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Antiatherosclerotic effect of the edible mushrooms *Pleurotus eryngii* (Eringi), *Grifola frondosa* (Maitake), and *Hypsizygus marmoreus* (Bunashimeji) in apolipoprotein E–deficient mice

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Abstract

In the present study, we examined the antiatherosclerotic effects of 3 edible mushrooms, *Pleurotus eryngii* (Eringi), *Grifola frondosa* (Maitake), and *Hypsizygus marmoreus* (Bunashimeji), in atherosclerosis-susceptible C57BL/6J, apolipoprotein E–deficient (apoE$^{-/-}$) mice. Male apoE$^{-/-}$ mice (6 weeks of age) were fed a normal diet (cholesterol concentration <66 mg/100 g) or a normal diet containing 3% dried Eringi, Maitake, or Bunashimeji mushroom powder for 10 weeks. Food intake, body weight, serum total cholesterol (TC), and serum triacylglycerols (TG) were measured periodically during the experimental period. At the end of the experiment (at 16 weeks of age), the atherosclerotic lesion area was measured in cross-sections of the aortic root. Serum TC concentrations in the Bunashimeji group were significantly lower than that in the control group at 8, 10, 12, 14, and 16 weeks of age. Serum TC concentrations in the Eringi, and Maitake groups were significantly lower than that in the control group only at 12 weeks of age. There was no significant difference in the serum TG concentrations in all groups during the experimental period. The atherosclerotic lesions were significantly decreased in the Eringi, Maitake, and Bunashimeji groups than that in the control group at the end of the experiment. Dietary supplementation with the Bunashimeji mushroom powder had the strongest antiatherosclerotic effect among 3 mushrooms. In conclusion, supplementation of the 3 edible mushrooms prevents the development of atherosclerosis, even normal diet. Antiatherosclerotic effect is partly via lowering of serum TC concentrations; further mechanisms should be investigated.

Keywords: Apolipoprotein E-deficient mice; Atherosclerosis; Mushroom; *Pleurotus eryngii*; *Grifola frondosa*; *Hypsizygus marmoreus*
Abbreviations: apoE$^{-/-}$,apolipoprotein E–deficient; TG,triacylglycerol; TC,total cholesterol.

1. Introduction

There are several thousand species of edible mushrooms found throughout the world. Edible mushrooms are considered to be an ideal food for the dietetic prevention of atherosclerosis because of their high fiber, protein, and microelement content and their low-fat (energy) content [1,2]. It is generally accepted that lowering high-serum total cholesterol (TC) concentrations is crucial for preventing atherosclerosis [3]. Several studies have demonstrated serum cholesterol–lowering effects of many types of mushrooms, such as *Grifora frondosa* (Maitake mushroom) [4,5],

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Lentinus edodes (Shiitake mushroom) [5], *Flammulina velutipes* (Enokitake mushroom) [5], *Pleurotus ostreatus* (Oyster or Hiratake mushroom) [6], *Polyporus confluens* [7], *Ganolucidum* (Reishi mushroom) [8], *Auricularia Auricula* [9], *Tremella Fuciformis* [9], and *Volvariella volvacea* (Straw mushroom) [10]. In most of these studies, however, a high cholesterol diet was given to elevate the control group serum TC concentrations [4,6,8-10], and none of these studies examined the inhibitory effect of edible mushrooms on atherosclerotic lesion formation. Yamada et al reported that atherosclerotic lesions induced in rabbit aorta by a 1% cholesterol diet for 8 weeks is inhibited by dietary supplementation with 1% *L edodes mycelia* [11]. We previously reported that green tea catechins supplementation prevents atherosclerotic lesion formation in the thoracic aorta in apolipoprotein E–deficient (apoE$^{-/-}$) mice fed an atherogenic diet without changing the serum cholesterol level [12]. Thus, the mushroom-induced inhibition of atherosclerotic lesion formation might not only be due to an effect on the serum TC level. It was important to determine if the mushrooms lower serum cholesterol level in a normal diet and to find the mechanism of antiatherosclerotic effect of mushrooms besides the effect of the inhibition of cholesterol absorption.

