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Comparative Study of Red Wine and Korean Black Raspberry Wine in Adipocyte Differentiation and Cardiovascular Disease Related Gene Expression

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Abstract Abilities of Korean black raspberry (KBR) wine to induce differentiation of 3T3 L1 adipocytes and express HepG2 cardiovascular disease-related genes were determined and compared with those of red wine. Red wine attenuated the differentiation of adipocytes faster than KBR wine and control. KBR wine decreased cholesterol concentration in HepG2 cells at a similar level to that of red wine, resulting in similar degrees of suppression in apolipoprotein B100, and enhancement in LDL receptor and 3-hydroxy-3-methylglutaryl coenzyme A reductase mRNA levels.

Keywords: Korean black raspberry wine, Adipocyte differentiation, ApoB 100, LDL receptor, HepG2

Introduction

The low rate of cardiovascular disease in France compared to other developed countries with a comparable intake of dietary saturated fat has been called the French paradox. Epidemiologic studies indicate that a moderate consumption of red wine lowers the risk of cardiovascular disease, with alcohol consumed as wine having a stronger inverse relationship with cardiovascular disease incidence than alcohol alone (1). The cardioprotective effect of red wine has been attributed to the polyphenols present in red wine. More recently, Pal et al. (2) elucidated that red wine reduced 50% of apolipoprotein B100 (ApoB 100), and increased 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase mRNA and LDL receptor-binding activities in HepG2 cells (2). However, in spite of the high correlation observed between lipid metabolism and cardiovascular disease-related metabolites, no report has yet been made on the effect of polyphenols of red wine on the adipocyte differentiation. Because phenolic compounds are also abundant in black raspberry (3, 4) and other plants (5, 6), this study was conducted to compare the abilities of red wine and Korean black raspberry (KBR) wine to induce the differentiation of 3T3 adipocytes and alter the expression of cardiovascular disease-related genes in HepG2 cells.

Materials and Methods

Cell culture and treatment 3T3-L1 fibroblasts (Cat. No. CL-173; American Type Culture Collection, Rockville, MD, USA) were cultured in 25-cm^3 flasks and induced to differentiate as described by Chen et al. (7). Briefly, the cells were placed in culture flasks and grown to confluence. They were then induced to differentiate into adipocytes in the presence of DMEM-F12 (Gibco BRL, Grand Island, NY, USA), 10% heat-inactivated fetal calf serum (FCS) and 5 μg/mL insulin with 390 ng/mL dexamethasone and 115 μg/mL 3-isobutyl-1-methyl-xanthine. Subsequently, the cells were differentiated in the treatment media, and the terminal differentiation occurred by day 10. The flasks were photographed on a phase-contrast microscope (Olympus 1X70-S8F2, Olympus Optical Co. Ltd, Tokyo, Japan) with magnification X100 at day 3 after induction. The treatment media were made by adding filter-sterilized 6 mg/L ethanol (control), 5 umol/L red wine (red wine; Premius Bordeaux Red® 2003, 12.5% alcohol; Yvonmau, Bordeaux, France), and 5 umol/L KBR (*Rubus coreanum* Miquel) wine (Bokbunjajoo® 2004, 15.0% alcohol; Bohae, Mokpo, Jeonnam, Korea) based on the gallic acid content of the wines. The HepG2 (Cat. No. HB-8069; American Type Culture Collection) cells were grown in 175-cm^3 flasks containing MEM (Gibco BRL), supplemented with 2 mg/L penicillin, 16 mg/L gentamycin, 1.5 g/L sodium bicarbonate, 110 mg/L sodium pyruvate, and 10% FCS. They were then subcultured from 175-cm^3 flasks into 25-cm^3 flasks and allowed to grow without disruption for 24 hr in MEM medium with FCS. The medium was replaced with fresh, prewarmed serum-free MEM medium, and the cells were incubated for additional 24 hr. Serum-free MEM was made by supplementing MEM with 80 g/L BSA (fatty acid free) complexed to 5 umol/L oleic acid (sodium salt), 22.2 mol/L glucose, 45.5 mol/L Na_2CO_3 and 1 mol/L sodium pyruvate as described by Pal et al. (2). The cells were then incubated with the treatment media for 24 hr.

