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Research article

Effect of Korea red ginseng on nonalcoholic fatty liver disease: an association of gut microbiota with liver function

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ABSTRACT

Background: Korea Red Ginseng (KRG) has been used as remedies with hepato-protective effects in liver-related condition. Microbiota related gut-liver axis plays key roles in the pathogenesis of chronic liver disease. We evaluated the effect of KRG on gut-liver axis in patients with nonalcoholic statohepatitis by the modulation of gut-microbiota.

Methods: A total of 94 patients (KRG: 45 and placebo: 49) were prospectively randomized to receive KRG (2,000 mg/day, ginsenoside Rg1+Rb1+Rg3 4.5mg/g) or placebo during 30 days. Liver function test, cytokeraton 18, and fatigue score were measured. Gut microbiota was analyzed by MiSeq systems based on 16S rRNA genes.

Results: In KRG group, the mean levels (before vs. after) of aspartate aminotransferase (53 ± 19 vs. 45 ± 23 IU/L), alanine aminotransferase (75 ± 40 vs. 64 ± 39 IU/L) and fatigue score (33 ± 13 vs. 26 ± 13) were improved ($p < 0.05$). In placebo group, only fatigue score (34 ± 13 vs. 31 ± 15) was ameliorated ($p < 0.05$). The changes of phyla were not statistically significant on both groups. In KRG group, increased abundance of *Lactobacillus* was related with improved alanine aminotransferase level and increased abundance of *Clostridium* and *Intestinibacter* was associated with no improvement after KRG supplementation. In placebo group, increased abundance of *Lachnospiraceae* could be related with aggravation of liver enzyme ($p < 0.05$).

Conclusion: KRG effectively improved liver enzymes and fatigue score by modulating gut-microbiota in patients with fatty liver disease. Further studies are needed to understand the mechanism of improvement of nonalcoholic steatohepatitis.

ClnicalTrials.gov: NCT03945123 (www.ClinicalTrials.gov).

1. Introduction

Fatty liver refers to the pathologic term in which triglycerides are accumulated in hepatocytes. Fatty liver is defined when fat accounts for more than 5% of total liver [1]. It can be usually classified into alcohol-induced fatty liver disease and nonalcoholic fatty liver disease (NAFLD) [2]. NAFLD has been known to be related to insulin resistance, obesity, metabolic syndrome, and hepatocellular carcinoma, considered a major world health problem, and thought to leading serious problems in social, economic, or medical aspects [3]. NAFLD includes a series of progressed liver diseases that range from steatosis to nonalcoholic steatohepatitis (NASH), fibrotic

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK18, cytokeratin 18; KRG, Korea Red Ginseng; LEfSe, Linear Discriminant Analysis Effect Size; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; OTUs, operational taxonomic units; PCoA, principal coordinate analysis.

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change, and cirrhotic change [4]. The pathophysiology of NAFLD has not been fully understood yet; however, several possible contributing factors to the progression of NAFLD has been proposed, including genetic factors, fat accumulation, and inflammatory stimulation [5]. In particular, the inflammatory mediators that are locally released or derived from organs, such as the gut and adipose tissue, may associated with the progression of steatosis to NASH [6].

Recently, the gut microbiota has been reported as a major contributor to the development of NAFLD [7,8]. The variations of gut microbiome are affected by various factors, including lifestyle, genotype, diet, and environment. A mutualistic relationship is formed between the gut microbiome and the human, and the microbiota may play a substantial role in host health. Several studies have recently suggested that NAFLD is related to disturbances in the composition of the gut microbiota, leaky bowel, and translocation of endotoxins [9]. The microbiota plays an important role in alternating the balance between pro-inflammatory and anti-inflammatory signals, contributing to inflammation [10,11]. As a result, steatosis process is induced by the metabolite or microbiome itself with hepatic inflammatory reaction. Therefore, there is a need to fully understand the contribution of microbiota to the pathogenesis of NAFLD and NASH.

The *Panax ginseng* Meyer root has been a commonly used herbal remedies for about 2,000 years in East Asia [12,13]. Ginsenoside Rb1 is the most abundant ginsenoside contained in ginseng, and it activates AMP-activated protein kinase, which, in turn, inhibits gene expression encoding lipogenesis-inducing enzymes in rats diagnosed with fatty liver disease [14]. Several studies have reported that Korean red ginseng (KRG) might have anti-neoplastic, neuro-protective, hepato-protective effects with anti-diabetic, anti-stress, anti-inflammatory, anti-hyperlipidemic, or anti-oxidative properties [12,15]. In particular, it is reported that KRG has beneficial effects on the progression of NAFLD and NASH [16,17]. However, there is limited data about the effect of KRG in patients with NAFLD and its influences on the gut microbiota. In particular, human data on liver disease have been extremely scarce. Therefore, in this study, we evaluated the therapeutic effects of KRG in patients with NASH and its influences on the gut microbiota for elucidating the association between therapeutic effects of KRG and the modulation of gut microbiota.

