Korean J. Environ. Biol. 22(3) : 405~410(2004)

Citrus junos Fractions Decrease Alcohol-induced Liver Damage and Influence Lipid Metabolism in Alcohol-fed Rats

Kap Joo Park

Department of Biological Sciences, Konkuk University, Seoul 143-701, Korea

Abstract - The effect of treatment with *Citrus junos* fractions (citron 3W, citron 3H, citron 4W and citron 4H) upon rat hepatocytes exposed to alcohol was investigated. We compared the serum biochemistry of rats administered both alcohol and *Citrus junos* fractions to control rats treated with alcohol alone. The effects of Alanine amino transferase (ALT) were significantly lower in the *citron* 3H extract group compared with the negative control group ($p<0.05$) and other experimental groups were not significantly low but a little low compared to negative control group. The levels of triglyceride (TG) were significantly low in all experimental groups compared with negative control group. Especially triglyceride level of *citron* 3H was lowest near to normal control group. The concentration of total cholesterol was significantly high in negative groups compared with normal control group but in all experimental groups, the concentration of total cholesterol was similar to that of negative control group. Total cholesterol of the citron 4W group was somewhat low compared with negative control group. In contrast, activities of alcohol dehydrogenase (ADH) were significantly higher in all experimental groups compared with the negative control ($p<0.05$) group. These data suggest that *Citrus junos* fractions may represents an excellent candidate for protection of rat hepatocytes from alcohol-mediated damage.

Key words : Fractions of Korea *Citrus junos*, rat liver cell, alcohol protection effect

INTRODUCTION

Much effort has been made to develop a reproducible and robust rodent model of alcohol-related liver disease in order to facilitate the study of the various factors involved in the initiation and progression of alcohol hepatotoxicity (Sherlock 1993; De la M Hall *et al.* 2001). Excessive intake of alcohol may severely damage such organs as liver and heart, resulting in dysfunction including derangement of blood pressure and triglyceride levels (Nakanishi *et al.* 2001). There have been numerous attempts to develop clinically useful compounds to ameliorate or cure alcohol-related disorders (Kono *et al.* 2001a,b). However, it is well documented that these compounds may exhibit severe cytotoxicity, reproductive toxicity and other important side effects. Therefore, in order to find an alternative to the traditional cure, studies have increasingly focused on the development of therapeutic agents based on natural products and medicinal herbs. Nowadays, researches for the development of liver function promotion medicines for cooling crapulence and of hyperlipidemia prevention medicines are lively put into practice. Mungbean have a medical function for detoxication and antiphlogistic. Choi's research (1997) showed that Aspartate aminotransferase (AST) and ALT activities are considerably dropped by having mungbean juice within rats which

* Corresponding author: Kap Joo Park, Ph.D. Tel. 02-447-5018, Fax. 02-3436-5432, E-mail. kkupkj@konkuk.ac.kr

have hurt liver by cadmium. Cho's research (1993) showed that the density of cholesterol and the content of triglyceride become lower by having mustad leaf (Brassica Juncea) juice within rats. Kim (1996) studied the mugwort influence to the rats that have hurt liver by ethanol injection. He reported that when *Artemissia selengensis* extract were treated to long-term alcohol fed rats, AST and ALT activities of blood serum of rats were considerably decreased. It showed that the internal peroxide injury caused by free radical that was formulated by ethanol was improved by *Pueraria radix* extract (Lee 1999) and *Pueraria labata* extract (Lee 2000). Ahn (1997) showed that *Phellinus linteus* extract made increased rat's alcohol ability to 13 percent. He also showed the same influence to the man and woman.

We have already reported the ameliorative effects of *Citrus junos* 3 and 4 on liver damage and hyperlipidemia in alcohol-fed rats. So in this research, we tested the *Citrus junos* fractions' influence whether it helps the liver function of alcohol fed rats or not and whether it helps the lipid metabolism of alcohol fed rats or not. And we found that *Citrus junos* fractions protects damage of alcohol-fed liver cells, may resulting in curing liver diseases and in improving lipid metabolism.