RNA isolation and real-time reverse transcriptase-PCR (real-time RT-PCR) The treated cells were cultured in 25-cm^3 flasks, and total RNA was extracted using the acid/guanidium thiocyanate/phenol chloroform method (8). Real-time RT-PCR was used to measure the concentrations of mouse PPAR-γ2 (Accession No. Y 12882), C/EBP-α (Accession No. NM 007678), and LPL (Accession No. BC 003305) mRNA relative to that of 18S (Accession No. S 56974) mRNA of 3T3 L1 cells, and the concentrations

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of LDL receptor (Accession No NM 000527) and HMG-CoA reductase (Accession No M 62633) relative to that of cyclophilin (Accession No. Y 00052) mRNA of HepG2 cells. Measurement of the relative concentration of cDNA was conducted using a SYBER Green real-time RT-PCR Master Mix (Qiagen, Cambridge, MA, USA), with the appropriate forward and reverse primers (0.5 μM) (Tables 1 and 2), and 0.2 μg RNA. Assays were performed in a Rotor-Gene 2000 Real-Time Cycler using the appropriate analysis software (Corbett Research, Sydney, Australia) and the thermal cycling parameters recommended by the manufactures (40 cycles of 15 sec at 94°C and 30 sec at 55 °C). Titrations of all forward and reverse primers of genes (0.5 μM) against increasing concentration of cDNA gave linear responses with slopes of -0.24.

Western blot and total cholesterol analyses After the completion of treatments in 25-cm^3 flasks, the HepG2 cells were washed twice with PBS, solubilized in Laemmli sample buffer (9), and assayed for protein contents using a BCA protein assay kit (Pierce, Rockford, IL, USA). They were then subjected to SDS-polyacrylamide gel electrophoresis on a 12% gel gradient and transferred electrophoretically onto a nitrocellulose membrane. The membrane was blocked for 2 h in a blocking buffer [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.05% Tween 20, 1% polyvinylpyrrolidone, and 0.1% bovine serum albumin] and incubated overnight with polyclonal anti-human LDL (Chemicon International Inc., Temecula, CA, USA) (1:2,000 dilution in the incubating buffer) or ApoB 100 (Biodesign International, Saco, ME, USA) (1:2,000 dilution in the incubating buffer) at 4°C in an incubating buffer [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.05% Tween 20, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin]. After a series of washes, the membranes were incubated in the incubating buffer containing goat anti-human antibodies (1:2,000 dilution in the incubating buffer) conjugated with horseradish peroxide (Sigma-Aldrich Inc., Saint Louis, MO, USA), washed three times for 30 min, immersed in an enhanced chemiluminescence solution (WEST-ZOL, Intron Biotechonology) Inc., Seoul, Korea), and exposed in a dark box (LAS-1000 plus, Fuji Photo Film Co. Ltd., Tokyo, Japan) for 10 sec. The data were normalized by blotting the same membranes with mouse anti-β-actin (Sigma-Aldrich Inc.) (1:10,000 dilution in the incubating buffer) as a house-keeping gene using image reading and analyzing programs (Image Leader LAS-1000 PRO V 2.1 and Science Lab 98, Fuji Photo Film Co.). To measure total cholesterol concentration in HepG2 cells, 1 mL of 1 mol/L KOH in methanol was added to an aliquot of cells to saponify the cholesterol esters. The tubes were then flushed with N_2 and heated at 45°C for 1 hr. After the completion of hydrolysis, the solution was diluted with 2 mL water, and the lipids were extracted twice with hexane (1 mL). Total cholesterol concentration was determined using an enzymatic cholesterol assay kit (BC 180-E, YD-Diagnostic Inc., Seoul, Korea). The absorbance was read using a spectrophotometer (Smartspec 3000, Bio-Rad Laboratories, Hercules, CA, USA) at 500 nm.

Table 1. Forward and reverse primers for real-time PCR for mouse PPAR-γ2, C/EBP-α and LPL

Item	Primer
PPAR-γ2	
Forward	5'-TGAACGTGAAGCCCATCGAGGAC-3'
Reverse	5'-TCTGTCATCTTCTGGAGCACCTTGG-3'
C/EBP-α	
Forward	5'-GGTGCGCAAGAGCCGAGATAAAG-3'
Reverse	5'-AGTTCACGGCTCAGCTGTTCCAC-3'
LPL	
Forward	5'-ACAAGGTCAGAGCCAAGAGAAGCAG-3'
Reverse	5'-GTTGCTTGCCATTCTCAGTCCCAG-3'
18S	
Forward	5' GATCCATTGGAGGGCAAGTCTGG 3'
Reverse	5' TACCCACTGAGCCATCTCACCAGC 3'