ApoE$^{-/-}$ mice are generated by inactivating the apolipoprotein E gene by gene targeting and are widely used for atherosclerosis studies [13,14]. In our preliminary studies, apoE$^{-/-}$ mice had markedly increased TC levels at 5 weeks of age and developed spontaneous arterial lesions at 10 weeks of age, even when fed a normal diet. The lesion formation in this mouse is similar to that in well-established larger animal models of atherosclerosis and in humans [15]. Therefore, this model is useful for studying the pathogenesis of atherosclerosis and potential therapies [16].

In the present study, the antiatherosclerotic effect of edible mushrooms *Pleurotus eryngii* (Eringi or King Oyster), *Grifola frondosa* (Maitake), and *Hypsizigus marmoreus* (Bunashimeji) was examined on serum TC and triacylglycerols (TG) concentrations and atherosclerotic lesion formation in the aortic valve using apoE$^{-/-}$ mice. To exclude the effect of dietary cholesterol on the development of atherosclerosis lesion formation, a normal diet was used.

2. Methods and materials

2.1. Materials

Eringi (Hokuto PLE-2 gou), Maitake (Hokuto NT-100), and Bunashimeji (Hokuto Shiro 1 goukin) mushrooms were cultivated by the Hokuto Corporation (Nagano, Japan). Fresh fruit mushroom bodies were freeze-dried and powdered using a mill (IFM-700G; Iwatani Corporation, Tokyo, Japan). The basic composition and ergosterol of each mushroom were analyzed by a foundation for analysis (Japan Food Research Laboratories, Tokyo, Japan) using standard methods. Ergosterol was analyzed by UV-HPLC method at 282 nm detection. Nova-Pak C-18 column (3.9 mm × 150 mm, Waters, Milford, MA) was used with a mixture of acetonitrile and methanol (1:1) as column eluant [17].

2.2. Animals and treatments

Animals were handled humanely according to the Guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka. The experimental protocol and animal use were approved by the Committee of the University of Shizuoka. ApoE$^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and bred in our laboratory under specific pathogen-free conditions. Two experiments, experiment 1 (Eringi and Maitake) and experiment 2 (Bunashimeji), were performed separately because we could not obtain enough mice to examine 3 mushrooms at once in our institution. Male apoE$^{-/-}$ mice at 6 weeks of age were divided into 3 groups in experiment 1 and into 2 groups in experiment 2, consisting of 5 mice/group, matched for their serum TC and TG concentrations and body weight. The control group was fed a normal diet (CRF-1; Oriental Yeast, Tokyo, Japan). The cholesterol content in the CRF-1 diet was less than 0.066% (data from Oriental Yeast). The Eringi, Maitake, and Bunashimeji groups were given the CRF-1 diet containing 3% of 1 of the mushroom powders ad libitum. Animals were given filtered water ad libitum and were maintained under controlled conditions at a temperature of 24 ± 1°C, relative humidity of 45 ± 5%, and 12-hour light cycle (0800-2000 hours). Mice were fed the experimental diet for 10 weeks. Food intake and body weight were measured every week during the experimental period. Blood was collected from the retro-orbital plexus every 2 or 4 weeks under light anesthesia with diethyl ether. At the end of the experiment, when mice were 16 weeks of age, they were anesthetized with diethyl ether, and blood was collected from the abdominal aorta. The heart was perfused with phosphate-buffered saline and 10 % formalin neutral buffer solution (pH 7.0-7.5), and the heart with aortic arch was excised and placed in 10% formalin neutral buffer solution for 2 weeks [18].

2.3. Measurement of serum TC and TG concentrations

The blood was allowed to stand at room temperature for 1 hour. The serum was obtained by centrifugation at 1000×*g*

Table 1
Composition of mushrooms

Components	Eringi	Maitake	Bunashimeji
Protein (g/100 g)	2.2	2.3	2.4
Carbohydrate (g/100 g)	3.5	3.5	3.0
Dietary fiber (g/100 g)	2.5	3.4	2.6
Crude fat (g/100 g)	0.3	0.5	0.5
Ash (g/100 g)	0.7	0.6	0.8
Ergosterol (mg/100 g)	45.5	49.3	52.8
Energy (kJ/100 g)	109	117	109

All data were analyzed by Japan Food Research Laboratories. Data indicate mean of duplicate determinations.