2. Materials and methods

2.1. Study subjects

We prospectively performed a multicenter, randomized, clinical trial between January 2017 and April 2018 that was aimed on evaluating the effect of KRG in patients with NAFLD (NCT03945123). Patients with aspartate aminotransferase (AST) or alanine aminotransferase (ALT) levels greater than or equal to 50 IU/L, fatty liver disease in abdominal ultrasound, and older than 20 years were included. During the study period, the patients were not permitted excessive alcohol drinking (male > 60 g/wk, female > 40 g/wk). The exclusion criteria were as follows: patients with virus related hepatitis, alcohol-induced hepatitis, autoimmune disease, pancreas problem, hemochromatosis, infiltrative liver diseases, drug-related liver injury, and cancers. This study was conducted in conformance with the ethical guidelines from the 1975 Helsinki Declaration as it is reflected by a priori approval of the institutional review board for human research in all hospitals participating in the trials (Hallym 2017-33 and 2017-03-014-001). Informed consent on enrollment was obtained from each participant.

A computerized procedure was used for the randomization. Patients were randomly assigned to get KRG or placebo during 30 days (Fig. 1A). A total of 100 patients were assigned to this study. Six patients were excluded because of exclusion criteria. The remaining 94 participants, who met the inclusion criteria, were randomly allocated either to KRG group or to the placebo group. Of these patients, 7 patients (KRG: 5 and placebo: 2 patients) were excluded: 3 patients refused to participate, 3 patients were lost to follow-up,

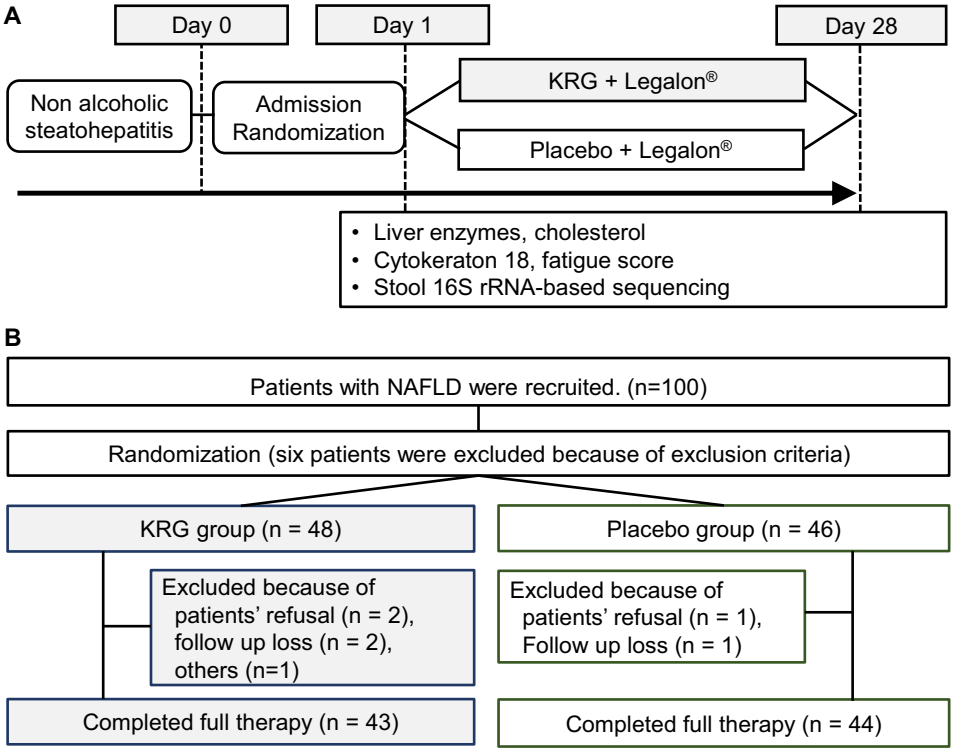

Fig. 1. Flow chart and study design. NAFLD, nonalcoholic fatty liver disease; KRG, Korean Red Ginseng.

and 1 patient was consuming alcohol (Fig. 1B). The blood tests, cytokeratin 18, and fatigue severity scores were checked at the pre and post 4 weeks of administration. Fecal samples were collected from each patient and transported in ice box to the laboratory within 24 hours. For gut microbiota analysis, two groups were divided into three subgroups (improve [improved by 10 IU/L or more], no change [changed less than 10 IU/L], and aggravate [worsened by 10 or more]) according to ALT change. The baseline evaluation was performed on family history, body mass index (BMI), liver ultrasound, routine blood test, liver enzymes, virus related marker, and human immunodeficiency virus.

An experienced hepatologist (K.T.S.) performed ultrasound examination of the liver with Sequoia 512 Acuson sonography machine (Siemens Healthcare, Erlangen, Germany) equipped with a 1.5-MHz convex probe. The ultrasound definition for the NAFLD was followed on guidelines of the Korean Association for the Study of the Liver [18]. Fifty-one patients (54%) performed CT scan for the diagnosis of NAFLD.

2.2. Medical treatment

Enrolled patients were administered with milk-thistle dried extracts powder (450 mg/d; Legalon®, Bukwang Pharmaceutical, Co., LTD, Seoul, Republic of Korea) and were recommended to have regular aerobic exercise for >30 min/day. All patients were educated about diet according to the recommendations [19,20]. Patients meeting the inclusion criteria were randomly allocated into two different treatment groups: KRG group with administration of a KRG powder capsule (ginsenosides Rg1 + Rb1 + Rg3 4.5 mg/g; 2,000 mg/d) and placebo group, 3 times a day for 4 weeks for both groups (Fig. 1A). The patients were not given any other medication. The placebos were made to be of the same size and shape as the KRG capsule powder (cellulose) manufactured at the Korea Ginseng Corporation (Seoul, South Korea).

