

# Wheat-bran autolytic peptides containing a branched-chain amino acid attenuate non-alcoholic steatohepatitis via the suppression of oxidative stress and the upregulation of AMPK/ACC in high-fat diet-fed mice

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**Abstract.** Whole-wheat intake is known to reduce the risk of metabolic syndrome. However, the active component remains unclear. Recently, we identified bioactive peptides [leucine-arginine-proline (LRP) and leucine-glutamine-proline (LQP)] from wheat bran autolytic hydrolysate. The present study aimed to investigate the effects of LRP and LQP on non-alcoholic steatohepatitis (NASH) in a mouse model. We also evaluated the effects of these peptides on oxidative stress and on the AMP-activated protein kinase (AMPK) signaling pathway, two major pathogenic factors of NASH. Seven-week-old male C57BL/6 mice were fed a high-fat diet for 10 weeks and administered water supplemented with 0.05% LRP, 0.20% LRP, 0.05% LQP, or 0.20% LQP (each n=5) or distilled water (control; n=5) *ad libitum*. Oxidative stress was evaluated by measuring the serum levels of diacron reactive oxygen metabolite (d-ROM) and biological antioxidant potential (BAP). Hepatic expression of phosphorylated AMPK and phosphorylated acetyl-CoA carboxylase (ACC) were evaluated by immunoblotting. The result showed that non-alcoholic fatty liver disease activity score was significantly decreased in all types of treatment. Serum d-ROM levels were significantly decreased in

the 0.20% LRP group, but not in the 0.05% LRP, 0.05% LQP, and 0.20% LQP groups. Serum BAP levels were significantly increased in the 0.05% LRP and 0.20% LRP groups, but not in the 0.05% LQP and 0.20% LQP groups. Immunoblotting analysis revealed that the expression of phospho-AMPK was increased whereas that of phospho-ACC was decreased in the 0.20% LQP group. In conclusion, we demonstrated that both LRP and LQP alleviated the severity of NASH in a high-fat diet-induced NASH mouse model. In addition, we showed that LRP and LQP modulated oxidative stress and upregulated AMPK/ACC, respectively. Thus, LRP and LQP may constitute clinically applicable therapeutic agents for NASH.

## Introduction

Non-alcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic syndrome. The prevalence of NAFLD is estimated to range from 25 to 37% of the general population (1-4), and it constitutes a common etiological factor of chronic liver disease worldwide. Non-alcoholic steatohepatitis (NASH) is a progressive form of NAFLD. Patients with NASH have a high risk of life-threatening complications, including cardiovascular disease, liver cirrhosis, and hepatocellular carcinoma (5,6). In addition, NASH is predicted to become the leading cause of liver transplantation in the USA by the year 2020 (2). Thus, NAFLD/NASH is becoming a major social issue worldwide.

The first-line therapy for NASH is life-style intervention. Although diet and exercise alleviate the severity of NASH, long-term adherence to life-style interventions is poor (7,8). Pioglitazone, an insulin-sensitizing anti-diabetic agent, leads to metabolic and histologic improvement in patients with NASH (9). However, the indication of pioglitazone is restricted to NASH patients with diabetes mellitus (10). Furthermore, severe liver disease is a contraindication of pioglitazone because of adverse events, including severe hepatitis. Vitamin E is also reported to attenuate histological changes of the liver in patients with NASH (11). However, its efficacy

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**Abbreviations:** LRP, leucine-arginine-proline; LQP, leucine-glutamine-proline; NASH, non-alcoholic steatohepatitis; AMPK, AMP-activated protein kinase; d-ROM, diacron reactive oxygen metabolite; BAP, biological antioxidant potential; ACC, acetyl-CoA carboxylase; NAFLD, non-alcoholic fatty liver disease; ACE, angiotensin I-converting enzyme

**Key words:** wheat, bran, non-alcoholic steatohepatitis, oxidative stress, AMP-activated protein kinase

is controversial (12). Furthermore, long-term administration of vitamin E is reported to increase all-cause mortality (13). Accordingly, the development of new, safe, easily obtainable, and low-cost therapeutic agents for NASH is required.

