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Effect of alcohol-free red wine concentrates on cholesterol homeostasis: An *in vitro* and *in vivo* study

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ABSTRACT

Polyphenolic composition of alcohol-free red wine concentrates (AFRWC) was determined by LC–MS/MS. The concentration of salicylic acid in non-flavonoid class and malvidin in flavonoid class was the highest among all the polyphenols determined in AFRWC. In the *in vitro* model using HepG2 cells, AFRWC was found to be more effective for the reduction of total cholesterol than lovastatin. For the *in vivo* model, animals were provided with AFRWC at ~750 mg of total polyphenols/kg body weight per day by oral administration. The amount of AFRWC was established by extrapolation to be equivalent to 375 ml/day wine consumption, which is ~2–3 glasses of wine per day for a 60 kg human. Despite a high cholesterol diet, a significant reduction in both total cholesterol and LDL-cholesterol was observed when supplemented with AFRWC, but the increase of HDL-cholesterol was not observed. The expression level of mRNA of some hepatic genes participating in cholesterol biosynthesis, cholesterol esterification was found to be influenced by AFRWC supplementation, whereas reverse cholesterol transport involved with HDL-cholesterol was seldom affected showing discrepancy in the expression of associated genes.

1. Introduction

Many epidemiological studies have associated a diet rich in fruits and vegetables with reduced risk of cardiovascular disease (CVD) [1]. This decreased risk may be attributed to the presence of natural antioxidants such as vitamin C, vitamin E, polyphenols, and carotenoids. In particular, polyphenols as a ubiquitous group of the secondary plant metabolites have received a lot of scientific and public interest, because phenolic compounds are broadly consumed in the form of beverages such as tea, coffee, cocoa, beer and red wine, as well as in the form of major dietary components including fruits, legumes, and cereals [2]. Red wine whose effect on CVD has been supported by the 'French Paradox' [3] contains a variety of polyphenols. The content and profile of red wine polyphenols (RWPs) are dependent upon many variables [4], including the type of yeast used for fermentation, the type of berry used, the vinification process used, and whether or not grape solids were in fact present during the maceration process. That is, RWPs are the sum of those directly originated from the skin and seeds of grape [5], those newly evolved by microorganism during fermentation [6], and those extracted from the oak barrel during aging [7]. Thus, the diversity of RWPs overwhelms polyphenols of other plant sources. The beneficial effect of red wine consumption against the development of atherosclerosis, a typical cause of CVD, was ascribed to both the antioxidant activity of its polyphenols [8,9] and its alcohol component [10,11]. Development of atherosclerosis is characterized by dysfunction of endothelial cells, oxidation of LDL, and foam cell formation from macrophage, migration of vascular smooth muscle cells (VSMC) from arterial media into intima, excessive proliferation of VSMC in the neointima, and increased extracellular matrix deposition [12]. An important property of red wine polyphenols to atherosclerosis is that RWPs can bind to plasma LDL and protect them from oxidation by scavenging free radicals derived from polyunsaturated fatty acid components of LDL [13]. Besides, ethanol of red wine is able to block or significantly reduce many of the key mechanisms that promote the formation of atheroma in arteries [14]. On the other hand, another typical cause of CVD is dyslipidemia. It is characterized by increased low-density lipoprotein cholesterol (LDLC) and decreased high-density cholesterol (HDLC), as well as increased total cholesterol and triglyceride in the blood plasma. The influence of red wine on improving dyslipidemia appears to be controversial and different from the case of atherosclerosis. Even moderate alcohol consumption (1–30 g/day) from alcoholic beverages other than wine has been shown to be consistently protective against

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coronary artery disease [15,16], and the alcohol protective effect on hypercholesterolemia is thought to be mediated in part (estimated at about 50%) by an increase in high-density lipoprotein (HDL) levels and an approximate 18% reduction in low-density lipoprotein (LDL) cholesterol [17,18]. Previous studies have suggested that alcohol can directly influence liver cholesterol by increasing apoA1, apoA2, and HDL-cholesterol synthesis, decreasing lipoprotein catabolism, or modifying the activity of enzyme that act on lipoprotein metabolism [19,20]. However, the effects of RWPs on hypocholesterolemia do not seem to be as clear as those of ethanol, although it has been well reported that other types of polyphenols, such as those which consisted of only a few compounds originated from grape skin or seeds [21], green tea [22], cacao [23], citrus [24], or blueberry [25] impact on the modulation of cholesterol metabolism. Therefore, because there have been few studies on the effects of RWPs as a whole product on cholesterol metabolism, we investigated the effects of alcohol-free red wine concentrates on cholesterol levels *in vitro*, and on the regulation of genes directly related to cholesterol homeostasis, as well as on plasma lipoprotein profiles, using an *in vivo* model fed a high-cholesterol diet.

2. Materials and methods

2.1. Materials

Human transformed hepatic HepG2 cells were purchased from ATCC (American Type Culture Collection, Rockville, MD, USA). Cell culture media, its reagents and fetal bovine serum (certified grade) were from Gibco BRL Life Technologies (Gaithersburg, MD, USA). All other reagents were commercially available, analytical grade chemicals, and unless otherwise stated, were purchased from Sigma Chemicals (St. Louis, MO, USA).

