

Caffeine attenuates lipid accumulation via activation of AMP-activated protein kinase signaling pathway in HepG2 cells

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The main purpose of this study is to examine the effect of caffeine on lipid accumulation in human hepatoma HepG2 cells. Significant decreases in the accumulation of hepatic lipids, such as triglyceride (TG), and cholesterol were observed when HepG2 cells were treated with caffeine as indicated. Caffeine decreased the mRNA level of lipogenesis-associated genes (SREBP1c, SREBP2, FAS, SCD1, HMGCR and LDLR). In contrast, mRNA level of CD36, which is responsible for lipid uptake and catabolism, was increased. Next, the effect of caffeine on AMP-activated protein kinase (AMPK) signaling pathway was examined. Phosphorylation of AMPK and acetyl-CoA carboxylase were evidently increased when the cells were treated with caffeine as indicated for 24 h. These effects were all reversed in the presence of compound C, an AMPK inhibitor. In summary, these data indicate that caffeine effectively depleted TG and cholesterol levels by inhibition of lipogenesis and stimulation of lipolysis through modulating AMPK-SREBP signaling pathways. [BMB Reports 2013; 46(4): 207-212]

INTRODUCTION

The liver plays an integral role in the coordination of fuel homeostasis since it is the major site for storage and release of glucose and lipid. Accumulations of lipid within liver are closely linked with the development of insulin resistance and non-alcoholic fatty liver disease and are subject to nutritional influence (1). AMP-activated protein kinase (AMPK) belongs to the serine/threonine kinase family and is a key enzyme that regulates glucose and lipid metabolism in the liver. AMPK is classically activated by an increase in the intracellular AMP : ATP ra-

tio, and phosphorylates a wide range of downstream targets, having the predominant effect of stimulating energy-producing pathways (fatty acid oxidation and glucose utilization), and inhibiting energy-consuming pathways (lipogenesis and gluconeogenesis) (2). In the liver, activation of AMPK phosphorylates and inactivates the rate-limiting enzymes of lipogenesis, such as acetyl-CoA carboxylase (ACC) (3). It is well documented that AMPK phosphorylation inhibits sterol regulatory element-binding protein-1 (SREBP-1), the key transcription factor responsible for fatty acid synthesis (4). Conversely, repressed AMPK activates anabolic pathways and inhibits catabolic pathways. In studies performed with hepatocytes and in the livers of ethanol-fed mice, You *et al.* demonstrated that the inhibition of AMPK leads to the activation of SREBP-1 mediated lipogenesis (5). AMPK positively regulated fatty acid oxidation by activating peroxisome proliferator-activated receptor- α (PPAR α) and PPAR γ coactivator (PGC)-1 (6). Thus, activation of AMPK by chemicals may contribute to the inhibition of intracellular accumulation of lipids.

Caffeine, a major ingredient found in a number of the most widely consumed non-alcoholic beverages worldwide (7, 8), is highlighted as a potent dietary-component associated with reduced risk of several chronic disease, including type 2 diabetes and its complication (9, 10). Several substances other than caffeine, e.g. chlorogenic acid and magnesium, have been suggested as being responsible for the protective effect of coffee in reducing the risk of type 2 diabetes (11). Although the inhibitory effects of caffeine alone or combination with other chemicals on intracellular lipid accumulation were reported in 3T3-L1 adipocytes (12) and in HepG2 cells (13), respectively, the effects of caffeine on triglyceride and cholesterol levels in liver cells have not been investigated yet. Here, we examined whether caffeine affects lipid metabolism via modulation of AMPK-SREBP signaling pathways in human hepatoma HepG2 cells.

