Wheat gluten hydrolysate alters the progress of hepatic pathology induced by prolonged carbon tetrachloride administration in rat

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Abstract

Wheat gluten hydrolysate (WGH) has been reported to mitigate chronic hepatitis in some patients. We aimed to reproduce and examine this phenomenon in a rat experimental model. Male Sprague-Dawley rats were injected with carbon tetrachloride twice a week for 25 weeks, and 3 weeks later WGH was added to the feed (none, 4%, and 8%) for the remaining 22 weeks. Transition of serum transaminases showed a temporary decrease in aspartate aminotransferase (AST) and a delay in the peaking of alanine aminotransferase (ALT) in the WGH groups. Macroscopically, at the end of the 25 weeks, the progress of cirrhosis was milder in the WGH groups, as indicated by less ascites fluid and the fewer tubercle formations. Histological and immunohistochemical analyses of the liver revealed fewer collagen fibers and less α-smooth muscle actin (SMA) in the 8% WGH group. The ethanol-soluble extract of liver tissue showed plasmin inhibiting activity in the 8% WGH group. The observed modification of the transition of serum transaminases during chronic CCl4 challenge supports the possibility that WGH mitigates chronic hepatitis, and the liver manifestations after 25 weeks of CCl4 treatment indicate that WGH ingestion alters the progress of cirrhosis.

Key words: Glutamine, cirrhosis, carbon tetrachloride, wheat gluten hydrolysate

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Introduction

Hepatitis is acute and chronic liver damage, which is characterized by hepatocyte necrosis and inflammation in the liver. Hepatitis is mainly caused by viral infection, excessive alcohol consumption, toxins and fatty liver. Although extensive studies have been carried out on the treatment of chronic hepatitis, it is still difficult to cure and control chronic hepatitis. Chronic hepatitis frequently causes fibrosis in the liver which consequently results in development of cirrhosis. Cirrhosis is believed to irreversibly progress, causing life-threatening damage to the liver. The treatment for hepatitis is primarily based on treatment of the underlying disease by alimentary-, exercise-, and drug-based therapies. In addition to these conventional therapies, there is increasing demand for alternative therapies, which is suitable for chronic use and has high compliance. Thus, there is great interest in food functionality to control hepatitis and subsequent fibrosis [1, 2].

Recently, a therapeutic effect of wheat gluten hydrolysate (WGH) on hepatitis in patients with different backgrounds was reported [3]. The objective of the present study was to reproduce and examine this possible liver protective effect of WGH on carbon tetrachloride-induced hepatitis and subsequent fibrosis in rat liver [7].

Materials and Methods

Animal experiment

Animal facilities and protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University. Male 7-week-old, specific-pathogen free Sprague-Dawley rats (n=18) were purchased from Japan SLC (Hamamatsu, Japan). They were housed individually in metabolic cages with lights on from 08:00 to 20:00, under controlled temperature (23 ± 2°C) and humidity (60% ± 10%). The animals had access to the standard 20% casein diet and water ad libitum.
for acclimation. To induce hepatitis, 50% carbon tetrachloride (CCL₄) (Wako Pure Chemical Industries, Osaka, Japan) dissolved in olive oil (Wako Pure Chemical Industries) was administered by subcutaneous injection at 0.1 ml/100 g body weight (approximately 0.8 g/kg) twice a week for 25 weeks. After three weeks, the rats were randomly assigned into three groups and fed a 4% WGH diet (4WGH, n=6), an 8% WGH diet (8WGH, n=6) or the control 20% casein diet (C, n=6) for 22 weeks. In the 4WGH and 8WGH diets, the same proportion of casein was replaced with WGH (Nissin Pharma, Tokyo, Japan). Body weight and food intake were measured every morning. To determine aminotransferase activity, blood was drawn from the caudal vein every week. On the 6th day of the 25th week, the rats were fasted overnight and euthanized under ether anesthesia by portal exsanguination. Ascites fluids and nodules were anatomically observed and recorded, and livers were collected, weighed, and frozen and stored in liquid nitrogen until use. The ascites fluid in 1 rat in the C group was indistinct, and thus excluded from the results.

**Biochemical assays**

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, and albumin and hyaluronic acid concentrations were determined using a CL-8000 Clinical analyzer (Shimazu, Kyoto, Japan).

