Short communication: Hypolipidemic and antiinflammatory effects of fermented Maillard reaction products by *Lactobacillus fermentum* H9 in an animal model

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ABSTRACT

This study examined the effects of Maillard reaction products reacted by casein and lactose (cMRP) and of cMRP fermented by *Lactobacillus fermentum* H9 (F-cMRP) on hypolipidemic and antiinflammatory effects in rats fed a high-fat and high-cholesterol diet (HD). The HD-fed rats had significantly increased hepatic triglyceride concentrations compared with the rats fed a normal diet. It was shown that treatment with simvastatin, *L. fermentum* H9 (H9), cMRP, and F-cMRP decreased total triglycerides in the liver compared with the HD group. On histological analysis, a reduction of lipid accumulation in the liver and aortic tissues was observed in the cMRP, F-cMRP, and H9-fed rats. Also, F-cMRP and cMRP reduced intima-media thickness in the HD group. In addition, the H9, cMRP, and F-cMRP treatments significantly reduced the expression levels of *ICAM-1* and *VCAM-1*, but not of *MCP-1*. In particular, the expressions of *ICAM-1* and *VCAM-1* were significantly decreased in the F-cMRP group compared with the HD group. These results of the present study suggest that cMRP and F-cMRP in dairy foods could potentially be used to prevent or treat cardiovascular diseases, especially atherosclerosis.

Key words: hypolipidemic and antiinflammatory effect, Maillard reaction product, *Lactobacillus fermentum*, reverse transcription-PCR, rat animal model

Short Communication

Cardiovascular disease (CVD) is the leading cause of global mortality and morbidity. Hypercholesterolemia has been established as one of the major risk

factors contributing to CVD, and the Framingham study reported that a 1% increase of plasma cholesterol is equivalent to a 2% elevation of coronary heart disease incidence (Jeon et al., 2007). Increased serum low-density lipoprotein cholesterol levels and decreased high-density lipoprotein cholesterol levels are the most important factors involved in CVD, especially coronary atherosclerosis, leading to inflammation and reduction of endothelial function and vascular lesions (Ross, 1993). Hyperlipidemia is a chronic metabolic disorder characterized by abnormalities in lipid and lipoprotein metabolism, which can cause many complications, such as atherosclerosis and hypertension (Lattanzio and Petrella, 2000). Several reports have studied the effect of fermented compounds on hyperlipidemia and inflammation, such as fermented mushroom milk for hyperlipidemia and fermented milk/soymilk for atherosclerosis (Jeon et al., 2004; Tsai et al., 2009).

The Maillard reaction, a chemical reaction between amino groups and reducing sugars, is significant for foods because it strongly affects quality (Van Boekel, 1998). Maillard reaction products (MRP) are naturally produced in food during thermal processing via chemical reduction (Hwang et al., 2011). In the case of milk, amino groups are mainly lysine residues in milk proteins (Walstra and Jenness, 1984). Recently, several studies have focused on the physiological effects of MRP derived from milk proteins and sugars such as antilipid peroxidation, antioxidative action, and antimicrobial action (Rufián-Henares and Morales, 2007; Gu et al., 2009).

Interest has been growing in the use of dairy hydrolysates containing bioactive peptides as agents for maintaining general health and preventing chronic human disease. Bioactive peptides from milk proteins can be released by enzymatic proteolysis, gastrointestinal digestion, or food processing. These peptides possess a wide range of properties, including antimicrobial action, antihypertensive action, antithrombotic action, immunomodulatory properties, and opioid properties, in ad-

Received April 7, 2016.

Accepted July 21, 2016.