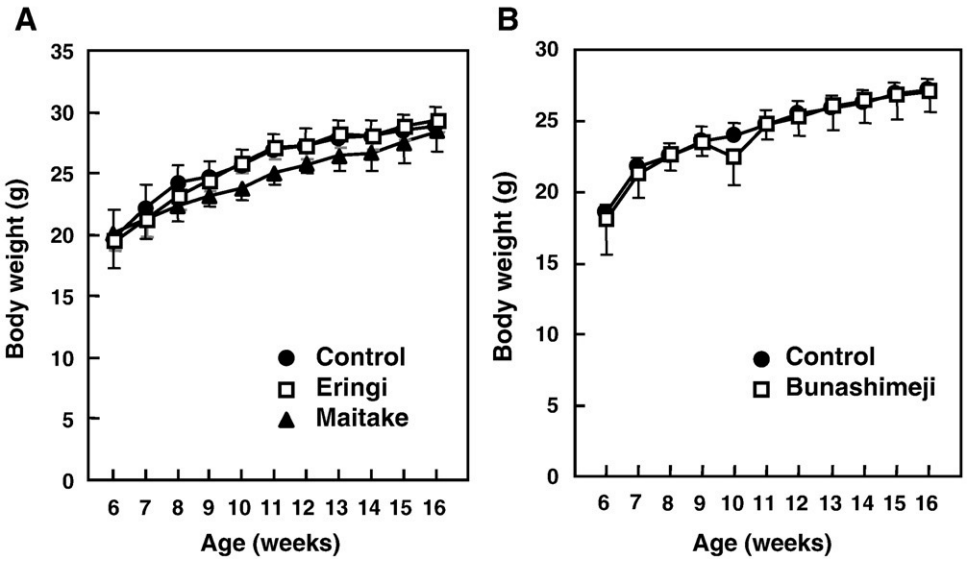

Fig. 1. Body weight changes in apoE$^{-/-}$ mice fed a normal or mushroom-supplemented diet. Male apoE$^{-/-}$ mice (6 weeks of age) were maintained on a normal diet (control) or a normal diet containing 3% mushroom powder for 10 weeks. Body weight was measured every week. Each point and vertical bar indicates mean ± SD for 5 mice. A, Control: filled circle, Eringi: open square, Maitake: filled triangle. B, Control: filled circle, Bunashimeji: open square.

for 10 minutes at 4°C and kept at −80°C until analyzed. Serum TC and TG concentrations were measured using commercial kits for measuring the levels of TC (Cholesterol E-test; Wako Pure Chemical Industries, Japan) and TG (Triglyceride E-test; Wako Pure Chemical Industries), according to the manufacturer's protocols.

2.4. Histologic analysis of atherosclerotic lesions

The formalin-fixed heart was horizontally cut just under the atrium [18]. The aortic root with the top of the heart was embedded in paraffin and sectioned serially at 5-μm intervals. Cross-sections were taken at the level of the aortic valves and stained with hematoxylin and eosin. The area of the atherosclerotic lesions from 6 cross-sections from each mouse was measured using image analysis software (Micro Analyzer; Japan Poladigital, Tokyo, Japan). Atherosclerotic lesions were calculated as the sum of lesion area across 6 cross-sections.

2.5. Statistical analysis

Data are expressed as mean ± SD except in Table 1. Serum TC and TG concentrations were analyzed by 2-factor