Whole-wheat consumption is known to reduce the risk of obesity, type 2 diabetes, and cardiovascular disease (14,15). Supplementation with bran, the outer layers of wheat grains, improves glycemic and lipid metabolism in patients with impaired glucose tolerance (16,17). Recently, we developed a simple peptide extraction method and identified two bioactive peptides [leucine-arginine-proline (LRP) and leucine-glutamine-proline (LQP)] from wheat bran by autolysis reactions (18). These peptides inhibit angiotensin I-converting enzyme (ACE), which causes oxidative stress (19,20). In addition, these peptides contain leucine, a branched-chain amino acid, which regulates lipid metabolism via activation of the AMP-activated protein kinase (AMPK) signaling pathway (21). Since increased oxidative stress and downregulation of AMPK are major pathogenic factors of NASH, these wheat-bran autolytic peptides may be potent therapeutic supplements for the treatment of NASH.

The aim of the present study was to investigate the therapeutic efficacy of LRP and LQP for NASH in a mouse model. In addition, we investigated the effects of these peptides on oxidative stress and the AMPK signaling pathway.

## Materials and methods

**Materials.** All the reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise indicated.

**Animals.** NASH was induced in 7-week-old male C57Bl/6 wild-type mice by feeding a high-fat diet containing 71% total calories as fat (CELA Japan, Tokyo, Japan) (22). The mice were housed individually in an air-conditioned room at 22±3°C and 55±10% humidity, and with a 12-h light/dark cycle. All mice were permitted *ad libitum* consumption of water throughout the experimental period.

All animal experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, and were approved by the University of Kurume Institutional Animal Care and Use Committee.

**Purification of two bioactive peptides, LRP and LQP, from wheat bran.** Two bioactive peptides, LRP and LQP, were purified from wheat bran using HPLC as previously described (18). Briefly, wheat samples were milled in a test mill to obtain bran (Fig. 1). Bran samples were suspended in 50 mM citrate-phosphate buffer (pH 3.2) and autolyzed at 40°C for 12 h, followed by high performance liquid chromatography purification (Shimadzu LC-10AD system; Shimadzu, Kyoto, Japan) (Fig. 1). The amino acid sequence was analyzed using a Shimadzu PPSQ-21 protein sequencer (18).

**Treatment protocol.** Seven-week-old male C57BL/6 mice were fed with a high-fat diet for 10 weeks to induce NASH, and were administered 0.05% LRP-supplemented water (0.05% LRP; n=5), 0.20% LRP-supplemented water (0.20% LRP; n=5), 0.05% LQP-supplemented water (0.05% LQP; n=5), 0.20%

LQP-supplemented water (0.20% LQP; n=5), or distilled water (control; n=5) *ad libitum* (Fig. 2). At week 17, body weight was measured. Then, the mice were sacrificed and the serum and livers were collected under anesthesia (Fig. 2).

**Liver histology.** Random histological sampling was performed throughout this study as previously described (23,24). Liver samples were fixed overnight in 10% buffered formalin and embedded in paraffin. All the sections were cut at 5 µm and stained with hematoxylin and eosin (25).

**NAFLD activity score.** Activity of NAFLD was evaluated by NAFLD activity score, in which the following findings were evaluated semi-quantitatively: steatosis (0-3 points), lobular inflammation (0-2 points), hepatocellular ballooning (0-2 points), and fibrosis (0-4 points) (26).

**Measurement of reactive oxygen metabolites and antioxidant capacity.** To analyze reactive oxygen metabolites and antioxidant capacity in serum, diacron reactive oxygen metabolite (d-ROM) and biological antioxidant potential (BAP) were measured by a free radical analyzer system (FREE Carpe Diem; Wismerll Co., Ltd., Tokyo, Japan) as previously described (27,28). The d-ROM measurements were determined based on the ability of transition metals to catalyze the formation of colored free radicals, and the results were expressed in arbitrary units (U.Carr), where 1 U.Carr was 0.8 mg/l of H<sub>2</sub>O<sub>2</sub>. In BAP measurements, 10 µl of serum was added to a solution containing FeCl<sub>3</sub>. Reduction of Fe<sup>3+</sup> to Fe<sup>2+</sup> caused a chromatic change, which was measured at 505 nm using a photometer. The results of BAP measurements were expressed in µmol/l of the reduced ferric ions.

