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Original Research

Peptides Obtained by Enzymatic Decomposition of Mackerel Prevent Nonalcoholic Steatohepatitis in Sprague-Dawley Rats fed a High-fat and High-cholesterol Diet

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Abstract:

***Background*:** The pathogenesis of nonalcoholic steatohepatitis (NASH) is thought to be multifactorial, and oxidative stress may play an important role in the development of NASH. Peptides obtained by enzymatic decomposition of mackerel (EMP) including selenoneine were recently reported to possess antioxidative activity. Thus, EMP is a potential dietary strategy for the prevention of NASH.

***Methods*:** Nine-week-old male Sprague-Dawley rats were fed a high-fat and high-cholesterol (HFC) diet with or without EMP (HFC supplemented with 1%, 2.5%, or 5% (w/w) EMP) for 9 weeks (n = 6-

7/group). Histopathology and serology were evaluated as well as expressions of genes in the liver involved in fibrogenesis, inflammation, oxidative stress, and lipid metabolism.

***Results*:** Histologically, the HFC diet with EMP tended to reduce NASH progression compared to the HFC diet without EMP (NASH diagnostic rate: 80% vs 100%). Serum leptins in rats fed the HFC diet with EMP tended to be lower than those in rats fed an HFC diet without EMP. Immunohistochemically, the expression of 4-hydroxynonenal as a marker of oxidative stress in the liver tended to be less evident in rats fed the HFC diet with EMP than without EMP. mRNA levels of *NF-κB* tended to be lower in rats fed the HFC diet with EMP than without EMP, whereas mRNA levels of *GPX-1* tended to be higher in rats fed the HFC diet with EMP than without EMP in an EMP-dose dependent manner. Histological findings and hepatic mRNA expressions indicate that the HFC diet containing 1% or 2.5% (w/w) EMP was more likely to reveal a preventive effect against NASH progression than the HFC diet with 5% (w/w) EMP.

***Conclusions*:** Our data suggests that EMP containing selenoneine may prevent NASH progression, presumably through its antioxidative activity. The optimal dose of EMP requires further study.

Keywords
Selenoneine; enzymatic decomposition of mackerel; nonalcoholic steatohepatitis; high-fat and high-cholesterol diet; antioxidative activity

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is currently recognized as the most common cause of chronic liver disease in many countries. NAFLD consists of two clinical entities: nonalcoholic fatty liver (NAFL), which is a mostly benign, nonprogressive disease and nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis or even hepatocellular carcinoma. NASH is histologically characterized by hepatic steatosis associated with liver cell injury (ballooning degeneration) and inflammation [1]. The pathogenesis of NASH is thought to be multifactorial, and oxidative stress may play an important role in the development of NASH [2, 3]. Nutritional factors are commonly associated with NASH; however, there are few fully proven treatments for NASH, making it imperative to develop novel dietary strategies that can prevent NASH.

Selenium is an essential micronutrient for humans and animals, and seafood is one of the major sources of selenium in humans [4, 5]. The antioxidant function of selenium plays protective roles in various human diseases including prostate, lung, or colon cancers; immunodeficiency; and heart diseases [6]. Selenoneine is the major form of selenium found in aqueous extracts and is the major selenium compound in the muscles of fish such as tuna, mackerel, and swordfish [7, 8]. Selenoneine has strong antioxidant properties and a detoxifying function against methylmercury toxicity [8]. Recently, peptides obtained by enzymatic decomposition of mackerel (EMP) containing selenoneine were reported to possess antioxidative activity and affect cytokine secretion such as interleukin-10 (IL-10) [9, 10]. Therefore, this EMP can be a potential dietary strategy for the prevention of NASH.

The objective of this study was to evaluate the preventive effect of EMP against NASH progression in Sprague-Dawley (SD) rats that were fed a high-fat and high-cholesterol diet (HFC; NASH model) [11]. The mechanisms underlying the effects of EMP, specifically in relation to fibrogenesis, inflammation, oxidative stress, and cholesterol and lipid metabolism in the liver were also investigated.

2. Materials and Methods

All procedures performed on the animals were approved by the Animal Use Committee of the University of Nagasaki, and the animals were maintained in accordance with the University of Nagasaki's Guidelines for the Care and Use of Laboratory Animals.

2.1 Animals and experimental design

Eight-week-old male Sprague-Dawley (SD) rats were purchased from Japan SLC (Hamamatsu, Japan) and housed individually in a temperature- and humidity-controlled room (22-24 °C and 50-60% relative humidity) with a 12-hour light-dark cycle. After a one-week acclimation period with standard rodent chow (MF; Oriental Yeast, Tokyo, Japan) and water *ad libitum*, the rats were randomly divided into five groups that were fed for 9 weeks as follows: the control group (n = 6) was fed MF as the normal diet, the HFC group (n = 6) was fed a high-fat and high-cholesterol (HFC) diet, the EMP1% group (n = 6) was fed a HFC diet supplemented with 1% (w/w) EMP, the EMP2.5% group (n = 7) was fed a HFC diet supplemented with 2.5% (w/w) EMP, and the EMP5% group (n = 7) was fed a HFC diet supplemented with 5% (w/w) EMP. The HFC diet was prepared by mixing MF with 28.75% (w/w) palm oil, 1.25% (w/w) cholesterol, and 0.5% (w/w) sodium cholate [11]. EMP was provided by L.S Corporation, Tokyo, Japan [12]. Mackerel meat was powdered and heated at 90 °C, and then was digested with proteases from *Bacillus subtilis* and *Aspergillus oryzae*. The hydrolysate was further digested with pancreatin and leucine aminopeptidase. After the subsequent processes of filtration, separation of oils and fats, concentration, removal of metals, and finally desiccation, approximately 20 g of EMP powder was obtained from 400 g of mackerel meat. Ultimately, 100 g of the EMP powder contained 90 g of protein, 0.2 g of lipid, 9.1 g of ash, and 951μg of selenium. The proximate dietary compositions of each diet fed to the rats are shown in Table 1. Daily energy intake and body weight were monitored throughout the study.