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dition to aiding the absorption of the mineral calcium (LeBlanc et al., 2002). Various probiotic strains possess proteolytic activity and milk protein hydrolysate via probiotic fermentation exhibited various biological functions. It has been shown that milk fermented by *Lactobacillus helveticus* R389, which has strong protease and peptidase activities, as compared with other lactic acid bacteria (Moineau and Goulet, 1991), is capable of exerting an antimutagenic effect whereas its protease-deficient derivative does not exert this effect (Matar et al., 1997). Other studies have shown that a proteinase from *L. helveticus* CP790 was able to release an antihypertensive peptide from casein hydrolysates (Yamamoto et al., 1994). Although the enzymatic and microbial hydrolysis of milk proteins have been thoroughly investigated (Ha et al., 2015), particularly various cardiovascular protective effects of milk-derived peptides, hydrolysis of MRP has not been well studied. Our group reported that the biological characteristics and antioxidant activity of milk proteins were improved by the combination of the Maillard reaction and enzymatic hydrolysis (with commercial proteases Alcalase, Neutrase, Protamex, and Flavorzyme; Oh et al., 2013). Furthermore, in previous in vitro and in vivo studies, we demonstrated that MRP produced from milk proteins and MRP fermented by *Lactobacillus gasseri* H10 and *L. fermentum* H9 the antithrombotic effects and anticardiovascular activities (Oh et al., 2014, 2015). Based on the results of our previous study, the aim of this study was to examine and verify the effects of MRP and fermented MRP on cardiovascular inflammation parameters, especially atherosclerosis, in a rat model.

The MRP formed from the reaction of casein with lactose (**cMRP**) and the product formed by fermentation of cMRP by *Lactobacillus fermentum* H9 (**F-cMRP**) were prepared in accordance with previous reports by Oh et al. (2015). Briefly, cMRP was prepared by incubating sodium caseinate and lactose at 55°C in a shaking water bath for a day, and then by extensively dialyzing and lyophilizing the reaction mixtures. Fermentation of cMRP was performed in an MRP medium containing 3% cMRP, 2% glucose, various minerals, and a small quantity of nitrogen sources such as yeast extract and peptone proteins at 37°C for 48 h. After fermentation, the supernatant of the MRP medium was freeze-dried.

A total of 30 male Sprague-Dawley rats [Sam: TacN(SD)BR, Samtako, Kyunggi, Korea] were obtained at the age of 5 wk (initially weighing 150 g). The animals were housed individually in mesh-bottom stainless-steel cages in a room with controlled temperature (23 ± 2°C), and a cycle of 12 h of light and 12 h of dark. The initial average animal BW did not differ

among the 6 groups. The rats always had free access to water. After 3 d of adaptation period, rats were divided into 2 groups: 5 rats fed normal diet (**ND**) and 25 rats fed a high fat, high cholesterol diet (**HD**). After 2 wk of normalization period, the HD group was once more divided into 5 groups for different feedings: group 1 received only the HD; group 2 received HD plus simvastatin (**SV**), which is widely used for treatment of vascular disease, as a positive control; group 3 received HD plus *L. fermentum* H9 (**H9**); group 4 received HD plus MRP reacted by casein and lactose (**cMRP**); and group 5 received HD plus cMRP fermented by *L. fermentum* H9 (**F-cMRP**). A total of 5 treatment groups were fed the following experimental diets for 6 wk after normalization. The HD contained 20% (wt/wt) fat and 0.5% (wt/wt) cholesterol, and diets of group 2, 3, 4, and 5 contained simvastatin 30 mg/kg per day, freeze-dried cells 1.4×10^8 cfu/d, cMRP 15,000 mg/kg per day, and F-cMRP 15,000 mg/kg per day. Their feed intake and BW were recorded weekly. All procedures were approved by the Institutional Animal Care and Use Committees of Chonbuk National University (accession number 2015–5).