**Immunoblotting.** Immunoblotting was performed as previously described (29,30) using the following antibodies: anti-phospho-AMPKα (Thr172; Cat. no. 07-681), anti-AMPKα-pan (Cat. no. 07-181) (both from Merck Millipore Corp., Darmstadt, Germany), anti-phospho-acetyl-CoA carboxylase (ACC) (Ser79; Cat. no. 3661) and anti-ACC (Cat. no. 3662) (both from Cell Signaling Technology Inc., Danvers, MA, USA). Equal amounts of protein (20 µg) from liver homogenates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resolved proteins were transferred electrophoretically onto polyvinylidene difluoride membranes. The membranes were incubated with the primary antibodies mentioned above at 1:1,000 dilution, and were subsequently incubated with the secondary antibodies at 1:10,000 dilution (Cat. no. NA9340-1ML; GE Healthcare Japan K.K., Tokyo, Japan). The membranes were then incubated with a chemiluminescence reagent (ECL kit) and analyzed by a luminescent image analyzer (LAS-4000) (both from GE Healthcare Japan K.K.).

Changes in immunoblotting intensity for phospho-AMPK, AMPK, phospho-ACC and ACC were quantitated by measuring pixel intensities using an image processing program, ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA) (<http://rsb.info.nih.gov/ij/>) (31). Changes in immunoblotting intensity for phospho-AMPK, AMPK, phospho-ACC and ACC were quantitated by measuring pixel intensities using an image processing program, ImageJ.

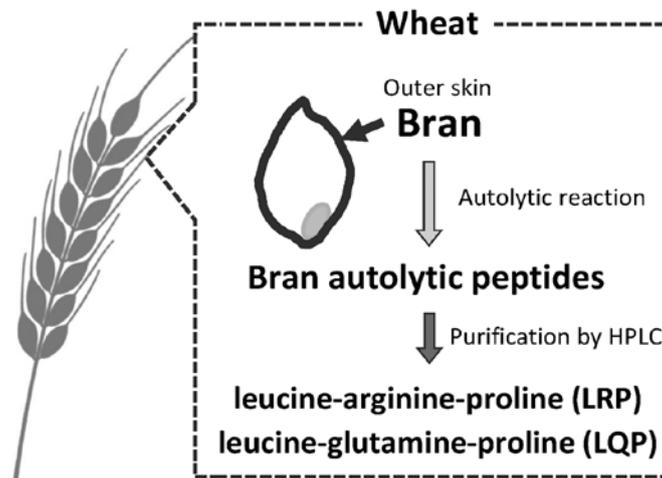


Figure 1. Purification process for LRP and LQP. Two bioactive peptides, LRP and LQP, were purified from wheat bran using HPLC. Wheat samples were milled in a test mill to obtain bran. Bran samples were suspended in 50 mM citrate-phosphate buffer (pH 3.2) and autolyzed, followed by HPLC purification. LRP, leucine-arginine-proline; LQP, leucine-glutamine-proline; HPLC, high performance liquid chromatography.