2.3. Fatigue severity scale

The Krupp fatigue severity scale was used [21]. The survey included 9 variables and all respondents were asked to score questions on a 7 points scale (1 = strong no, 4 = neither no nor yes, and 7 = strong yes). All scores for each question were collected, and two nurses from the department of gastroenterology calculated the total scores.

2.4. Measurement of cytokeratin 18 fragment

Cytokeratin 18 fragment (CK18) has been reported as a biomarker that is useful marker for the differentiation of NASH and others in patients with NAFLD [22]. The CK18, which reacts to the M-30 monoclonal antibody, is a serological marker of apoptosis and has been related with the progression of liver disease in patients with hepatitis C virus infection and NAFLD [23,24]. The serum level of CK18-F was checked at the pre and post 4 weeks of administration using the M30-Apoptosense ELISA kit (PEVIVA AB, Bromma, Sweden).

2.5. Microbiota sequencing using MiSeq system

Metagenomic DNA was extracted from fecal samples of subjects using the RNeasy Power Microbiome kit following the company's instructions (Qiagen, Valencia, CA, USA). The extracted DNA was purified using the DNeasy PowerClean Pro Cleanup Kit (Qiagen, Valencia, CA, USA). DNA in each sample was amplified with adaptor primers targeting the V1-V3 region of the 16S rRNA gene. PCR amplification was performed according to the protocol for preparing a 16S metagenomics sequencing library with the MiSeq system (Illumina, Inc., San Diego, CA, USA). After amplification, PCR products were verified with a 2% agarose gel electrophoresis, and amplicons of target size were selected and purified using AMPure XP bead (Beckman Coulter, Brea, CA, USA). The purified amplicon library was quantified using the TaKaRa PCR Thermal Cycler Dice Real Time System III with the GenNext NGS Library Quantification kit (Toyobo, Osaka, Japan). Equimolar concentrations of each library were pooled and sequenced using the Illumina MiSeq System (300bp paired end).

2.6. Measurement of bacterial amounts by quantitative real-time PCR

The relative amounts of total bacteria were measured by quantitative real-time PCR based on 16S rRNA gene. The 16S rRNA gene was amplified using the primers 340F (5′- TCCTACGGGAGG-CAGCAG-3′) and 518R (5′- ATTACCGCGGCTGCTGG-3′) with the TaKaRa PCR Thermal Cycler Dice Real Time System III (TaKaRa, Shiga, Japan). Triplicate reactions were performed for each sample with a final volume of 25μL comprising 12.5μL of 2X SYBR Premix Ex Taq (TaKaRa), 10 μM of primers, and 2μL of DNA template (10-fold diluted metagenomic DNA) or distilled water (negative control). Standard graphs were made from parallel PCRs with continuous log-concentrations (1×10^2–$1x10^8$) of the copy number of 16S rRNA gene from *Escherichia coli* w3110. The differences between samples were analyzed using the Student's t-test in the R software (ver. 3.2.0). Results with p value < 0.05 were considered statistically significant.

2.7. Sequence analysis

Raw sequence reads were merged, and sequences with short read lengths (<430 bp of merged reads) or low quality score and chimeric reads were trimmed using USEARCH (ver. 11.0.667). Primer sequence was deleted from the merged sequences. Then, final sequences were stepwise collected into operational taxonomic units (OTUs) by 97% identity with EzTaxon-e database, and taxonomic positions of representative sequences in each OTU cluster were assigned [25]. To compare diversity indices among samples, read number were normalized by random subsampling, and the diversity indices were calculated using Mothur [26]. Principal coordinate analysis (PCoA) plots were generated to compare microbiota composition among samples using Calypso [27]. The significantly differential taxa between groups were determined using Linear Discriminant Analysis Effect Size (LEfSe) [28]. The correlation of genera with AST and ALT levels was analyzed by the Spearman's rank correlation and visualized using heatmap by R software. Significantly correlated genera with AST and ALT levels were selected by p value < 0.05.

2.8. Statistical analysis

In this clinical trial, we evaluated the therapeutic effect of KRG through alteration of gut microbiota in patients with NASH. Hence, we designed the sample number by calculating with the difference (0.7) and standard deviation (1) in the fatigue score. At least 60 patients were needed for the study. The primary aim was an improvement in liver enzymes and fatigue scale scores at 4 weeks. The secondary aims were changes in cytokeratin 18 and gut microbiota (composition and diversity).

Unless otherwise stated, all clinical variables were expressed as mean ± standard deviation. A paired t-test, independent-samples t-test, and ANCOVA were used to assess the significance of data. The differences of microbiota between groups were analyzed using

the Mann-Whitney U-test and Kruskal-Wallis test in R software. Permutation tests were used to determine the significance of PCoA plot result. It is regarded as statistically significant when the p value is < 0.05. Routine blood test data were analyzed using statistical software (SPSS, version 19.0, SPSS, Inc., Chicago, Ill, USA) and GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Baseline group characteristics and changes in clinical outcomes

Baseline findings and improvements in clinical variables in each group after 4 weeks are presented in Table 1. Overall 87 patients (44 patients in KRG and 43 patients in placebo) completed the study. The mean age was 49.8 ± 13.1 years and 52 patients were men. The mean BMI was 27.7 ± 4.1 kg/m^2. The mean levels of AST and cholesterol showed improvement ($p < 0.05$), and the fatigue score was significantly improved after 4 weeks of administration.