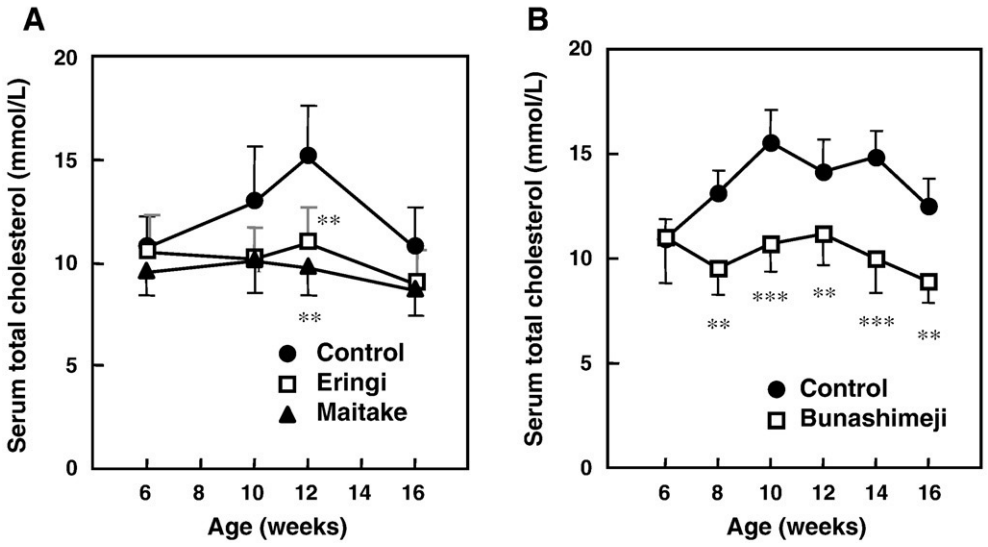

Fig. 2. Changes in the serum TC concentration in apoE$^{-/-}$ mice fed a normal or mushroom-supplemented diet. Male apoE$^{-/-}$ mice (6 weeks of age) were maintained on a normal diet (control) or a normal diet containing 3% mushroom powder for 10 weeks. Blood was collected from the retro-orbital plexus every 2 or 4 weeks. Each point and vertical bar indicates mean ± SD for 5 mice. $^{**}P < .01$ and $^{***}P < .001$ vs control by 2-factor factorial analysis of variance followed by a Bonferroni/Dunn test. A, Control: filled circle, Eringi: open square, Maitake: filled triangle. B, Control: filled circle, Bunashimeji: open square.

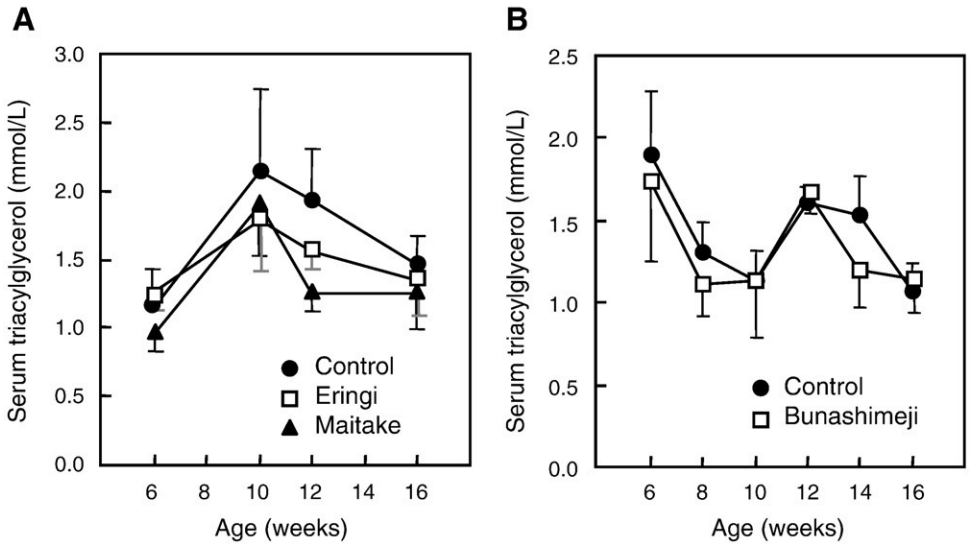

Fig. 3. Changes in the serum TG concentration in apoE$^{-/-}$ mice fed a normal or mushroom-supplemented diet. Male apoE$^{-/-}$ mice (6 weeks of age) were maintained with a normal diet (control) or a normal diet containing 3% mushroom powder for 10 weeks. Blood was collected from the retro-orbital plexus every 2 or 4 weeks. Each point and vertical bar indicates mean ± SD for 5 mice. A, Control: filled circle, Eringi: open square, Maitake: filled triangle. B, Control: filled circle, Bunashimeji: open square.

factorial analysis of variance, followed by a Bonferroni/Dunn test. Data of the area of the atherosclerotic lesions were analyzed by Student *t* test. Statistical analysis was performed using Prism 4 (GraphPad Software Inc, San Diego, Calif).