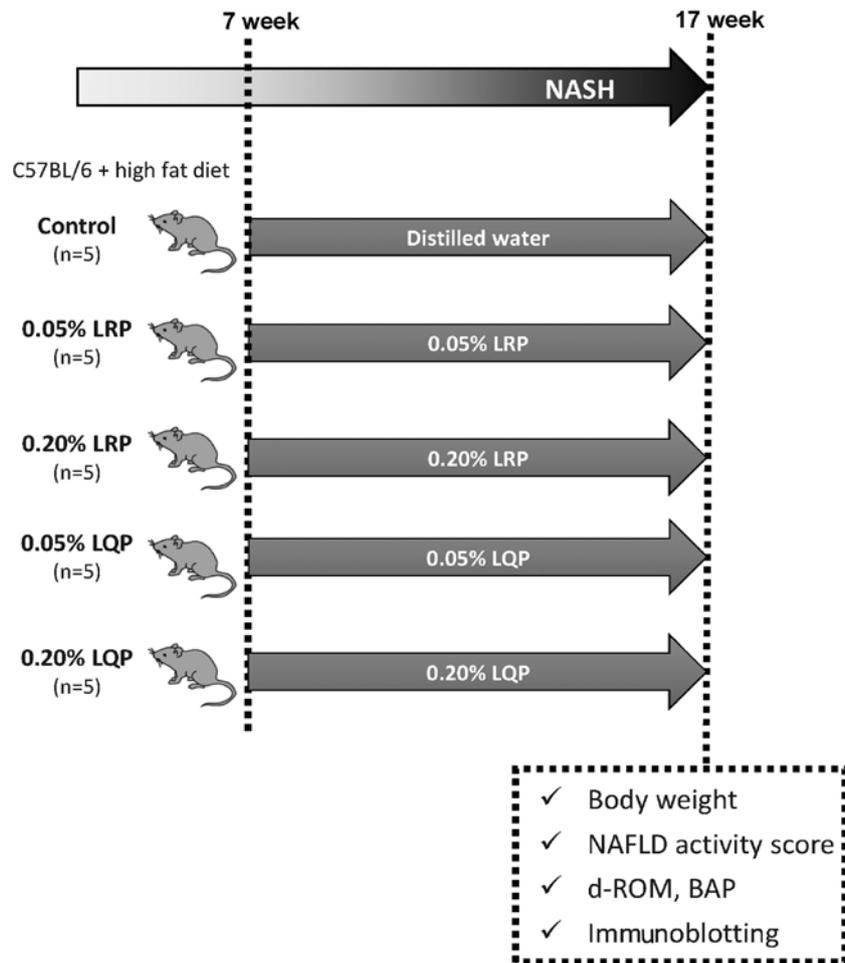


Figure 2. Study protocol. NASH, non-alcoholic steatohepatitis; LRP, leucine-arginine-proline; LQP, leucine-glutamine-proline; d-ROM, diacron reactive oxygen metabolites; BAP, biological antioxidant potential.

**Statistical analysis.** Data are expressed as mean  $\pm$  SD. Statistical comparisons among multiple groups were performed by ANOVA followed by Scheffe's post-hoc test (JMP Pro version 12.01; SAS Institute Inc., Cary, NC, USA).  $P < 0.05$  was considered significant.

**Results**

**Effects of LRP and LQP on body weight.** Body weight in the 0.05% LQP group was significantly lower compared to that in the control group ( $46.0 \pm 3.0$  vs.  $38.0 \pm 3.3$  g/body,

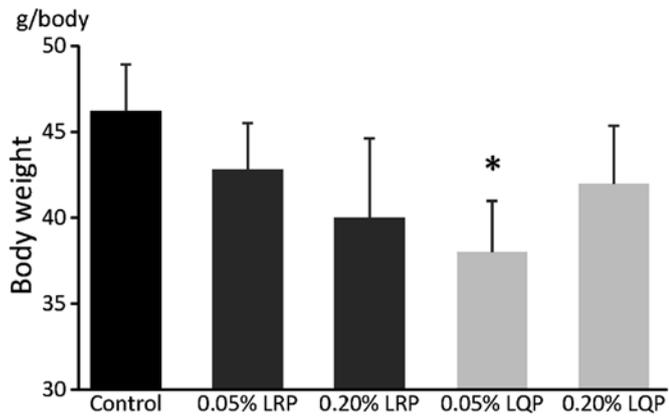


Figure 3. Effect of LRP and LQP on body weight. \* $P < 0.05$ . LRP, leucine-arginine-proline; LQP, leucine-glutamine-proline.

$P = 0.02$ ) (Fig. 3). No significant differences were seen in body weight between the control group and the 0.05% LRP, 0.20% LRP or 0.20% LQP groups (Fig. 3).

*Effects of LRP and LQP on liver histology and NAFLD activity score.* Severe hepatic steatosis was identified in the Control and 0.05% LQP groups (Fig. 4A and D). However, amelioration of hepatic steatosis was observed in the 0.05% LRP, 0.20% LRP and 0.20% LQP groups (Fig. 4B, C and E). Hepatic fibrosis was rarely seen in any group, including the control group.

The grade of steatosis was significantly decreased in the 0.05% LRP, 0.20% LRP, and 0.20% LQP groups compared to that in the control group (Fig. 5A). The grade of lobular inflammation was significantly decreased in the 0.05% LRP,