The mean levels (before [day1] and after [4 weeks]) of AST, ALT, and gamma glutamyl transferase (γ-GT) were improved after KRG therapy ($p < 0.05$). However, the levels of cholesterol, triglyceride, fasting glucose did not show significant difference between the before and after of KRG administration. In the placebo group, the mean levels of cholesterol improved after placebo administration ($p < 0.05$). In the comparison between KRG and placebo groups, mean levels of ALT, γ-GT, and triglyceride were significantly different ($p < 0.05$) (Table 1).

The mean fatigue scores were 33 ± 13 in before and 26 ± 13 in after ($p < 0.001$) for the KRG group and 34 ± 13 in before and 31 ± 15 in after ($p = 0.042$) for the placebo group. Statistical difference was present in fatigue score between KRG and placebo groups ($p = 0.027$). Fatigue improvement rate in the KRG group was significantly higher than that of placebo group ($p = 0.002$). The mean levels (before vs. after) of CK18 (743 ± 477 vs. 807 ± 506 pg/ml) increased significantly after placebo administration ($p = 0.038$). Although not statistically significant, the KRG group had an elevated cytokeratin 18 level after KRG treatment ($p = 0.175$) (Table 1).

3.2. Gut microbiota of subjects

Gut microbiota of 130 fecal samples from 65 subjects before and after ingestion (35 subjects for KRG intake, 30 subjects for placebo intake) were analyzed by MiSeq system based on 16S rRNA gene. A total of 10,491,312 reads (5,513,843 for KRG intake; 4,977,467 for placebo intake) after trimming were analyzed. Diversity indices were calculated after normalized read number to 9,100 in each sample by random sampling (Table S1). The coverage of microbiota was over 92% in Good's coverage result. The highest diversity was observed in Pre_23 (subject #23 before ingestion) of KRG, and the lowest diversity was observed in Pre_11 (subject #11 before ingestion) of KRG. The number of observed OTUs and diversity were decreased after ingestion of KRG, whereas those were increased after 4 weeks in placebo group (Fig. 2A). However, the bacterial amounts were increased after 4 weeks in both groups. The composition of microbiota was not clearly separated between KRG and placebo ingestion groups in PCoA plot (Fig. 2B). However, the UniFrac distances of microbiota were different between KRG group and placebo group. The inter-variations of gut microbiota in KRG groups were increased after ingestion, and the gut microbiota was different between before and after ingestion comparing to before intra-variation of microbiota. The distances of microbiota were similar in before and after ingestion of placebo group.

The composition of microbiota was compared between groups at phylum and genus levels (Fig. 3). Firmicutes was the predominant phylum in all groups, and *Bacteroidetes, Proteobacteria*, and *Actinobacteria* were dominant in all groups. Although the proportion of *Bacteroidetes* was decreased in both groups (24.0→23.2% and 21.4→18.7%), the proportions of other phyla were differently changed after 4 weeks in both of KRG and placebo group. In particular, the proportion of predominant phylum, *Firmicutes*, was decreased after 4 weeks in KRG ingestion group, whereas that was increased in placebo group. However, the changes of phyla were not statistically significant ($p > 0.05$). *Faecalibacterium*, *Bacteroides*, *Prevotella*, *Bifidobacterium*, and *Escherichia* were dominant genera (over 5% of microbiota in at least 1 group) in all samples. The relative abundances of dominant genera were differently changed between KRG and placebo groups after ingestion (Fig. 3A).

Differences in microbiota between KRG and placebo groups were analyzed by LefSe analysis (Fig. 3B). *Akkermansia*, *Verrucomicrobiae, Akkermansiaceae, Verrucomicrobia, Verrucomicrobiales, Sprobacter*, *Clostridium*_g7, and *Clostridium*_g8 were different genera between KRG and placebo groups before ingestion, and their proportions were higher in placebo group. The proportions of *Holdemanella* and *Eubacterium*_g4 were higher in placebo group than KRG group after ingestion, whereas those of *Paraprevotella*, *Fusobacterium*, and *Lachnospira* were higher in KRG group. Dominant genera in microbiota (Fig. 3A) did not detected in LefSe analysis.

Table 1
Clinical characteristics of patients

Variables (mean [SD])	KRG group (n = 44)			Placebo group (n = 43)			p-value*
Male (%)	57			63			0.570
Age (years)	50.0 ± 13.3			49.7 ± 13.2			0.915
BMI (kg/m^2)	28.0 ± 4.6			26.9 ± 3.4			0.211
	before	after	p-value	before	after	p-value	p-value*
AST (IU/L)	53 (19)	45 (23)	0.014	53 (21)	48 (23)	0.060	0.550
ALT (IU/L)	75 (40)	64 (39)	0.021	73 (36)	77 (38)	0.500	0.048
γ-GT (IU/L)	93 (76)	81 (71)	0.011	90 (108)	92 (129)	0.687	0.043
Cholesterol (mg/dL)	185 (40)	179 (46)	0.320	191 (34)	184 (41)	0.047	0.658
Triglyceride (mg/dL)	240 (35)	189 (154)	0.145	166 (67)	214 (98)	0.077	0.023
Fasting glucose (mg/dL)	124 (35)	122 (37)	0.734	120 (25)	118 (28)	0.593	0.988
Fatigue score	33 (13)	26 (13)	0.000	34 (13)	31 (15)	0.042	0.027
CK18 (pg/ml)	939 (580)	960 (540)	0.175	743 (477)	807 (506)	0.038	0.867

Data are presented as % or mean ± SD. All data are calculated from male and female.
n, number; SD, standard deviation; KRG, Korean Red Ginseng; BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GT, gamma glutamyl transferase; CK18, cytokeratin 18 fragment.
p-value, difference between pre and post.
p-value*, difference between placebo group and KRG group.

