An Animal Study to Compare Hepatoprotective Effects Between Fermented Rice Bran and Fermented Rice Germ and Soybean in a Sprague-Dawley Rat Model of Alcohol-Induced Hepatic Injury

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Abstract: We compared hepatoprotective effects between fermented rice bran (FRB) and fermented rice germ and soybean (FRS) in a Sprague-Dawley (SD) rat model of alcohol-induced hepatic injury (AIHI). To establish an SD rat model of AIHI, the SD rats were given 30% ethanol or water without ethanol treatment. Then, they were given 30% ethanol followed by FRB or FRS at concentrations of 15% or 30%. Our results indicate that the FRB might be more effective in lowering serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP), malondialdehyde (MDA) levels in the serum and liver mitochondria, serum triglyceride levels and ALDH levels at a concentration of 15%, serum levels of lactate dehydrogenase (LDH), GSH levels at a concentration of 30% and MDA levels in the liver homogenate and microsome, and hepatic triglyceride levels at both concentrations as compared with the FRS. It can therefore be concluded that FRB might also be considered as an alternative to FRS in improving the AIHI.

Keywords: fermented foods; rice; soybean

1. Introduction

Foods or beverages are fermented through regulation of microbial growth or actions of enzymes [1,2]. Thus, fermented foods are produced depending on factors such as nature of microorganisms, ingredients and environmental conditions [3–5]. In more detail, there might be different kinds of fermentation processes; these include alcoholic fermentation (yeasts), lactic acid fermentation (lactic acid bacteria) and acetic acid fermentation (Bacillus or molds) [6–10]. It is well known that fermented foods can be characterized by enhanced nutritional properties and health-promoting effects [11–16].

Rice bran (RB) is a by-product of the rice milling process, and it contains diverse bioactive chemicals. Its anti-oxidative and anti-inflammatory activities have been well described in the literature [17,18]. RB is a rich source of dietary fibers and bioactive molecules, whose constituents are known to raise treatment effects of functional foods against chronic diseases [19,20]. Moreover, animal studies have shown that foods or beverages supplemented with RB are effective in improving dyslipidemia [21–23]. Beneficial effects of RB arising from several active compounds such as oryzanols, tocopherols, tocotrienols, phytosterols and nucleotides have been identified in rice bran [24–26]. Rodent models have demonstrated its efficacy against chronic diseases when treated with enzymes [27,28]. The effect of protein hydrolysates from RB in improving insulin resistance is one of the best examples in this case [29]. Thus, there is a growing evidence of the potential effects of RB in treating chronic diseases and their application to human health [30].
In an effort to improve nutraceutical activities of rice bran, several techniques have been employed. Of these, the fermentation of RB has been applied to the appropriate management of metabolic syndrome [31]. Thus, it has been reported to be effective against stress and fatigue when fermented with *Saccharomyces cerevisiae* and sodium dextran sulfate-induced colitis when fermented with *Aspergillus oryzae* [32,33]. Moreover, hepatoprotective effects of fermented rice bran (FRB) have also been described in the literature; Park et al. fermented the RB with *Bacillus sp.* (KCTC11351BP), *Bacillus subtilis* (KCTC11352BP), *Bacillus sonolensis* (KCTC11354BP) and *Bacillus circulans* (KCTC 11355BP) and then showed that the FRB was effective in protecting the liver from toxic chemicals in mice [34].

Currently, a natural ingredient extracted from fermented rice germ and soybean (FRS) is commercially available as an anti-hangover agent. It has been reported to be effective in improving alcoholic hangovers by lowering blood alcohol levels through the modulation of alcohol-metabolizing enzymes and the anti-oxidative activity [35].

In our preliminary in vitro experiment, we compared the total amount of phenolic compounds and flavonoids, the degree of free radical-scavenging activity, the effects in inhibiting lipid peroxidation in liver microsome and the antioxidative effects between the FRB and the FRS. Thus, we found that the FRB had significantly better findings compared with the FRS [36].

Given the above background, we conducted this experimental study to compare hepatoprotective effects between the FRB and the FRS in a Sprague-Dawley (SD) rat model of alcohol-induced hepatic injury (AIHI).