At 18 weeks of age, the rats were fasted for 6 hours and sacrificed under anaesthesia with pentobarbital sodium. Blood samples were taken from the inferior vena cava or heart and kept at -20°C until analysis. The epididymal fat pad and liver were removed, washed in cold saline, and weighed. Liver tissues were either placed in 10% neutral buffered formalin or snap frozen in liquid nitrogen and stored at -80°C.

Table 1 Proximate dietary compositions.

Ingredient\Group	Control	HFC	EMP1%	EMP2.5%	EMP5%
Water (g)	7.90	5.49	5.46	5.42	5.35
Crude protein (g)	23.10	16.05	16.79	17.89	19.73
Crude lipid (g)	5.10	3.54	3.51	3.46	3.37
Crude ash (g)	5.80	4.03	4.08	4.16	4.28

Crude fiber (g)	2.80	1.95	1.93	1.90	1.85
Nitrogen-free extract (g)	55.30	38.43	38.04	37.45	36.48
Palm oil (g)	0.00	28.75	28.46	28.02	27.29
Cholesterol (g)	0.00	1.25	1.24	1.22	1.19
Sodium cholate (g)	0.00	0.50	0.49	0.49	0.47
Total (g)	100.00	100.00	100.00	100.00	100.00
Protein energy ratio (%)	25.70	12.63	13.25	14.18	15.76
Lipid energy ratio (%)	12.77	57.15	56.74	56.13	55.10
Carbohydrate energy ratio (%)	61.53	30.23	30.01	29.69	29.14
Energy (kcal/100g)	359.50	508.60	507.03	504.68	500.76

Control group rats were fed MF diet as a normal diet.

HFC, high-fat and high-cholesterol diet; EMP, peptides obtained by enzymatic decomposition of mackerel; EMP1%, EMP2.5% and EMP5%, HFC diet supplemented with 1%, 2.5% and 5% (w/w) EMP, respectively.

2.2 Serum biochemical analysis

Serum triglyceride (TG), free fatty acid (FFA), total cholesterol (TC), free cholesterol (FC), glucose, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were determined using Triglyceride E test Wako, NEFA C test Wako, Cholesterol E test Wako, Free cholesterol E test Wako, Glucose C II test Wako, and Transaminase C II test Wako (Wako Pure Chemical Industries, Osaka, Japan), respectively. Serum insulin, leptin, and adiponectin levels were measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science Inc., Yokohama, Japan), a mouse/rat leptin ELISA kit (Morinaga Institute of Biological Science Inc.), and a mouse/rat adiponectin ELISA kit (Otsuka Pharmaceuticals Co., Ltd., Tokyo, Japan), respectively.

2.3 Hepatic lipid analysis

Hepatic lipids were extracted from the frozen liver using the method of Folch et al. [13]. The extract was dissolved in isopropanol and analyzed for TG and TC with a kit, as described above.

2.4 Histopathological examinations

Liver tissues stored in 10% neutral-buffered formalin were embedded in paraffin, sectioned at 4 μm, and processed for hematoxylin-eosin (HE) and Azan staining for histopathological examination. Histological steatosis (0-3), lobular inflammation (0-3), and hepatocyte ballooning (0-2) were assessed semi-quantitatively to determine the NAFLD activity score (NAS) according to the NASH Clinical Research Network Scoring System [14]. NAS scores ≥ 5 and ≤ 2 were considered diagnostic and not diagnostic, respectively, for steatohepatitis. Liver fibrosis (0-4) was also assessed according to this system [13]. For the immunohistochemical analysis of 4-hydroxynonenal (4-HNE), deparaffinized liver specimens were incubated with rabbit polyclonal antibodies against 4-HNE (OXIS International, Foster City, CA, USA) to detect oxidative stress-induced lipid peroxidation. Normal rabbit serum was used as a negative control. EnVision peroxidase was used as the second antibody (DAKO, Glostrup, Denmark), and 3,3’ diaminobenzidine (DAB) was used as the substrate.

The intensity of 4-HNE was scored as 0, 1, and 2, indicating no positive staining or slightly positive in the perivenular zone, slightly positive in the periportal zone, and positive staining in hepatocytes or strongly positive in the periportal zone, respectively. All histopathological examinations were performed by a pathologist (K.T.) who was blinded to the experimental and serological data.

2.5 Quantification of mRNA using real-time polymerase chain reaction

Total RNA from the liver was extracted using RNAiso Plus (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA templates using a commercial kit (PrimeScript RT Master Mix, Takara Bio). Real-time polymerase chain reaction (PCR) analysis was performed as described previously [11]. Specific primers were designed using the Primer-BLAST primer designing tool (National Center for Biotechnology Information [NCBI], Bethesda, MD, USA) and were synthesized by Greiner Bio-One Japan (Tokyo, Japan) (Table 2).

Table 2 Primer sequences for real-time polymerase chain reaction.