2.2. Preparation of alcohol-free red wine concentrates (AFRWC) and determination of total phenolic content

Red wine used in this study was produced from *Vitis vinifera* var. Cabernet Sauvignon grown in the Maule region in Chile during 2009 vintage following standard wine-making procedures and wood contact of one year. The red wine was selected on the basis of total polyphenol content ranged from 2.0 to 2.2 g/l and was concentrated 10-fold by weight, removing all alcohol, by a centrifugal thin film evaporator (Okawara MFG Co., Shizuoka, Japan) at a maximum temperature of 45 °C. Gas chromatography was then used to identify any traces of ethanol in wines from which alcohol had been removed. The average composition of the alcohol-free red wine concentrates (AFRWC) was as follows: 17.8% carbohydrate, 2% protein, 0.2% fat, 2% ash, 78% moisture, and 0.1% glucose as a sole sugar. The total polyphenolic content of AFRWC was assayed by the Folin–Ciocalteau method and was expressed as gallic acid equivalents [26], with an average value of 25.2 mg/g.

2.3. LC–MS/MS analysis for determination of phenolic compounds composition in AFRWC

Analysis of the polyphenolic composition of AFRWC was performed using liquid chromatography (Thermo Scientific, San Jose, CA, USA) coupled to tandem (triple quadrupole) mass spectrometry (LC–MS/MS). AFRWC was separated on a Zorbax Extend-C18 (2.1 mm × 150 mm, 5 μm, Agilent). The mobile phase consisted of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B) and the flow rate was 200 μl/min. The gradient condition was set to the following conditions: 10–90% B for 10 min, isocratic 90% B for 3 min and 10% B for 6 min for equilibration of column. The injection volume was 10 μl. AFRWC was monitored in the negative ion mode with electrospray ionization (ESI). The optimum MS conditions were as follows: spray voltage 3000, capillary temperature 300 °C, and sheath gas pressure and aux gas pressure of 40 (psi) and 10 (Arb), respectively. For quantitative analysis of AFRWC, reference solutions of individual phenolic compounds (c = 2–100 μg/ml in methanol) were used for external standardization, and selective reaction monitoring was used (*i.e.*, gallic acid: parent ion m/z, 163.00; daughter ion m/z, 125.06 and caffeic acid: parent ion m/z, 179.00; daughter ion m/z, 135.07). Data processing was performed using the Xcalibur software (Thermo Scientific, San Jose, CA, USA).

2.4. Cell culture and preparation for treatment

The HepG2 cells were grown in DMEM (Dulbecco's Modified Eagle's Medium; Sigma) supplemented with 10% fetal bovine serum (FBS) at 37 °C under a humidified 5% CO_2 atmosphere. The cells were subcultured at a density of ~5×10^5 cells in a petri dish of 60 mm diameter, and the medium was changed every two or three days until the cells were 80–90% confluent. The subcultured cells were allowed to grow without disruption in 12-well plates with new fresh media for 24 h, and treatments were initiated by supplementing the cells with alcohol-free red wine concentrates (AFRWC) to reach the level of 0.1 mg/ml, 0.2 mg/ml, and 0.4 mg/ml in the media. The value of 0.1 mg/ml was designed as equivalent to a dilution of 1000-fold of red wine, according to the experiment of Frankel et al. [27], which showed that the non-alcoholic compounds of red wine diluted 1000-fold had potent antioxidant properties toward oxidation of human LDL, and 0.2 and 0.4 mg/ml correspond to 500-fold and 250-fold, respectively, by dilution.

2.5. Determination of intracellular triglyceride and total cholesterol

Cells were grown under the same conditions as described above. They were seeded in 12-well plates at 3×10^5 cells per well and were grown for 24 h. The following day, the cells were lipid-loaded with 400 μM of palmitic acid and oleic acid in DMEM containing 0.5% bovine serum albumin, and were incubated for 6 h. Cells which were lipid-loaded as a positive control were treated with three concentrations of AFRWC: 0.1, 0.2, and 0.4 mg/ml, as well as 2 μM of lovastatin (an HMG-CoA reductase inhibitor), for 24 h, and were then lysed in RIPA buffer. The intracellular triglycerides and cholesterol were measured using a Roche COBAS c111 analyzer.

2.6. Animals and diets

ICR mice (12 week old, n = 30) obtained from Semtako Laboratory Animal Inc. (Seoul, Korea) were acclimatized and given free access to water and chow for one week prior to the initiation of the experimental diets. Mice weighing ~35 g were used for experiments. The mice were housed in plastic cages in temperature-controlled rooms (21–23 °C), with 45–60% humidity, subjected to a 12 h light–dark cycle, and allowed free access to both food and water, with uneaten food being weighted daily. The mice were randomly divided into three groups of 10, and were fed one of the following diets for eight weeks: standard normal diet (Group ND), cholesterol-rich (2%, w/w) diet (Group HC), or cholesterol-rich (2%, w/w) diet plus alcohol-free red wine concentrates (Group HCRW). Body weights were recorded weekly, and food intake was monitored twice a week. The amount of alcohol free red wine concentrates (AFRWC) administered through gavage was adjusted to the weight of the mice. The amounts were maintained at ~750 mg/kg body weight per day by oral administration, established by extrapolation from a wine consumption of ~375 ml/day equivalent to ~ 2 or 3 glasses of wine per day for a 60 kg human. All experimental protocols were reviewed and approved by the Ethical Committee of Korea University, in accordance with NIH guidelines outlined in 'Principles of Laboratory Animal Care' (NIH publication no. 85-23 revised 1985).