### 2. Materials and Methods

#### 2.1. Experimental Setting and Design

The current experimental study was approved by the Institutional Animal Care and Use Committee (IACUC) of Dong-A University, Busan, Korea (IAUAC approval number: DIACUC-11-27).

For the current experiment, we used active constituents of the FRB and FRS, both of which were provided by the Korea Bio-Solutions Co. Ltd. (Busan, Korea). The FRB was characterized in our previous study; the RB was fermented using *Bacillus subtilis* [36]. The FRS was the RSE® (DuhanBio Co., Ltd., Seoul, Korea). In addition, we also purchased 42 white male SD rats aged four weeks (Hyochang Science Animals Co., Daegu, Korea). They were housed individually in suspended wire-mesh stainless steel cages at a room temperature of 21–24 °C with lights on between 08:00 and 20:00. During a 1-week period prior to the experimental procedure, they were allowed free access to a semi-purified basal diet. During a 4-week experimental period, all the SD rats were evaluated daily for food intake and water consumption. They were also evaluated for body weight gain once weekly.

The SD rats were assigned to the following experimental groups:

1. The 15% FRB group (*n* = 6): The SD rats which were given 30% ethanol (*v/v*) and 1.5% FRB (*w/w*).
2. The 30% FRB group (*n* = 6): The SD rats which were given 30% ethanol (*v/v*) and 3.0% FRB (*w/w*).
3. The 15% FRS group (*n* = 6): The SD rats which were given 30% ethanol (*v/v*) and 1.5% FRS (*w/w*).
4. The 30% FRS group (*n* = 6): The SD rats which were given 30% ethanol (*v/v*) and 3.0% FRS (*w/w*).

#### 2.2. Experimental Procedures

##### 2.2.1. Validation of an SD Rat Model of AIHI

To establish an SD rat model of AIHI, the experimental rats were given 30% ethanol or water without ethanol treatment. Then, they were sacrificed by withdrawing blood from the abdominal aorta under light diethyl ether anesthesia. To examine whether we successfully established an SD rat model of AIHI, we performed histopathologic examinations of the liver sample. To do this, the liver sample was fragmented and then fixed using 4% paraformaldehyde and 0.1 M phosphate buffered saline (pH = 7.4). After that, it was embedded in a paraffin block and then sectioned at a thickness of approximately 6 µm. This was followed by staining with a hematoxylin and eosin dye. Then,
we examined histopathologic specimens using a light microscope (Olympus BS41; Olympus Co., Tokyo, Japan). Thus, we compared the number and volume of fat globules between the SD rats ingesting 30% ethanol and those ingesting water without ethanol treatment.

2.2.2. Quantification of Serum Hepatic Enzymes

After sacrifice, the SD rats were evaluated for the collection of serum samples. Then, the samples were collected, centrifuged at 1026×g for 15 min at 4 °C and stored at −70 °C for further laboratory procedure.

We measured serum levels of hepatic enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyltranspeptidase (γ-GTP) and lactate dehydrogenase (LDH) using the Chemiclinical Chemistry Analyzer (Samkwang Medical Laboratories, Seoul, Korea).

2.2.3. Preparation of Liver Homogenate Fractions

After the SD rats were sacrificed, their liver was extracted and then perfused in situ with saline (8.5 g NaCl/L). Then, it was weighed, frozen immediately in liquid nitrogen and stored at −70 °C for further laboratory procedure. As previously described, subcellular liver fractions were prepared [37]. In detail, the liver was homogenized in nine volumes of ice-cold potassium phosphate buffer (0.1 M potassium phosphate containing 1 mM sodium EDTA and 1 mM dithiothreitol, pH = 7.4) using an IKA-ULTRA-TURRAX T25 basic homogenizer (Ika-Werke GmBH and Co., Staufen, Germany). The homogenate was centrifuged at 800×g for 10 min, and the supernatant was centrifuged at 10,000×g for 20 min. The pellet was resuspended in buffer as the mitochondrial fraction. The supernatant was further centrifuged at 105,000×g for 60 min. The resulting clear supernatant was used as the cytosolic fraction. The precipitate was resuspended in the same buffer and then centrifuged again at 105,000×g for 60 min. The pellet was resuspended in buffer as the microsome fraction. Then, subcellular fractions of mitochondria, cytosol and microsomes were stored at −70 °C for further laboratory procedure.

