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RESEARCH ARTICLE

Hempseed Water Extract Ameliorates Atherosclerosis in Apolipoprotein E Knockout Mice

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Abstract The principal objective of this experiment was to evaluate the anti-atherosclerotic activity of hempseed water extract in apolipoprotein E knockout (ApoE KO) mice. Fourteen male ApoE KO mice were divided into 2 groups and were treated via intragastric inoculation for 14 weeks. The hempseed water extract (HWE) inoculation group exhibited greater gains in weight as compared to the control group, which was inoculated with distilled water. Plaque lesion areas in the aortic sinus were reduced in the HWE group. Total plasma cholesterol, LDL-cholesterol, atherosclerotic index, and cardiac risk factor were all reduced in the HWE group. These results demonstrate that HWE is an excellent nutritional resource and evidences anti-atherosclerotic activity in ApoE KO mice. Further studies will be required to assess the pharmacological mechanisms underlying these effects.

Keywords: hempseed, apolipoprotein E knockout mice, atherosclerosis, low-density lipoprotein

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Introduction

Cardiovascular disease is a principal cause of mortality in many countries, and accounts for up to 16.7 million deaths annually (1). Cardiovascular disease is primarily caused by atherosclerosis, a chronic inflammatory disease of the arteries that generally clinically manifests as thrombosis (2). In the past, atherosclerosis was thought to be due to a passive accumulation of cholesterol in the blood vessel wall. However, current data corroborate the hypothesis that atherosclerosis involves chronic inflammatory features (3). In vascular walls predisposed to atherosclerosis, hypercholesterolemia is associated with increased LDL transcytosis through the endothelium. This results in an accumulation of LDL in the intima, the innermost layer of the artery, where they are prone to oxidative modification, thereby increasing atherogenicity and retention within the vascular intima (4). Increased plasma concentration and oxidative modification of LDL are important factors in early atherogenesis (5). Although few effective therapies are currently available to treat the disease because of its pathological complexity, the previous data may prove to be very important in reducing plasma LDL levels, regulating oxidative modification, and inhibiting the inflammatory response (6).

Hempseed contains approximately 20-25% protein, providing all of the essential amino acids, and 35% oil, and is very rich in essential fatty acids and other polyunsaturated fatty acids (PUFAs). It also contains 20-30% carbohydrate, 10-15% insoluble fiber, minerals, and vitamin E (7). The predominant fatty acids in hempseed are linoleic acid (ω6) and α-linoleic acid (ω3) (8). The ratio of ω6 to ω3 fatty

acids is 3:1. This ratio is much lower than the recommendations of the Nutrition Society of Germany, Austria, and Switzerland (D-A-CH Recommendations for Nutrition) (9). Current theories on the subject recommend that the ingestion of ω6 be curtailed; from this perspective, hempseed oil is clearly an appropriate resource (10). Additionally, previous study has demonstrated that an intracellular deficiency in essential fatty acids plays a central role in the atherogenic process (11). Therefore, we believed that hempseed might prove to be a promising candidate for the anti-atherosclerotic effect. Although several previous studies have attempted to characterize the effects of dietary hempseed intake, its effects on atherosclerotic heart disease have yet to be thoroughly elucidated, owing to the political issues surrounding its cultivation (12,13)

To study the pathophysiology of atherosclerosis, inbred and genetically modified mice are being increasingly used. In apolipoprotein E knockout (ApoE KO) mice, total plasma cholesterol (TC), and triacylglycerol (TG) spontaneously increase and conversely, HDL-cholesterol (HDL-C) spontaneously decreases. The atherosclerotic lesions forming in ApoE KO mice have been found to evidence a morphology reminiscent of those in humans (14). Therefore, to evaluate the effects of hempseed water extract (HWE) on the anti-atherosclerotic effect, we used ApoE KO mice for this experiment.

Materials and Methods

Preparation of hempseed extract Hempseed extract was manufactured as described previously, with some modifications (13). Hempseeds were washed and ground using an electric grinder. The polar and nonpolar components were extracted from the ground hempseeds with distilled water and hexane, respectively. First, 100 g of ground hempseed was dissolved in 300 mL of hexane. This solution was then filtered through 0.45-μm Whatman nylon membrane filters to divide the residues and extracted solutions. The filtered residue was freeze-dried and mixed with 300 mL of distilled water for extraction. Then, 2.5 g of dried distilled water extract was dissolved in 50 mL of distilled water. This hempseed water extract (HWE) was utilized in further experiments.