Table 2. Forward and reverse primers for real-time PCR for Human LDL receptor and HMG-CoA reductase

Item	Primer
LDL receptor	
Forward	5'-CACAGCCGTAAGGACACAGCACAC-3'
Reverse	5'-GCCCAGAGCTTGGTGAGACATTC-3'
HMG-CoA reductase	
Forward	5'-GCCTGGGCCAGAGAAGATAATGTTC-3'
Reverse	5'-GCACAGTTCTAGGGCCATTCACG-3'
Cyclophilin	
Forward	5'-CAGGGTTTATGTGTCAGGGTGGTG-3'
Reverse	5'-AGATGCCAGGACCCGTATGCTTTAG-3'

Statistical analysis Values presented in the figures are expressed as means±SEM of at least three independent experiments. Differences between groups were analyzed by the Duncan's multiple range test using SAS (SAS Inst. Inc, Cary, NC, USA).

Results and Discussion

Adipocyte differentiation Expressions of LPL at day 10 after the induction were 52.4 and 99.1% of the control for red wine and KBR wine, respectively (Fig. 1). Red wine also reduced (30%, $p<0.05$) the expression of C/EBP-α

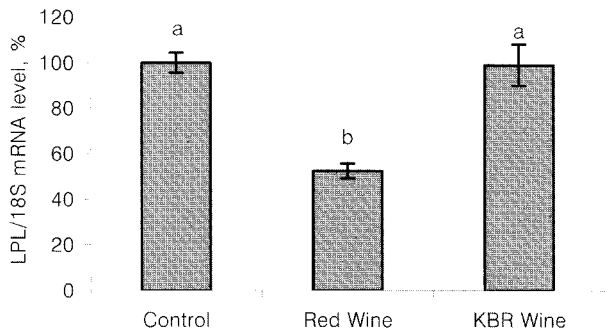

Fig. 1. The effects of red wine and Korean black raspberry (KBR) wine on LPL mRNA level in 3T3 L1 cells. The bar graph indicates the mean±SEM of three independent analyses. [a,b] Means above the columns followed by different letters differ significantly ($p<0.01$).

(Fig. 2). In addition, KBR and red wines reduced the expression of PPAR-γ2 to 20 and 54% ($p<0.05$) of the control (Fig. 3). Inhibition of PPAR-γ2 by both wines is consistent with the roles of these genes; PPAR-γ2 is a major adipogenic transcription factor expressed during the differentiation period, while LPL is one of the genes expressed by adipocytes during the terminal differentiation period (10). However, it is unclear why, in the case of KBR wine, inhibition of C/EBP-α was lower than that of LPL, given that both genes are major adipogenic transcription factors expressed during the entire differentiation period.

After treating 3T3 cells with 6 mg/L ethanol (control), red wine, and KBR wine for 3 days, numerous colonies of adipose cells were evident. The control showed many small fat vacuoles, while smaller populations of adipose cells were found in the two wine treatments, as observed by a phase-contrast microscope (Fig. 4).

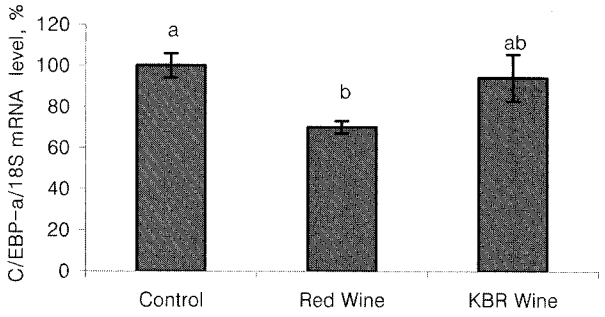

Fig. 2. The effects of red wine and Korean black raspberry (KBR) wine on C/EBP-α mRNA level in 3T3 L1 cells. The bar graph indicates the mean±SEM of three independent analyses. [a,b] Means above the columns followed by different letters differ significantly ($p<0.05$).