2.7. Determination of plasma lipoprotein profiles

At the end of the eight-week experimental period, body weights were measured and the mice were sacrificed by guillotine. Blood was collected in 1.5 ml E-tubes containing 1 mg of EDTA, and plasma was prepared by centrifugation at 2000 × g for 10 min at 4 °C, and then stored at −80 °C until analysis. Plasma total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), and triglycerides (TG) were determined using commercially available assay kits (Bioassay Systems, USA), according to the manufacturer's instructions.

2.8. Determination of AST, ALT, and TBARS in the plasma

The plasma AST (aspartate amino-transferase) and ALT (alanine amino-transferase), which are useful markers of assessing the level of liver cell damage, were measured in the blood of mice using a Roche COBAS c111 analyzer, following the method described in Section 2.5. Total of thiobarbituric acid reactive substances (TBARS) in plasma was measured by malondialdehyde (MDA), using a Cayman TBARS Assay Kit (Cat #10009055) for lipid peroxidation. The protocol was performed according to the direction of the manufacturer, following the method described in Section 2.5.

2.9. Reverse transcription polymerase chain reaction

Immediately after sacrifice, liver tissue was frozen in liquid nitrogen and stored at −80 °C prior to use. Total RNA was extracted from liver tissue using TRIzol and an RNA purification kit (Invitrogen, Carlsbad, CA, USA), and 1 μg of total RNA was converted to complementary DNA (cDNA) using AMV reverse transcriptase (Promega, WI, USA) in accordance with the manufacturer's protocol. PCR reactions for this study were performed using the primer sequences listed in Table 1. The PCR products were resolved by 1.5% agarose gel electrophoresis. Differences in mRNA expression were calculated after normalization to β-actin expression.

2.10. Statistical analysis

All experiments were performed in triplicate at least 2 times, independently, and the data are expressed as means ± SD. Statistical analyses were carried out using SPSS statistical software version 12.0 (SPSS Inc., Chicago, IL, USA). A two-sample t test for independent analysis of samples was used to compare

Table 1
Primer sequences for the genes used in RT-PCR analyses.

Genes		Product-length (bp)	Sense primers	Antisense primers
Group 1	SREBP2	247	CACCATGTCAGGCGTCCGCC	CAAGCCTAAAGACATAATTAT
	LDLR	688	TGTCGCTGAGGTCTGAAGCT	GCTTTCAGGTCAGACATGTCC
	HMGCR	618	TGCCGGCCCACATGCTCCAAG	GCTTTCAGGTCAGACATGTCC
Group 2	ACAT2	499	GTGAGACACCCAAGGCTA	TCTGACTAGAACAGCTGGA
Group 3	ABCA1	447	CAGGAGGTGATGTTTCTGACCA	TTGGCTGTTCTCCATGAAGGTC
	SR-B1	499	GAACTAGGTCTTTGCACCA	ACTTCTATGGGAGGTAGGA
	PPAR γ	499	AAATGCCAGGAGTGTCGA	GTAACTGAAGTCCTTGAGGA
Control	β-actin	540	GTGGGCCGCTCTAGGCACCAA	CTCTTTGATGTCACGCACGATTTC

between the values obtained from the treatment and control groups. When ANOVA revealed $p < 0.05$, the data were further analyzed using Duncan's test for multiple comparisons.

3. Results and discussion

3.1. Polyphenolic composition in AFRWC

Table 2 shows the polyphenolic composition of alcohol-free red wine concentrates (AFRWC) used in this study. A total of 21 polyphenolic compounds were quantitatively identified in our sample. Among the non-flavonoid group, the level of salicylic acid was the highest among phenolic acids, whereas malvidin, belonging to the class of anthocyanin, was the highest in the flavonoid group. Following these compounds, caffeic acid, quercetin, hesperetin, cyanidin, peonidin, and epicatechin were determined by LC–MS/MS to be major compounds over 25 mg/l. Salicylic acid (SA), which had the lowest molecular weight among polyphenols in the AFRWC, has been used in the synthesis of acetylsalicylic acid (ASA, *i.e.* aspirin), which is rapidly deacetylated to SA *in vivo*, and prolongs its anti-inflammatory effect by a reversible inhibition of cyclooxygenase-1 enzyme and by inhibition of cycloxygnease-2 gene transcription [28]. A regular ASA consumption at low-dose level is associated with a reduced risk of cardiovascular disease and colon cancer [29,30]. On the other hand, although malvidin, which is major pigment of red wine color, existed at the highest level among all the polyphenols in the AFRWC, few beneficial effects toward improving lipid profile *in vivo* have been reported for this compound. The other six polyphenols that existed in relatively low concentration against malvidin and salicylic acid were found to be effective in improving cholesterol metabolism, as well as in increasing antioxidant capacities, through both *in vitro* and *in vivo* experiments of the single compound [31–36].