3. Results

3.1. Basic composition of mushrooms

The basic composition of the mushrooms was summarized in Table 1. Protein and ash contents were the same for all

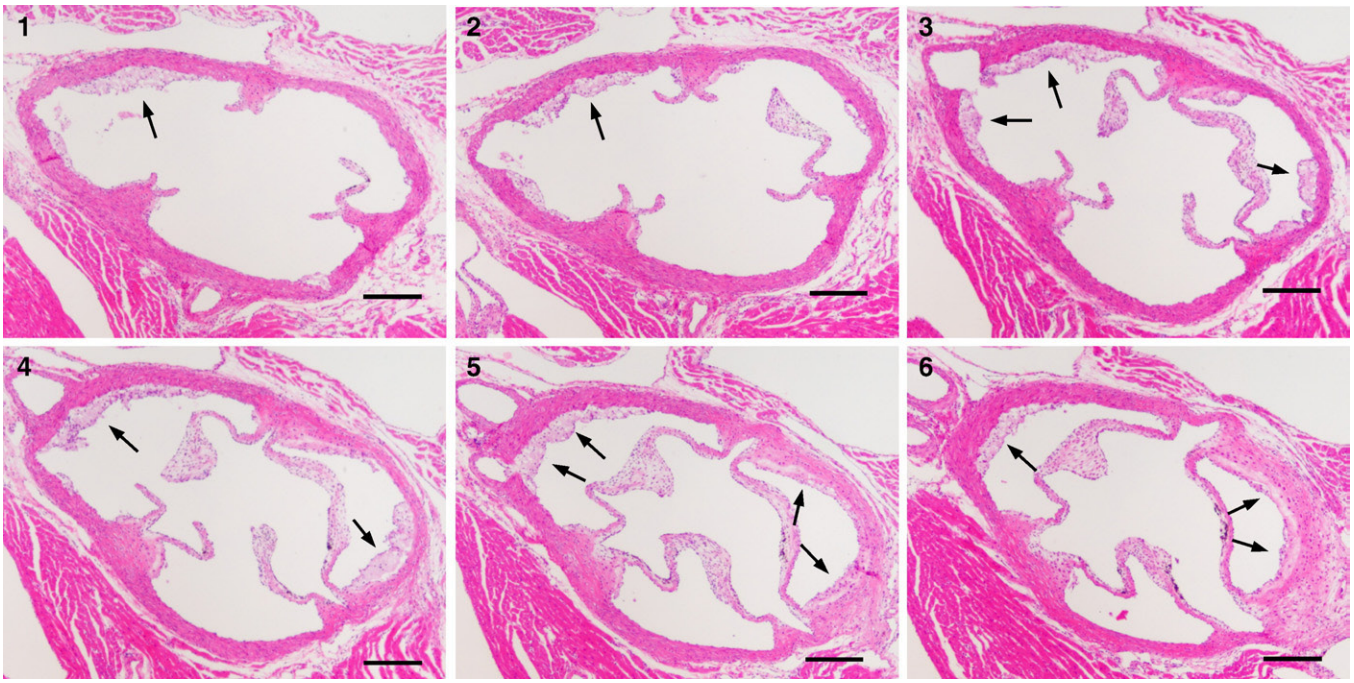

Fig. 4. Cross-sections of the aortic sinus from apoE$^{-/-}$ mouse at 16 weeks of age. Cross-sections of the aortic sinus were prepared and stained with hematoxylin-eosin. Atherosclerotic lesions were detected as areas of light staining in the wall of the aortic sinus (arrows). Each bar shows 200 μm.

