (++~+++)	Severe (+++)

[a] : Fatty change in the perilobular area
[b] : Collagen in the interlobular connective tissue
[c] : Myofibroblast in the interlobular connective tissue
[d] : Cytochrome P4502E1 in the centrilobular area

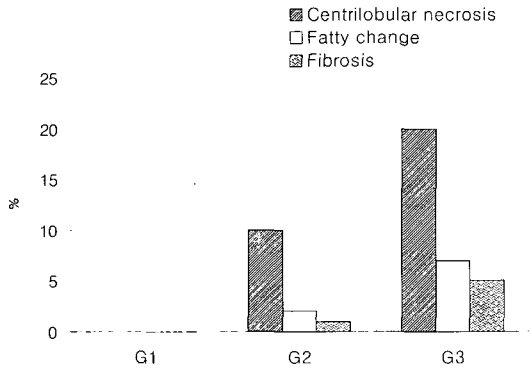
Alcohol is oxidized primarily in the liver and the main pathway involves alcohol dehydrogenase. The oxidation of ethanol via the alcohol dehydrogenase pathway results in the production of acetaldehyde with loss of hydrogen ions. Reducing NAD^+ in large numbers to NADH. Reducing equivalents overwhelm the hepatocyte's ability to maintain redox homeostasis, and a number of metabolic disorders ensue(Text-Fig 2). Increased NADH opposes lipid oxidation and promotes fatty acid synthesis with hepatic fat accumulation[26]. In addition to elevated levels of NADH, microsomal induction has been incriminated in lipogenesis which was found to be associated with enhanced production of low(LDL) and very low density lipoproteins(vLDL)[39]. A toxicological break-through was achieved with the discovery of a microsomal ethanol oxidizing system and its interactions with xenobiotics. This second pathway for alcohol metabolism is separate from that of alcohol dehydrogenase and catalase and may be characterized and reconstituted by a semi-purified preparation of cytochrome P-450[40]. The role of microsomes in ethanol metabolism and their increase after chronic ethanol consumption has been demonstrated in rats[41]. An ethanol-inducible form of cytochrome P-450 was discovered and subsequently purified by the livers of different species, including rats and humans. MeOS was found to be strikingly inducible by chronic ethanol consumption, with its key component, namely 2E1, increased 4- to 10-fold in liver biopsies of recently drinking subjects[42].

Ethanol oxidation, whether by the ADH or the microsomal pathway, results in acetaldehyde production, which may cause, damage throughout the cells, including within mitochondria. Administration of ethanol, particularly at high levels and in animals fed alcohol chronically, was accompanied by a 10-fold increase in splanchnic acetaldehyde release within the hepatic vein, and a striking leakage of the mitochondrial enzyme glutamic dehydrogenase into hepatic venous blood. Acetaldehyde also contributes to depletion of glutathione and its potentiation of lipid peroxidation[43].

Several animal models of alcoholic liver disease have been established[23,28]. However, ethanol intake has been mainly performed by force feeding in most animal models. Here, we established a new animal model of alcoholic liver fibrosis in the pig. Our model is based on the predilect for swine + voluntarily consumption ethanol in the diet. Our study was conducted by feeding twelve pigs with 15% ethanol, resulting in increase of collagen depositions within the interlobular connective tissues of liver. Furthermore, in our study, myofibroblasts and activation of CYP2E1 increased in the interlobular connective tissues and centrilobular areas, respectively. Ethanol produces fatty liver in both rats and humans, and in non-human primates[44]. Both the ADH and the MeOS pathways generate the toxic metabolite acetaldehyde, which forms adducts with proteins, resulting in enzyme inactivation, decreased DNA repair, antibody production, glutathione depletion, mitochondrial toxicity, impaired utilization of oxygen and increased collagen synthesis (Text-Fig 2).

Recently, some studies of micropig models of alcohol-induced liver disease have been performed[45,46]. Halsted et al.[45] described that livers from ethanol-fed micropigs showed increased centrilobular CYP2E1 and protein adducts with acetaldehyde and malondialdehyde(MDA). They also found that steatohepatitis occurred in five of six pigs

fed ethanol for 14 weeks. One study reported that perivenous fibrosis was present in three of five micropigs fed ethanol at 12 months and more extensive pericentral and intralobular fibrosis was noted in one micropig fed ethanol for 21 months[46].