3.2. Effect of AFRWC on intracellular triglyceride and total cholesterol in HepG2 cells

Fig. 1 shows that AFRWC at each concentration level of 0.1, 0.2, and 0.4 mg/ml, as well as 2 μM lovastatin, significantly decreased triglyceride and total cholesterol in treated cells compared with control cells. Here, dose-dependent effects of AFRWC were not observed. The triglyceride concentration in Fig. 1(A) was reduced by around 38–40% by AFRWC and by about 48% by lovastatin, respectively, in cells, whereas the attenuated amount of total cholesterol in Fig. 1(B) was around 22–33% for AFRWC and about 14% for lovastatin. AFRWC was more effective in decreasing triglyceride than total cholesterol. However, in terms of decreasing total cholesterol, AFRWC ($p < 0.001$) was more effective than lovastatin ($p < 0.05$). With regard to polyphenol concentration, the applied concentration was 1000-fold diluted, and based on this, salicylic acid of both a relatively low molecular weight and a high concentration among the polyphenols analyzed in AFRWC would be an important factor in the reduction of cholesterol or triglyceride. Takagi et al. [37] reported that salicylic acid was taken up rapidly across the lipid bilayer of liposome consisting of egg yolk phosphatidyl choline and cholesterol, showing overshoot phenomena, and the protonated SA in the extraliposomal solution (pH 5.8) was rapidly taken up by liposomes, followed by a redisssociation to anion according to the intra-liposomal pH (pH 5.8). Interestingly, the pH in the medium of grown HepG2 cells treated with AFRWC was around 5.6. Besides, the experiment conducted by Nakagawa et al. [38] demonstrated that the total cholesterol content in serum of rats administered

Table 2
Molecular weights and concentrations of individual polyphenolic compounds quantified in alcohol-free red wine concentrates (AFRWC).

	Compounds	Molecular weight	Concentration (μg/ml)
Non-flavonoid	*p*-Coumaric acid	164.16	10.19 ± 1.21
	Gallic acid	170.12	3.03 ± 0.25
	Caffeic acid	180.16	26.63 ± 2.39
	Ferulic acid	194.18	0.82 ± 0.04
	Gentisic acid	154.12	2.18 ± 0.18
	Syringic acid	198.17	5.08 ± 1.34
	Salicylic acid	138.12	80.08 ± 4.65
	Vanillic acid	168.14	5.45 ± 1.37
	trans-resveratrol	228.24	1.75 ± 0.42
Flavonoid	Luteolin	286.24	0.17 ± 0.05
	Kaempferol	286.23	0.65 ± 0.09
	Quercetin	302.24	34.23 ± 3.16
	Myricetin	318.24	8.62 ± 2.30
	Hesperetin	302.27	43.54 ± 5.32
	Delphinidin	303.24	3.21 ± 0.64
	Cyanidin	287.24	88.99 ± 7.31
	Peonidin	301.27	54.38 ± 3.29
	Malvidin	331.30	1381.98 ± 82.16
	Epicatechin (EC)	290.27	27.72 ± 4.20
	Epicatechin gallate (ECG)	442.37	2.89 ± 0.68
	Epigallocatechin (EGC)	306.27	0.05 ± 0.01

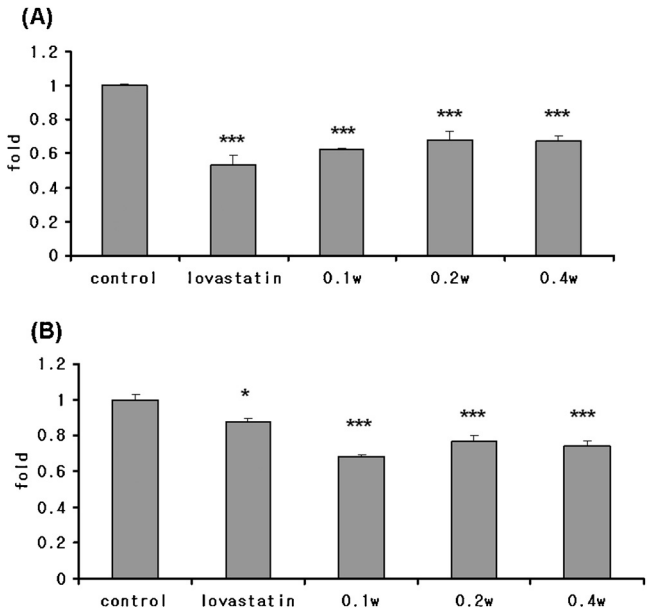

Fig. 1. Effects of alcohol-free red wine concentrates (AFRWC) on intracellular triglyceride (A) and total cholesterol (B) in HepG2 cells. The cells were incubated with 2 μM lovastatin, as well as 0.1, 0.2, and 0.4 mg/ml AFRWC, for 24 h in a DMEM media. Values are expressed as a ratio of control, mean ± SEM of three separate experiments done with replicates. *, **, and *** denote values which are $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared to the control group (Student's *t*-test). Abbreviations on X-axis are as follows: 0.1 w, 0.1 mg/ml AFRWC; 0.2 w, 0.2 mg/ml AFRWC; 0.4 w, 0.4 mg/ml AFRWC.

aspirin or salicylic acid was decreased to 79–92% of that of control rats.