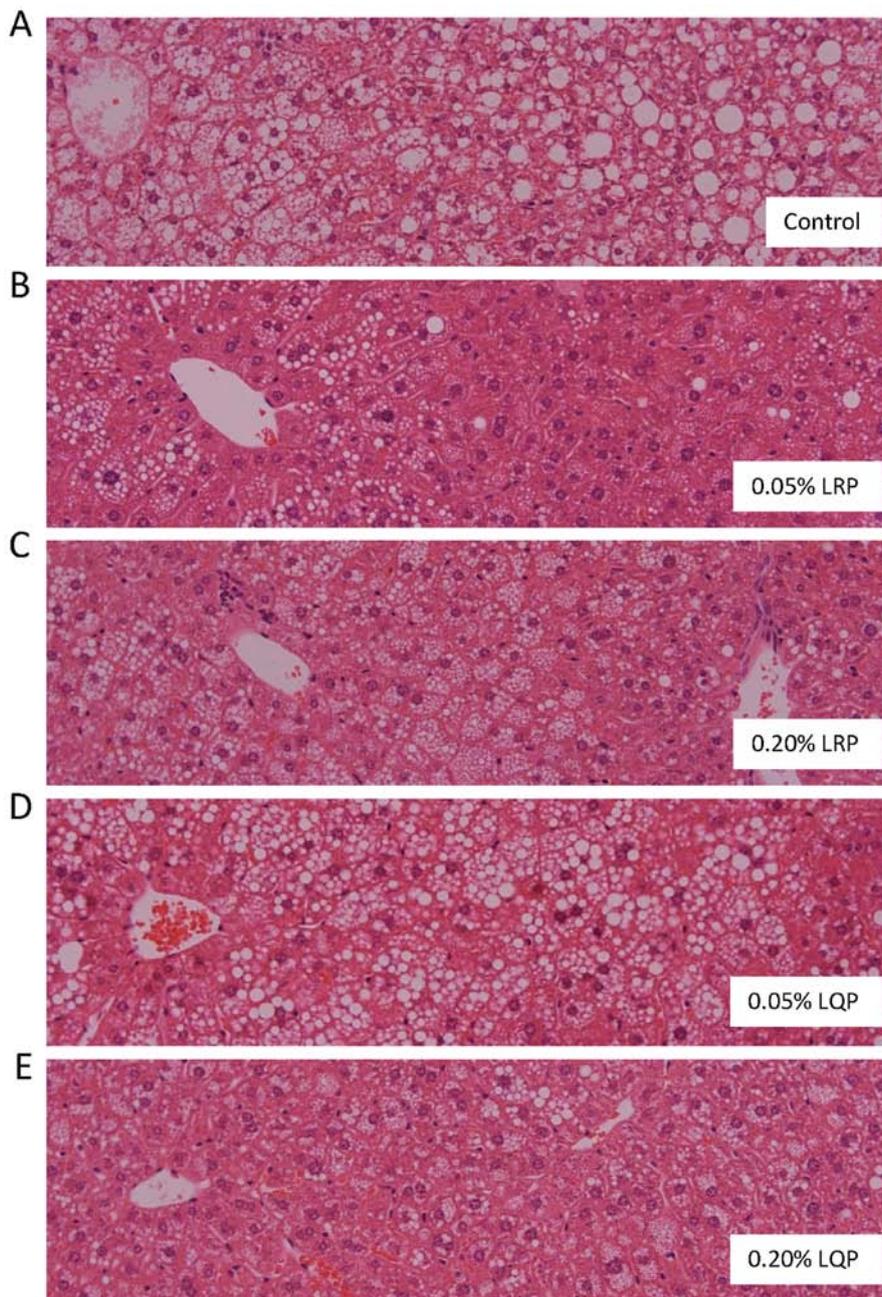


Figure 4. Effect of LRP and LQP on liver histology. (A) Control, (B) 0.05% LRP, (C) 0.20% LRP, (D) 0.05% LQP, (E) 0.20% LQP.