Primer	Forward (5' to 3')	Reverse (5' to 3')
COL1A1	GCGTAGCCTACATGGACCAA	AAGTTCCGGTGTGACTCGTG
TGF-β	CTTTGTACAACAGCACCCGC	TAGATTGCGTTGTTGCGGTC
α-SMA	GCCAAGAAGACATCCCTGAAGT	TGTGGCAGATACAGATCAAGCAT
TNF-α	TGATCGGTCCCAACAAGGA	TGGGCTACGGGCTTGTCA
MCP-1	TCTGTCACGCTTCTGGGCCTGT	GGGGCATTAACTGCATCTGGCTGAG
IL-1β	CTCCAGTCAGGCTTCCTTGTG	GGTCATTCTCCTCACTGTCGAAA
IL-6	GATACCACCCACAACAGACCAGTA	TGCACAACTCTTTTCTCATTTCCA
NF-κB	TGACATCATCAACATGAGAAACGA	CCCCAACCCTCAGCAAGTC
CYP2E1	CCCATCCTTGGGAACATTTTT	GCCAAGGTGCAGTGTGAACA
HO-1	CACAGGGTGACAGAAGAGGCTAA	GGGACTCTGGTCTTTGTGTTCCT
GPX-1	GCTGCTCATTGAGAATGTCG	GAATCTCTTCATTCTTGCCATT
MnSOD	GACCTGCCTTACGACTATG	TACTTCTCCTCGGTGACG
LXR-α	CAGGACCAGCTCCAAGTAGA	GAACATCAGTCGGTCGTGG
MTP	CAAGCTCAAGGCAGTGGTTG	AGCAGGTACATCGTGGTGTC
FAS	CAACATTGACGCCAGTTCCG	TTCGAGCCAGTGTCTTCCAC
GPAT	GCTACCTGAAGGTGAGCCAG	AGGTACTCAGACTCCGGGAC
CPT-1	AACCTCGGACCCAAATTGC	GGCCCCGCAGGTAGATATATT
AOX	CCACTGAACAAAACAGAGGTCC	GTCCCAGGGAAACTTCAAAGC

All quantifications were normalized using the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). For studies in rats, hepatic expressions of genes involved in fibrosis [collagen type I alpha 1 (COL1A1), transforming growth factor-β (TGF-β), and α-smooth muscle actin (α-SMA)], inflammation [tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β), interleukin-6 (IL-6), and nuclear factor-κB (NF-κB)], oxidative stress [cytochrome P450 family 2 subfamily E polypeptide 1 (CYP2E1), heme oxygenase-1 (HO-1), glutathione peroxidase-1 (GPX-1), and manganese superoxide dismutase (MnSOD)],

cholesterol metabolism [liver X receptor-α (LXR-α)], and lipid metabolism [microsomal triglyceride transfer protein (MTP), fatty acid synthase (FAS), glycerol-3-phosphate acyltransferase (GPAT), carnitine palmitoyltransferase-1 (CPT-1), and acyl-CoA oxidase (AOX)] were quantified. All data were expressed as a fold change relative to the control group.

2.6 Statistical analysis

All values were expressed as mean ± standard error (SE). Differences between groups were tested for statistical significance using one-way analysis of variance (ANOVA), followed by Scheffe's post hoc test, chi-square test, or Fisher's exact probability test. Correlations between two variables were determined by Spearman's rank correlation coefficient. All analyses were performed using IBM SPSS statistics software program, version 24 (IBM Co., Somers, NY, USA) on a Windows computer. A p value of less than 0.05 was considered to be statistically significant.

3. Results

3.1 Cumulative energy intake, body weight, and relative organ weights

During the 9-week study (9 to 18 weeks of age), cumulative energy intake was significantly higher in the EMP1% group than in the control and EMP5% groups (p = 0.003 and 0.023, respectively), whereas body weight at 18 weeks of age and weight gain during the 9-week study were not significantly different among the groups. The liver weight/body weight ratio at 18 weeks of age was significantly higher in the HFC, EMP1%, EMP2.5%, and EMP5% groups than in the control group (p < 0.001), and this ratio was notably similar between the HFC, EMP1%, EMP2.5%, and EMP5% groups. The epididymal fat pad weight/body weight ratio was not significantly different among the groups (Table 3).

Table 3 Cumulative energy intake, body weight, relative organ weights, serum parameters and hepatic lipid concentrations at 18 weeks of age.

Item\Group	Control (n=6)	HFC (n=6)	EMP1% (n=6)	EMP2.5% (n=7)	EMP5% (n=7)
Cumulative energy intake (kcal)	5187±86^{a}	5907±165^{ab}	6253±277^{b}	5782±130^{ab}	5426±97^{a}
Final body weight (g)	511±11	525±10	527±13	506±13	489±9
Body weight gain (g)	210±9	223±10	225±12	208±12	191±7
Liver/body weight (%)	2.8±0.1^{a}	5.5±0.2^{b}	5.5±0.1^{b}	5.4±0.2^{b}	5.6±0.2^{b}
Epididymal fat pad/body weight (%)	1.9±0.1	2.2±0.2	2.2±0.1	2.3±0.1	1.7±0.2
Serum triglyceride (mg/dL)	72±8	55±8	52±3	63±5	52±9
Serum free fatty acid (mEq/L)	0.28±0.06	0.28±0.08	0.28±0.04	0.26±0.04	0.22±0.04
Serum total cholesterol (mg/dL)	65±5^{a}	91±4^{ab}	83±5^{ab}	92±9^{ab}	95±6^{b}
Serum free cholesterol (mg/dL)	22±2^{a}	27±2^{a}	26±2^{a}	29±2^{a}	40±3^{b}
Serum glucose (mg/dL)	134±8	152±14	130±9	159±15	181±45
Serum insulin (ng/mL)	6.4±1.3	7.2±1.1	4.6±1.5	5.7±0.9	6.1±1.0
Serum AST (IU/L)	56±12	137±22	111±19	114±13	120±22
Serum ALT (IU/L)	11±1^{a}	44±8^{ab}	51±13^{ab}	83±8^{b}	92±14^{b}