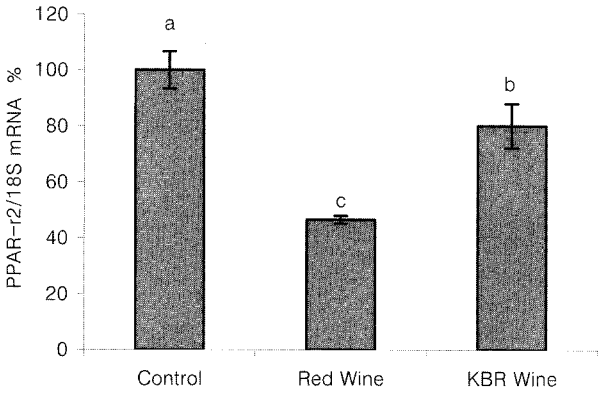

Fig. 3. The effects of red wine and Korean black raspberry (KBR) wine on PPAR-γ2 mRNA level in 3T3 L1 cells. The bar graph indicates the mean±SEM of three independent analyses. [a,b] Means above the columns followed by different letters differ significantly ($p<0.05$).

Cardiovascular disease related gene expression and cholesterol analyses KBR wine significantly ($p<0.05$) increased the level of HMG-CoA reductase mRNA compared to the control cells (Fig. 5). On the other hand, red wine treatment had no effect on the expression of HMG-CoA reductase. In addition, both wines had no effect on the mRNA expression and the number of proteins in the LDL receptors (Figs. 6 and 7). One aim of the present study was to determine whether KBR wine reduces the expression of apoB 100 in HepG2 cells, as observed in red wine (2). When the cells were incubated with red wine and KBR wine, apoB 100 productions were suppressed by 12 and 15%, respectively, compared to the control cells (Fig. 8). Both red wine and KBR wine significantly decreased ($p<0.05$) the cholesterol availability, which regulates VLDL synthesis and secretion (11), in the cells (Fig. 9). In general, a decrease in the intracellular cholesterol level triggers the cell to upregulate the rate-limiting enzyme in the cholesterol biosynthesis, HMG-CoA reductase, as well as LDL receptor gene exression to increase the cholesterol concentration in the cell (12). Collectively, our data suggest that KBR wine has a significant ($p<0.05$) effect on decreasing the availability of cholesterol to these cells, which resulted in a suppression of apoB 100, and significantly increasing the expression of

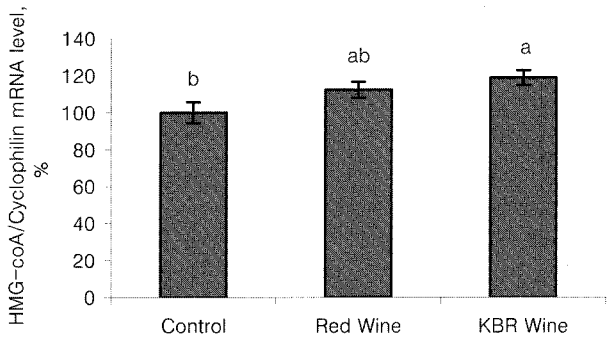

Fig. 5. The effects of red wine and Korean black raspberry (KBR) wine on HMG-CoA reductase mRNA level in HepG2 cells. The bar graph indicates the mean±SEM of three independent analyses. [a,b] Means above the columns followed by different letters differ significantly ($p<0.05$).

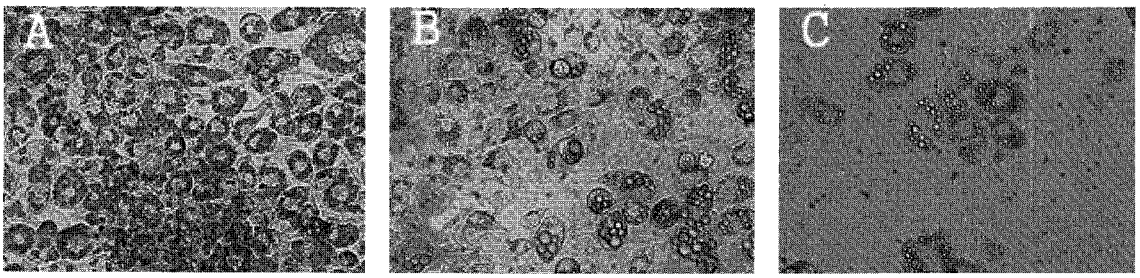

Fig. 4. Phase-contrast micrograph of differentiated 3T3 L1 adipocytes obtained by treatment of control (A), red wine (B) and Korean black raspberry (KBR) wine (C). Magnification X100.