For quantification of triglycerides (**TG**) in liver tissue, 350 to 400 mg of liver tissue were homogenized with 2 mL of lysis buffer (1% NP-40, 50 mmol/L Tris-base, 0.1% SDS, 0.5% deoxycholic acid, 150 mmol/L NaCl, pH 7.5) supplemented with protease inhibitor. All mixtures were centrifuged at $10,360 \times g$ for 10 min at 4°C. Assay was performed with TG colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Aorta tissues were dissected and immediately placed on ice in RNAlater (Qiagen, Mississauga, ON, Canada) and stored at –20°C until RNA extractions were performed. Total RNA was isolated with a GeneJET RNA Purification Kit (Fermentas, Burlington, ON, Canada) following the manufacturers' protocol. Final RNA concentrations were determined by optical density reading at 260 nm using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Subsequently, cDNA was synthesized from 2 µg of total RNA with cDNA synthesis (LeGene Biosciences, San Diego, CA) carried out at 45°C for 50 min following incubation at 85°C for 5 min. Primer sets used for reverse-transcription-PCR are shown in Table 1. The PCR products were resolved by electrophoresis on 1.5% agarose gels, visualized by ethidium bromide staining. The intensity of each band was determined by densitometric analysis of gels using a Kodak DC290 zoom digital camera and Kodak 1D image analysis software (Eastman Kodak Company, Rochester, NY) and ImageJ software (NIH Image, Bethesda, MD). The mRNA expression levels of each gene were normalized to that of the housekeeping gene (β -actin).

A histological examination was performed on the samples of the liver and aorta from each animal at the end of the study. The samples were fixed in 10% buffered formalin, dehydrated in ethanol, and then embedded in paraffin. Sections (2 μ m thick) of paraffin-embedded tissue were then prepared and stained with hematoxylin and eosin. After fixation, tissues were infiltrated with a 20% sucrose solution overnight at 4°C. Cryosections on glass slides were fixed with 10% (vol/vol) formaldehyde in PBS for 1 h at 23°C, rinsed twice with water, and then stained with 0.1% (wt/vol) Oil Red O in 75% (vol/vol) isopropanol at 23°C. After 2 h, the stained cryosections on glass slides were rinsed twice with water to remove unincorporated dye. The slides were examined with an Olympus CH30 microscope (Olympus Corp., Tokyo, Japan), and digital images were recorded. In all experiments, data were analyzed statistically with SPSS Inc. software (version 12.0, SPSS Inc., Chicago, IL) and differences between groups were assessed for statistical significance ($P < 0.05$) using a one-way ANOVA followed by Duncan's test.

The concentrations of hepatic TG in the control and experimental rats are shown in Figure 1. The HD-fed rats had significantly increased concentrations of hepatic TG as compared with the ND-fed rats. It was shown that treatment with SV, H9, cMRP, and F-cMRP significantly decreased total TG levels (121.1 ± 11.4 , 133.6 ± 12.4 , 98.4 ± 15.7 , and 87.7 ± 10.7 mg/g of tissue, respectively) in comparison to the HD group (177.8 ± 29.6 mg/g of tissue). Among the treatment groups, F-cMRP especially significantly decreased the concentration of hepatic TG by 50% in comparison to the HD group, whereas the HD-fed rats were observed to have 190% increased total TG levels compared with the ND group (Figure 1A). This result coincided with the findings of Kobayashi et al. (2012), which determined that plasma TG levels were continuously de-

creased from 1 to 5 wk of administration of fermented soymilk. Decreased plasma TG levels contributed to the reduction of liver weight and the decrease of hepatic cholesterol and TG levels. Therefore, treatment with F-cMRP was expected to prevent liver damage caused by lipid accumulation.

Next, livers from rats fed 6 different diets were analyzed histologically, and stained sections were analyzed by light microscopy. The effect of H9, cMRP, and F-cMRP on the protection of the liver from the progression of lipid accumulation and hepatic steatosis was further verified by hematoxylin and eosin (H&E) stains. As shown in Figure 1B, the HD group's liver tissues demonstrated obvious fat depositions, in which the size and the number of lipid droplets were increased, compared with the ND group. In addition, it seems that the liver tissues of the HD group exhibited massive fatty changes, ballooning degeneration, and loss of cellular boundaries. Treatment with H9, cMRP, and F-cMRP could mitigate these defective cellular phenomena, which had similar results in the SV group. Remarkable reduction of lipid droplets and lipid accumulation were observed, which means that an obvious restriction occurred of fat accumulation in the section of liver tissue after treatment with H9, cMRP, and F-cMRP. Consequently, the livers of the rats in all treated groups showed noticeable recovery from high-fat and high-cholesterol-induced liver damage when compared with the untreated group, which is in agreement with a previous study (Chen et al., 2012).