RESULTS

Effects of caffeine on lipid levels and transcription factors in hepatocytes

To examine the anti-hyperlipidemic effects of caffeine, its ef-

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fect on lipid contents in human hepatoma HepG2 cells was investigated. HepG2 cells were treated with caffeine for up to 24 h. Caffeine did not show any cellular toxicity up to 8 mM, but significant cytotoxicity was shown at > 25 mM (data not shown). Triglyceride and cholesterol levels were significantly decreased by caffeine in concentration-dependent manners. Compared to the basal level, triglyceride and cholesterol levels were decreased by 40% and 35%, respectively, upon treatment of cells with 4 mM caffeine (Fig. 1A and B). ER-bound SREBPs regulate lipid homeostasis by controlling the expression of genes required for cholesterol, triglyceride and phospholipid (14). Enzymatic triglyceride and cholesterol syntheses in the liver have been known to be under the control of SREBP1c and SREBP2 transcription factors, respectively. Therefore, we investigated the effects of caffeine on the gene expression of transcription factors responsible for lipid biosynthesis. As shown in Fig. 1C-E, caffeine markedly attenuated gene expression of SREBP1c in both time- and concentration-dependent manners. Lipogenic genes such as fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1), well known target enzymes of SREBP1c, were also marginally suppressed in time- and concentration-dependent manners. In contrast, gene expression of CD36, responsible for inducing

lipid uptake and catabolism, was increased in a concentration- and time-dependent manner. Next, the effect of caffeine on gene expression of SREBP2 was examined. Caffeine also suppressed gene expression of SREBP2 in a time- and concentration-dependent manner (Fig. 1F-H). In addition, genes for hydroxymethyl glutaryl CoA reductase (HMGR) and low density lipoprotein receptor (LDLR), known target molecules of SREBP2, were both suppressed in time- and concentration-dependent manners. HMGR and LDLR are associated with the formation of cholesterol precursors and cholesterol uptake into cell, respectively. These results suggest that caffeine attenuates TG and cholesterol storage through the regulation of gene expression responsible for lipid metabolism in liver cells.

Effects of caffeine on protein expression of AMPK, upstream kinase for SREBPs

Next, we investigated whether phosphorylation of AMPK is induced by caffeine since AMPK plays a key role in regulating carbohydrate and fat metabolism in the liver. According to studies of the fatty liver mice model, it is known that a high-fat diet reduces the basal activity of AMPK, suggesting that a certain level of AMPK basal activity may be necessary to prevent fat accumulation (15). Of further relevance is the role of AMPK