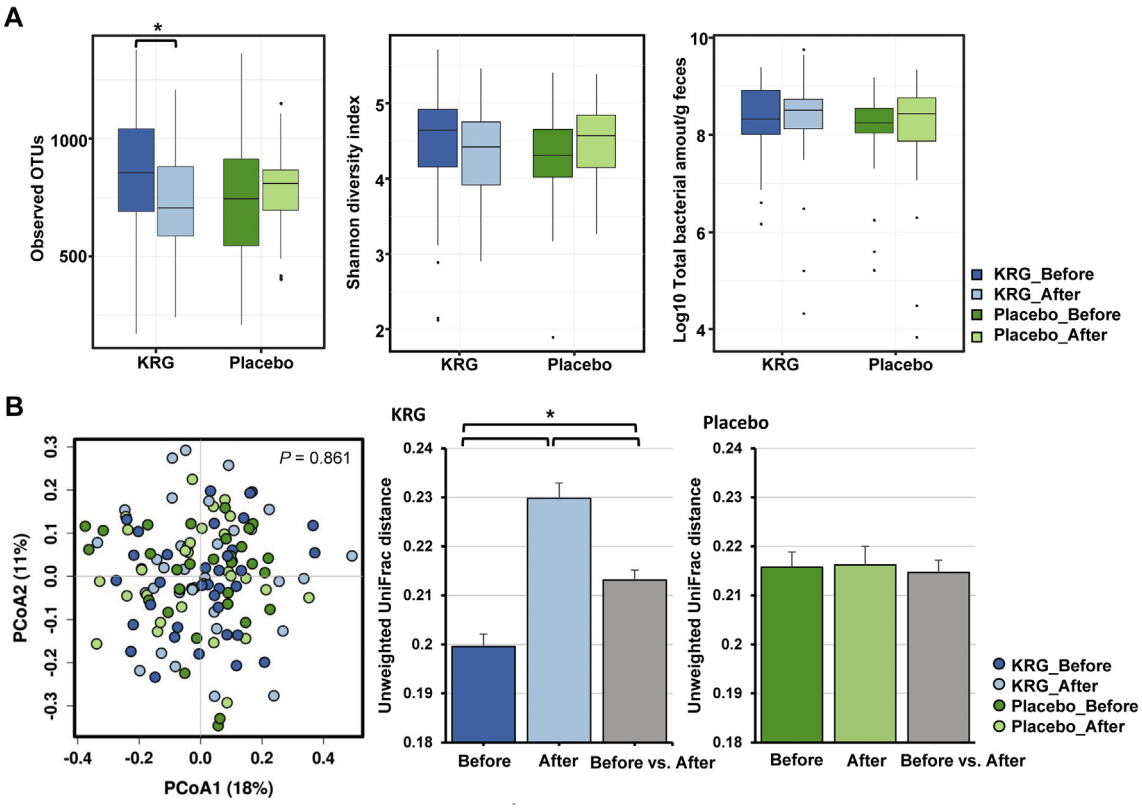

Fig. 2. Comparison of diversity indices and total bacterial amounts between before and after ingestion of KRG (n = 35) and placebo (n = 30) groups. (A) The number of observed OTUs, Shannon diversity indices, and total bacterial amounts were compared between before and after ingestion of KRG and placebo. (B) The difference of gut microbiota was analyzed in PCoA plot. The inter-variation and intra-variation of gut microbiota were compared in both of KRG and placebo group based on unweighted UniFrac distance. *p value < 0.05.

The significantly different genera after ingestion in three subgroups (improve, no change, and aggravate) according to ALT changes were compared between KRG ang placebo groups (Fig. 4). The number of subjects and mean values of ALT changes in each sub-group were summarized in Table S2.

In KRG group, the proportion of *Lactobacillus* was increased after treatment in improved sub-group and the proportions of *Clostridium* and *Intestinibacter* were increased in no change sub-group ($p < 0.05$). In placebo group, the proportion of uncultured *Lachnospiraceae* was increased in aggravated sub-group after treatment ($p < 0.05$). The changes of these genera after treatment could be related to the changes of ALT level in each sub-group.

3.3. Correlations of gut microbiota with measurements of liver enzyme

The elevation of ALT level was related to the liver injury. Thus, we analyzed the correlation between gut microbiota and the values of AST and ALT in KRG and placebo groups. The significant correlations were selected and shown in heatmap analysis (Fig. 5). The correlated genera with values of AST and ALT were different between KRG and placebo groups. In KRG group, 5 genera were positively correlated with values of AST or ALT, whereas 6 genera were negatively correlated with only ALT values. In placebo group, 7 genera were positively correlated with values of AST or ALT, and another 7 genera were negatively correlated with those values. Although the numbers of correlated genera were more in placebo group than KRG group, more numbers of genera were negatively correlated with values of ALT (6 genera in KRG group and 4 genera in placebo group), which was significantly different between groups in KRG group than placebo group. Among correlated genera, the proportion of uncultured *Prevotellaceae* was significantly higher in gut microbiota of KRG group after ingestion than placebo group in LefSe analysis (Fig. 3B).