In addition, the transcriptional levels of inflammation-related genes in the aorta were determined using reverse transcription (RT)-PCR analysis. The HD group showed upregulated transcriptional levels of endothelial intercellular adhesion molecule-1 (*ICAM-1*), vascular cell adhesion molecule-1 (*VCAM-1*), and monocyte chemotactic protein-1 (*MCP-1*) compared with the ND group, whereas treatment with H9, cMRP,

Table 1. Reverse transcription-PCR primers and PCR conditions used in analysis of inflammation-related genes in aorta

Gene	Sequence ¹ (5'-3')	Product size (bp)	Annealing temperature (°C)	Accession number
<i>ICAM-1</i>	F: CTGGAGAGCACAAACAGCAGAG R: AAGGCCGCGAGGCAAAAGAAGC	377	60.0 60.0	NM_012967.1
<i>VCAM-1</i>	F: CCACTTGTACACGGGAGACC R: CACAGGATTTTGGGAGTTGG	398	59.5 55.4	NM_012889.1
<i>MCP-1</i>	F: ATGCAGGTCTCTGTCACG R: CTAGTTCTCTGTCATACT	447	54.3 47.5	NM_031530.1
<i>TGF-β1</i>	F: CTTCAGCTCCACAGAGAAGAACTGC R: CACGATCATGTTGGACAACTGCTCC	298	62.2 62.2	NM_021578.2
E-Selectin	F: GCGAGCAATCAGGAACACAAATGC R: ATTCCAGAGTGACGAAGAGTCAGG	307	60.4 60.4	NM_138879.1
β -Actin	F: AGCCATGTACGTAGCCATCC R: AGGAAGGAAGGCTGGAAGAG	411	57.5 57.5	NM_031144.3

¹F = forward; R = reverse.

Figure 1. (A) Hepatic triglyceride concentration and (B) rat hepatic tissues stained with hematoxylin and eosin assay (magnification $\times 100$). Values are means $\pm$ SD, $n = 3$. Mean values in the column with different letters (a–d) are significantly different as determined by Duncan's multiple range tests ($P < 0.05$). ND = normal diet; HD = high fat and cholesterol diet; SV = HD + simvastatin; H9 = HD + *Lactobacillus fermentum* H9; cMRP = HD + Maillard reaction products (MRP) reacted by casein and lactose; F-cMRP = HD + cMRP fermented by *L. fermentum* H9. Liver-tissue sections were prepared from (a) ND, (b) HD, (c) SV, (d) H9, (e) cMRP, and (f) F-cMRP. Color version available online.

Figure 2. (A) Gel intensity and (B) relative mRNA expression levels related to inflammation in aorta. The mRNA expressions were measured by reverse transcription-PCR. The quantitative analysis of relative intensities was performed by using ImageJ software (National Institutes of Health, Bethesda, MD). β -Actin signals were used for the normalization. Values are means $\pm$ SD, $n = 3$. Mean values in the column with different letters (a–f) are significantly different as determined by Duncan's multiple range tests ($P < 0.05$). ND = normal diet; HD = high fat and cholesterol diet; SV = HD + simvastatin; H9 = HD + *Lactobacillus fermentum* H9; cMRP = HD + Maillard reaction products (MRP) reacted by casein and lactose; F-cMRP = HD + cMRP fermented by *L. fermentum* H9.

Figure 3. Rat aorta tissues stained with hematoxylin and eosin assay (magnification 100 $\times$). Aorta tissue sections prepared from (A) normal diet (ND), (B) high fat and cholesterol diet (HD), (C) HD + simvastatin (SV), (D) HD + *Lactobacillus fermentum* H9 (H9), (E) HD + Maillard reaction products (MRP) reacted by casein and lactose (cMRP), and (F) HD + cMRP fermented by *L. fermentum* H9 (F-cMRP). The arrows indicate the intimal thickness of each group and the scale bar represents 200 μ m. Color version available online.