Animal Six-week-old, male ApoE KO (B6.129P2-*Apoe*^{tm1Unc}/J) mice, purchased from the Jackson Laboratory (Bar Harbor, ME, USA) were used in all experiments. The mice were bred and maintained at the Laboratory Animal Facility in Konkuk University (Seoul, Korea). All mice were housed on woodchip bedding with a 12 h light-dark photocycle. The room temperature was controlled at 22±2°C with a relative humidity of 50±10%. The animals were fed a sterilized regular pellet (2918C; Harlan Teklad, Madison, WI, USA) and had access to autoclaved water *ad libitum* through drinking bottles. All procedures were approved by Institutional Animal Care and Use Committee of Konkuk University. The ApoE KO mice were divided into 2 groups, the HWE inoculation group and distilled water inoculation (control) group. Each group was composed of 7 mice. The mice of each group were administered 300 μL of corresponding solution via intragastric inoculation every day for 14 consecutive weeks. The body weights of all mice were measured at weekly intervals.

Assessment of lipid accumulation in aortic sinus The heart and major organs of each mouse were harvested and washed in phosphated buffered saline (PBS). All hearts were embedded in optimal cutting temperature (OCT) compound and frozen in liquid nitrogen. For analysis of aortic sinus plaque lesions, cryostat sections were stained with oil red O and counterstained with hematoxylin. For quantification of the plaque lesions in the aortic sinus, images of each cryostat section were captured through a 10× objective using a DP71 digital camera (BX51; Olympus, Tokyo, Japan). Image analysis was conducted using Metamorph version 7.5.6.0 software.

Oil red O staining A 0.5%(w/v) solution of oil red O in 2-propanol was prepared by heating the reagents to 56°C for 1 h and then filtering them through filter paper. A working solution was prepared via dilution of the stock solution at a ratio of 3:2 with distilled water including 1% dextrin. Prior to use, the working solution was filtered through a 0.2-μm filter. Each cryosection was stained on a slide for 30 min in oil red O working solution and quickly rinsed in distilled water, then counterstained with hematoxylin and returned to distilled water.

Blood sampling and biochemical analyses At the end of the experiment, blood samples were obtained from mice via the caudal vena cava under 1.25% Avertin anesthesia, and sera were prepared via 10 min of centrifugation at 14,000×*g*. Analysis of serum biochemical parameters including glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), TC, HDL-C, and LDL-cholesterol (LDL-C) were determined using an automated chemistry analyzer (Clinical Analyzer 7020; Hitachi, Kobe, Japan). Then, the atherogenic index (AI) and cardiac risk factor (CRF) were calculated via the previous method (15).

$$AI = \frac{TC-(HDL\text{-}C)}{HDL\text{-}C}$$

$$CRF = \frac{TC}{HDL\text{-}C}$$

Cytokine enzyme-linked immunosorbent assay (ELISA) The level of tumor necrosis factor (TNF)-α in serum a was quantified by using commercially available sandwich ELISA kits (eBioscience, San Diego, CA, USA). The serum samples were diluted 1:3 in ELISA diluent buffer to quantify mouse TNF-α level. TNF-α level was calculated with reference to a standard curve. The detection limit of TNF-α is 8 pg/mL.

Statistical analysis All results were expressed as means ±standard deviation (SD). Significant differences between the groups were evaluated using unpaired Student's *t*-test. Data analysis was conducted using Prism 5 for Windows software (Graphpad Software, Inc., San Diago, CA, USA). A *p*-values of <0.05 were considered statistically significant.

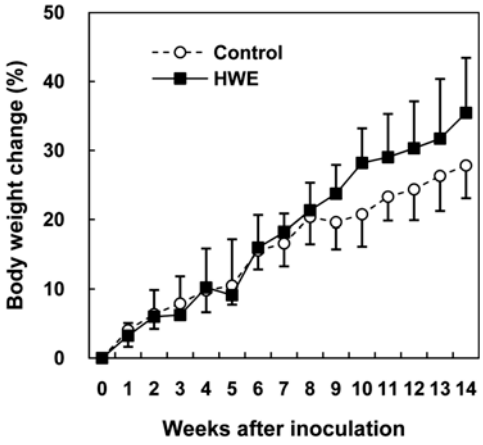

Fig. 1. Body weight changes of ApoE KO mice for 14 weeks. HWE, hempseed water extract. Data are expressed as the mean± SD of 7 mice/group.

Results and Discussion

Body weight changes The body weight changes over a 14-week period are shown in Fig. 1. Body weights generally increased over time. The mice fed on HWE exhibited greater weight gains than the control mice inoculated with distilled water. However, there was no significant difference in increase between both groups. The liver weights of the control group did not differ significantly from that of the HWE group (data not shown). Histopathological lesions in the livers of both groups (data not shown) are observed. These results indicate that HWE exerted no negative effects on the liver. Therefore, this outcome might be 1 piece of evidence supporting the proposition that dietary HWE ingestion is safe.