4. Discussion

Although intestinal microbial imbalance is known to be related to NAFLD, few clinical trials regarding the alteration of intestinal microbiota in patients with NAFLD and NASH have been reported. KRG is touted for its effect on cancer, neuro degeneration, diabetes mellitus, stress, inflammation, hyperlipidemia, and oxidative stress; however, few studies were analyzed the effect of KRG in patients with NAFLD by the modulation of intestinal microbiota. To our knowledge, this has been the first randomized controlled study to examine the effect of red ginseng on NAFLD patients through the alteration of intestinal microbiota. Here, we analysed the shift of gut microbiota from patients with NAFLD after KRG ingestion and compared them with those in placebo group. The values of ALT and γ-GT were significantly different between KRG and placebo groups. The gut microbiota were changed more in KRG group than placebo group, and more number of genera were correlated with the decreased ALT values in KRG group.

After 4 weeks of KRG treatment, the KRG group showed significant decreases in biochemical markers such as AST, ALT, triglyceride, and γ-GT. Previous studies have shown that rats with liver fibrosis had significant improvement in insulin resistance and ALT levels after *P. ginseng* intake [29] and that rats with alcohol-induced liver damage had significant decreases in γ-GT levels after KRG extract intake [30]. Thus, it is suggested that the KRG supplement can improve AST, ALT, and γ-GT levels in patients with

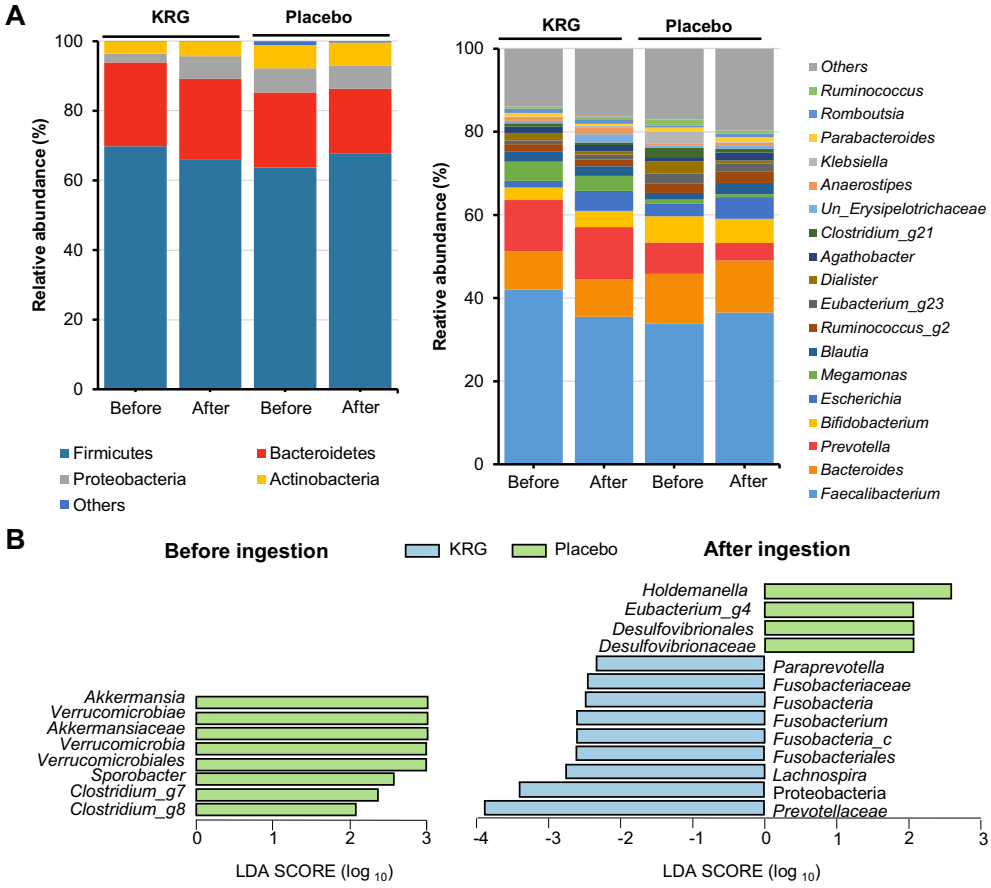

Fig. 3. Different members of gut microbiota between KRG (n = 35) and placebo (n = 30) groups. (A) Comparison of gut microbiota composition between before and after ingestion in both of KRG and placebo group at phylum and genus level. (B) Different taxa of gut microbiota between KRG and placebo group before and after ingestion detected in LefSe analysis.

NAFLD and may reduce liver toxicity. In a meta-analysis, triglyceride was effectively controlled by using ginseng in patients with diabetes [31]. Our previous clinical trial showed improvement in the level of adiponectin [13]. In light of these findings, KRG supplement can be an effective treatment for NAFLD with abnormal findings.

This study also showed that both the groups, KRG and placebo, presented a significant decrease in fatigue severity between baseline and 4 weeks. The KRG supplement may help reduce fatigue in patients with NAFLD. There was a study suggesting that *Panax ginseng* Meyer can be used as a therapeutic agent for idiopathic chronic fatigue patients [32]. On the basis of our result, KRG may be effective in treating fatigue in NAFLD patients.