3 mushroom types. The carbohydrate content of Bunashimeji was lower than that of Eringi and Maitake. The dietary fiber content of Maitake was 1.3-fold higher than that of the Eringi and Bunashimeji. The crude fat of the Eringi mushroom was lower than that of the Maitake and Bunashimeji mushrooms. The ergosterol content was highest in Bunashimeji mushrooms. The energy content was the same for all 3 mushroom types.

3.2. Body weight, food intake, serum cholesterol, and TG concentrations

There was no significant difference in the change in body weight between the mushroom groups and control group (Fig. 1). Food intake was approximately 4 g/d per mouse throughout the experimental period in all groups, and there was no significant difference between any mushroom group

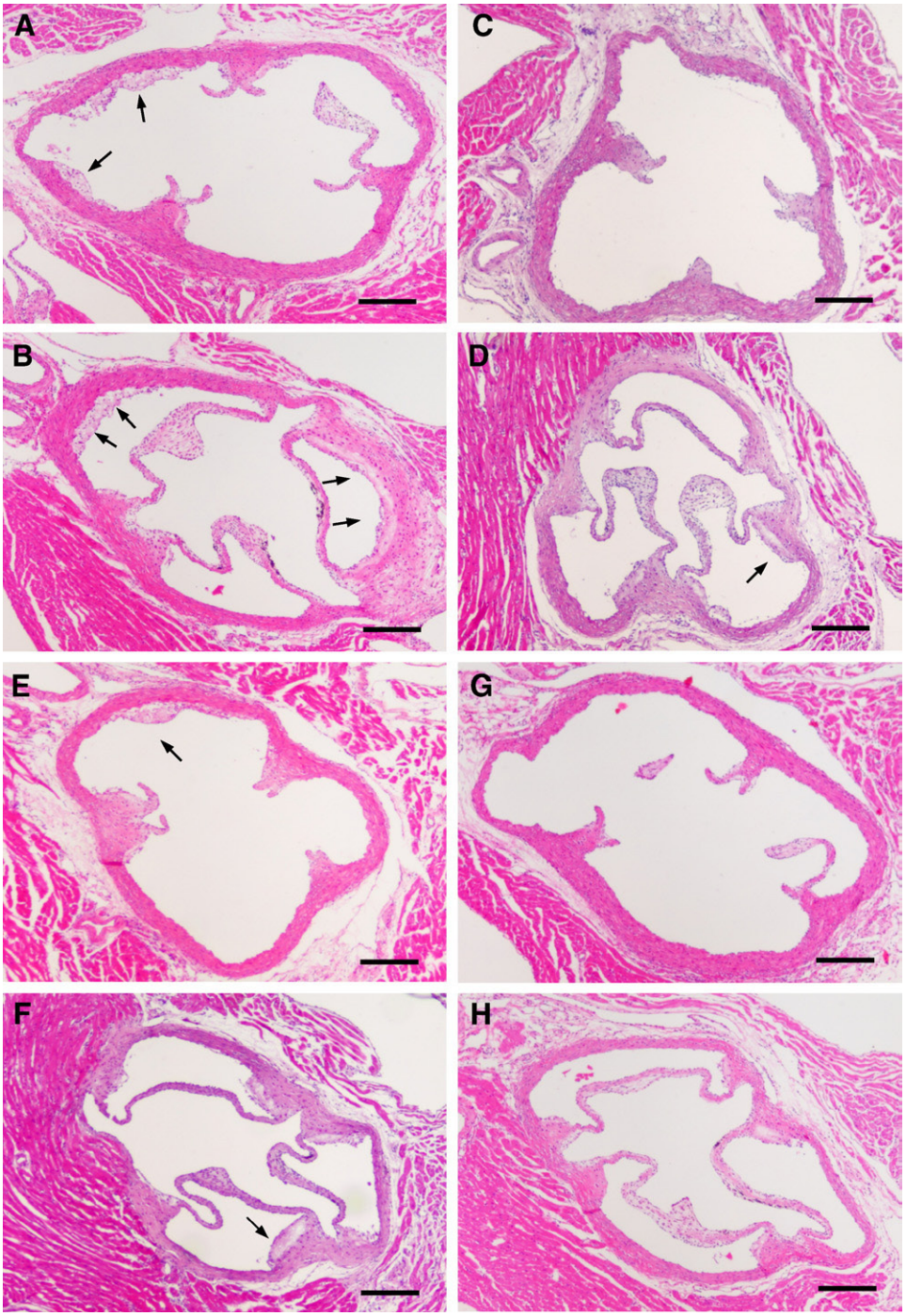

Fig. 5. Cross-sections of the aortic sinus from control (A and B), Eringi (C and D), Maitake (E and F), and Bunashimeji (G and H)–treated apoE$^{-/-}$ mouse at 16 weeks of age. Atherosclerotic lesions were detected as areas of light staining in the wall of the aortic sinus (arrows). Each bar shows 200 μm.

and control group (data not shown). Serum TC concentrations in the Eringi and Maitake groups were significantly lower than that in the control group at 12 weeks of age, but not at 10 and 16 weeks of age (Fig. 2A). There was no significant difference in the serum TC concentrations between the Eringi and Maitake groups. Serum TC concentrations in the Bunashimeji group were significantly lower than that in the control group at 8 to 16 weeks of age (Fig. 2B). Serum TC in Bunashimeji group decreased rapidly and consecutively than that in either Eringi or Maitake group. Serum TG concentrations among the groups were not significantly different (Fig. 3).

3.3. Atherosclerotic lesion area

Atherosclerotic lesions were observed in 16-week-old male apoE$^{-/-}$ mice fed a normal diet from the end of the aorta to the aortic sinus. Six sections were selected for measurement of the atherosclerotic lesion area (Fig. 4). Fatty streak lesions composed primarily of foam cells were observed at the aortic sinus. The atherosclerotic lesion area of each section was measured, and the lesion