Serum leptin (ng/mL)	7.4±0.5[ab]	9.4±1.1[b]	5.3±0.6[ac]	5.5±0.4[ac]	3.6±0.4[c]
Serum adiponectin (μg/mL)	4.4±0.4	4.0±0.6	3.0±0.4	3.5±0.4	4.0±0.5
Hepatic triglyceride (mg/g tissue)	22±4[a]	95±14[b]	127±18[bc]	151±4[c]	150±10[c]
Hepatic total cholesterol (mg/g tissue)	3.8±0.2[a]	78.8±9.9[b]	101.0±12.7[b]	75.6±4.9[b]	78.6±12.2[b]

Values are expressed as means±SE.

[abc]Values not sharing the same lowercase letter in a row are significantly different among groups ($p<0.05$). HFC, high-fat and high-cholesterol diet; EMP, peptides obtained by enzymatic decomposition of mackerel; EMP1%, EMP2.5% and EMP5%, HFC diet supplemented with 1%, 2.5% and 5% (w/w) EMP, respectively; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

3.2 Serum and hepatic biochemical parameters

At 18 weeks of age, serum TG, FFA, glucose, insulin, AST, and adiponectin levels were not significantly different among the groups. Serum TC levels were significantly higher in the EMP5% group than in the control group ($p = 0.039$), and serum FC levels were significantly higher in the EMP5% group than in the control, HFC, EMP1%, and EMP2.5% groups ($p < 0.001$, 0.005, 0.004, and 0.018, respectively). Serum ALT levels were significantly lower in the control group than in the EMP2.5% and EMP5% groups ($p = 0.001$ and <0.001, respectively). Serum leptin levels were significantly higher in the HFC group than in the EMP1%, EMP2.5%, and EMP5% groups ($p = 0.006$, 0.005, and <0.005, respectively); the serum leptin levels were also significantly higher in the control group than in the EMP5% group ($p = 0.007$). Hepatic TG values were significantly lower in the control group than in the HFC, EMP1%, EMP2.5%, and EMP5% groups ($p = 0.003$, <0.001, <0.001, and <0.001, respectively), and were also significantly lower in the HFC group than in the EMP2.5% and EMP5% groups ($p = 0.025$ and 0.029, respectively). Hepatic TC values were significantly lower in the control group than in the other 4 groups ($p < 0.001$) (Table 3).

3.3 Histopathological findings of the liver

Representative histopathology of the rat liver and histological assessments are shown in Figure 1 and Table 4. No obvious findings of hepatic steatosis, lobular inflammation, hepatocyte ballooning, or fibrosis were seen in any of the rats in the control group. In contrast, severe steatosis (score 3), moderate or severe lobular inflammation (score 2 or 3), and mild or perisinusoidal and portal/periportal fibrosis (score 1a or 2) were observed in all 6 rats of the HFC group, despite no evidence of hepatocyte ballooning. In the EMP1%, EMP2.5%, and EMP5% groups, severe steatosis (score 3) was observed in 19 (95%) of the rats; moderate or severe lobular inflammation (score 2 or 3) was observed in 17 (85%) of the rats; and perisinusoidal, portal/periportal, or bridging fibrosis (score 2 or 3) was observed in 12 (60%) of the 20 rats. According to the NAS [14], none of 6 rats of the control group (0%), all of 6 rats of the HFC group (100%), 5 of 6 rats of the EMP1% group (83%), 5 of 7 rats of the EMP2.5% group (71%), and 6 of 7 rats of the EMP5% group (86%) were diagnosed with NASH (i.e., having a NAS of 5 of greater).

Representative immunohistochemistry of the rat liver and immunohistochemical assessments are also shown in Figure 1 and Table 4. Negative staining (score 0) was seen in all 6 rats of the control group, whereas positive staining (score 2) was observed in 5 of 6 rats (83%) in the HFC group. In the 20 rats of EMP1%, EMP2.5% or EMP5% group, negative staining (score 0) was

observed in 5 (25%) rats, and positive staining (score 2) was also observed in 5 (25%) rats. The 4-HNE staining score was significantly correlated with the histological lobular inflammation score and the diagnostic result of NAS (p=0.005 and p=0.001, respectively) (Table 5).