3.3. ALT, AST, and TBARS in the plasma

ALT and AST, which are kinds of hepatocellular cytoplasmic enzymes, and serve as indicators of liver function, are released into systemic circulation by liver damage, such as hepatocellular necrosis [39]. As shown in Fig. 2(A) and (B), their levels were significantly lower in the HCRW group than in the HC group. The reduced level is more clear in the ALT ($p < 0.01$) than in the AST ($p < 0.05$). TBARS in Fig. 2(C), which is indicative of malonaldehyde and peroxidation break-down product formation from numerous substances, including polyunsaturated fatty acid, was significantly lower in the HCRW group than in the HC group. The level of TBARS in the HCRW group was attenuated by about 62% against the HC group. In addition to the cholesterol lowering effects observed for AFRWC, the reduction of AST and ALT values, as well as TBARS level, demonstrates that AFRWC can exert increased antioxidative capacities with no harmful effects on the liver.

3.4. Plasma lipoprotein profile

The results of plasma cholesterols and triglycerides in the three diet treatment groups were summarized in Table 3. All biomarkers, including triglycerides, total cholesterol, HDL-cholesterol, and LDL-cholesterol in the HCRW group were significantly lower than those in the HC group. These plasma cholesterol-related findings, except for the result of HDL-cholesterol, agree well with many previous studies from an animal model employing polyphenol-rich products originated from grape, such as grape extracts or products [40–43], grape seed [44], and dealcoholized red wine [45,46]. As regards HDL-cholesterol, some studies in animals [41,42,47] have reported its increased figures after supplementation with products derived from grape, whereas many other conflicting studies

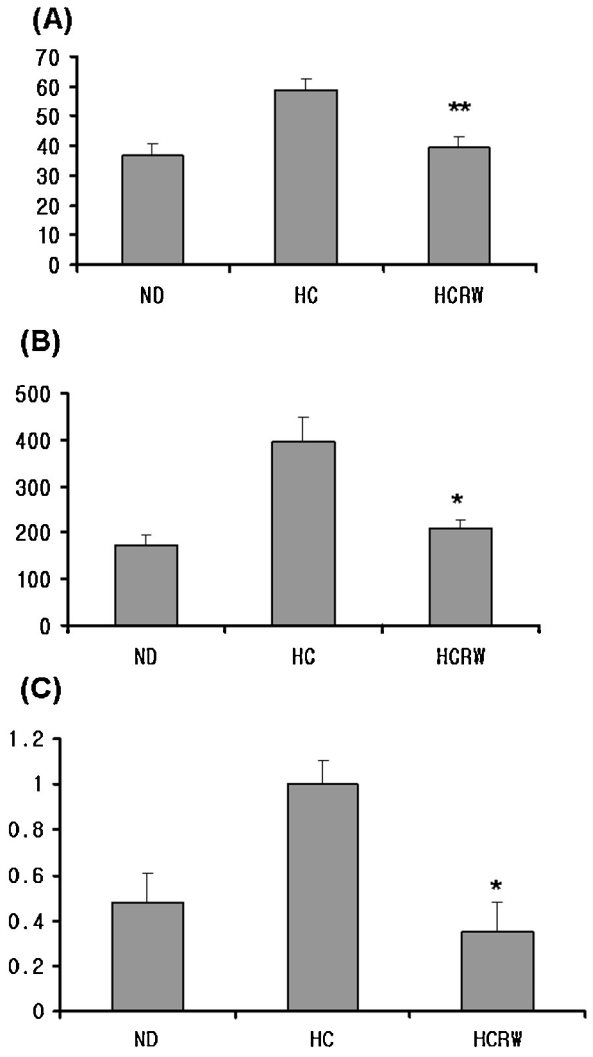

Fig. 2. Effects of alcohol-free red wine concentrates (AFRWC)-supplemented diets on the plasma levels of alanine amino transferase (ALT) (A), aspartate amino transferase (AST) (B), and thiobarbituric acid reactive substances (TBARS) (C) in ICR-mice fed a cholesterol-rich diet for eight weeks. (A) and (B) data are given as mean ± SEM ($n = 10$), and *, and ** denote values which are $p < 0.05$ and $p < 0.01$, compared to the HC group (Student's *t*-test). (C) Data are expressed as a ratio of HC group, mean ± SEM ($n = 10$). * denotes $p < 0.05$, compared to the HC group (Student's *t*-test).

[43,44,46,48–52] have also reported no change or even a decrease in the HDL level in the experiments. The sources of polyphenols used in the conflicting studies, including the result in the present study, appear not to be significantly different from those of polyphenols in the works showing an increase in HDL-cholesterol. Nevertheless, such disputing results for HDL-cholesterol might be due to the difference in quantitatively dominant polyphenolic compounds in each source, difference between the doses to animal models, and disparity of animal models or overall experimental protocols. However, works related to gene expression operated in cholesterol metabolism are needed in order to elucidate these contrary results.