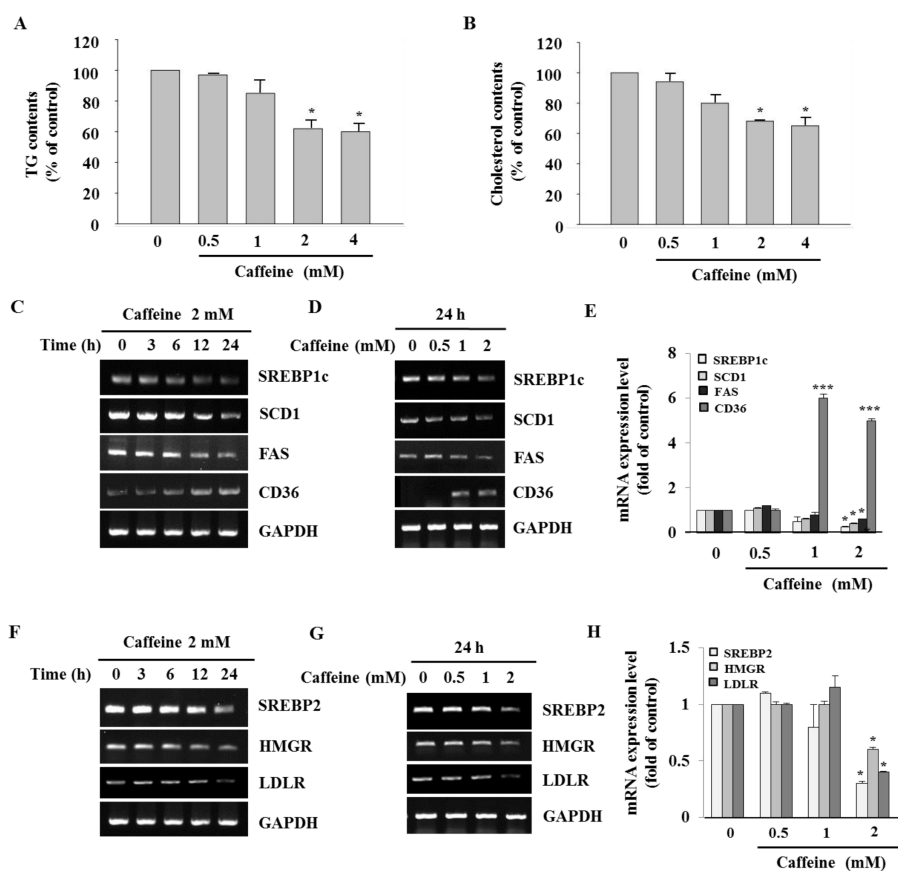


Fig. 1. Effects of caffeine on lipid accumulation and lipogenic or lipolytic gene expressions in HepG2 cells. HepG2 cells were treated with different concentrations (0.5-4 mM) of caffeine for 24 h. Triglyceride (TG) and cholesterol levels were measured (A, B). Each bar represents the mean \pm SEM of triplicate determinations. * $P < 0.05$ compared to control (0 concentration in this figure). Cells were treated with 2 mM caffeine for the indicated times (C, F) or treated with different concentrations of caffeine for 24 h (D, G). (E and H) Bar graphs are densitometric determinants for panels (D and G) respectively. * $P < 0.05$, *** $P < 0.001$ compared to control.

in reducing cholesterol synthesis, which occurs by the direct inhibition of HMGCR, a rate-limiting enzyme in hepatic cholesterol biosynthesis (16, 17). As shown in Fig. 2, caffeine significantly induced AMPK and ACC phosphorylation in time- and concentration-dependent manners. These effects were reversed in the presence of compound C, a selective AMPK inhibitor (Fig. 3A and B). In addition, the caffeine induced-gene suppressions of SREBPs and their target molecules were also reversed by treatment with compound C (Fig. 3C and D). Caffeine-induced suppressions of TG and cholesterol levels in liver cells were also blunted when the cells were pretreated with compound C (Fig. 3E and F). These results strongly suggest that caffeine inhibits hepatic triglyceride and cholesterol levels via modulation of AMPK-SREBP signaling pathways.

DISCUSSION

Coffee is a complex mixture of more than a thousand substances, including caffeine (primary source), phenolic compounds (chlorogenic acid and quinides -primary source), minerals and vitamins (magnesium, potassium manganese, chromium, niacin), and fiber (18), and several of these coffee constituent have a possible role in lipid and glucose metabolism. Recently, Nakabayashi *et al.* reported that caffeine reduces the accumulation of lipid in murine 3T3-L1 adipocytes (12). In addition, Murosaki *et al.* reported that a combination of caffeine, arginine, soy isoflavones, and L-carnitine enhances both lipolysis and fatty acid oxidation in 3T3-L1 and HepG2 cell, and in KK mice (13). However, the specific lipid lowering effect of caffeine in the liver cell has not yet been reported. Here, we examined whether caffeine affects lipid metabolism in human hepatoma HepG2 cells. Fatty acids in the liver are esterified into triglyceride or oxidized to produce energy. Stores in the form of triglyceride are inert and harmless to the cells.

However, an excess of triglyceride reduces insulin sensitivity (19) and is hydrolyzed back to fatty acyl-CoA at a rate that exceeds the oxidative needs of the cells, resulting in the un-oxidized excess being subject to more harmful pathways of fatty acid metabolism, such as *de novo* ceramide formation (20).

SREBPs are known as transcription factors that are conserved from fission yeast to man, and regulate the expression of genes required to maintain cellular lipid homeostasis. In mammals there are two SREBP genes, SREBP1 and 2. Most data suggest that the two SREBP1a and 1c primarily regulate fatty acid metabolism, and that SREBP2 is the main regulator of cholesterol metabolism (21). SREBP1c is the predominant isoform in most adult non-dividing metabolic tissues, such as liver and adipose. As an inhibitor of triglyceride and cholesterol accumulation in the liver cell (Fig. 1A and B), the effects of caffeine on gene expression of SREBP1c and 2 were examined in HepG2 cells. The expressions of both SREBPs and their target molecules were significantly suppressed or enhanced by caffeine (Fig. 1C-H). These data could result from alterations in the synthesis and/or uptake of fatty acids. It is well documented that AMPK phosphorylation inhibits SREBP-1 through the mammalian target of rapamycin (mTOR) and liver X receptor- α (LXR α) (4). Regulation of hepatic SREBPs *in vivo* is largely dependent on nutritional status. Under conditions of fasting, the activation of AMPK reduces lipogenesis in the liver by suppressing SREBPs activity. Conversely, activation of LXR increases SREBP expression under insulin-stimulated conditions and leads to hepatic lipogenesis. Thus, identifying pharmacological agents that inhibit the activity of LXR or stimulate AMPK activity in hepatocytes may provide effective treatment options for fatty liver disease. The effect of caffeine on phosphorylation of AMPK and ACC was examined. AMPK inhibits the accumulation of fat by modulating downstream-signaling components like ACC. ACC is a rate-controlling enzyme for the synthesis of malonyl-CoA,