Hempseed has been used for thousands of years as an excellent nutritional resource. Whole hempseed contains protein, fat, carbohydrate, vitamin, and minerals. The protein content of hempseed is the second only to that of the soybean (16). Hempseed protein does not contain the trypsin inhibitors and oligosaccharides found in soy. Thus, hempseed protein is absorbed more easily and does not cause stomach upset or gas (17). Hempseed also provides high-quality protein in the diet, due to the fact that the amino acids of hempseed include all components required for human health. Hempseed oil contains essential fatty acids, including ω6, ω3, γ-linoleic acid, and phytosterol (7,10). In previous studies, these fatty acids have been shown to be extremely important for human health (18-20). As mentioned previously, because hempseed is an excellent nutrition source, the ingestion of dietary HWE results in greater body weight gains as compared to the control group.

Quantification of oil red O stain To quantify the results of oil red O staining, the serial sections obtained between the aortic valve and the aortic sinus were measured. Representative oil red O stained aortic root sections from ApoE KO mice are shown in Fig. 2. The cross-sectional area of the lesion is smaller in the HWE group. Quantification of the oil red O stained section of the aortic lesions in ApoE KO mice is depicted in Fig. 3. The relative lipid content was represented as a percentage of the oil red O stained lesions found in the total area through the 10×

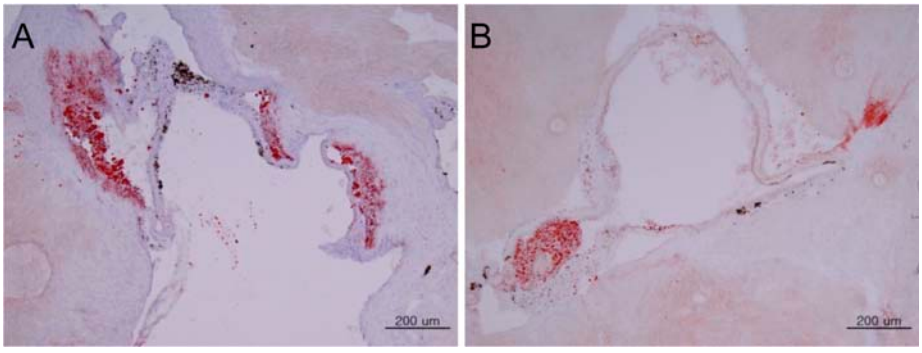

Fig. 2. Representative photomicrographs of the aortic sinus stained by oil red O in ApoE KO mice. A, control group; B, hempseed water extract group

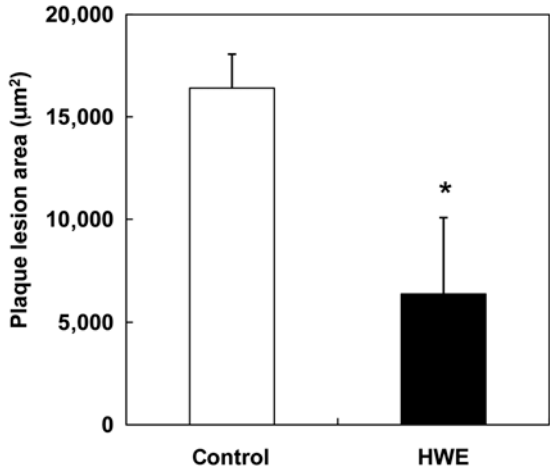

Fig. 3. Quantification of oil red O stained section of aortic lesions in ApoE KO mice. HWE, hempseed water extract. Data are expressed as the mean±SD of 7 mice/group; *Significantly different from control by unpaired Student's *t*-test ($p<0.05$)

objective. The oil red O stained area was significantly smaller in the HWE group than in the control group (mean±SD: 6,386±3,699 μm^2 in HWE group and 16,406 ±1,654 μm^2 in control group, $p<0.05$). These results indicate that HWE effectively reduces atherosclerotic plaque formation in the aortic sinuses of ApoE KO mice.