CK18 is a filament protein which contain keratin and can be detected in the cytoplasmic skeleton of tissue [33]. If liver tissue is exposed to oxidative stress or toxic insults, hepatocytes show balloon and Mallory bodies by accumulating fat and disrupting cytokeratin intermediate filament network [34]. In previous studies, and dysbiosis is associated with cytokeratin and *Lactobacillus* induced bacteriocin binds to cytokeratin of intestinal epithelial cells [35,36]. Another study suggested that level of cytokeratin was changed by intestinal disease [37]. In our results, *Lactobacillus* is increased in improved sub-group of KRG group. Even if there is no difference between the two groups in change of CK18, CK18 proposed to be suppressed by KRG since CK18 was significantly increased in the control group. Taken together, we can suppose that KRG might ameliorate CK18 elevation in the progression of NASH.

In our previous clinical trial, tumor necrosis factor-α was decreased in KRG group and serum adiponectin levels increased in the KRG group, especially obese patients [13]. Ginsan (isolated from aqueous Korean *P. ginseng* extract) reduced tumor necrosis factor-α in control group [38]. In other study, *P. ginseng* extract increases adiponectin levels [39]. Based on these findings, KRG might have anti-inflammatory and fat-regulating activity. For the evaluation of exact mechanism of KRG, more research is required to further investigate the therapeutic effect of KRG

Recently, some studies have reported the association of gut microbiome with NAFLD. The influence of gut microbiota on the pathophysiology of NAFLD was reported by Janssen et al [40]; however, different results in gut microbiota in participants with NAFLD were also reported [41]. In this study, Firmicutes was the predominant phylum in gut microbiota, followed by *Bacteroidetes*. This result was partly in line with the results of a study by Mousaki et al, who demonstrated decreased *Bacteroidetes* composition in NASH patients with higher BMI than in those in controls with lower BMI and patients with simple steatosis [41]. Because BMI is one of influencing factors on gut microbiota composition, the categorizing participants with NAFLD according to BMI could be analyzed as a potential role of the gut microbiota in NAFLD patients [42]. However, we did not find significant difference in BMI between KRG and placebo after ingestion in the results.

Previous studies with NAFLD patients or mice showed that intestinal microbial imbalance may exacerbate the progression of NAFLD [43]. Altered metabolic response of gut microbiota in dysbiosis were reported to be related to NAFLD severity [43]. In our

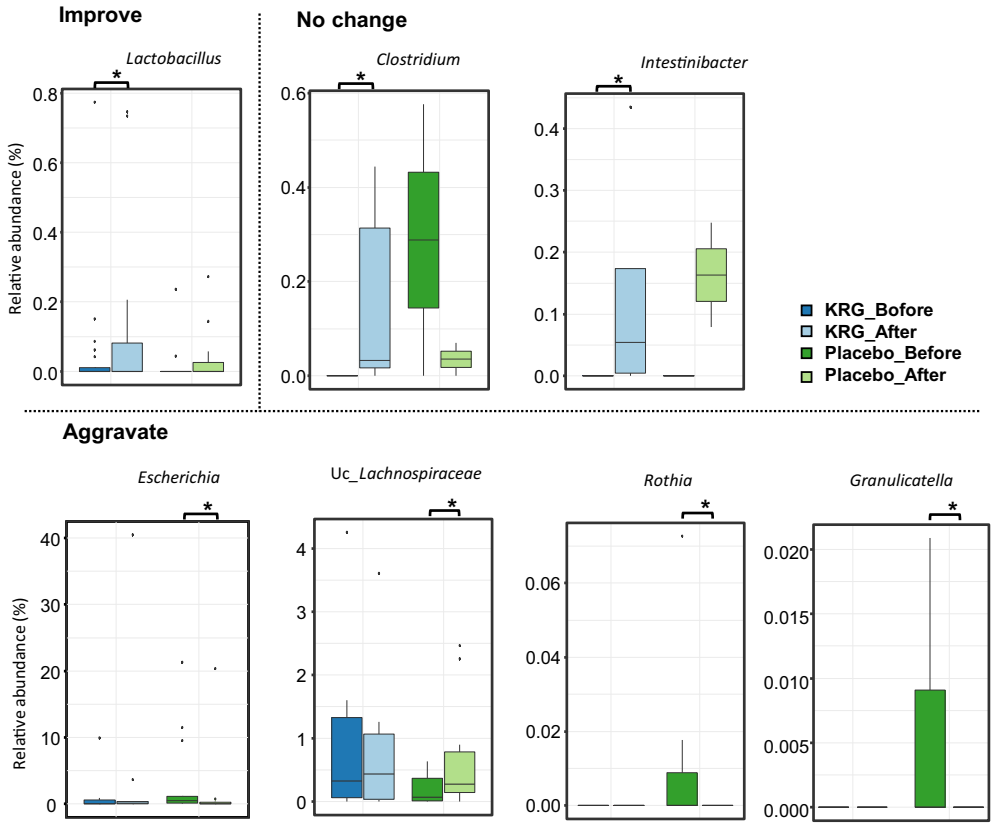

Fig. 4. Significantly different genera between before and after ingestion in both of KRG and placebo sub-groups. Subjects in each treatment group were divided into three subgroups (improve, no change, and aggravate) according to ALT changed values. *p value < 0.05. Two groups were divided into three subgroups (improve [improved by 10 IU/L or more], no change [changed less than 10 IU/L], and aggravate [worsened by 10 or more]) according to ALT change.

study, more genera of gut microbiota in KRG group were correlated with the decrease level of ALT than placebo group. The level of ALT was different between KRG and placebo groups. Different genera were also correlated with the decrease and increase level of AST and ALT in placebo group. In addition, the gut microbiota after KRG ingestion was significantly different to before ingestion, and the variation of gut microbiota in placebo group was similar between before and after ingestion based on UniFrac distance analyses. However, the significant difference of genera proportion between KRG and placebo groups was detected only for uncultured *Prevotellaceae*.