3.5. Effect of AFRWC on mRNA fluctuation with cholesterol biosynthesis

As mentioned in Table 1, Group 1 consisted of genes responsible for cholesterol biosynthesis [22,53–55], such as HMGCR (HMG-CoA reductase), LDLR (LDL receptor protein), and SREBP2 (sterol regulatory element binding protein number 2). HMGCR is the

Table 3
Effects of alcohol-free red wine concentrates (AFRWC) supplementation on plasma lipid and cholesterol concentrations in ICR-mice fed a high cholesterol diet for 8 weeks.[a]

Biomarkers	ND group ($n = 10$)	HC group ($n = 10$)	HCRW group ($n = 10$)
Triglycerides (mmol/l)	1.23 ± 0.004^{a}	1.10 ± 0.008^{b}	0.77 ± 0.001^{c}
Total cholesterol (mg/dL)	125.3 ± 0.32^{b}	132.0 ± 0.31^{a}	118.6 ± 0.36^{c}
HDL cholesterol (mg/dL)	114.4 ± 0.22^{c}	126.5 ± 0.27^{a}	119.8 ± 0.34^{b}
LDL cholesterol (mg/dL)	11.5 ± 0.32^{c}	15.6 ± 0.42^{a}	13.5 ± 0.30^{b}

[a] Values are expressed as means ± SD. The means ± SD with different superscripts in the same row for each biomarker are significantly different, $p < 0.05$.