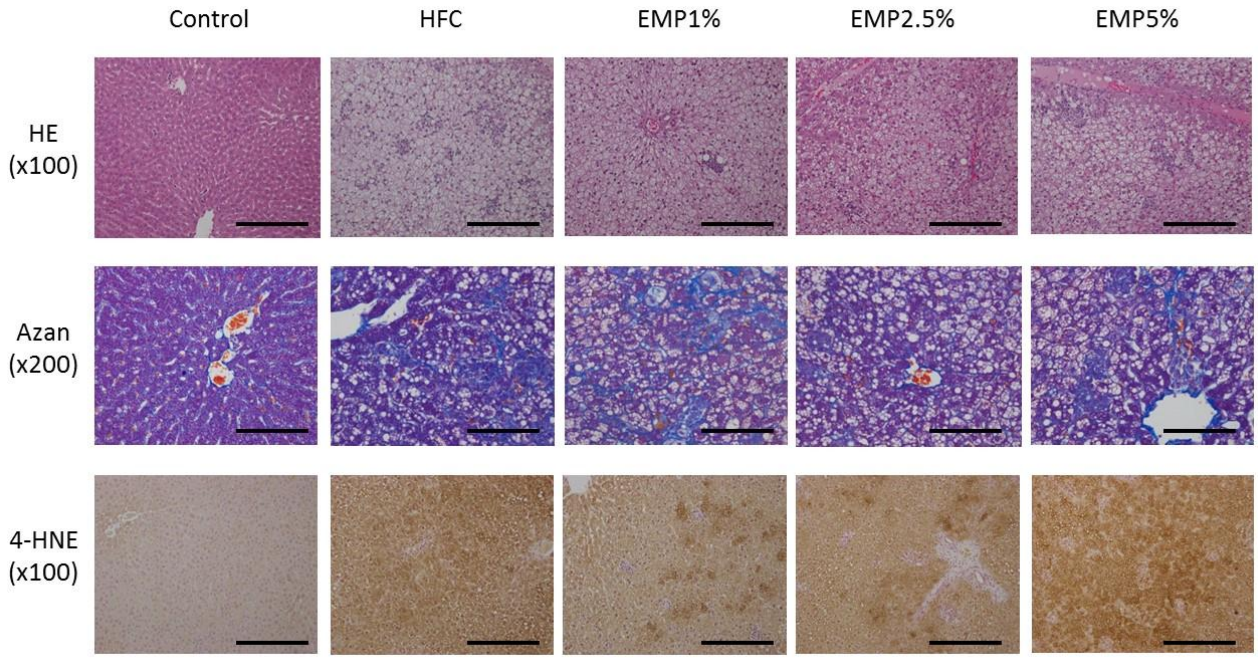

Figure 1 Representative histopathology of the rat liver in the control, HFC, EMP1%, EMP2.5%, and EMP5% groups at 18 weeks of age. Upper panels: HE-stained sections (original magnification, x100; scale bars=400µm). Middle panels: Azan-stained sections (original magnification, x200; scale bars=200µm). Lower panels: Immunohistochemical 4-HNE-stained sections (original magnification, x100; scale bars=400µm). HFC indicates high-fat and high-cholesterol diet; EMP indicates peptides obtained by enzymatic decomposition of mackerel; EMP1%, EMP2.5%, and EMP5% were fed HFC diet supplemented with 1%, 2.5%, and 5% (w/w) EMP, respectively.

Table 4 Histopathological and immunohistochemical assessments of the liver at 18 weeks of age.

Item\Group	**Score**	**Control (n=6)**	**HFC (n=6)**	**EMP1% (n=6)**	**EMP2.5% (n=7)**	**EMP5% (n=7)**
Steatosis*	0	6	0	0	0	0
	2	0	0	0	0	1
	3	0	6	6	7	6
Lobular inflammation*	0	6	0	0	0	0
	1	0	0	1	2	0
	2	0	5	2	3	5
	3	0	1	3	2	2
Hepatocyte ballooning*	0	6	6	6	7	7
NAFLD activity score (NAS)*	0-2	6	0	0	0	0
	3-4	0	0	1	2	1

	5-8	0	6	5	5	6
Fibrosis*	0-1	6	5	2	5	1
	2-3	0	1	4	2	6
4-HNE staining	0	6	0	1	2	2
	1	0	1	3	5	2
	2	0	5	2	0	3

Values are expressed as the number of rats.

*Scores are according to the NASH Clinical Research Network Scoring System proposed by Kleiner et al. [14].

HFC, high-fat and high-cholesterol diet; EMP, peptides obtained by enzymatic decomposition of mackerel; EMP1%, EMP2.5% and EMP5%, HFC diet supplemented with 1%, 2.5% and 5% (w/w) EMP, respectively; 4-HNE, 4-hydroxynonenal.

Table 5 Correlations between 4-HNE staining score and histological lobular inflammation score or NAS score.

Item\4-HNE staining score	**0**	**1**	**2**
Histological lobular inflammation*			
Score 0	6	0	0
Score 1	1	2	0
Score 2	2	5	8
Score 3	2	4	2
NAFLD activity score (NAS)*			
0-2 (non-NASH)	6	0	0
3-4 (borderline)	1	3	0
5-8 (NASH)	4	8	10

Values are expressed as the number of rats.

*Scores are according to the NASH Clinical Research Network Scoring System proposed by Kleiner et al. [14].

4-HNE, 4-hydroxynonenal; NASH, nonalcoholic steatohepatitis.

3.4 Hepatic mRNA expression

To reveal the effect of EMP on NASH on a molecular level, we evaluated key molecular markers of fibrosis, inflammation, oxidative stress, and cholesterol and lipid metabolism in the liver. As shown in Figure 2, the mRNA levels of *COL1A1* and *α-SMA*, which are both involved in fibrogenesis, tended to be lower in the EMP1% and EMP2.5% groups than in the HFC group; however, the mRNA level of *TGF-β* (which is a key inducer of fibrogenesis), was similar between these groups and the HFC group. The mRNA levels of *TNF-α* and *IL-1β*, which are involved in inflammation, tended to be higher in the EMP groups (EMP1%, EMP2.5%, and EMP5%) than the HFC group in an EMP-dose dependent manner. The mRNA levels of *MCP-1* and *IL-6*, which are also involved in inflammation, tended to be lower in the EMP1% group and EMP2.5% group compared to the HFC group, but were the highest in the EMP5% group. In contrast, the mRNA level of *NF-κB*, which is a central regulator of inflammation, tended to be lower in the EMP groups than in the HFC group in

an EMP-dose dependent manner. The mRNA levels of *CYP2E1* and *MnSOD*, which is a member of the cytochrome P450 mixed-function oxidase system and a member of the iron/manganese superoxide dismutase family, respectively, did not significantly differ between the HFC group and