The effects of KRG on gut microbiota in adult with NAFLD and clinical importance concerning manipulation were provided in this study. Red ginseng may help prevent the progression of NAFLD by alteration of gut microbiota. Further longitudinal study with larger number of participants and detailed clinical information including diets are necessary to understand the mechanism of improvement liver functions by KRG intake.

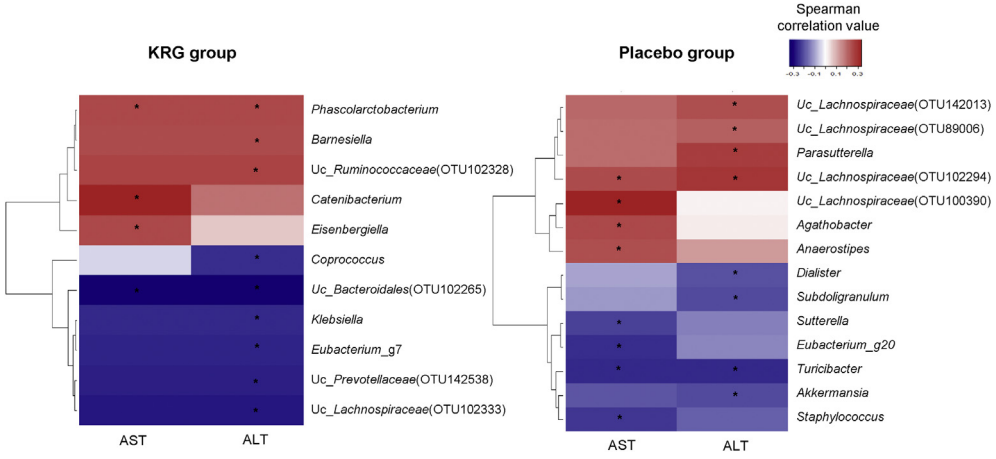

Fig. 5. Correlated genera with the values of AST and ALT in both of KRG (n = 35) and placebo (n = 30) groups. The correlation was determined by the Spearman's rank correlation using the proportions of genus in gut microbiota of each group and the values of AST and ALT. *p value < 0.05.

Genus *Lactobacillus* looks like rod and forms biofilms in the vaginal and gut microbiota [44]. It has various functions and roles in human health such as preventive effect on cancer, reduction of inflammation, or immune modulation [45,46]. *Clostridium* has been reported that it is related with inflammation of bowel [51]. In our report, the proportion of *Lactobacillus* was increased in improved sub-group of KRG group. On the other hand, *Clostridium* was increased in no change group. *Akkermansia* is increased in KRG group and decreased in placebo group. We can suppose that KRG related with modulation of gut microbiota and responsiveness of KRG is dependent on microbiota in bowel. Since gut microbiome is ecosystem influenced by multiple factors, multifactorial approaches are need for the evaluation of KRG mechanism on liver enzyme improvement.

In performing this study, we did not carry out a liver biopsy to all patients. To reduce selection bias in the diagnosis of NASH, we performed liver enzyme tests, a medical history, and liver ultrasonography. In addition, we excluded patients with uncertain NASH in the clinical data [47]. Another limitation is that all of the patients' stool were not collected both before-after feces were not collected in some cases because patients did not bring stool on time. In addition, some stools did not pass quality control. In this study, proportion of some microbiota are different at the baseline between two groups. Because microbiomes vary greatly depending on the individual characteristics of the patients, matching groups with same proportion in species level is very difficult in the clinical trial, suggesting that changes in each group may be more important.

In summary, this study provides evidence for a link between KRG and gut microbiota in NAFLD individuals. The values of AST, ALT, γ-GT, and fatigue in patients with NAFLD were effectively reduced after KRG ingestion comparing to placebo group. The gut microbiota was differently changed between KRG and placebo groups after ingestion. In particular, genera in gut microbiota were correlated to the improvement of ALT values, which was significantly different between KRG and placebo groups. On the basis of these results, oral supplementation with KRG might be associated with improvement of NAFLD by gut microbiota modulation. Further studies are needed to understand the mechanism about the improvement of liver function by gut microbiome alteration.

Author's contribution

Ji Taek Hong and Min-Jung Lee: analysis and interpretation of data, collection and assembly of data, and drafting the article. Ki Tae Suk and Bong-Soo Kim: conception and design, critical revision of the article for important intellectual content, and final approval of the article. Other authors: Provision of study materials or patients.

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Declaration of competing interest

The authors declare that there is no conflict of interest with regard to relevant financial interests, activities, relationships, affiliations, or any other as explicitly and implicitly expressed in the Editorial Policies for Authors.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jgr.2020.07.004.

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