MATERIALS AND METHODS

1. Preparation and treatment of fractionated extract of *Citrus junos*

All Korean *Citrus junos* used were purchased from Seungil farm (Wando, Korea) at October (ripened for three month) of 2003 and November (ripened for four month) of 2003. Less mellowed citron (ripened for three months, so not mellowed completely, afterward it will be marked citron 3) and mellowed citron (ripened for four months, so mellowed completely, it'll be marked by citron 4) were grinded properly by using mixer (Hanil Co. Ltd., Korea). The grinded Citron 3 and 4 samples were centrifuged at 6,000 × g for 15 minutes and supernatant were filtered by using gauze, and lyophilized. The dried sample was dissolved in 400 mL of distilled water (concentrated sample) and the concentrated sample and equal volume of hexane were added to separate funnel and mixed for 20 minutes. When the mixture has completely fractionated layer, mixed them again for 20 minutes and re-fractionated. And each fractionated solutions (water layer and hexane layer) was vaccume concentrated by using rotavapo R-200 (Buchi, Germany), and lyophilized and re-dissolved in distilled water. Each fractionated extract of *Citrus junos* were administered orally at dose of 0.6 g kg^{-1} rat body weight for 4 weeks. The following abbreviations are used for the extracts: the letter W represents water fraction extract and the letter H represents hexane fraction extract. For example, water fraction extract of *Citrus junos* 3 was named citron 3W and hexane fraction extract of *Citrus junos* 4 was named citron 4H, *etc*

2. Animal models

Young adult male Sprague Dawley rats, initial weight 130-150 g, were purchased from the Daehan Biolink Co. Ltd. (Seoul). Animals were housed in individual cages under conditions of constant temperature (22±2°C) and humidity (55±5%). They were kept on a 12 h light/dark cycle and acclimatized to the housing situation for four weeks before experiments. Rats were divided into seven groups (n = 6). In normal control group, rats were fed with water. For negative control group rats were pair-fed with alcohol/water. Rats in positive control group were pair-fed with alcohol and hangover cure solution (Condition : Cheil je dang Co. Ltd., Seoul). In each group of experiments, rats were pair-fed with ethanol and experimental materials for the same period (Table 1). Ethanol-fed rats were induced to consume 40% ethanol in water. An intake of 5 g ethanol kg day^{-1} was achieved. The body weight and general condition of

Table 1. Composition of groups

Group	No. of exam	Treatment
Normal Control	6	Non-alcohol
Negative Control	6	Alcohol+Distilled Water
Positive Control	6	Alcohol+HCS*
Experiment group 1	6	Alcohol+citron 3W
Experiment group 2	6	Alcohol+citron 3H
Experiment group 3	6	Alcohol+citron 4W
Experiment group 4	6	Alcohol+citron 4H

HCS*: Hangover cure solution (Condition: Cheil je dang Co. Ltd., Seoul).

the animals were monitored twice weekly. Rats were sacrificed for determination of biochemical test by anesthetizing with diethyl ether. After blood was obtained from abdominal vein, the liver was rapidly removed and rinsed in cold physiological saline. Liver weight on the each animal was measured after blotting with filter paper and stored at −70°C.

3. Biochemical determinations

Blood was set aside in an EDTA-freed tube for half an hour. The serum was separated from the blood with a centrifuge at 3,000 × g for 15 minutes. AST or ALT activity in the serum was determined using the AST kit (Boehringer Mannheim, Germany) or ALT kit (Boehringer Mannheim, Germany). Triglyceride was measured using the TG kit (Boehringer Mannheim, Germany), and the enzymatic colorimetric test for cholesterol content was also conduced using the Total Cholesterol kit (Boehringer Mannheim, Germany).

4. Determination of ADH in the liver

The rats were killed by decapitation and bled, and then liver cells were transferred to 0.25 M sucrose buffer and homogenized for determination of the ADH. Homogenized solution centrifuged at 14,000 × g for 15 min. Achieved supernatant was filtered by a 0.45 μm membrane filter (Millipore, France) and stored at 4°C.

5. Statistical analysis

All results were shown as mean standard deviation. Statistical evaluation of data was performed by Duncan's multi-range test to make comparisons between groups.

RESULT AND DISCUSSION

1. Weight gain and ratio of liver weight to body weight

Table 2 shown that the body weight change and liver damage were observed after chronic alcohol administration. The experimental group 2 (72.8±13.26 g), 3 (73.2±11.13 g) and 4 (70.8±11.00 g) showed a greater gain than any other controlled groups in body weight. The normal control group was determined 59.8±5.49 g, the negative control group was determined 58.5±12.49 g and the positive control group was determined 56.2±11.50 g.

Table 2. Total body weight gains and the weight of liver

Group	Body weight change (g)	Liver index (liver/body weight %)
Normal control	59.8±5.49[a] [1) 2)]	3.98±0.40[a]
Negative control	58.5±12.49[a]	4.06±0.24[bc]
Positive control	56.2±11.50[a]	4.34±0.13[b]
Experiment group 1	58.5±9.75[a]	4.40±0.24[b]
Experiment group 2	72.8±13.26[c]	3.79±0.22[a]
Experiment group 3	73.2±11.13[c]	4.17±0.48[bc]
Experiment group 4	70.8±11.00[c]	4.13±0.07[bc]

[1)] Value are Mean±S.D. (standard deviation) of six rats.
[2)] Means with different superscript letters within a column and significantly different from each other at $P<0.05$ as determined by Duncan's multiple-range test.