2.2.4. Quantification of Lipid Peroxidation

The concentration of lipid peroxides was measured based on thiobarbituric acid reactive substances (TBARS) in both hepatic subcellular fractions and serum, as previously described [38–40]. The reaction mixture was a solution containing homogenates, subcellular fractions and thiobarbituric acid (TBA). Then, it was incubated in boiling water for 30 min. After centrifugation at 1000×g for 10 min, its upper layer was measured for the absorbance at a wavelength of 532 nm. The concentration of TBARS was expressed as nM of malondialdehyde (MDA) per g liver or mL serum.

2.2.5. Quantification of Hepatic Enzymes Involved in The Alcohol Metabolism

The activity of ADH was measured using a spectrophotometry based on its indicator, the degree of the conversion of NAD (Sigma Chemical Co., Louis, MO, USA) to nicotinamide adenine dinucleotide dehydrogenase (NADH), as described previously [41,42]. In addition, the activity of ALDH was measured using the methods of Koivula and Koivusalo [43,44]. Then, on noticing the reaction of enzymes, we measured the absorbance at a wavelength of 340 nm for 5 min.

2.2.6. Western Blot Analysis

For the Western blot analysis, we isolated the protein of the liver homogenate fraction containing ADH or ALDH using the 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (20 and 100 µg for ADH and ALDH proteins per lane, respectively), as described previously [45]. The protein samples were transferred electrophoretically to a nitrocellulose membrane at 4 °C for 16 h at a constant current of 300/240 mA/cm², and non-specific binding was inhibited using a blocking buffer (10% fat-free milk in Tris-Buffered Saline and Tween 20 (TBST) buffer) at room temperature for 1 h. The membrane
was repeatedly washed with the TBST buffer (10 mM Tris, pH = 7.5, 100 mM NaCl, 0.1% Tween 20) for 5 min. Then, it was probed with a rabbit ADH- or ALDH1A1-conjugated antibody (diluted at a ratio of 1:1000, Abcam, UK) overnight at 4 °C. After the primary antibody reaction, the membrane sample was washed with the TBST buffer. Then, it was incubated with an anti-mouse or anti-rabbit IgG-HRP (diluted at a ratio of 1:5000, DBUSA) at room temperature for 1 h. Equivalent loading of the protein samples was confirmed by re-probing membranes with mouse monoclonal GADPH IgG (diluted at a ratio of 1:1000; Milford). Proteins in the nitrocellulose membranes were detected by using the image analysis method using the SuperSignal West Pico Chemiluminescent Substrate and densitometrically quantified. The relative density of the ADH or ALDH protein band in each reaction was quantified by a densitometer (Lumi-Imager F1, Roche, Bazel, Switzerland).

2.2.7. Quantification of GSH

The concentration of γ-glutamyl-cysteinyl-glycine (glutathione, GSH) was determined, as previously described [46–48]. A 0.2-mL aliquot of liver homogenate was well mixed with EDTA solution 1.8 mL. This was followed by the addition of precipitating reagent 3.0 mL (1.67 g of metaphosphoric acid, EDTA disodium salt 0.2 g, sodium chloride 30 g and distilled water 1 L). The sample was preserved at 4 °C for 5 min and then centrifuged at 3000× g for 5 min. A 2-mL of the supernatant was mixed with 0.3 M disodium hydrogen phosphate solution 4 mL and 5,5′-dithiobis (2-nitrobenzoic acid) reagent 0.1 mL. This was followed by the spectrophotometry at a wavelength of 412 nm. Finally, the total concentration of GSH was expressed as nM per g liver.

2.3. Statistical Analysis

All data was expressed as mean±SE (SE: standard error) and was analyzed using one-way analysis of variance (ANOVA). Statistical analysis was done using the Duncan’s new multiple-range test. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Validation of an SD Model of AIHI

On histopathologic examinations of the liver samples, the number and volume of fat globules were increased in the SD rats ingesting 30% ethanol as compared with those ingesting water without ethanol treatment (Figure 1). This confirms that we successfully established an SD model of AIHI.