area of each of the 6 sections was summed to define the size of the atherosclerotic lesion. In Fig. 5, 2 typical sections of the 6 sections containing atherosclerotic lesions are shown for the control and the 3 mushrooms groups. The atherosclerotic lesion area of the 3 mushroom groups was smaller than that of the control group. The total atherosclerotic lesion size in the Eringi, Maitake, and Bunashimeji groups was significantly smaller than that in the control groups, respectively (Fig. 6). The Bunashimeji mushroom group had the smallest atherosclerotic area of all groups. The atherosclerotic area was similar between the Eringi and Maitake mushroom groups. Atherosclerotic lesion areas in the control groups in experiments 1 and 2 were 0.244 ± 0.032 and 0.405 ± 0.185 mm^2 (mean ± SD; n = 5), respectively. There were no significant differences between the control groups in experiments 1 and 2 by Student *t* test.

4. Discussion

Previously, it was reported that many types of mushrooms including Maitake have serum cholesterol–lowering effects [4-10]. However, it has not been studied whether these mushrooms have effects to inhibit atherosclerotic lesion formation. In this study, a normal diet supplemented with 3% Eringi, Maitake, or Bunashimeji mushroom powder reduced not only serum TC concentrations but also atherosclerotic lesion formation in apoE$^{-/-}$ mice. Serum TC in Bunashimeji group decreased rapidly and consecutively than that in Eringi and Maitake groups. Moreover, the supplementation of Bunashimeji mushroom was the most effective among the 3 mushrooms on decreasing atherosclerotic lesion area. It is likely that the cholesterol-lowering effect of the mushrooms may inhibit the formation of atherosclerotic lesions. Kay [19] reviewed the effect of dietary fiber, a plant-derived material, on plasma cholesterol concentrations. The cholesterol-lowering effect of dietary fiber might be mediated by increased fecal steroid excretion, enhanced fecal excretion of bile acids, and lowered insulin response [19]. Maitake and Enokitake fibers (5% of cholesterol-free diet) decreased serum TC levels by enhancing fecal cholesterol excretion [5]. Thus, a possible reason for the