Text-Fig 7. Ratio of hepatic injury in each group (Values are arbitrarily expressed as percentage of hepatic injury)

Alcoholic hepatitis is characterized by extensive necrosis and inflammation in the livers, furthermore, these lesions trigger fibrosis as well suggesting that this is the main mechanism for the development of alcoholic cirrhosis. Because of the potential role of inflammatory factors in the pathogenesis of fibrosis and cirrhosis, therapeutic anti-inflammatory treatments have been proposed. Furthermore, in view of the potential fibrogenic role of cytokines[47], anticytokine therapy is also being contemplated, with either antibodies, inhibitors of cytokine binding or regulators of cytokine production[48]. Colchicine may provide a useful approach as anti-inflammatory agent for the treatment of alcoholic liver injury[48]. In patients with alcoholic liver disorders and hepatitis C, no specific antiviral therapy is currently available as alpha-interferon is contradicted in alcoholics.

Increase in collagen accumulation induced by acetaldehyde has been prevented by PPC extracted from soybeans[49] and by its main phosphatidylcholine series, namely dilinoleophosphatidyl choline(DLPC)[50]. More recently, PPC was also found to prevent alcohol-induced steatosis and hyperlipemia[51] and to exert an unexpected but potent antioxidant effect[52] of possible relevance to the fibrosis, since the latter is known to be stimulated by products of lipid peroxidation. PPC is now being tested in man[47]. However, there have been no investigations on anti-fibrotic efficacy of ceramic water on alcohol induced hepatic fibrosis models in pig.

The purpose of this study was to develop animal model for ethanol-induced hepatic fibrosis and to identify a possible new anti-fibrogenic agent. Twelve male Landrace pigs were divided into 3 groups of 4 animals each. Group 1, 2 and 3 were fed with active ceramic water only, active ceramic water + liquid diet containing 15% ethanol and normal tap water + liquid diet containing 15% ethanol for 12 weeks, respectively. At week 12, all pigs were immediately sacrificed with collection of tissue and blood. Serologically, serum ALT and AST levels were significantly reversed in group 2, as compared to group 3. They were normal range in pigs of group 1. Microscopical results are shown in Text-Fig 7. Macrovesicular lipid droplets and moderate necrosis were evident in the tap water + ethanol fed group 3. However, active ceramic water intake group 1 exhibited normal architecture. Moreover, in group 2, mild fatty change and necrosis was observed. Collagen fibers were detected in spaces surrounding periportal and interlobular areas in the group 3 of tap water + ethanol, but collagen synthesis and its thickness of fibrotic septa

connecting portal tracts were markedly reduced in the group 2 of active ceramic water + ethanol. In immunohistochemistry, myofibroblasts were detected in the interlobular connective tissues in livers of group 3, which were treated ethanol and tap water. A few or no myofibroblasts were observed in groups 1 and 2. CYP2E1 was rarely detected in group 1 fed active ceramic water. However, group 2 showed slightly activation of CYP2E1 in the area ofpericentral veins, while CYP2E1 was significantly activated in group 3 fed tap water and ethanol.

Based on the above data, we believe that we have developed a unique alcohol induced fibrosis model in pig, which will be useful in developing anti-fibrotic agents and drugs. Furthermore, the active ceramic water used in our study had an inhibitory and may be protective against ethanol induced hepatic toxicity and fibrosis.

Legends for Figures

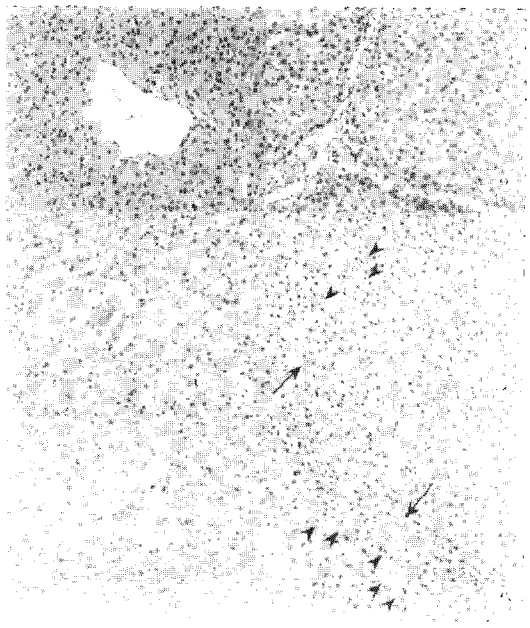


Fig 1. Pig liver of group 1 fed with active ceramic water only for 12 weeks. Central vein shows normal condition. H-E. ×66.