**Histology and immunohistochemistry**

A portion livers was fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 48 h at 4 °C, embedded in paraffin, sectioned in a 4 µm thickness, and stained with Azan-Mallory for collagen visualization. Deparaffinized sections were immunostained using peroxidase conjugated anti-α-smooth muscle actin (α-SMA) antibody (Dako Epos system) according to the manufacturer's protocol. Diaminobenzidine was used as the chromogen, and counter staining was performed with hematoxylin. The stained area was measured in 10 microscopic visual fields (×25).

**Plasmin inhibitory activity of liver ethanol soluble fraction**

The ethanol-soluble fraction of liver extract was prepared as follows. Liver was homogenized with 2 volumes of phosphate-buffered saline; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.2, containing 0.1% Tween 20, 5 mM ethylene diamine tetra-acetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF), in a glass homogenizer. The homogenate was centrifuged at 5,000×g for 10 min. The residue was rehomogenized and centrifuged, and the supernatants combined and mixed with 3 volumes of ethanol. The resultant precipitate was then removed by centrifugation. A 100 µL aliquot of the ethanol soluble fraction was dried under vacuum in a 1.5 mL centrifuge tube and then dissolved in 100 µL of 67 mM sodium phosphate buffer, pH 7.4 and used as liver extract.

Plasmin activity was estimated using casein as the substrate. Plasmin (500 µg, MO) was dissolved in 1.5 mL of 50 mM Tris-HCl buffer, pH 7.2 containing 100 mM NaCl. This stock solution was kept at -80°C until use. The stock solution was diluted with 34 volumes of the same buffer. The reaction mixture of 10 µL of the diluted plasmin solution, 10 µL of 0.3% casein dissolved in 67 mM sodium phosphate buffer, pH 7.4, and 30 µL of the liver extract dissolved in 67 mM sodium phosphate buffer, pH 7.4, was incubated at 37°C for 20 min. The enzyme reaction was terminated by addition of 10 µL of 100 mM PMSF and then 10 µL of 10% sodium dodecyl sulfate (SDS), 50 µL of 8 M urea, and 10 µL of 0.1% bromophenol blue (BPB) were added. Next, a 20 µL aliquot of the mixture was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using a 15% separation gel by the method of Laemmli. Proteins in the gel were developed with Coomassie Brilliant Blue R-250 staining. The staining intensity of the bands in the substrate were determined using RFLPscan plus ver.3.0 (Scanalytics, Billerica, MA).

**Statistical analysis**

All values express the mean±SD or SE as described. The animal study data was analyzed using Tukey’s test. Immunohistochemical data was analyzed using the Kruskal-Wallis test, followed by multiple t-test with Bonferroni correction. A probability value of less than 0.05 was considered to be statistically significant.

**Results**

**Development of chronic hepatitis**

The mean food intake of the 4WGH and 8WGH groups was higher than that of the C group for most of the study period, although no statistically significant difference was found (Fig. 1). On the other hand, the 4WGH group showed significantly higher weight gain than the other groups (Fig. 2), indicating that the feed efficiency ratio of the 4WGH group was higher than that of the other groups.

In the C group, plasma AST activity increased upon CCL₄ treatment and reached a plateau after 5-6 weeks of administration, and then slightly decreased after 15 weeks, whereas a temporary decrease in plasma AST was seen in both the 4WGH and 8WGH groups around 8-11 weeks (Fig. 3). Similarly, plasma ALT also increased up to 7-8 weeks and then began to decline rapidly in the C group, while plasma ALT retained its peak levels for a few weeks in the WGH groups (Fig. 4).

On the autopsy day, ascites fluid was observed in 3 of the 5 in the C group rats, whereas only 1 rat showed ascites fluid in 1 rat in the C group was indistinct, and thus excluded from the results.

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fluid in the WGH groups. The number of liver nodule was apparently fewer and the liver was heavier in both WGH groups (Table 1). Analysis of drawn blood was characterized by low plasma albumin and high plasma hyaluronic acid levels in all groups compared to normal conditions (Table 2). The mean plasma hyaluronic acid concentration decreased by WGH in a dose-dependent manner, although not statistically significantly.

Histology and immunohistochemistry

Azan-Mallory staining of liver showed excess collagen fibers and lipid droplets deposited in all groups, indicating that cirrhosis had been induced by the CCl4 treatment. The aniline blue-positive, stained collagen, area showed dose-dependent suppression of fibrosis by WGH: C, 15.2%; 4WGH, 12.2%; and 8WGH, 9.1%; P<0.05 for C vs. 8WGH (Fig. 5). However, no significant differences in the accumulation of lipid droplets were observed.

Cells positively stained by anti-α-SMA antibody showed suppression of α-SMA expression in the 8WGH group, although this was not statistically significant: C, 0.49%; 4WGH, 0.51%, and 8WGH, 0.36% (Fig. 6).