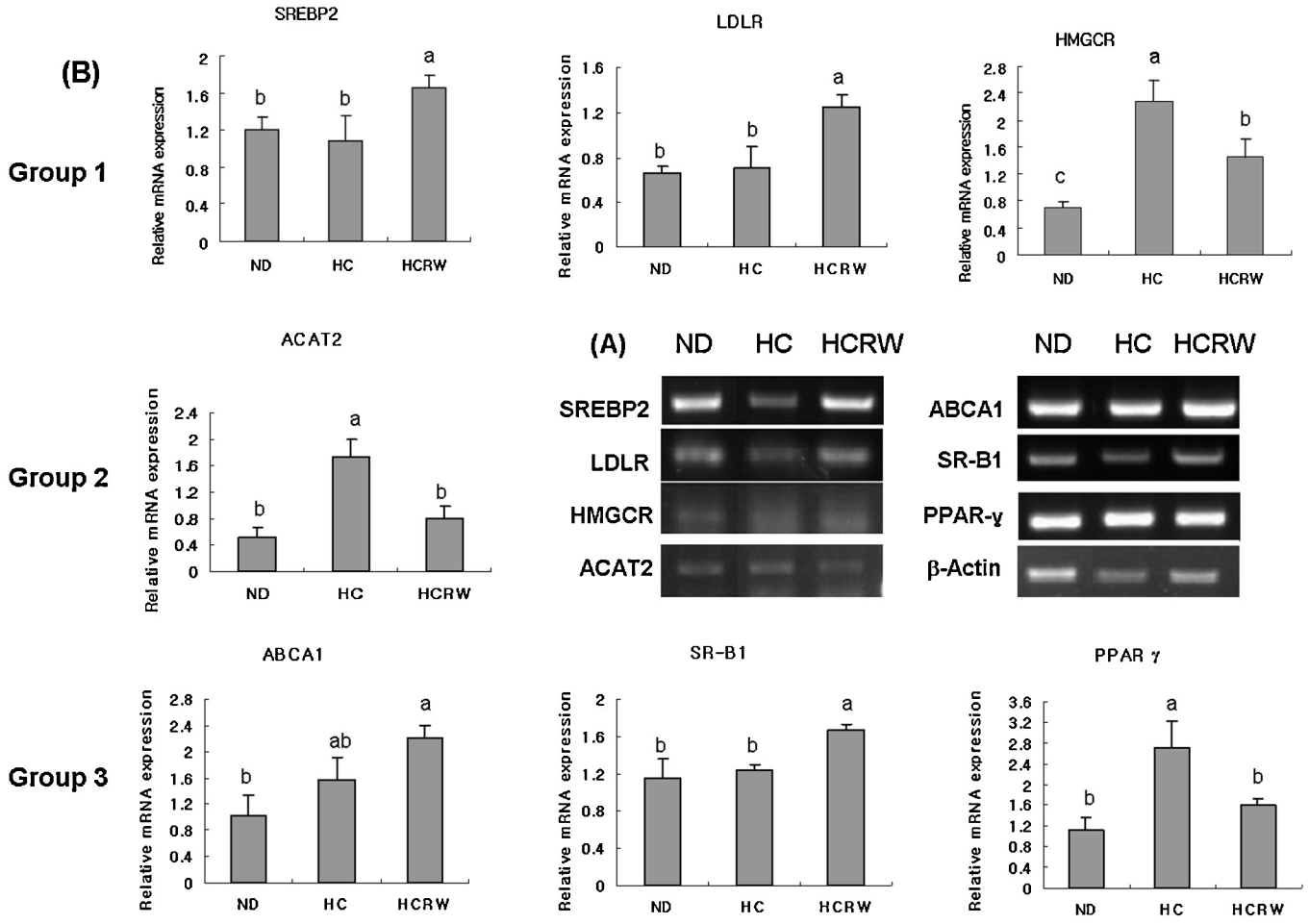

Fig. 3. Effects of alcohol-free red wine concentrates (AFRWC) on the mRNA expression of genes involved in cholesterol homeostasis in the liver, as determined by RT-PCR after feeding experimental diets to ICR-mice for eight weeks. As described in Table 2, the genes belonging to Group 1 are SREBP2, LDLR, and HMGCR, which are involved in cholesterol biosynthesis; ACAT2 of Group 2, cholesterol esterification; SR-B1, ABCA1, and PPAR-γ in Group 3, reverse cholesterol transport. (A) Total RNA was isolated, reverse transcribed, and used as a template for PCR, using β-actin specific primers. PCR products were electrophoresed on agarose gels and visualized with ethidium bromide. (B) Each bar represents mean ± SEM ($n = 10$), and relative expressions were normalized to β-actin. Different letters above bar graphs indicate significant difference at $p < 0.05$ by one-way ANOVA.

proteins involved in biosynthesis of mevalonate from 3-hydroxy-3-methylglutaryl coenzyme A, acting as a key regulatory enzyme in cholesterol biosynthetic pathway, and LDLR can mediate the lowering of plasma cholesterol by enhancing the uptake of LDL cholesterol from the circulation into liver. It has been well reported that when the cholesterol content of the cell decreases, SREBPs, which reside in an inactive form in the cytoplasm, are cleaved by protease and activated as transcription factors. The active SREBPs then migrate to the nucleus of the cell and bind to the genes directly or indirectly involved in cholesterol biosynthesis, such as HMGCR and LDLR, thereby modulating expression or transcription of these genes [56,57]. As can be seen in Fig. 3, the level of mRNA of both SREBP2 and LDLR was significantly increased in the HCRW group in comparison with the HC group, while the expression of HMGCR mRNA was significantly lower in the HCRW group than in the HC groups. This result well agreed with the result shown in Table 3 indicating that the reduction of total cholesterol could be explained by attenuation of gene expression of HMG-CoA reductase that stimulates the conversion of HMG-CoA into mevalonic acid, which is a rate-limiting step in cholesterol biosynthesis, and the decrease of LDL-cholesterol in plasma could be supported by an increase of LDLR mRNA, which is associated with the uptake of LDL-cholesterol into liver. Several studies have demonstrated that substances modulating cholesterol metabolism exert their influence *in vivo* and *in vitro* by two different mechanisms. One mechanism [23,58–61] interferes with the normal reabsorption of both cholesterol and bile acid and causes their simultaneous excretion along with the excretion of substances rich in polyphenols. The other mechanism [22,55,57,62] indicates that polyphenols prevent *de novo* synthesis of cholesterol in the liver by acting like a competitive inhibitor

in several steps of the cholesterol biosynthesis pathway. In other words, cholesterol metabolism works on the biological system by coordinating the absorption of dietary cholesterol in the small intestine and *de novo* synthesis of cholesterol in liver cells. Hepatic *de novo* synthesis of cholesterol decreases when sufficient cholesterol is absorbed in the intestine, whereas its biosynthesis increases in the case of insufficiency of cholesterol absorption [61]. In this context, the significant increase of mRNA levels of both SREBP2 and LDLR in the HCRW group indicates that AFRWC could be excreted in the form of a complex of both AFRWC and cholesterol from the body, following combination with cholesterol, and liver cells start to stimulate the mRNA of SREBP2 and LDLR genes in order to compensate for the deficiency in liver. In contrast to that of SREBP2 and LDLR, the expression of HMGCR mRNA in the HCRW group could be explained in two cases: being stimulated to make up for the cholesterol deficiency in the whole system in order to maintain cholesterol homeostasis; or similarly to the result of lovastatin identified in the *in vitro* model as a inhibitor of HMGCR, being inhibited by AFRWC to decrease total cholesterol. Thus, in terms of obtaining the same result in decrease of total cholesterol both *in vitro* and *in vivo*, it is likely that AFRWC have an inhibitory effect on the biosynthesis of HMGCR at a transcriptional level.

3.6. Effect of AFRWC on mRNA fluctuation with cholesterol esterification

In Table 1, Group 2 consisted of ACAT2 (acyl CoA:cholesterol acyltransferase number 2), which catalyzes the intracellular esterification of cholesterol and provides the cholesteryl esters (CE) for lipoprotein assembly in the liver and intestines [63]. ACAT2 esterifies excess free cholesterol to form CE, which makes cholesterol more hydrophobic and suitable for storage and transportation, and consequently improves the availability of CE to apolipoprotein B (apoB) secretion to make VLDL in further maturation [64,65]. In Fig. 3 the expression of ACAT2 mRNA was significantly increased in the HC group compared to that in the HCRW groups. This result can be extrapolated into the result of Table 3, which indicates that the highest level of total cholesterol determined was that of the HC group, as well as that AFRWC contributed to decreased level of total cholesterol in the HCRW group. Thus, we could infer that AFRWC caused some restriction to esterification of free cholesterol, which is an essential step for cholesterol adsorption to the intestine and liver, and accelerated excretion of free cholesterol from the body. This finding was in agreement with previous studies using polyphenolic fractions derived from apples [66] and citrus [67], as materials for the experiments.