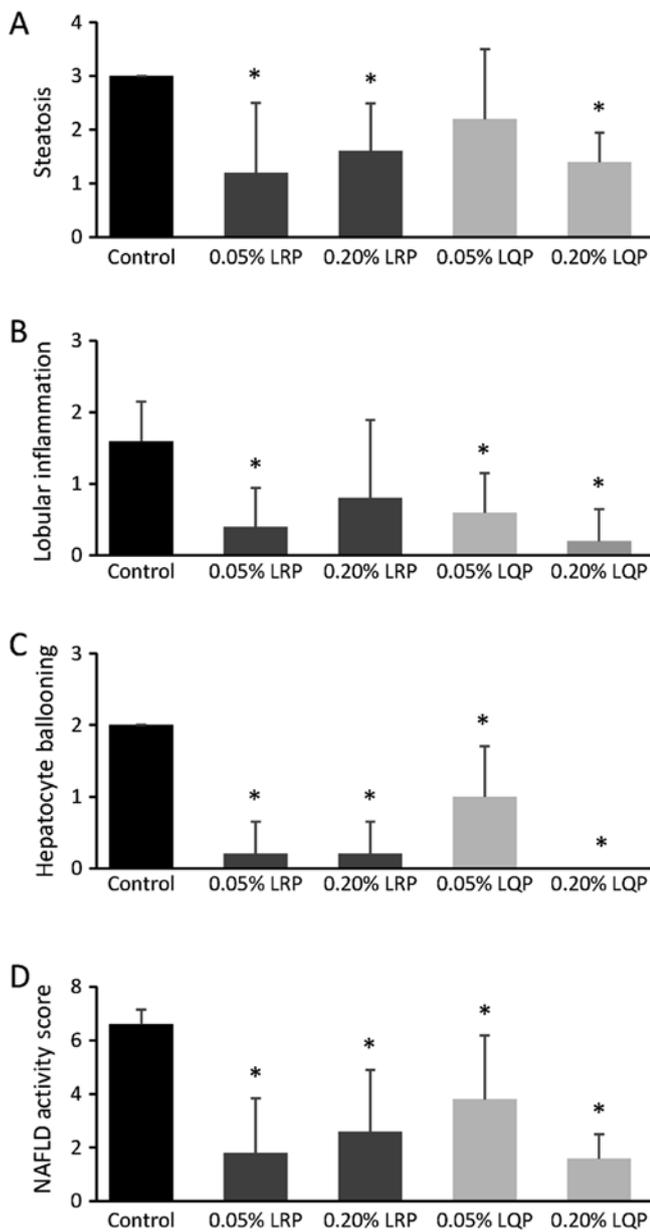


Figure 5. Effect of LRP and LQP on the NAFLD activity score and its components. (A) Steatosis, (B) lobular inflammation, (C) hepatocyte ballooning, and (D) NAFLD activity score. \* $P < 0.05$ . LRP, leucine-arginine-proline; LQP, leucine-glutamine-proline; NAFLD, non-alcoholic fatty liver disease.

0.05% LQP and 0.20% LQP groups compared to that in the control group (Fig. 5B). The grade of hepatocyte ballooning and NAFLD activity score were significantly decreased in all the treated groups compared to that in the control group (Fig. 5C and D).

**Effects of LRP and LQP on d-ROM and BAP.** Serum d-ROM levels were significantly decreased in the 0.20% LRP group, but not in the 0.05% LRP, 0.05% LQP, and 0.20% LQP groups, compared to that in the control group (Fig. 6A). Serum BAP levels were significantly higher in the 0.05% LRP and 0.20% LRP groups, but not in the 0.05% LQP and 0.20% LQP groups, compared to that in the control group (Fig. 6B).

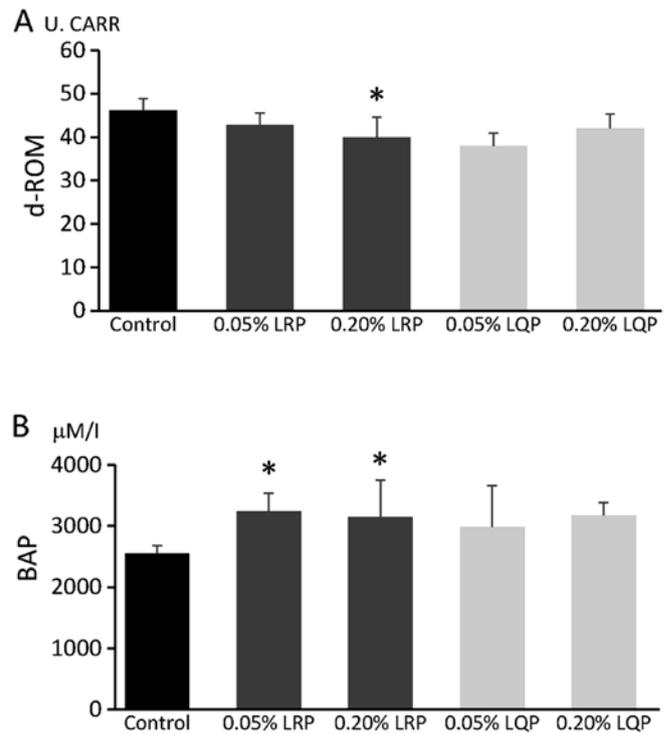


Figure 6. Effect of LRP and LQP on serum d-ROM and BAP. (A) d-ROM, and (B) BAP. \* $P < 0.05$ . LRP, leucine-arginine-proline; LQP, leucine-glutamine-proline; d-ROM, diacron reactive oxygen metabolite; BAP, biological antioxidant potential.