![Figure 1](image.png)  
Figure 1. Validation of a Sprague-Dawley (SD) rat model of alcohol-induced hepatic injury on histopathologic examinations. The number and volume of fat globules were increased in (right) the SD rats ingesting 30% ethanol as compared with (left) those ingesting water without ethanol treatment (Hematoxylin & eosin, ×400).
3.2. Effects of FRB and FRS on Serum Levels of ALT, AST, ALP, γ-GTP and LDH

Serum levels of AST, ALT and ALP were significantly lower in the 15% FRB and 30% FRB group compared with the 15% FRS and 30% FRS group in the corresponding order \((p < 0.05)\). Moreover, serum levels of γ-GTP were significantly higher in the 15% FRB group as compared with the 15% FRS group, but were significantly lower in the 30% FRB group compared with the 30% FRS group \((p < 0.05)\). Furthermore, serum levels of LDH were significantly lower in the 15% FRB group compared with the 15% FRS and 30% FRS group in the corresponding order \((p < 0.05)\) (Figure 2). These results indicate that the FRB might be more effective in lowering serum levels of AST, ALT and ALP at a concentration of 15% and those of LDH at a concentration of 30% compared with the FRS.

![Figure 2](image-url)

**Figure 2.** The effects of fermented rice bran (FRB) and fermented rice germ and soybean (FRS) on serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyltranspeptidase (γ-GTP) and lactate dehydrogenase (LDH). * Statistical significance at \(p < 0.05\). ** Statistical significance at \(p < 0.05\).

3.3. Effects of FRB and FRS on TBARS

MDA levels in the liver homogenate and microsome were significantly lower in the 15% FRB and 30% FRB group compared with the 15% FRS and 30% FRS group in the corresponding order \((p < 0.05)\). Moreover, MDA levels in the serum and liver mitochondria were also significantly lower in the 30% FRB group compared with the 30% FRS group \((p < 0.05)\), but there were no significant differences between the 30% FRB group and the 30% FRS group \((p > 0.05)\) (Figure 3). These results indicate that the FRB might be more effective in lowering MDA levels in the liver homogenate and microsome at both concentrations as compared with the FRS. Moreover, it can also be inferred that the FRB might be more effective in lowering MDA levels in the serum and liver mitochondria at a concentration of 15% as compared with the FRS.
Figure 3. Effects of fermented rice bran (FRB) and fermented rice germ and soybean (FRS) on thiobarbituric acid reactive substances (TBARS) in the liver and serum. * Statistical significance at $p < 0.05$. ** Statistical significance at $p < 0.05$.

3.4. Effects of FRB and FRS on GSH

There were no significant differences in GSH levels in the liver between the 15% FRB group and the 15% FRS group ($p > 0.05$). However, they were significantly lower in the serum in the 30% FRB group compared with the 30% FRS group ($p < 0.05$) (Figure 4). These results indicate that the FRB might be more effective in lowering GSH levels in the serum at a concentration of 30% compared with the FRS.

Figure 4. Effects of fermented rice bran (FRB) and fermented rice germ and soybean (FRS) on glutathione concentrations in the liver and serum. * Statistical significance at $p < 0.05$. ** Statistical significance at $p < 0.05$. 

3.5. Effects of FRB and FRS on Levels of Triglyceride

Serum triglyceride levels were significantly lower in the 15% FRB group compared with the 15% FRS group and higher in the 30% FRB group compared with the 30% FRS group \( (p < 0.05) \). Moreover, hepatic triglyceride levels were significantly lower the 15% FRB and 30% FRB group compared with the 15% FRS and 30% FRS group \( (p < 0.05) \) (Figure 5). These results indicate that the FRB might be more effective in lowering serum triglyceride levels at a concentration of 15% and hepatic triglyceride levels at both concentrations compared with the FRS.

![Figure 5](image1.png)

Figure 5. Effects of fermented rice bran (FRB) and fermented rice germ and soybean (FRS) on triglyceride concentrations in the liver and serum. * Statistical significance at \( p < 0.05 \). ** Statistical significance at \( p < 0.05 \).