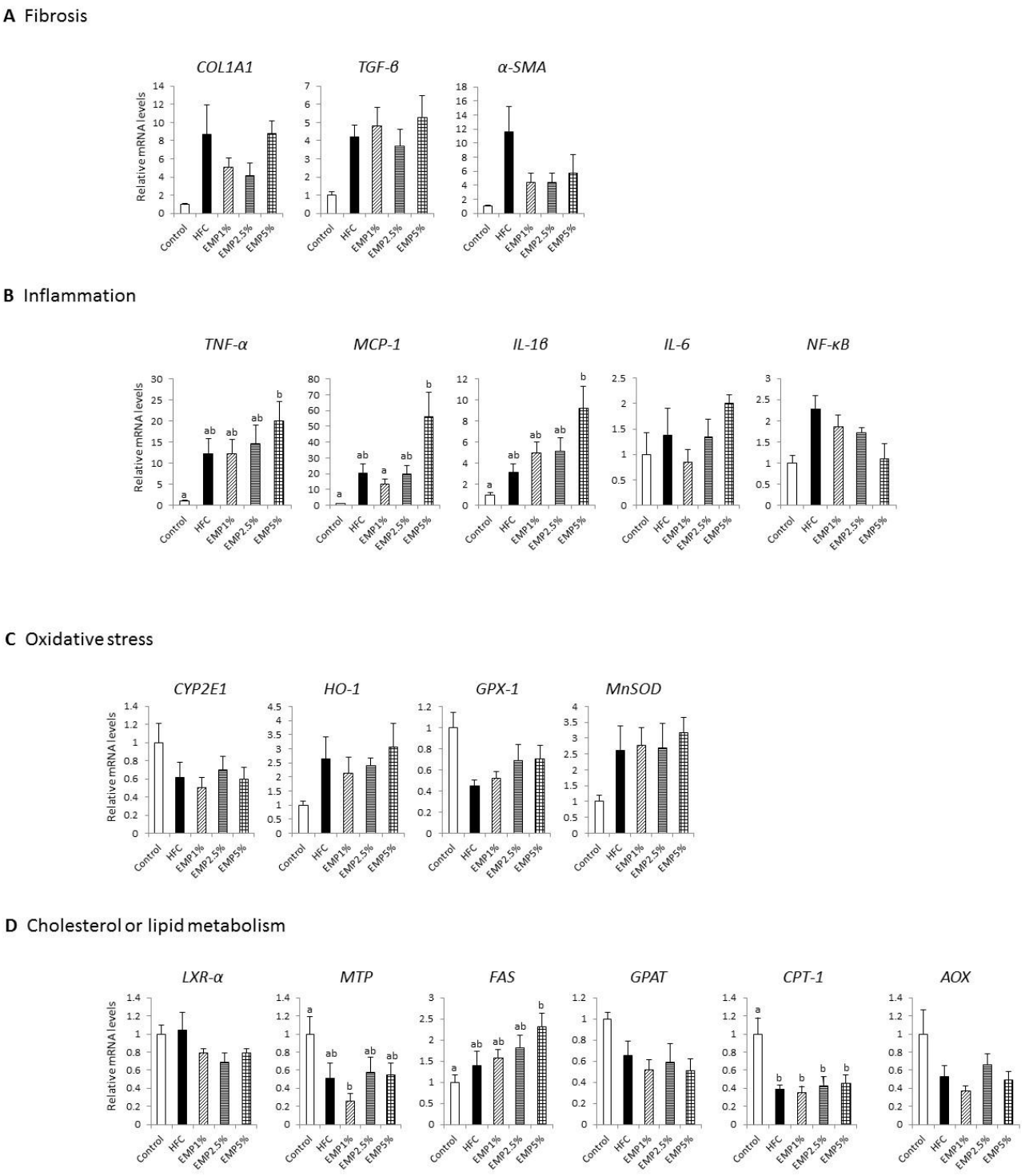

Figure 2 Hepatic gene expression involved in (A) fibrosis, (B) inflammation, (C) oxidative stress, and (D) cholesterol or lipid metabolism in the control, HFC, EMP1%,

EMP2.5%, and EMP5% groups (n = 6-7/group) at 18 weeks of age. mRNA levels are expressed relative to the control group (mean ± SE). abValues not sharing the same lowercase letter in a row are significantly different among groups ($p < 0.05$). HFC indicates high-fat and high-cholesterol diet; EMP indicates peptides obtained by enzymatic decomposition of mackerel; EMP1%, EMP2.5%, and EMP5% were fed HFC diet supplemented with 1%, 2.5%, and 5% (w/w) EMP, respectively.

EMP groups. The mRNA level of *HO-1*, which is an inducible enzyme in response to oxidative stress, tended to be lower in the EMP1% group than in the HFC group. In contrast to this, the mRNA level of *GPX-1*, which is an intracellular antioxidant enzyme, tended to be higher in the EMP groups than in the HFC group in an EMP-dose dependent manner, although the difference was not statistically significant. The mRNA level of *LXR-α*, which is involved in hepatic lipid homeostasis, tended to be lower in the EMP groups than in the HFC group. The mRNA level of *MTP*, which is a rate-limiting protein in the synthesis and excretion of very-low-density lipoprotein (VLDL) from the liver, was lower in the EMP1% group than in the control group. The mRNA level of *FAS*, which is a multi-enzyme protein that catalyzes fatty acid synthesis, tended to be higher in the EMP groups than in the HFC group in an EMP-dose dependent manner. The mRNA levels of *GPAT*, *CPT-1,* and *AOX*, which are involved in lipid metabolism, were not significantly different between the HFC group and the EMP groups.