According to previous research (Mezey 1980; Pikarr 1987) alcohol-fed rats had a loss weight than normal groups in body weight. In case of Pikarr (1987), the loss weight is due to loss of body fat, reduction of food-intake and high consumption of energy as a result of alcohol-fed. In this study, the body weight of negative control group was a little decreased compared to that of normal control group. And in the citron 3H, citron 4W and citron 4H groups, significant recovery of body weight was appeared. This may indicate a recovery of liver function by these three groups.

The ratio (%) of liver weight to body weight was shown in table 2. In the ratio (%) of liver weight to body weight, the normal control group was 3.98±0.40%, the negative control group was 4.06±0.24% and the positive control group was 4.34±0.13%. In alcohol-fed animal, the ratio (%) of liver weight to body weight was increased because lipid and fiber were accumulated in liver (Leo *et al.* 1983). in this study, relative liver weight to body of the negative control group was higher than that of normal control group. In the experimental group, relative liver weight to body of citron 3H was much more lower (3.79±0.22%) than that of negative control group ($P<0.05$). It seemed that citron 3 hexane extract was prevent accumulating fiber and lipid in the ethanol fed rat liver cells.

2. Activity of AST and ALT

As to the AST level in the blood serum, the normal group was determined at 60.33±4.98 unit, the negative group determined at doubled 117.00±20.02 unit and the positive group was determined 95.33±4.46 unit (Table 3). The AST level of experimental group 1, 2, 3 and 4 was measured 127.83±21.35 unit, 125.00±20.06 unit, 109.33±8.64 unit and 103.33±22.24 unit. The AST level of experimental group 3 and 4 was a little lower than that of negative group ($p > 0.05$).

As shown in Table 3, the ALT amount was 19.17± 2.14 unit for the normal group, whereas the negative control group were significantly ($p < 0.05$) high, at 45.83 ±7.14 unit. The ALT level of experimental group 1, 2, 3 and 4 was measured 34.17±3.37 unit, 28.17±4.62 unit, 31.33±4.18 unit and 32.50±4.93 unit. The experimental group 2, citron 3H, demonstrated significantly low ALT level compared to negative control groups at near-normal levels. And citron 4W and citron 4H groups demonstrated somewhat low ALT levels.

The AST and ALT are essential enzymes determining the status of liver diseases. In general, decreasing function of the liver is signaled by the increased AST and ALT amount in blood serum (Kien and Ganther 1983; Thompson and Scott 1970). It is reported that the activities of AST and ALT was increase in the result of problem with liver metabolism and loss of liver cell by alcohol intake (Zimmerman 1981). In negative control group, these two enzymes were higher value than normal control group. This result was caused with alcohol-intake. However, as to the AST enzyme in table 3, the intake of experimental group 4 (citron 4H) with alcohol can somewhat effectively decrease the activities of AST enzymes. And experimental group 2, citron 3H, was significantly low compared with negative control group for ALT enzyme activity. Summing up, in case of the activity of AST enzyme, all of experimental groups have no powerful efficacy to decrease this enzyme compared with positive control group. But the AST level of citron 4H groups have a little effect to decline the activity of AST enzyme. As to the amount of ALT, all of experimental group were decreased compared with negative group. Especially for experimental group 2, citron 3H, was lowest level near to normal control group. This fact was suggested that citron 3H may recovery agent for the damaged liver induced by alcohol.

3. Serum triglycerides and total cholesterol content

The triglyceride contents in the blood serum were determined for each group in order to examined the citron effects on the lipid metabolism of an animal with liver lesions. A number of reports have demonstrated that alcohol intake increases the triglyceride and total cholesterol level significantly in the liver and the blood (Belfrage 1977). Given the results of the studies in this report (Table 4), alcohol intake leads to increase a significant amount of triglyceride and cholesterol in the blood and fatty liver. The triglyceride amount in the normal, negative and positive control group was 14.17± 6.80 mg dL^{-1}, 71.33±44.27 mg dL^{-1} and 76.33±23.57 mg dL^{-1}, respectively, whereas in all experimental groups, triglyceride amount was significantly reduced compared with negative control group ($p < 0.05$). Especially for the experimental group 2, citron 3H, was lowest content at 20.50±9.145 mg dL^{-1}.