3.6. Effects of FRB and FRS on Levels of ADH and ALDH

ADH levels were significantly higher in the 15% FRB and 30% FRB group as compared with the 15% FRS and 30% FRS group \( (p < 0.05) \). Moreover, ALDH levels were significantly lower in the 15% FRB group compared with the 15% FRS group and higher in the 30% FRB group compared with the 30% FRS group \( (p < 0.05) \) (Figure 6). These results indicate that the FRB might be more effective in lowering ALDH levels at a concentration of 15% compared with the FRS.

![Figure 6](image2.png)

Figure 6. Effects of fermented rice bran (FRB) and fermented rice germ and soybean (FRS) on the activity of alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) in the liver. Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase. * Statistical significance at \( p < 0.05 \). ** Statistical significance at \( p < 0.05 \).
4. Discussion

Fermentation is the key process where bread, cheese and alcoholic beverages are produced. It not only improves the usability of nutrients, texture and taste but also increases the flavor of foods [49,50]. It involves microbes and thereby produces compounds that inhibit the growth of contaminating microorganisms; these include organic acids, ethanol, short-chain fatty acids, and bacteriocins [51]. According to several previously published studies, fermented foods have beneficial effects on the gastrointestinal tract system. Moreover, recent studies have shown that supplementation of fermented foods had beneficial effects in improving symptoms of type 2 diabetes mellitus, impaired glucose metabolism, obesity, irritable bowel syndrome, hyperlipidemia, hypertension and osteoporosis [2,3,7,51–57].

Antioxidative compounds are also produced during fermentation [58]. Fermented soybean is incubated with Aspergillus oryzae, Bacillus subtilis and Rhizopus oligosporus, and it has a higher degree of anti-lipid peroxidative effects compared with steam soybeans [56,59,60]. Its active constituents include free isoflavones, such as daidzein and genistein, which are considered major antioxidants [61–63].

Our results indicate that the FRB might be more effective in lowering serum levels of AST, ALT, and ALP at a concentration of 15% and those of LDH at a concentration of 30% compared with the FRS. This is in agreement with previously published studies showing that the FRB had a positive effect on serum levels of hepatic enzymes [30,31,64–73].

According to our experiment, the FRB might be more effective in lowering MDA levels in the liver homogenate and microsome at both concentrations compared with the FRS. Moreover, it can also be inferred that the FRB might be more effective in lowering MDA levels in the serum and liver mitochondria at a concentration of 15% compared with the FRS. Our results are in agreement with previous studies showing that the FRB is useful to lower both MDA levels [74–78].

We found that the FRB might be more effective in lowering GSH levels in the serum at a concentration of 30% compared with the FRS. This is also seen in prior publications [79–84].

Levels of lipid peroxides and GSH are increased and decreased, respectively, in association with chronic alcohol consumption [85–87]. Moreover, levels of TBARS are elevated in association with acute or chronic alcohol consumption, which is followed by the involvement of aldehyde in increasing the synthesis of free radicals [86]. With the actions of aldehyde on xanthine oxidase in the cytoplasm, there is an increase in the synthesis of superoxidase. This eventually leads to damages to the liver tissue [88]. Furthermore, reactive oxygen species are increasingly formed in association with the chronic alcohol consumption. This is responsible for the inactivation of hepatic enzymes, followed by the structure and functions of mitochondria [89–91]. Our results showed that there was an increase in levels of lipid peroxides in fractions of homogenate, mitochondria and microsome in the liver, which is in agreement with previously published studies [92–94]. This also leads to the speculation that lower levels of lipid peroxides in the liver and serum might have a relationship with elevated levels of anti-oxidants [72].

Our results indicate that the FRB might be more effective in lowering serum triglyceride levels at a concentration of 15% and hepatic triglyceride levels at both concentrations compared with the FRS. Moreover, we found that the FRB might be more effective in lowering ALDH levels at a concentration of 15% compared with the FRS. These results are in agreement with previous studies [64,65,67,70,79,95–97].

5. Conclusions

Based on our results, it can be concluded that the FRB might also be considered as an alternative to the FRS in improving the AIHI, but this deserves further experimental and clinical studies.

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