Atherosclerotic plaques are characterized by an abnormal accumulation of circulating LDL particles within the artery wall. This accumulation of LDL is an important initiation factor in atherogenesis (21). LDL particles in the intima are prone to oxidative modifications. Machrophage adhesion to the subendothelium induced by oxidized LDL (Ox-LDL) results in the ingestion of cholesterol and transformation into foam cells, the hallmark of early atherosclerosis (22). The ω3, phytosterols, and vitamin E contained in hempseed function to suppress these processes. The effect of ω3 in atherosclerosis involves the reduction of LDL levels in serum, the modulation of oxidative stress in macrophages, and the stabilization of atherosclerotic plaque (23). Phytosterols also reduce LDL levels in serum. As a result of these reductions, phytosterols inhibit cardiovascular disease, including atherosclesosis (19,24). Because oxidative stress accelerates Ox-LDL formation, anti-oxidants have been determined to prevent atherosclerosis (25). In previous study, vitamin E evidences a protective effect against LDL oxidation (26). Collectively, these results demonstrate that HWE effectively inhibits atherosclerosis.

Serum biochemistry The concentrations of TC, HDL-C, and LDL-C are provided in Table 1. In this study, serum GOT concentration in the HWE mice was lower than that in the control group. Serum GPT concentration between the control and HWE group was identical (data not shown). GOT and GPT are the enzymes that exist in the liver and other organs. Their concentration in the blood is maintained at constant levels due to normal cellular destruction. If many cells of the liver and other organs are damaged, these enzymes are secreted to the outside of cell. The levels of enzymes are subsequently increased in the serum (27). Therefore, HWE is harmless to the liver and other organs compared with the control group.

Atherosclerotic disease and its complications are associated with TC and LDL-C and are inversely associated with HDL-C (28). An atherogenic lipid profile of diabetes patients shows increased levels of TG and apolipoprotein B, including a reduced level of HDL-C (29). A low concentration of HDL-C is associated with an increase in cardiovascular disease (30). Our data demonstrated that TC in serum was higher in the control group than in the HWE group ($p=0.233$). The level of HDL-C in serum was lower in the control group than in the HWE group ($p=0.188$). The level of LDL-C was lower in the HWE group than in the control group ($p=0.171$). The proper balance of ω3 and ω6, and the comparatively high phytosterols contained in hempseed, could partially explain these results. In a previous study, ω3 and phytosterols were shown to lower TC and LDL-C in the serum. These components have been theorized to lower the risk of atherosclerosis and cardiovascular disease (23,24). Omega 6 also inhibits the inflammatory response, which plays a key role in atherogenesis, via metabolism to anti-inflammatory molecules, prostaglandins and leukotrienes (31). Recently, ω6 has been shown to lower plasma LDL levels and reduce the risk of atherosclerosis when the ω6/ω3 ratio is low (32,33). Additionally, the reduced AI and CRF observed in the HWE group (Table 1) supports the previously mentioned results. These results reflect the possibility that HWE suppresses atherosclerosis and related diseases.

TNF-α production in serum Pro-inflammatory cytokine, in particular TNF-α, play an important role in low-grade systemic inflammatory condition in atherogenesis. When expression of TNF-α was increased, atherosclerosis is initiated and progressed (34). The level of TNF-α is shown

Table 1. Analysis of serum biochemistry, atherogenic index (AI), and cardiac risk factor (CRF)

Group	TC (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	AI	CRF
Control	641±127[1)]	26±6	100±21	26±13	27±13
HWE	526±153	31±5	77±26	16±6	17±6

[1)]Data are expressed as the mean±SD of 7 mice/group.

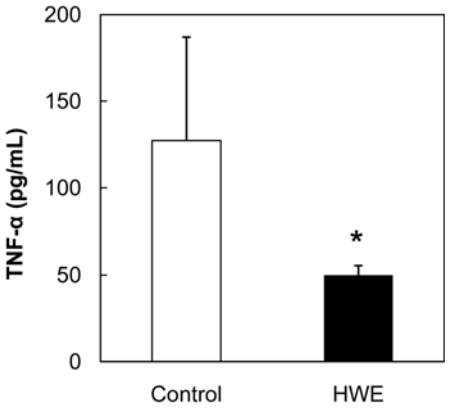

Fig. 4. Levels of TNF-α in serum of ApoE KO mice. HWE, hempseed water extract. Data are expressed as the mean±SD of 7 mice/group; *Significantly different from control by unpaired Student's *t*-test ($p<0.05$)

in Fig. 4. The level of TNF-α in serum was higher in the control group than in the HWE group ($p<0.05$). These results could be explained partially by PUFAs contained in hempseed. In previous studies, PUFAs have been known to have anti-inflammatory properties (11,35,36).

In conclusion, our experiments demonstrate that dietary HWE ingestion exerts an anti-atherosclerotic effect by inducing a reduction in TC and LDL-C levels, regulating oxidative stress, and inhibiting the inflammatory response. Although further details of the pharmacological mechanism underlying HWE's effects remain to be elucidated, we suggest that HWE may be a promising candidate for the treatment of atherosclerosis, or may prove useful toward the development of a novel anti-atherosclerotic treatment.

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