Table 1 Anatomic observations

<table>
<thead>
<tr>
<th>Group</th>
<th>Ascites(^a)</th>
<th>Liver weight (g)</th>
<th>Nodules(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>3/5</td>
<td>3.27±0.53</td>
<td>+++</td>
</tr>
<tr>
<td>4WGH</td>
<td>1/6</td>
<td>4.14±0.68</td>
<td>+</td>
</tr>
<tr>
<td>8WGH</td>
<td>0/6</td>
<td>4.00±0.34</td>
<td>+</td>
</tr>
</tbody>
</table>

Liver weights are expressed as mean ± SD. Superscripts indicate as follows: \(a\); ascites positive animal per total, \(b\); visual determination of the grade of nodules ranged from +++ (severe) to + (slight), and \(c\); P<0.05 vs. C.

Table 2 Plasma albumin and hyaluronic acid concentration on the day of euthanasia

<table>
<thead>
<tr>
<th>Group</th>
<th>Albumin (g/dl)</th>
<th>Hyaluronic acid (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.2±0.1</td>
<td>208±150</td>
</tr>
<tr>
<td>4WGH</td>
<td>2.1±0.2</td>
<td>210±91</td>
</tr>
<tr>
<td>8WGH</td>
<td>2.2±0.1</td>
<td>166±115</td>
</tr>
<tr>
<td>Normal Range</td>
<td>4.18±0.169</td>
<td>-</td>
</tr>
</tbody>
</table>

Data express mean ± SD. Normal range of albumin is quoted from the data sheet provided by Japan SLC.

Food Intake

Figure 1. The mean food intake of the 4WGH and 8WGH groups was higher than that of the C group for most of the experimental period. Error bars indicate the standard error. Abbreviations: + and ++ indicate P<0.05 and P<0.01 compared to C, respectively.
Figure 2. Mean body weight of the rats during the experimental period. The 4WGH group showed higher weight gain than the other groups. Error bars indicate the standard error. Abbreviation: + indicates P<0.05 compared to C.

Figure 3. Plasma aspartate aminotransferase (AST) activity during the experimental period. A temporary decrease was seen in both the 4WGH and 8WGH groups around 8-11 weeks. Error bars indicate the standard error. Abbreviations: + and ++ indicate P<0.05 and P<0.01 compared to C, respectively.
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Figure 4. Plasma alanine aminotransferase (ALT) activity increased up to 7-8 weeks and then declined rapidly in the C group, while they retained their peak levels for a few weeks in the WGH groups. Error bars indicate the standard error. Abbreviations: + and ++ indicate P<0.05 and P<0.01 compared to C, respectively.

Figure 5. Azan-Mallory staining of the liver: a, control; b, 4WGH; c, 8WGH; d, aniline blue positive area. WGH suppressed the expression of collagen fibers, the aniline-blue stained area, in a dose-dependent manner, whereas WGH did not affect accumulation of lipid droplets.
Cells positively stained by α-SMA antibody showed suppression of SMA expression in the 8WGH group, although this was not statistically significant.

Inhibition of plasmin by the ethanol-soluble fractions of livers from the control (C), 4WGH, and 8WGH groups. M, molecular markers; N, negative control (casein substrate only); P, positive control (plasmin digest of casein). The two major bands, α- and β-casein, observed in N were digested by the plasmin in P. Addition of the liver extract of the 8WGH group inhibited plasmin digestion.
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0.00 0.05 0.10 0.15 0.20 0.25 0.30

Staining intensity

Figure 8. Intensity of α- and β-casein. The liver extract from the C group did not inhibit plasmin. On the other hand, addition of the liver extract from the 8WGH group increased the amounts of undigested casein bands.

Plasmin inhibitory activity of liver extract

The inhibitory activity of the ethanol soluble fraction of liver against plasmin was examined using casein as the substrate. As shown in Figure 7, two major bands corresponding to α- and β-casein were observed in the substrate solution. Plasmin degraded these bands into smaller molecular weight fragments. The liver extract from the C group did not inhibit plasmin, while the addition of liver extract from the 8WGH group increased the amounts of undigested casein bands. These findings indicate that the livers of the 8WGH group contained an ethanol-soluble plasmin inhibitor (Fig. 8).

Discussion

The rats in the WGH groups consumed more food than those in the C group throughout the experiment, whereas body weight did not differ significantly between the C and 8WGH groups. The progress of cirrhosis was apparently mild in the WGH groups and occurred in a dose-dependent manner, which may have resulted in greater physical activity, which consequently may have affected energy consumption and body weight.