The mRNA level of *TGF-β* was positively correlated with histological hepatic fibrosis ($p = 0.001$, $r = 0.553$). The mRNA levels of *NF-κB* and *HO-1* were positively correlated with histological lobular inflammation of the liver ($p = 0.003$, $r = 0.513$ and $p = 0.005$, $r = 0.481$, respectively). The mRNA level of *GPX-1* was negatively correlated with immunohistochemical 4-HNE staining (Figure 3).

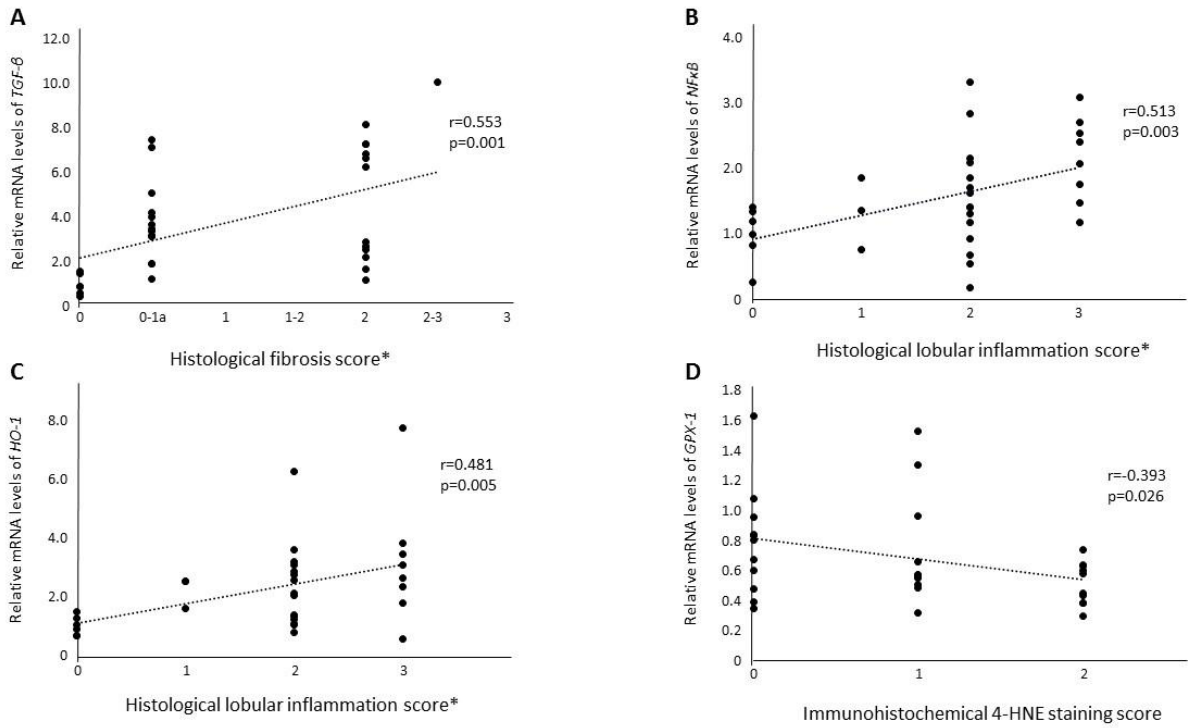

Figure 3 Correlations between hepatic histological findings and gene expressions. Histological fibrosis score is positively correlated with the relative mRNA level of *TGF-β* (A). Histological lobular inflammation scores are positively correlated with the relative mRNA levels of *NFκB* (B) and *HO-1* (C), respectively. Immunohistochemical 4-HNE staining score is negatively correlated with the relative mRNA level of *GPX-1* (D). *Scores are according to the NASH Clinical Research Network Scoring System proposed by Kleiner et al. [14].

4. Discussion

The daily doses of EMP in this study were determined based on human doses. The average daily selenium intake of Japanese people is reported to be approximately 100 μg [15]. Because EMP contains 951 μg selenium in 100 g, daily doses of EMP1%, 2.5%, and 5% in rats would be equivalent to daily doses of approximately 77, 193, and 385 μg in humans, respectively, after taking into account the human equivalent dose.

In this study, there were no significant differences in liver/body weight, epididymal fat pad weight/body weight, or serum metabolic parameters such as serum glucose, insulin, or adiponectin at 18 weeks of age between the HFC group and the EMP groups; however, serum leptin levels were lower in EMP groups than in the HFC group. Leptin possesses a number of effects on the immune system and plays an important role in modulating inflammatory responses, regulation of food intake, and energy expenditure [16]. Indeed, serum leptin levels were negatively correlated with serum ALT levels in this study ($p = 0.003$, $r = -0.510$, data not shown). No significant difference in epididymal fat pad weight/body weight, serum triglyceride levels, or free cholesterol levels between the HFC groups and the EMP groups was detected; this indicates that the leptin-lowering component of EMP may not largely affect obesity or visceral fat tissue. Further studies examining the insulin-leptin axis and the role of endotoxins are needed to determine the mechanism of this effect [16, 17].