**Effects of LRP and LQP on the AMPK signaling pathway.** Immunoblotting analysis showed that the hepatic expression of phospho-AMPK was increased in the 0.20% LQP group compared to that in the control group (Fig. 7A and B). The hepatic expression of phospho-ACC, a downstream molecule of AMPK, was suppressed in the 0.20% LQP group compared to that in the control group (Fig. 7D).

## Discussion

We demonstrated that LRP and LQP alleviated the severity of NASH in high-fat diet-fed mice. In addition, we revealed that LRP suppressed oxidative stress, while LQP upregulated the AMPK/ACC signaling pathway. Thus, LRP and LQP may attenuate NASH through different mechanisms. LRP and LQP are easily obtainable at a low cost; therefore, these wheat-bran autolytic peptides may be clinically applicable therapeutic agents in patients with NASH.

In general, 7-10% weight loss is required to attenuate hepatic steatosis in patients with NASH (32). In this study, although significant weight loss was observed in the 0.05% LQP group, no significant attenuation of hepatic steatosis was observed. On the other hand, a significant attenuation of hepatic steatosis without weight loss was identified in the 0.05% LRP, 0.20% LRP and 0.20% LQP groups. These findings indicate that the mechanisms underlying LRP- and LQP-induced attenuation of hepatic steatosis are independent of weight loss. Increase in oxidative stress is a major NASH pathogenic factor (33). Recently, Ni *et al* reported that silymarin, derived from the milk thistle plant, upregulates NADPH oxidase