The serum transaminases increased with the prolonged CCl₄ treatment. After 11 weeks, serum ALT activity began to decline in both the WGH groups, whereas AST remained elevated. This decline in ALT transition may reflect the progress of cirrhosis; the start of fibrosis and atrophy in addition to the chronic hepatitis. For the first 8 weeks, WGH did not affect the increase in serum transaminases induced by CCl₄ treatment, and thus WGH did not interfere with the CCl₄-induced radical formation and subsequent liver damage. On the other hand, after 10-11 weeks, the serum transaminases temporarily showed a different transition pattern in the WGH groups; lower AST and higher ALT activities compared to the C group. This temporary decrease in AST and delayed turning point in ALT observed in the WGH groups indicates that WGH ingestion altered the progression of the hepatic pathology. Horiguchi et al. reported patients whose chronic hepatitis was mitigated by oral administration of WGH [3]. The transition of the transaminases around 8-11 weeks in the WGH groups is in accordance with the findings in those cases. The representation of the transient improvement in rat chronic hepatitis supported the anti-inflammatory effect of WGH as indicated in some patients. However, the effect was temporal and the hepatic lesion could not be examined at the exact timing when the inflammation was suppressed.

Macroscopically, the cirrhosis of the liver was dose-dependently milder in the WGH groups. The incidence of ascites fluid and tubercle formation was lower in the WGH groups. In addition, the greater liver weight indi-
cated less hepatic atrophy in the WGH groups. However, alleviation by WGH was not apparent in the serum albumin and hyaluronic acid levels.

Histological and immunohistochemical examinations were performed to confirm the macroscopic observations. The amount of collagen fibers was lower in the WGH groups in a dose-dependent manner, which is in accordance with the macroscopic tubercle formation findings. Thus, WGH ingestion was confirmed to mitigate hepatic fibrogenesis. Hepatic stellate cells play a major role in hepatic fibrogenesis, and hepatic injury activates hepatic stellate cells to transform into myofibroblasts and to express α-SMA and various extracellular matrix components [5]. Immunohistochemical staining showed lower expression of α-SMA in the 8WGH group, suggesting that activation of hepatic stellate cells was suppressed in this group. The hepatic lesion indicated the suppression of fibrosis in WGH group, which was supported by immunohistochemical examination. The modification of the progression of cirrhosis could be account for the transient improve in chronic hepatitis. However, the relation between serum transaminase and hepatic pathological change has not been understood.

Transforming growth factor (TGF)-β has been shown to play a central role in the activation of hepatic stellate cells and subsequent fibrosis [5]. TGF-β is synthesized in a high molecular weight, latent (inactivated) form and attached to the cell surface. The latent TGF-β on the cell is converted to the active form by proteolytic cleavage [6]. In the development of liver fibrosis, plasmin has been demonstrated to be involved in activation of TGF-β [7]. Therefore, plasmin inhibiting activity in the liver was examined using α- and β-caseins as the substrate. The ethanol-soluble liver fraction was added to the assay system and the undigested substrates then analyzed by SDS-PAGE. The extract from the 8WGH group inhibited plasmin activity, but since this inhibitory activity was countered by a 75% ethanol-soluble fraction, a low molecular weight component derived from WGH may be contributing to the inhibitory activity. The exact nature of this inhibitory activity remains to be elucidated.

As demonstrated in this report, the CCl₄ rat cirrhosis model could reproduce the anti-inflammatory activity of WGH. Therefore, it is worthwhile to clarify the transition of serum transaminase with the progression of the hepatic lesion in this model to understand the human pathology and elucidate the mechanism of WGH. The plasmin inhibiting activity observed in the liver lesion might account for the anti-inflammatory activity of WGH. The examination of the chemical nature of this suppression will not only reveal the mechanism of WGH but also lead a novel anti-inflammatory agent.

Conclusion

Some patients with chronic hepatitis reported to show mitigation by WGH administration. In this report, we reproduced the phenomenon in experimental animal model to confirm that WGH could moderate the liver inflammation temporally. The hepatic lesion indicated the suppression of fibrosis in WGH group, which was supported by immunohistochemical examination. The liver lysate displayed the plasmin inhibiting activity in 75% ethanol soluble fraction. However the chemical nature of the anti-inflammatory activity remained to be elucidated.

Conflict of interest

A part of this work was granted by Nisshin Pharma Inc. which is the manufacturer of the wheat gluten hydrolysate investigated in this paper.

References


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