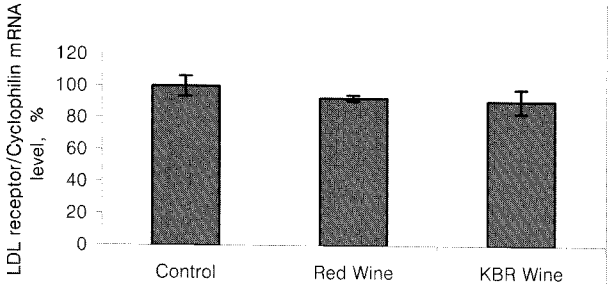

Fig. 6. The effects of red wine and Korean black raspberry (KBR) wine on LDL receptor mRNA level in HepG2 cells.

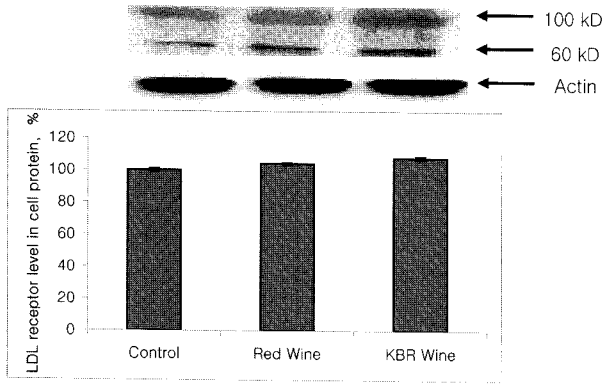

Fig. 7. The effects of red wine and Korean black raspberry (KBR) wine on LDL receptor level in cell protein of HepG2 cells.

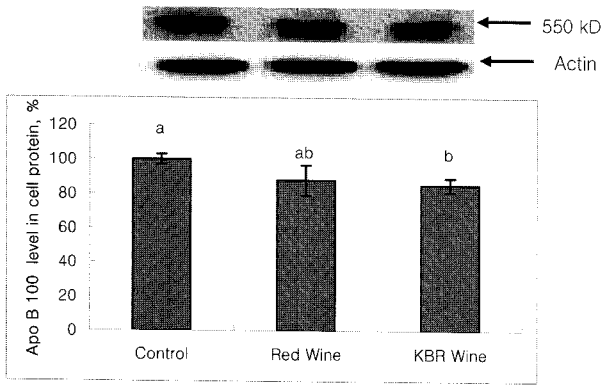

Fig. 8. The effects of red wine and Korean black raspberry (KBR) wine on ApoB 100 level in cell protein of HepG2 cells. The bar graph indicates the mean±SEM of three independent analyses. [a,b] Means above the columns followed by different letters differ significantly ($p<0.05$).

HMG-CoA reductase. However, the inverse relationship between the expression of LDL receptor mRNA and protein abundance in HepG2 cells after the wine treatments has yet to be elucidated.

Conclusions

Our present findings suggest that both red wine and KBR wine reduce the differentiation of 3T3 L1 adipocytes via down-regulation of the major adipogenic transcription factor and adipocyte-expressed gene such as PPAR-γ2. Furthermore, KBR wine also significantly ($p<0.05$) decreased the cholesterol availability in HepG2 cells, which resulted in a suppression of apoB 100 and a significant increase in the expression of HMG-CoA reductase.

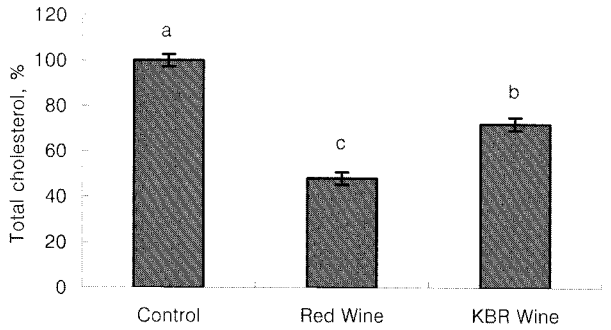

Fig. 9. The effects of red wine and Korean black raspberry (KBR) wine on total cholesterol concentrations in HepG2 cells. The bar graph indicates the mean±SEM of three independent analyses. [a,b,c] Means above the columns followed by different letters differ significantly ($p<0.05$).

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