Fig 2. Pig liver of group 1 fed with active ceramic water only for 12 weeks. Periportal area and interlobular connective tissue are normal. H-E. ×66.

Fig 3. Pig liver of group 2 fed with active ceramic water + liquid diet containing

15% ethanol for 12 weeks. Mild centrilobular necrosis is observed. H-E. ×66.

Fig 4. Pig liver of group 2 fed with active ceramic water + liquid diet containing 15% ethanol for 12 weeks. Mild fatty changes are observed around interlobular area. H-E. ×132.

Fig 5. Pig liver of group 3 fed with tap water + liquid diet containing 15% ethanol for 12 weeks. Moderate necrosis is observed in the centrilobular vein. H-E. ×66.

Fig 6. Pig liver of group 3 fed with tap water + liquid diet containing 15% ethanol for 12 weeks. Mild fatty change is observed in the interlobular connective tissues. H-E. ×66.

Fig 7. Pig liver of group 1 fed with active ceramic water only for 12 weeks. Collagen fibers are detected in the periportal area and interlobular connective tissue. Azan-Mallory. ×33.

Fig 8. Pig liver of group 1 fed with active ceramic water only for 12 weeks. Few α-SMA-positive cells are detected and observed in the central vein, portal triad and interlobular connective tissue. Streptavidin-Biotin peroxidase method and Mayer's counter stain. ×33.

Fig 9. Pig liver of group 2 fed with active ceramic water + liquid diet containing 15% ethanol for 12 weeks. Collagen fibers are observed similar to those in the liver of group 1. Azan-Mallory. ×33.

Fig 10. Pig liver of group 3 fed with active ceramic water + liquid diet containing 15% ethanol for 12 weeks Few α-SMA-positive cells are detected in the central vein, portal triad and interlobular connective tissue. Streptavidin-Biotin peroxidase method and Mayer's counter stain. ×132.

Fig 11. Pig liver of group 3 fed with tap water + liquid diet containing 15% ethanol for 12 weeks. Collagen fibers are increased in the interlobular connective tissue. Azan- Mallory. ×33.

Fig 12. Pig liver of group 3 fed with tap water + liquid diet containing 15% ethanol for 12 weeks. α-SMA- positive cells are also increased in the interlobular connective tissue. Streptavidin-Biotin peroxidase method and Mayer's counter stain. ×13.

Fig 13. Pig liver of group 1 fed with active ceramic water only for 12 weeks. Few CYP2E1-positive cells are observed. Streptavidin-Biotin peroxidase method and Mayer's counter stain. ×13.

Fig 14. Pig liver of group 1 fed with active ceramic water only for 12 weeks. Few CYP2E1-positive cells are also observed in the centrilobular vein. Streptavidin-Biotin peroxidase method and Mayer's counter stain. ×66.

Fig 15. Pig liver of group 2 fed with active ceramic water + liquid diet con- taining 15% ethanol for 12 weeks. CYP2E1-positive cells are increased in the centrilobular area. Streptavidin-Biotin peroxidase method and Mayer's counter stain. ×13.

Fig 16. Pig liver of group 2 fed with active ceramic water + liquid diet containing 15% ethanol for 12 weeks. CYP2E1-positive cells are mildly increased in the centrilobular area. Streptavidin-Biotin peroxidase method and Mayer's counter stain. ×66.

Fig 17. Pig liver of group 3 fed with tap water + liquid diet containing 15% ethanol for 12 weeks. CYP2E1-positive cells are markedly increased in the centrilobular area. Streptavidin-Biotin peroxidase method and Mayer's counter stain. ×13.

Fig 18. Pig liver of group 3 fed with tap water + liquid diet containing 15% ethanol for 12 weeks. CYP2E1- positive cells are markedly increased around the central vein. Streptavidin-Biotin peroxidase method and Mayer's counter stain. ×66.

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