The serum FC levels were significantly higher in the EMP5% group than in the control, HFC, EMP1%, and EMP2.5% groups in this study. The reason for this result is unclear. Lecithin cholesterol acyltransferase (LCAT) is synthesized by the liver, and esterifies FC on the surface of HDL, forming cholesteryl esters [18]. Because the histological hepatic fibrosis was more progressive in the EMP5% group, it is conceivable that LCAT synthesis and esterification of FC can be stagnated.

Histopathological findings of the liver revealed that all 6 rats in the HFC group were diagnosed with NASH; contrastingly, 4 out of 20 rats (20%) in the EMP groups had borderline scores according to the NASH Clinical Research Network Scoring System [14] and were not diagnosed as fully-developed NASH in this study. Because the histopathological findings of steatosis and hepatocyte ballooning were similar between the HFC group and the EMP groups, this difference in the NASH diagnostic rate between the HFC group and the EMP groups (100% vs 80%) was mainly due to the histopathological lobular inflammation score. Moreover, immunohistochemical staining intensities of 4-HNE tended to be lower in the EMP groups than the HFC group; the 4-HNE staining score was significantly correlated with the histological lobular inflammation score and the diagnostic result of NAS. Oxidative stress seems to be one of the most important mechanisms leading to hepatic injury in NAFLD, as augmented generation of reactive oxygen species (ROS) can

induce lipid peroxidation, leading to inflammation [19]. Thus, our results indicate that EMP reduced oxidative stress, which decreased lobular inflammation and NASH progression.

The TGF-β pathway is a significant modulator of *COL1A1*, the type I collagen gene [20,21], while Smads are a family of proteins that operate downstream of various members of the TGF-β superfamily [20]. In this study, the mRNA level of *TGF-β* was positively correlated with histological hepatic fibrosis. mRNA levels of *TGF-β* in the EMP groups were similar to that in the HFC group, and mRNA levels of *COL1A1* and *α-SMA* tended to be lower in the EMP1% and EMP2.5% groups than in the HFC group. However, histological hepatic fibrosis was more progressive in the EMP groups than in the HFC group. The reason for these discrepancies is not clear but may be attributable to the non-Smad signaling pathways [22].

Antioxidants can inhibit NF-κB activation [23]. In this study, the mRNA levels of *NF-κB* were positively correlated with histological lobular inflammation of the liver and tended to be lower in the EMP groups (EMP1%, EMP2.5%, and EMP5%) than in the HFC group in an EMP-dose dependent manner. In contrast, the mRNA level of the intracellular antioxidant enzyme *GPX-1* was negatively correlated with immunohistochemical 4-HNE staining and tended to be higher in the EMP groups than in the HFC group in an EMP-dose dependent manner, although the difference was not statistically significant. These results indicate that the lobular inflammation in EMP groups can be reduced by *NF-κB* suppression via *GPX-1* expression. However, the histological lobular inflammation score tended to be lower in the EMP1% and EMP2.5% groups but not in the EMP5% group. The reason for this discrepancy is not obvious, but inflammation-related cytokines such as TNF-α, MCP1, IL-1β, and IL-6 in the EMP5% group can be induced by non-NF-κB signaling pathways, which may offer an explanation [24].

Our results from the histological fibrosis score, NAS score, and hepatic mRNA expressions indicate that the EMP5% group did not reveal a preventive effect on NASH progression. Supranutritional selenium intake or high plasma selenium concentration was reported to be associated with type 2 diabetes or insulin resistance [8, 25, 26]. Selenium supplementation induces selenoproteins such as intracellular antioxidant enzyme GPX-1 and selenium transporter selenoprotein P, which are both implicated in insulin resistance due to the impairment of insulin signaling in the liver and skeletal muscle [8, 25, 27-29]. In this study, the mRNA level of *HO-1*, which is an inducible enzyme in response to oxidative stress, was positively correlated with histological lobular inflammation of the liver and tended to be lower in the EMP1% group but higher in the EMP5% group when compared to the HFC group. ROS can induce lipid peroxidation, leading to fibrogenesis through the activation of stellate cells, and also inhibit hepatocyte secretion of VLDL, which induces liver fat accumulation [19]. Malaguarnera et al. reported that a significant correlation between the increased levels of *HO-1* and lipid peroxidation as well as the increased *HO-1* expression reflected the severity of NASH [30]. Therefore, 5% (w/w) supplementation of EMP to a HFC diet was an excessive dose for SD rats in the prevention of NASH.

5. Conclusions

Our results demonstrate that EMP may possess a preventive effect on NASH progression, presumably through its antioxidative stress activity. The EMP1% and 2.5% groups, which were fed a HFC diet supplemented with 1% and 2.5% (w/w) EMP for 9 weeks, respectively, were more

effective in prevention than the EMP5% group, which was fed a HFC diet supplemented with 5% (w/w) EMP for 9 weeks. The optimal dose of EMP should be further researched in future studies.

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Author Contributions

Katsuhisa Omagari created the study design, adjusted results, and finalized the manuscript.
Ayumi Fukuda carried out the experiments and performed the data analysis.
Machiko Suga carried out the experiments and performed the data analysis.
Ayumi Ogata carried out the experiments and performed the data analysis.
Shinta Nishioka performed the data interpretation and revised the manuscript.
Kazuhito Suruga carried out PCR for mRNA expression and performed the data interpretation.
Mayuko Ichimura carried out the experiments and data collection.
Koichi Tsuneyama performed the histopathological assessment and corrected the manuscript.

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Competing Interests

Katsuhisa Omagari received a research grant from L.S Corporation for this work. The other authors have declared that no competing interests exist.

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