3.7. Effect of AFRWC on mRNA fluctuation with reverse cholesterol transport

As indicated in Table 1, Group 3 consisted of genes for promoting reverse cholesterol transport (RCT) [68–70], which is a complex process that ensures the efflux of cholesterol from peripheral cells as well as its transport back to the liver for its metabolism and biliary excretion. Three major genes involved with RCT, such as ABCA1 (ATP-binding cassette transporter A1), SR-B1 (scavenger receptor class B number 1), and PPARγ (peroxisome proliferators-activated receptor gamma), were studied.

ABCA1 is involved in the control of high density lipoprotein and apolipoprotein AI (apoAI)-mediated cholesterol efflux from macrophage, and plays a major role in translocating cholesterol from intracellular cholesterol pools into the extracellular space and liver [71]. Besides, SR-B1 can induce cholesterol efflux by enabling HDL to bind to both liver and cholesterol-rich domains in the plasma membrane [69]. The key mechanism by which RCT operates *in vivo* is [70] to help lipid-poor apolipoprotein AI (apoA-I) particles, which later become mature HDL, to promote the efflux of cellular free cholesterol *via* ABCA1 transporter to the liver. The mature HDL can also promote the transport of cholesterol *via* SR-B1, which is a cell surface receptor that mediates selective HDL uptake into (liver) cells, with cholesteryl esters of HDL ultimately being absorbed in the liver [69]. As shown in Fig. 3, in this study, the expression of ABCA1 mRNA and SR-B1 mRNA was significantly increased in the HCRW group compared to that in the HC group. Therefore, it was assumed that AFRWC could affect the regulation of ABCA1 mRNA and SR-B1 mRNA in order to transport accumulated cholesterol from the plasma into the liver for excretion. An unexpected result from the RCT study occurred for PPAR-γ mRNA, which is a member of the nuclear receptor superfamily that regulates genes involved in lipid homeostasis, including adipocyte differentiation and lipid storage, as well as glucose homeostasis [72]. PPAR-γ have been reported to induce the expression of ABCA1 gene in macrophages through a transcriptional cascade mediated by the nuclear receptor, liver X receptor (LXR) [73,74]. We hypothesized that the expression of PPAR-γ mRNA in the HCRW group would be significantly higher than that in the HC group, since the level of ABCA1 mRNA in the HCRW group was significantly increased compared to that in the HC group. However, mRNA expression of PPARγ was significantly higher in the HC group than in the HCRW group. This result did not agree with studies by Xia et al. [35] and Gijsbers et al. [75], which used cyanidin and peonidin, tomato extracts, respectively, as polyphenolic sources *in vitro*. This discrepancy with the expression of PPAR-γ gene could be attributed to the difference between polyphenolic compositions studied, as well as critical disparities between methods. That is, we employed *in vivo* model and a whole polyphenolic product originated from red wine instead. The controversy over HDL-cholesterol that we mentioned in the introduction, including both many previous results in Section 3.4 that suggested no increase in HDL-cholesterol level and the result described in Table 3, could be in part explained by the inconsistency in the expression of three genes involved with RCT. Therefore, given that cholesterol is a crucial component of cellular membranes and essential to the survival of animals, yet can be cytotoxic when it accumulates to excess, reverse cholesterol transport is much more likely to work in a complex manner at a transcriptional level to maintain the appropriate cellular cholesterol level.

4. Conclusions

The present study demonstrated that AFRWC, a concentrated source of polyphenols from red wine, was involved in the reduction of plasma cholesterol, including total cholesterol and LDL cholesterol in an animal model, as well as a decrease in intracellular total cholesterol and triglyceride *in vitro*. The polyphenolic composition determined by LC–MS/MS revealed that salicylic acid for non-flavonoid group and malvidin for flavonoid group reached the highest concentration in each group, while caffeic acid, quercetin, hesperetin, cyanidin, peonidin, and epicatechin were also present in relative abundance. As determined through an *in vitro* model using HepG2 cells, AFRWC was more effective in decreasing total cholesterol than lovastatin, which is an inhibitor of HMG-CoA reductase concerning the rate limiting step of cholesterol biosynthesis in the liver. On the other hand, the animal model showed a significant reduction in total cholesterol and LDL-cholesterol, but not an increase in HDL-cholesterol. These results can be explained by the evidence that hepatic genes associated with cholesterol biosynthesis and esterification were easily affected by AFRWC at a transcriptional level, but genes with reverse cholesterol transport to the liver were not affected as easily. Therefore, in order logically to explain the fluctuation of HDL-cholesterol in connection with sustaining cholesterol homeostasis *in vivo*, further studies,

such as those relating to the conversion of cholesterol into bile acids or to other mechanisms in RCT pathway, including cholesteryl ester transfer protein and lecithin-cholesterol acyltransferase, are needed.

Conflict of interest statement

The authors declare no conflict of interest.

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