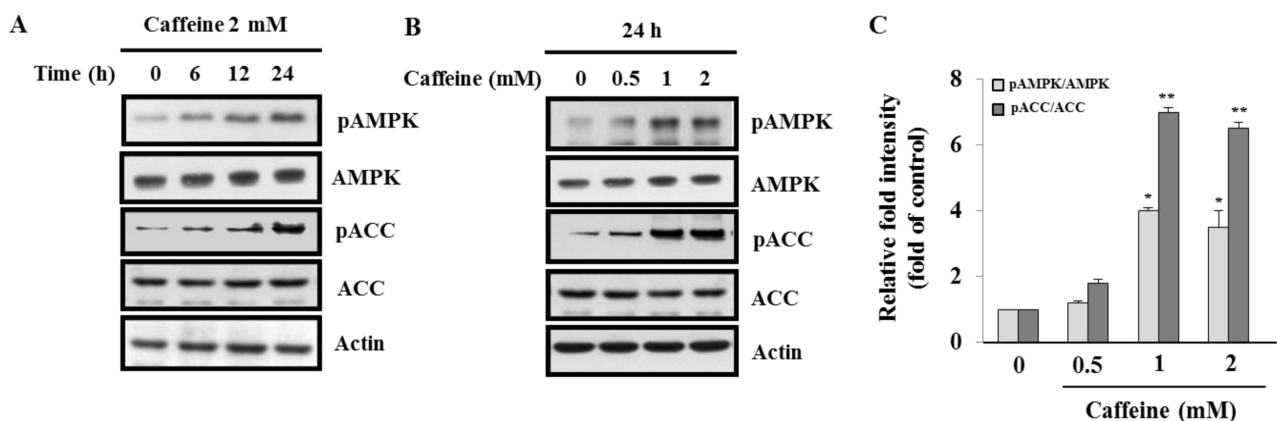


Fig. 2. Effects of caffeine on protein expressions of AMPK and ACC in HepG2 cells. HepG2 cells were treated with 2 mM caffeine for different times (A), or were treated with caffeine at the indicated concentrations for 24 h (B). After immunoblotting, total and phospho-protein levels of AMPK and ACC were identified by their specific antibodies. Actin was used as an internal control to evaluate the relative expression of protein. Bar graph (C) represents the densitometric determinant for panel (B). *P < 0.05, **P < 0.01 compared to control.