Table 3. Enzyme activity of AST and ALT in serum

Group	AST (U L^{-1})	ALT (U L^{-1})
Normal control	60.33 ± 4.98^{a} [1) 2)]	19.17 ± 2.14^{a}
Negative control	117.00 ± 20.02^{b}	45.83 ± 7.14^{b}
Positive control	95.33 ± 4.46^{c}	43.50 ± 8.62^{b}
Experiment group 1	127.83 ± 21.35^{b}	34.17 ± 3.37^{bc}
Experiment group 2	125.00 ± 20.06^{b}	28.17 ± 4.62^{a}
Experiment group 3	109.33 ± 8.64^{b}	31.33 ± 4.18^{b}
Experiment group 4	103.33 ± 22.24^{bc}	32.50 ± 4.93^{b}

[1)] Value are Mean±S.D. (standard deviation) of six rats.
[2)] Means with different superscript letters within a column and significantly different from each other at $P < 0.05$ as determined by Duncan's multiple-range test.

Table 4. Concentration of serum lipid in rat

Group	Triglyceride (mg dL^{-1})	Total cholesterol (mg dL^{-1})
Normal control	14.17 ± 6.80^{a} [1) 2)]	42.83 ± 13.99^{a}
Negative control	71.33 ± 44.27^{b}	70.50 ± 6.19^{b}
Positive control	76.33 ± 23.57^{b}	81.17 ± 7.00^{b}
Experiment group 1	29.83 ± 14.57^{cd}	$70.00 \pm 4.43b^{cd}$
Experiment group 2	20.50 ± 9.145^{cd}	74.67 ± 6.09^{bc}
EΩxperiment group 3	21.67 ± 10.25^{de}	64.50 ± 10.05^{ad}
Experiment group 4	21.33 ± 11.22^{de}	73.00 ± 5.10^{bd}

[1)] Value are Mean±S.D. (standard deviation) of six rats.
[2)] Means with different superscript letters within a column and significantly different from each other at $P < 0.05$ as determined by Duncan's multiple-range test.

The serum cholesterol concentration for normal control group was 42.83±13.99 mg dL^{-1}, for negative control group was 70.50±6.19 mg dL^{-1}, and for positive control group was 81.17±7.00 mg dL^{-1}. In positive control group, triglyceride and cholesterol levels were not much different from negative group significantly ($p>0.05$). But the result of the tests proved that all experimental groups were significantly lower than negative control group ($p<0.05$) in the triglyceride contents of blood serum. In the concentration of total cholesterol, all experimental groups were not significantly different from negative and positive groups except for the experimental group 3, citron 4W. In this context, this report suggests that the some material of citron 3H and 4W may be great alternative in preventing and treating the high triglyceride contents of the blood.

4. Activity of ADH

In Table 5, the ADH (alcohol dehydrogenase) level of the negative control group was determined at 1.05± 0.44 U L^{-1} and of the normal control group was determined with 0.54±0.62 U L^{-1}, while the ADH level was about four fold as high as that of the normal control group at the experimental groups.

ADH is an enzyme that oxidizes ethanol, and the enzyme's activity changes by the length of ethanol administration (Koivula and Lindors 1975). Kim (1985) and Lee (2000) reported that the ADH activity increases resulting from a chronic intake of alcohol in animal tests. It is widely known that poisons caused by alcohol is closely related to alcohol metabolism (Nanji and Zakim 1996). The damages to organic tissue are caused by the acetaldehyde created during the metabolic process. The results (Table 5) shows that a long-term intake of alcohol may lead to an accumulation of acetaldehyde, and that some material of citron fractions promotes alcohol metabolism. It is suggested that the experimental materials can enhance alcohol metabolism, so they discourage the accumulation of acetaldehyde and prevent alcohol poisoning.

Table 5. Activity of alcohol dehydrogenase in rat

Group	ADH (U L^{-1})
Normal control	0.54 ± 0.62^{a} [1) 2)]
Negative control	1.05 ± 0.44^{b}
Positive control	1.31 ± 0.60^{b}
Experiment group 1	2.55 ± 0.54^{c}
Experiment group 2	2.02 ± 0.40^{c}
Experiment group 3	2.28 ± 0.64^{c}
Experiment group 4	1.85 ± 0.32^{b}

[1)] Value are Mean±S.D. (standard deviation) of six rats.
[2)] Means with different superscript letters within a column and significantly different from each other at $P<0.05$ as determined by Duncan's multiple-range test.

ACKNOWLEDGEMENT

This work was supported by Korea Research Foundation Grant (KRF-2002-075-E00005).

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Manuscript Received: April 29, 2004
Revision Accepted: August 20, 2004
Responsible Editorial Member: Jae Seok Lee
(Konkuk Univ.)