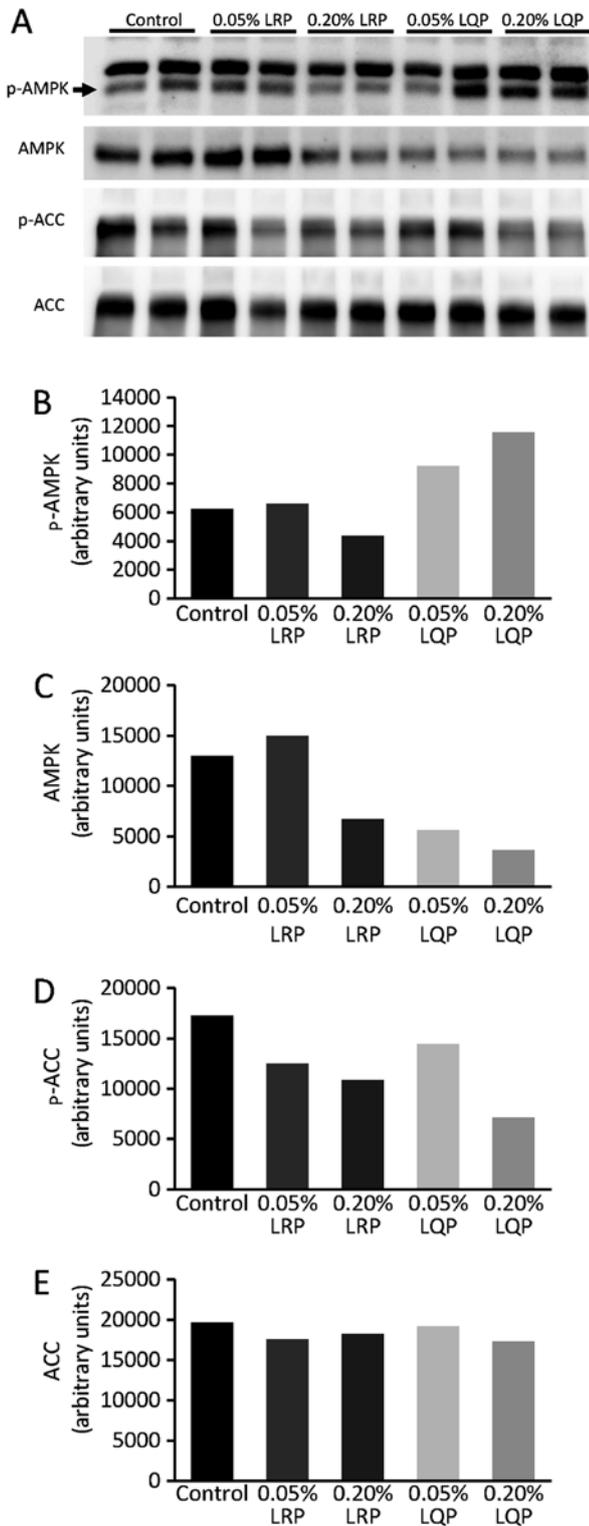


Figure 7. Effect of LRP and LQP on hepatic expression of AMPK signaling pathway components. (A) Immunoblotting image. Relative density of (B) p-AMPK, (C) AMPK, (D) p-ACC, and (E) ACC. LRP, leucine-arginine-proline; LQP, leucine-glutamine-proline; p-AMPK, phosphorylated AMP-activated protein kinase; p-ACC, phosphorylated acetyl-CoA carboxylase.

components and antioxidant enzymes, and attenuates hepatic steatosis without significant weight loss in a mouse model of NAFLD (34). This led us to investigate the effects of LRP and LQP on oxidative stress.

There are several markers for evaluating oxidative stress, including 8-hydroxyguanosine, 4-hydroxynonenal protein, and activity of glutathione and superoxide dismutase (35,36). Additionally, the levels of d-ROM and BAP have been used to evaluate oxidative status. d-ROM levels are the total levels of peroxidized metabolites, whereas BAP reflects serum antioxidant capacity. These markers are known to correlate with the development of metabolic syndrome, cardiovascular disease, and hepatocellular carcinoma (37-39). In this study, a significant decrease in d-ROM was observed in the 0.20% LRP group, and a significant increase in BAP was observed in the 0.05% LRP and 0.20% LRP groups. These data indicate that LRP upregulates antioxidant capacity, leading to an attenuation of NASH. Although the mechanisms underlying LRP-induced upregulation of antioxidant capacity remain unclear, we previously reported that LRP inhibits the activity of angiotensin I-converting enzyme (ACE) (18). Fiordaliso *et al* reported that an ACE inhibitor reduces oxidative stress in the heart and aorta in a rat model of diabetes mellitus (19). In addition, Privratsky *et al* reported that ACE inhibition reduces the activation of NADPH oxidase and suppresses the production of oxygen radicals in a rat model of hypertension (20). Thus, LRP may attenuate NASH through the downregulation of oxidative stress in high-fat diet-fed mice.

LRP and LQP contain leucine, a branched-chain amino acid, which regulates lipid metabolism via the AMPK signaling pathway (21). Therefore, we evaluated the effects of LRP and LQP on phosphorylation of AMPK and ACC, a downstream lipogenic molecule of AMPK. In the 0.20% LQP group, the expression of phospho-AMPK and phospho-ACC was increased and decreased, respectively. These data indicate that 0.20% LQP activates the AMPK/ACC pathway, leading to the suppression of *de novo* lipogenesis. Recently, leucine, along with metformin or a phosphodiesterase 5 inhibitor, was reported to attenuate hepatic steatosis, insulin sensitivity, and glycemic control via the upregulation of AMPK in obese mice (40,41). These findings support our hypothesis that LQP attenuates NASH through activation of the AMPK/ACC signaling pathway.

There are several limitations in this study. Our results showed that liver function and glucose metabolisms were improved. However, we did not perform any biochemical examination for liver function and glucose metabolisms such as glucose tolerance test and insulin tolerance test. In addition, although LRP and LQP may attenuate NASH through upregulation of the transporter of fatty acid, we did not evaluate the expression and function of the transporter of fatty acid such as CD36 and CPT1. These are uncertainty areas in this field, and should be clarified in further research.

In conclusion, we demonstrated that LRP and LQP attenuate NASH in mice fed a high-fat diet. LRP and LQP may attenuate NASH through different mechanisms. LRP suppressed oxidative stress, while LQP upregulated the AMPK/ACC signaling pathway. Since the worldwide prevalence of NASH is high, treatment availability and cost are important issues. LRP and LQP are easily obtainable at a low cost; therefore, these wheat-bran autolytic peptides may represent clinically applicable therapeutic agents in patients with NASH.

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