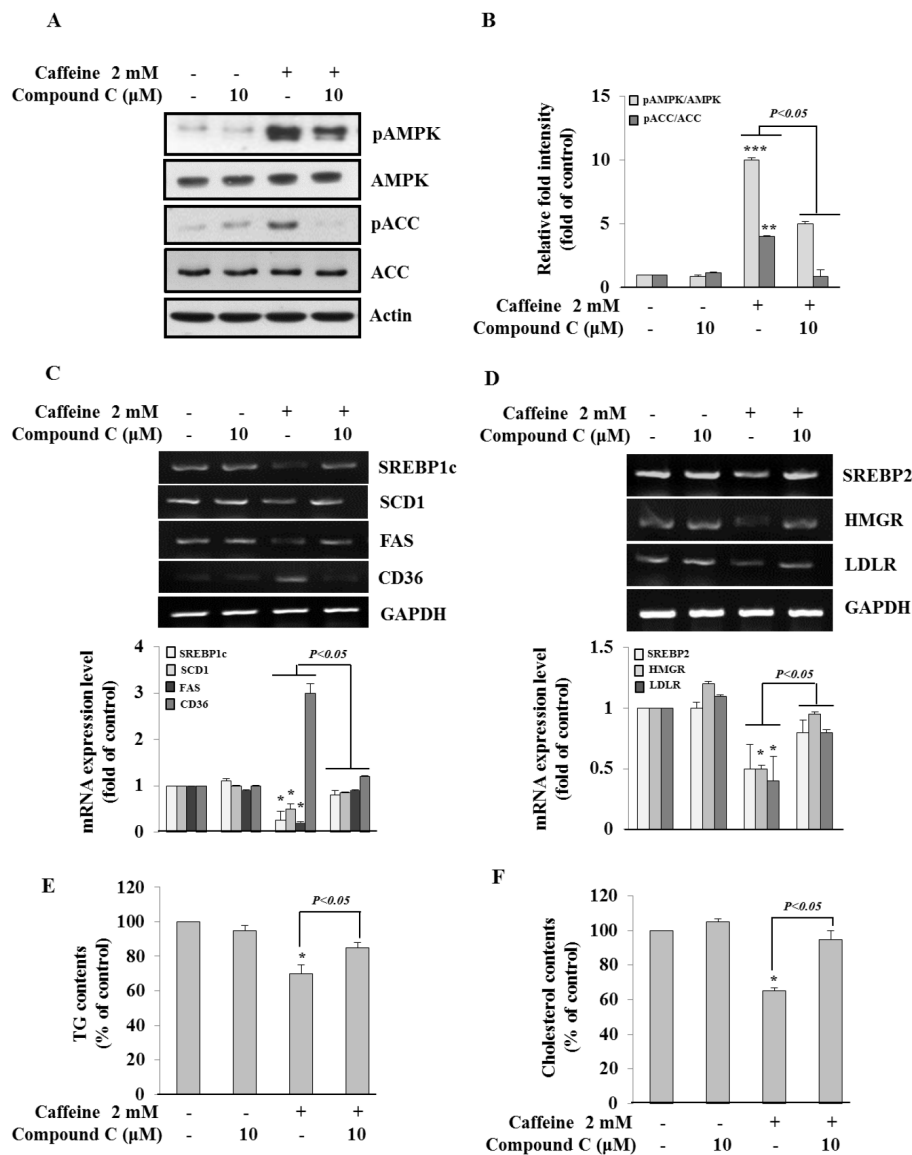


Fig. 3. Effects of compound C on AMPK protein, lipogenic and lipolytic gene expression in HepG2 cells. HepG2 cells were pretreated for 2 h with compound C, and were then treated with 2 mM caffeine for 24 h. The phosphorylation levels of AMPK and ACC were analyzed by immunoblotting using the corresponding antibodies (A), and densitometric analysis (B), and lipogenic or lipolytic genes were subjected to RT-PCR (C, D) as described in Materials and Methods. Hepatic TG and cholesterol contents were determined in the absence and presence of 10 μM compound C (E and F). *P < 0.05, **P < 0.01, ***P < 0.001 compared to control.

which is a critical precursor in the biosynthesis of fatty acids and a potent inhibitor of mitochondrial fatty acid oxidation (22, 23). Activation and inhibition of AMPK and ACC activities were experimentally proven by enhancement of phosphorylated forms for both proteins, and these results were confirmed through the presence of the AMPK inhibitor, compound C (Fig. 3). We have not yet determined the mechanism through which caffeine activates the AMPK signaling pathway in HepG2 cells. The activation of AMPK by caffeine either directly or indirectly through modulation of the AMP : ATP ratio in mitochondria is a legitimate possibility, and deserves further investigation.

In conclusion, caffeine, a major component of coffee, plays a significant role in reducing hepatic lipid accumulation by mod-

ulating AMPK-SREBP signaling pathways. These results broaden our understanding of how caffeine shows anti-hyperlipidemic activity in the liver, and caffeine itself or caffeine-containing beverages could represent a promising dietary supplement to prevent fatty liver disease and hypercholesterolemia.

MATERIALS AND METHODS

Chemicals

Caffeine was purchased from Sigma (St. Louis, MO, USA) and triglyceride and cholesterol measuring kits were from ASAN Pharmaceutical (Seoul, Korea). Antibodies against AMPK, phospho-AMPK, ACC, phospho-ACC were from Cell Signaling

Technology (Beverly, MA, USA) and anti-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Reverse transcriptase and Taq polymerase were supplied by Promega (Madison, WI, USA), and compound C was from Calbiochem (Darmstadt, Germany). Protein extraction, EASY-BLUE total RNA extraction and ECL-reagent kits were from Intron Biotechnology Inc. (Beverly, MA, USA) and the protein assay kit was from Bio-Rad (Hercules, CA, USA). Other reagents and chemicals were of analytical grade.