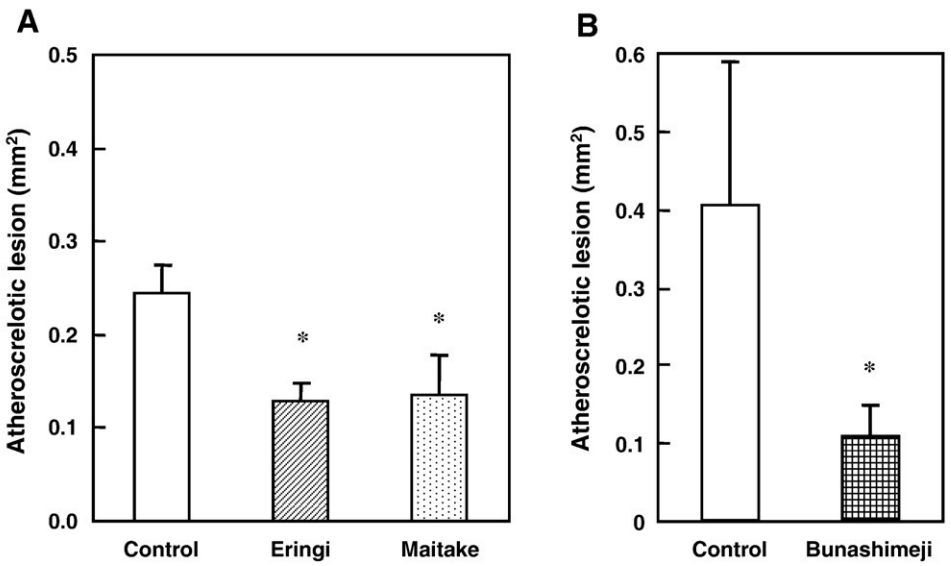

Fig. 6. Inhibitory effects of edible mushrooms on the atherosclerotic lesion formation. The area of the atherosclerotic lesions from 6 cross-sections from each mouse was measured using image analysis software. Atherosclerotic lesions were calculated as the sum of lesion areas across 6 cross-sections. Each column and vertical bar indicates mean ± SD for 5 mice of experiment 1 (A) and experiment 2 (B). *$P < .05$ vs control by Student *t* test.

antiatherosclerotic effect of mushrooms might be their dietary fiber content. However, the cholesterol content of the diet used in the present study was very low (<66 mg/100 g) compared with previous reports using a high-cholesterol diet [4-10]. Furthermore, the dietary fiber content was highest in the Maitake mushroom, relative to that in the Bunashimeji mushroom, which has dietary fiber content equivalent to that in Eringi mushroom. The serum cholesterol–lowering effect of the mushrooms, therefore, did not correlate with their dietary fiber content in the present study. These results suggest the possibility that the supplementation of mushrooms reduces atherosclerotic lesion formation via endogenous cholesterol. The cholesterol-lowering effect of mushrooms may not be due to the inhibition of cholesterol absorption. However, we could not completely exclude dietary cholesterol. Further studies are needed to determine the fecal excretion of cholesterol and bile acids to clarify the mechanisms for the antiatherosclerotic effect of the mushrooms.

Although the fiber content in Maitake was higher than that in Bunashimeji, the antiatherosclerotic effect of the Bunashimeji mushroom powder was greater than that of the Maitake mushroom powder. Matsuzawa et al reported that oral administration of Bunashimeji induced an antioxidant effect in plasma of mice [20,21]. A major lipoprotein in the serum of apoE$^{-/-}$ mice is beta-very low-density cholesterol (β-VLDL) [14]. This lipoprotein is highly atherogenic [22] and is taken up abundantly by both macrophages [23] and smooth muscle cells [24,25] to form foam cells. In addition, oxidatively modified β-VLDL is degraded by macrophages at about twice the rate of unmodified β-VLDL [26], and the oxidation of β-VLDL by endothelial cells enhances its metabolism by smooth muscle cells [27]. These reports suggest that oxidative modification of β-VLDL enhances the development of atherosclerosis in apoE$^{-/-}$ mice, and the antioxidative effect of Bunashimeji might prevent atherosclerotic lesion formation without changing the serum cholesterol concentration.

In conclusion, a normal diet containing dry Eringi, Maitake, or Bunashimeji mushroom powder lowered serum TC levels and decreased the formation of atherosclerotic lesions in apoE$^{-/-}$ mice. The antiatherosclerotic effect of these mushrooms should be investigated in the human diet for application in preventing atherosclerosis.

Acknowledgment

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