and F-cMRP significantly reduced the transcription of *ICAM-1* and *VCAM-1* (Figure 2). In particular, the transcriptions of *ICAM-1* and *VCAM-1* were decreased to a significant degree with F-cMRP (59.1 and 69.1%, respectively) compared with the HD group ($P < 0.05$). In hypercholesterolemia, oxidized low-density lipoprotein could stimulate the inflammatory cascade in neighboring endothelial cells via the release of cytokines and chemokines, such as tumor necrosis factor- α , IL-1 β ,

IL-6, and IL-8. At the same time, this inflammogen stimulates the expression of adhesion molecules, such as MCP-1, ICAM, and VCAM (Webb, 2008). These genes are involved in leukocyte recruitment and adhesion, and could increase the adhesiveness of the endothelium with leukocytes and platelets. Also, they could stimulate the migration and proliferation of smooth-muscle cells and thicken the artery walls (Ross, 1999) as well as promote the development of atherosclerotic

lesions. Moreover, the induction of endothelial adhesion molecules by inflammatory cytokines depends on the activation of transforming growth factor- β 1 (TGF- β 1; Lee et al., 2010). Similarly, TGF- β 1, as a pro-fibrotic cytokine, associates transiently with the upregulation of the vascular extracellular matrix, which induces the pathology of diabetes-associated atherosclerosis

(Kolachala et al., 2007; Pham et al., 2010). As the transcriptions of *ICAM-1* and *VCAM-1* were decreased in the treatment group, TGF- β 1 expression was significantly downregulated at 28.4% and 60.1% by H9 and F-cMRP, respectively, compared with the HD group. Inflammation plays an important role in both the initiation of atherosclerosis and the development of

Figure 4. Rat aorta tissues stained with Oil Red O (magnification 40 $\times$). Aorta-tissue sections prepared from (A) normal diet (ND), (B) high fat and cholesterol diet (HD), (C) HD + simvastatin (SV), (D) HD + *Lactobacillus fermentum* H9 (H9), (E) HD + Maillard reaction products (MRP) reacted by casein and lactose (cMRP), and (F) HD + cMRP fermented by *L. fermentum* H9 (F-cMRP). Color version available online.

atherothrombotic events. The involvement of vascular inflammation in the pathogenesis of atherosclerosis is well established (Tribolo et al., 2008). Therefore, it was determined that the administration of H9, cMRP, and F-cMRP significantly downregulated the transcriptional levels of the *ICAM-1*, *VCAM-1*, and *TGF-1 β* genes related to inflammation in the aorta.

To examine histopathological changes in the descending aorta, segments were stained with H&E assay and the stained sections were analyzed by light microscopy. As shown in Figure 3, treatment with H9, cMRP, and F-cMRP reduced intima-media thickness (IMT) values compared with the HD-fed rats. Administration of HD thickened the intima-media as in the study by Renju et al. (2014), which suggests that the high-cholesterol diet causes a thickened intima, large plaques in the lumen, and mild degeneration in the deeper layers. Atherosclerotic lesions actually first begin to develop in the abdominal aorta (Jarvisalo et al., 2001). Measurement of aortic IMT was helpful for the understanding of the extent of coronary atherosclerosis (Couturier et al., 2006). Treatment with H9, cMRP, and F-cMRP could alleviate the development of atherosclerosis through the reduction of aortic IMT in the treatment groups; especially, the F-cMRP-treated group exhibited the greatest reduction of aortic IMT. This result was consistent with the results of mRNA expression. In addition, the effect of MF27, cMRP, and F-cMRP in protecting the aorta from the progressive lipid accumulation was further verified by Oil Red O staining. As shown in Figure 4, the aorta of rats fed a high-fat high-cholesterol diet showed mild lipid accumulation compared with the ND group. All treatment groups showed a slight reduction of lipid accumulation. This result was consistent with H&E stain analysis.

In conclusion, we found that fermented MRP by *L. fermentum* H9 have profound hypolipidemic and anti-inflammatory activity and these functional components may be applied to various dairy foods and used as dairy-based adjuncts for preventing chronic metabolic disorders including cardiovascular risk.

ACKNOWLEDGMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2014R1A1A1006872) and the High Value-Added Food Technology Development Program of the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (iPET), the Ministry for Food, Agriculture, Forestry, and Fisheries of the Republic of Korea (115006-03-1-SB010).

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