Cell culture and viability assay

Human hepatoma HepG2 cell line was purchased from Korean Cell Line Bank (Seoul, Korea). HepG2 cells were grown in DMEM (GibcoBL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and antibiotics (100 unit/ml penicillin and 100 µg/ml streptomycin). Cells were maintained at sub-confluent conditions in a humidified incubator at 37°C, with ambient oxygen and 5% CO₂. For the cytotoxicity test, HepG2 cells were cultured in 96-well culture plates, and were treated with the indicated concentrations of caffeine for 24 h. The cytotoxicity of caffeine was determined by CellTiter 96 Aqueous One solution Cell Proliferation Assay kit (Promega, Madison, WI, USA).

Determination of TG and cholesterol

TG and cholesterol levels were determined in cell lysates using a colorimetric assay, and were expressed as µg of lipid per mg of cellular protein. The levels of TG and cholesterol in cell lysates were measured according to the instructions of the manufacturer's of InfinityTM TG/cholesterol reagents.

Western blot

Cells were washed with ice-cold phosphate buffered saline (PBS) and were lysed in a protein extraction kit. Insoluble protein was removed by centrifugation at 15,000 rpm for 20 min and soluble protein concentrations were measured using a Bio-Rad protein assay kit. Equal amounts of protein (40 µg/lane) were resolved by 8% SDS-polyacrylamide gel electrophoreses (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Massachusetts, MA, USA), and hybridized with primary antibodies (diluted 1 : 1,000) overnight at 4°C. After incubation with a 1 : 2,000 dilution of horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature, protein bands were detected using an enhanced chemiluminescence Western blot detection kit (Amersham, Uppsala, Sweden), exposed to X-ray film, and then quantified by densitometric analysis.

RNA extraction and RT-PCR

Total RNAs from HepG2 cells were prepared by using EASY-BLUE total RNA extraction kit. mRNAs were reversely transcribed into cDNA, and were amplified with PCR and normalized to the endogenous GAPDH. The primers used in this experiment were as follows: SREBP1c sense 5'-GTGGCGG

CTGCATTGAGAGTGAAG-, antisense 5'-AGGTACCCGAGGG CATCCGAGAAT-3'; SREBP2 sense 5'-CGCCACCTGCCCT CTCCTTCC-3', antisense 5'-TGCCCTGCCACCTATCCTCTCAC G-3'; FAS sense 5'-CAAGAAGTGCACGGAGGTGT-3', antisense 5'-AGCTGCCAGAGTCGGAGAAC-3'; SCD1 sense 5'-TTGCCAGCTCTAGCCTTTAAATTC-3', antisense 5'-TCCTG GTAGCATTATTCAGTAGTT-3'; CD36 sense 5'-GGGCTATA GGGATCCATTTTTG-3', antisense 5'-CCTTTCAGATTAACGT CCGATTC-3'; HMGR sense 5'-TACCATGTCAGGGGTACGT C-3', antisense 5'-CAAGCCTAGAGACATAATCATC-3'; LDLR sense 5'-CCCCGAGATCAAACCCACC-3', antisense 5'-AG ACCCCCAGGCAAAGGAGACGA-3'; GAPDH sense 5'-TCCA CCACCTGTGCTGTA-3', antisense 5'-ACCACAGTCCATGC CATCAC-3'. PCR was performed at 95°C for 30 sec, followed by 50°C (FAS, HMGR), 52°C (GAPDH), 56°C (SCD1, CD36, LDLR, SREBP2) or 64°C (SREBP1c) for 30 sec, and 72°C for 1 min. The last cycle was followed by a final extension step at 72°C for 10 min. The RT-PCR products were electrophoresed in 0.8% agarose gels under 100 V, and were stained with 0.5 µg/ml ethidium bromide. Scanning densitometry was performed with an i-MAXTM Gel Image Analysis System (Core Bio, Seoul, Korea).

Statistical analysis

All data were expressed as a mean values ± standard error (S.E.) and differences between groups were analyzed using Student's t-test. Mean values were considered significantly different when P < 0.05.

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